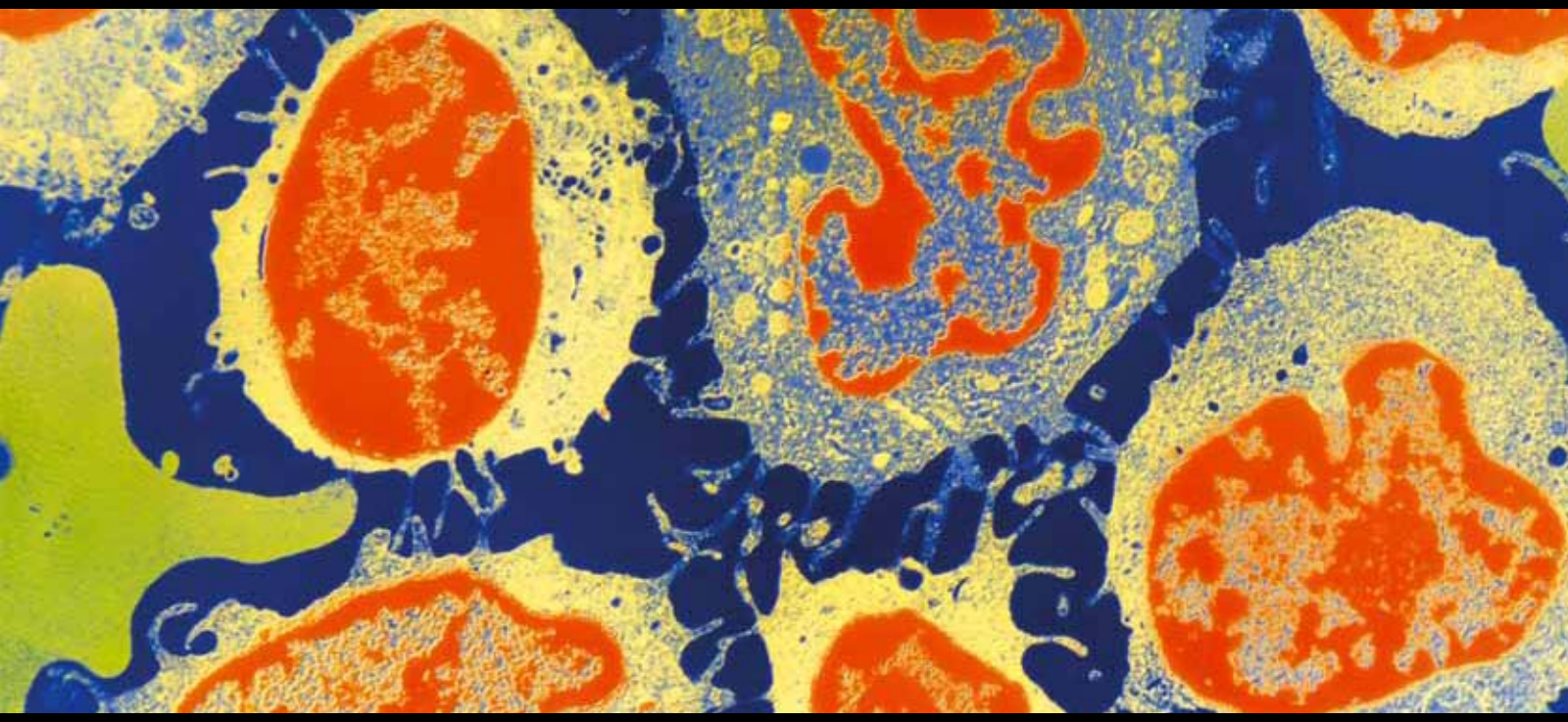
Journal of Oncology

Papillomavirus from the Bench to the Clinics

Guest Editors: Adhemar Longatto Filho, Luisa Lina Villa, and Kari Syrjänen



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Editorial Board

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Editorial

Papillomavirus from the Bench to the Clinics

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Human papillomavirus (HPV) represents an exciting subject of study because it is currently established as an essential etiological factor of uterine cervical cancer and strongly implicated in the development of other genital cancers as well, in addition to benign genital warts. Additionally, substantial amount of new data have been elaborated linking HPV with head and neck cancer and, more tentatively, also with esophageal, breast, prostate, and lung cancers. Despite the existing controversies, the possible link of HPV infection with these nongenital carcinomas opens a new fascinating era of HPV research.

Concomitantly, the HPV vaccination has emerged as a new paradigm to cancer prevention programs worldwide. As much as 10% of all cancers can be related to certain HPV types, and therefore we can anticipate a substantial reduction in cancers worldwide with the implementation of HPV prophylactic vaccines. HPV vaccination provides a realistic option to reduce cervical cancer incidence and mortality in poor and developing countries, where the secondary prevention options (i.e., the screening by Papanicolaou smears and HPV testing) are not easily implemented due to lacking infrastructure, low human resources, lack of population adherence, and lack of political commitment. We can anticipate substantial reduction of HPV-related diseases.

During the past decades, HPV research has been pursued along different lines, which has resulted in an ever-increasing number of publications dissecting the multifaceted mechanisms of HPV infections and the complexity of biological cascade related to HPV-associated human carcinogenesis.

We are pleased to introduce this special issue dedicated to Papillomavirus, from the bench to the clinics, providing us an opportunity of entering this fascinating world that integrates a plethora of specialties studying the relation between this virus and human cancer.

Adhemar Longatto Filho
Luisa Lina Villa
Kari Syrjänen

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Research Article

Burden of Human Papillomavirus among Haitian Immigrants in Miami, Florida: Community-Based Participatory Research in Action

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Background. Haitian immigrant women residing in Little Haiti, a large ethnic enclave in Miami-Dade County, experience the highest cervical cancer incidence rates in South Florida. While this disparity primarily reflects lack of access to screening with cervical cytology, the burden of human papillomavirus (HPV) which causes virtually all cases of cervical cancer worldwide, varies by population and may contribute to excess rate of disease. Our study examined the prevalence of oncogenic and nononcogenic HPV types and risk factors for HPV infection in Little Haiti. *Methods*. As part of an ongoing community-based participatory research initiative, community health workers recruited study participants between 2007 and 2008, instructed women on self-collecting cervicovaginal specimens, and collected sociodemographic and healthcare access data. *Results*. Of the 242 women who contributed adequate specimens, the overall prevalence of HPV was 20.7%, with oncogenic HPV infections (13.2% of women) outnumbering nononcogenic infections (7.4%). Age-specific prevalence of oncogenic HPV was highest in women 18–30 years (38.9%) although the prevalence of oncogenic HPV does not appear to be elevated relative to the general U.S. population. The high prevalence of oncogenic types in women over 60 years may indicate a substantial number of persistent infections at high risk of progression to precancer.

1. Introduction

Haitian immigrant women residing in Little Haiti, a large ethnic enclave in Miami-Dade County, experience the highest cervical cancer incidence rates in South Florida. Between 2007–2009, disease incidence in Little Haiti (34 per 100,000 women) was nearly four times higher than that reported for the Miami metropolitan area overall (9 per 100,000 women) [1]. This disparity reflects lack of access to the formal healthcare system and screening with cervical cytology (Papanicolaou test) [2, 3]. While cytology-based screening programs have effectively reduced cervical cancer incidence and mortality in the United States [4, 5], Haitian women in Little Haiti encounter multiple barriers to routine Pap testing, and often cannot comply with screening recommendations or necessary follow up for detected abnormalities [6, 7].

Underutilization of screening may not solely account for the excess burden of cervical cancer observed among Haitian women. Epidemiologic and virologic studies have shown that persistent cervical infection with oncogenic "high risk" human papillomavirus (HPV) types cause virtually all cases of cervical cancer worldwide [8], but the prevalence of HPV

varies by population. Knowledge of the burden of HPV by type and age will be necessary to establish the utility of vaccines against HPV infection and for the effective design of screening protocols with HPV DNA testing, which may improve uptake of screening in populations with access barriers if self-sampling of HPV DNA specimens proves acceptable and feasible. Using a community-based participatory research (CBPR) approach, our primary objective was to document the prevalence and type distribution of HPV in Little Haiti, which to date has not been described. We also examined risk factors associated with oncogenic HPV infection in our sample.

2. Methods

2.1. Overview of Patnè en Aksyon. The current study was conducted as part of an ongoing CBPR initiative in Little Haiti, which has been described in previous publications [2, 3, 9]. Briefly, CBPR is a research methodology, increasingly popular in the field of public health, which invites community participation throughout the research process, from study conceptualization to dissemination of findings [10–12]. This approach helps dissuade community suspicion about the intent of inquiry, which is prevalent in Little Haiti and other underserved communities that are largely disenfranchised from the formal healthcare system.

In Little Haiti, CBPR efforts are governed by a campus-community partnership known as *Patnè en Aksyon* (Partners in Action). This partnership, which involves active participation of community leaders from Little Haiti and an interdisciplinary team of investigators from a large university in the Miami metropolitan area, strives to reduce the excess burden of cervical cancer experienced by Haitian women and to improve the health status of women in Little Haiti. To this end, community health workers (CHWs), who are formally employed by a large community-based organization whose leadership is active in Patnè en Aksyon, play a central role.

2.2. Participant Recruitment and Data Collection. In the present study, female CHWs of Haitian descent (fluent in English and Haitian Kreyol) were trained to recruit study participants and collect data using a standardized manual created by one of the academic partners. As part of the training, each CHW also completed an online certification program for conducting human subjects research (CITI), as mandated by the University of Miami's Institutional Review Board (IRB). The university IRB approved the study.

Between September 2007 and March 2008, CHWs recruited participants primarily through the extensive network of the community-based organization (CBO) where they were formally employed, and by canvassing community venues including flea markets, health clinics, and laundromats across Little Haiti to identify women meeting study eligibility criteria (i.e., were 18 years of age and older, had no prior history of cervical cancer or surgical hysterectomy, and reported having no Pap smear within the past year). The CHWs approached all women in such venues who appeared to be of Haitian descent and at least 18 years of age ($n = 362$) and told them about the study. For women who were interested and eligible ($n = 290$), the CHWs scheduled an interview to have women self-sample for HPV and respond to a short survey.

Interviews and self-sampling took place wherever the participant felt most comfortable, usually at her home or the home of a close friend, and were conducted in English or Haitian Kreyol according to the participant's preference. The CHWs were instructed to (1) obtain informed consent, (2) teach women how to appropriately self-sample using visual aids, and (3) interview participants about their experience with self-sampling. To monitor adherence to this research protocol, CHWs were required to log the time that they completed each step on data collection forms. Given widespread skepticism about research in Little Haiti, the CHWs spent one hour (on average) explaining to participants the benefits of participation, explaining the purpose of collecting specimens and genotyping those positive for HPV, and assuring women that results would be kept confidential. Following informed consent, CHWs instructed women on how to appropriately use the device using a pictorial brochure that visually demonstrated each step in the process (Figure 1). The participants then collected their sample in private and gave it to the CHW. Immediately afterwards, the CHW administered a brief questionnaire, which assessed participant's impressions about self-sampling for HPV sociodemographic background, Pap smear screening history, and risk factors for HPV. Questionnaire items were adapted from previously validated women's health behavior surveys and were translated and back-translated from English to Haitian Kreyol for monolingual Kreyol participants.

The CHWs notified all participants of their results, and assisted those with cytological abnormalities and/or infections (e.g., candida, gardnerella, trichomoniasis) in obtaining timely and appropriate followup care. Nearly all women with abnormalities obtained follow-up at a free gynecologic oncology clinic in Little Haiti with the assistance of CHWs.

2.3. Specimen Collection. Women self-collected cervicovaginal specimens with the Fournier device, which has demonstrated high concordance with physician-collected cervical specimens and can provide specimens for cytology assays [13, 14]. This device is currently only approved for research purposes, but is under review by the FDA for clinical application. Mechanically similar to a tampon, the device includes an outer sheath to prevent cross-collection of unwanted vaginal cells that may compromise specimen quality and reliability. Women were instructed to insert the device into their vagina, eject the Dacron tip to obtain a sample of cervical cells for cytology, and then retract the tip to avoid cross-collection of vaginal cells during removal (Figure 1). Specimens were shipped to Select Diagnostics (Greensboro, North Carolina) for HPV testing, genotyping, and cytological evaluation. Cytology was performed using standard Thin Prep technology [15].

2.4. Human Papillomavirus Detection and Typing. Cervicovaginal specimens were genotyped using a polymerase chain

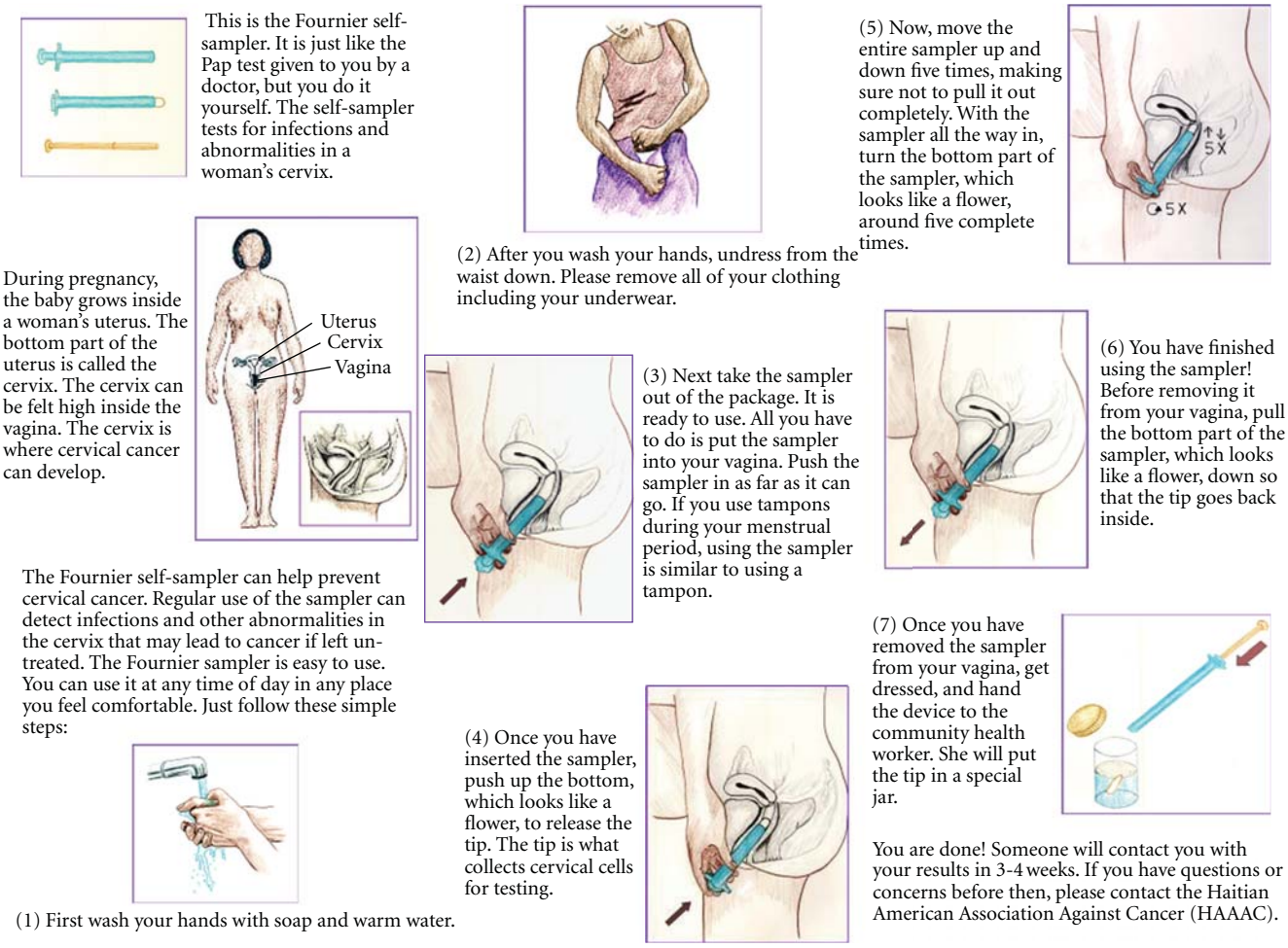

Figure 1: Self-sampling device brochure.

reaction restriction fragment length polymorphism (PCR-RFLP) assay (*Access Genetics*, Eden Prairie, MN), which amplifies the highly conserved L1 region of the HPV genome to detect approximately 80 HPV types. Genomic DNA was extracted from ecto- or endocervical epithelial cells obtained via the Fournier device and fixed in alcohol-based liquid ThinPrep™ (Cytyc Inc, Boxborough, MA) solution using standard techniques. In brief, cellular material was concentrated and the alcohol preservatives removed by distilled water dilution. Each sample was evaluated for cellularity to determine the dilution volume that equalizes the concentrations across all samples followed by an enzymatic incubation to achieve increased cell membrane permeability. An aliquot of each cellular sample was then combined with Cellerate *Access Genetics*, Eden Prairie, MN. This mixture was subjected to thermal cycling incubation resulting in protein degradation and the release of purified DNA.

PCR amplification of HPV products used degenerate primers specific for the consensus regions of the L1 gene in the HPV genome. The assay was performed in two parts: HPV detection (presence/absence) and HPV identification (RFLP genotyping). The first reaction included two unique and specific primers, one for the HPV genome, and the other for the β-globin housekeeping gene. The latter was used as an internal control to document the presence of human nucleated cells in the sample analyzed. PCR products were then simultaneously separated and stained for visualization using 3% agarose gels prestained with ethidium bromide. Electrophoresis was performed in 1X TBE buffer at ~95 V for 90 minutes. Images of each gel were digitally captured. HPV type was determined by RFLP. A PCR product from each HPV-positive sample underwent endonuclease digestion with restriction enzymes *Pst I, Rsa I,* and *Hae III*. The digestion products were separated using 5% polyacrylamide gel electrophoresis (PAGE). The resultant fragment band patterns determined the specific HPV types present in each sample.

For this analysis, oncogenic "high-risk" (HR) HPV types included 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82 [16, 17]. All other HPV types, including unknown types, were considered low risk (LR).

2.5. Statistical Analyses. Among the 290 women eligible for the study, 246 completed self-sampling and the subsequent

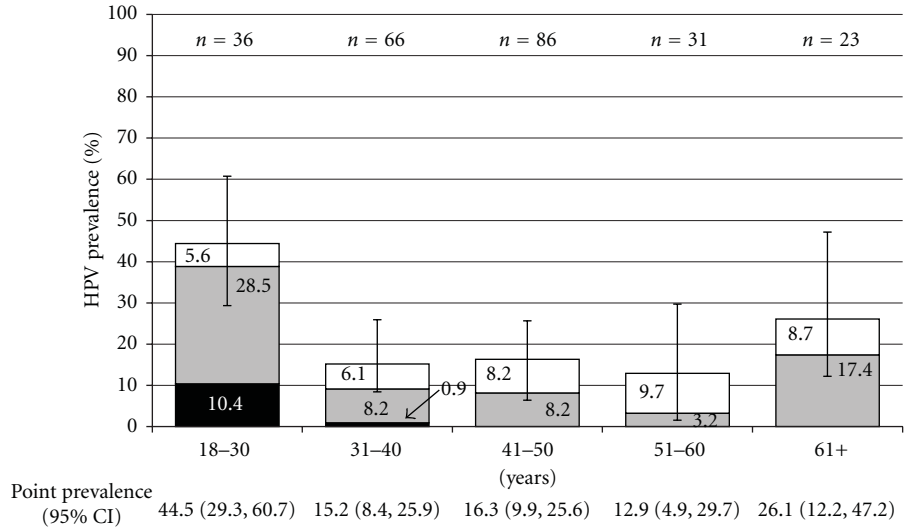

Figure 2: Age-specific prevalence of cervical human papillomavirus (HPV) DNA by LR, HR, and HR Types 16 and 18. *Vertical bars* indicated 95% confidence intervals of overall HPV prevalence. (Black) HPV 16 and/or 18 (including co-infection), (grey) all other HR-HPV, (white) LR-HPV only.

questionnaire. Of these, we included 242 women in the present analysis; the four excluded women did not collect adequate specimens for HPV testing. All data were managed and analyzed in SAS version 9.2 for Windows (SAS Institute Inc., Cary, NC, USA). Frequency distributions of HPV infection and HPV types were calculated for the entire sample and then stratified according cytology result (normal versus abnormal [ASC-US+]). We also estimated HPV prevalence by age group (18–30; 31–40; 41–50; 51–60; >60 years) according to risk class (HPV 16/18, other HR, and LR types). To determine factors associated with prevalent HR HPV infection, we calculated odds ratios (ORs) and 95% confidence intervals with unconditional logistic regression models (univariate and adjusted for age). The reference group consisted of women with LR or no HPV. Women with multiple infections were included in the HR HPV group if at least one infection was with a HR HPV type.

3. Results

The vast majority of women (85.1%) were over 30 years of age (Table 1). Most participants (97.1%) were not born in the United States, but 74% had been in the USA for more than 5 years. While 49.6% reported a regular place for receiving healthcare, only 14.9% had health insurance. A significant proportion of women (79.3%) reported having had a Pap smear in their lifetime and 60.3% had been screened within the past 3 years. Approximately half (52.5%) of the sample were married or cohabitating, were employed part or full-time (55.8%), and reported more than 3 pregnancies in their lifetime (49.6%). Inflammation was present in cytological specimens for 45.9% of women, and the prevalence of vaginal infections with candida, gardnerella, or trichomoniasis was 33.1% (data not shown).

Table 2 reports the overall rates of HPV infection and prevalence by type among participants. Of the 50 women infected with HPV (20.7%), 8 were infected with multiple HPV types (3.3% of all women, 16% of HPV-positive women). HR HPV infections (13.2%) were considerably more common than LR infections (7.4%) among all women. The most prevalent HPV types, detected as either single or multiple infections, were HPV 53, 82, and 61 (each detected in 5 women). HPV 16 or 18 were detected in 5 specimens. Two samples were HPV positive but could not be genotyped and were subsequently classified as LR.

Overall, 21 women (8.7%) had abnormal cytology results, including 14 with atypical cells of undetermined significance (ASC-US), 1 with atypical glandular cells not otherwise specified (AGC-NOS), 4 with low-grade intraepithelial lesions (LSIL), and 2 with high-grade intraepithelial lesions (Table 3). Of women with abnormal cytology results, 16/21 (76.1%) had detectable HPV; of women with normal cytology results, 34/221 were HPV-positive (15.3%). The most common HPV types among women with abnormal cytology were HPV 82, 35, and 61 (each found in 2 women). The two women with HSIL were infected with HPV 35 and 82. There was no association between cytology result and risk class of HPV ($P = 0.88$).

Figure 2 shows the prevalence of HPV (by HPV 16 or 18, other HR HPV, and LR types) across age strata. The age-specific prevalence of HPV decreased from 44.5% (95% CI: 29.360.7%) in those aged 18–30 to 15.2% (95% CI: 8.4–25.9%) among women 31–40, 16.4% (95% CI: 9.9–25.6%) in women 41–50, and 12.9% (95% CI: 4.9–29.7%) in 51–60 year olds. Prevalence increased to 26.1% (95% CI: 12.2–47.2%) among women aged 61 years and over. Similarly, the prevalence of HR HPV infections was highest in 18–30 year olds (38.9%), falling to 9.1%, 8.2%, and 3.2% in women aged 31–40 years, 41–50 years, and 51–60 years, respectively, and

Table 1: Characteristics of 242 cervical self-sampling participants.

		N	%
Age	18–30	36	14.9
	31–40	66	27.3
	41–50	86	35.5
	51–60	31	12.8
	>60	22	9.1
Education	< High school	119	49.2
	High school	49	20.3
	> High school	74	30.6
Years in USA	<5	63	26.0
	6–10	82	33.9
	>10	97	40.1
Marital Status	Never been married	71	29.3
	Married/living with partner	127	52.5
	Divorced/widowed/separated	44	18.2
	Employed	134	55.8
	Unemployed	95	39.6
	Homemaker/Student/Other	11	4.6
Income <15 K		121	50.0
Have health insurance		36	14.9
Have a regular place for healthcare		120	49.6
Read/Speak Creole Only		98	40.7
		Mean	SD
Number of pregnancies		3.8	2.6
Age at first pregnancy		22.9	5.1

then increasing to 17.4% among women 61 years and over. By contrast, the prevalence of LR infections was lowest in 18– 30 year olds (5.6%) and highest in older women (9.7% in 51–60 year olds). HPV 16 and 18 were found exclusively among women between 18 and 40 years, with 5 out of 6 infections occurring in women aged 30 and under. HR HPV infections outnumbered LR infections in the youngest and oldest age groups, while LR infections were at least as prevalent as HR infections in women between 41 and 60 years.

Table 4 shows the crude and age-adjusted ORs from logistic regression models for prevalent infection with HR HPV and the major characteristics of women in the study. In univariate analyses, younger age, never being married, being born outside the USA, never having been pregnant, and ever being exposed to tobacco were significantly associated with HR HPV infection. However, when we adjusted for age group, only ever exposure to tobacco smoke at home remained significantly associated with HR HPV infection (OR = 4.05, 95% CI: 1.2–12.8; $P < .01$). There was a low prevalence of concomitant sexually transmitted infections (STIs) in our sample including 13 cases of Chlamydia and 1 case of Gonorrhea. There was no significant association between STI infection and any HPV infection, HR HPV infection, or abnormal cytology. There were no significant associations between HR HPV positivity and vaginal infection.

4. Discussion

These data are the first to estimate the prevalence of HPV and distribution of infections by type and age in Haitian immigrants living in the USA. The crude prevalence of any HPV (20.7%) and HR HPV (13.2%) in this sample was slightly lower than estimates from a representative sample of USA women aged 15–49 years who provided self-collected HPV specimens (any HPV: 26.8%; HR HPV: 15.2%) [18]. Our estimates of any or HR HPV prevalence, both overall and for ages 60 and under, tended to be slightly lower than those reported by other USA-based studies [19–22]. Differences in study populations, statistical uncertainty, and HPV sampling and detection methods likely account for subtle differences.

We found that HR HPV types 53, 82, 52, and 68 and LR HPV types 61 and 62 were the most prevalent in the study population. This type distribution contrasts with findings from a meta-analysis of other USA-based studies, which found HPV 16, 52, 18, 51, and 58 to be the most prevalent types among women with normal cytology [23]. While the prevalence of HPV 16 tends to increase with the degree of cytologic abnormality (both in the USA and worldwide) [24], we found a relatively low prevalence of HPV 16 (1.2%), and none of these infections were associated with abnormal cytology. Cancer registry data aggregates individuals by broad race-based classifications and

Table 2: Cytology results and type-specific HPV prevalence among 242 residents of Little Haiti.

Cytology results		Normal	Abnormal*
		n (% of row total)	
Total		221 (91.3%)	21 (8.7 %)
HPV −		187 (97.4%)	5 (2.6%)
HPV +		34 (68%)	16 (32%)
HR HPV +		22 (69%)	10 (31%)
LR HPV +/ Unknown		12 (67%)	6 (33%)
HR infections	Type	No. of infected women	
	53	4	0
	82	1	2
	52	3	0
	68	2	1
	16	2	0
	35	1	2
	18	1	1
	33	2	0
	45	2	0
	66	0	1
	31	0	1
LR infections			
	61	2	2
	62	0	0
	83	2	1
	CP108	1	0
	11	1	1
	72	1	0
	84	1	0
	Unknown	2	0
	42	0	1
	44	0	1
Multiple HR infections		4	2
	16 & 84	1	0
	52 & 53	0	1
	62 & 66	1	0
	62 & 68	1	0
	82 & CP108	1	1
Multiple LR infections		2	0
	62 & 61	1	0
	62 & 72	1	0

*Abnormal cytology includes the following diagnoses: ASC-US ($n = 14$), AGS-NOS ($n = 1$), LSIL ($n = 4$), and HSIL (CIS, CIN2, and moderate dysplasia, $n = 2$).

Table 3: Distribution of HR HPV¥ types and cytology results.

Total HR HPV positive women	32 (13.2%)		
Type	No. of infected women *n* (%)	Abnormal ($n = 10$)	Normal ($n = 22$)
16	2 (5.2)	0	2
18	2 (5.2)	1	1
31	1 (2.6)	1	0
33	2 (5.2)	0	2
35	3 (7.9)	2	1
45	2 (5.2)	0	2
52	3 (7.9)	0	3
53	4 (10.5)	0	4
66	1 (2.6)	1	0
68	3 (7.9)	1	2
82	3 (7.9)	2	1
HR coinfections*	6 (15.8)	2	4
			n/total (%)
Differential cytology results	ASC-US	6	6/14 (42.8)
among HR-HPV positive	LSIL	1	1/4 (25)
women	HSIL	1	1/2 (50)

¥ HPV: human papillomavirus, HR: high risk (oncogenic HPV type).
*Coinfections by types 16 & 84, 52 & 53, 62 & 66, 62 & 68, ($n = 1$ for each listed) and CP6108 & 82 ($n = 2$).

ethnicity data is often lacking and incomplete. Therefore, the HPV type distribution among cervical cancer cases in the Haitian immigrant population of Miami is unknown. The distribution of HPV types among women in Little Haiti bares similarity to study results from Dunne and colleagues [18], which also found HPV 53 to be the most prevalent HR HPV type in a representative USA sample, followed by HPV 52; HPV 62 was the most prevalent LR type. While we used a different self-sampling device than Dunne et al. and the Fournier device used in our study was designed to improve collection of cervical cells while minimizing sampling from the vagina, it is possible that the similar respective type distributions are an artifact of self-sampling. However, a study of paired vaginal and cervical specimens did not find significant differences in prevalence of HR HPV types (including the most prevalent ones in our study) based on sampling location; differences in HPV type distribution between cervical and vaginal specimens are more likely to arise among LR types in phylogenetic groups $\alpha 3$ (which includes HPV 61 and 62) and $\alpha 15$ [25]. Thus, the relatively high prevalence of HR HPV 53, 82, and 68 among our study population relative to meta-analysis of other USA studies would not appear to be a function of inadvertent sampling of vaginal cells.

Data on the type distribution of HPV in Haiti is currently unavailable [26], although we are collecting in-country data that will allow for estimation of HPV burden and comparison between Haitian immigrants in the U.S. and women living in Haiti. Elsewhere in the Caribbean, overall HPV prevalence is higher than in our study. HPV 45 appears to be the most common HR type in Jamaica and Tobago [27, 28], while HPV 52 is the most prevalent HR type in Trinidad [29]. Geographical differences in the relative prevalence of HPV types—among Caribbean countries and the United States—may be influenced by an interaction between HPV types and host immunogenetic profile (for instance, human leukocyte antigen [HLA] polymorphisms) [24, 30]. HPV 16, the most oncogenic HPV type, appears to evade immune surveillance more effectively than other types [31]. Impairment of cellular

Table 4: Crude and age-adjusted odds ratio estimates of HR HPV infection to negative and LR HPV infection ($n = 242$).

Age		%	Crude OR¥ (95% CI)	*P* value	Age-adjusted *OR (95% CI)	*P* value
	18–30	14.9	1			
	31–40	27.3	**0.16 (0.05–0.51)**		—	—
	41–50	35.5	**0.14 (0.04–0.43)**		—	—
	51–60	12.8	**0.05 (0.01–0.41)**		—	—
	>60	9.1	0.34 (0.07–1.32)	<.001	—	—
Educational attainment						
	< High school graduate	49.2	1		1	
	=> High school graduate	50.8	1.5 (0.70–3.17)	0.3	1.04 (0.38–2.88)	1
Marital status						
	Never married	29.3	1		1	
	Married/cohabitating	52.5	**0.39 (0.18–0.86)**		0.63 (0.24–1.65)	
	Divorced/Widowed/Separated	18.2	**0.07 (0.01–0.58)**	<.01	0.15 (0.01–1.15)	0.12
Place of birth						
	US born	2.9	1		1	
	Born abroad	97.1	**5.33 (1.14–25.0)**	<.001	1.22 (0.15–8.8)	1
Years in the United States						
	<5 years	26.0	1		1	
	5–10 yrs	33.9	0.73 (0.30–1.82)		1.02 (0.35–3.07)	
	>10 years	40.1	0.54 (0.22–1.37)	0.43	1.05 (0.33–3.36)	1
Employment status ($n = 240$)						
	Unemployed	39.6	1		1	
	Employed part or full time	55.8	1.19 (0.53–2.64)		1.27 (0.50–3.35)	
	Homemaker/Student/Other	4.6	2.86 (0.66–12.4)	0.37	1.19 (0.16–7.01)	0.90
Menopause status						
	Premenopausal	68.6	1		1	
	Postmenopausal	31.4	2.17 (0.85–5.51)	0.08	2.68 (0.47–29.8)	0.31
Number of pregnancies						
	None	9.5	1		1	
	1–3	40.9	**0.24 (0.09–0.65)**		0.34 (0.1–1.19)	
	>3	49.6	**0.14 (0.05–0.41)**	<.01	0.29 (0.07–1.26)	0.10
Age at first pregnancy ($n = 215$)						
	Under 18	12.1	1		1	
	18–25	51.6	1.88 (0.40–8.76)		2.67 (0.51–27.5)	
	26–41	36.3	1.0 (0.19–5.3)	0.38	1.61 (0.24–19)	0.33
Ever tobacco use						
	Nonsmoker	86.8	1		1	
	Former or current smoker	13.2	0.93 (0.30–2.85)	0.90	0.71 (0.16–2.5)	0.78
Ever exposed to tobacco smoke at home						
	Nonexposed		1		1	
	Exposed	10.3	**3.11 (1.18–8.21)**	0.03	**4.05 (1.2–12.8)**	<.01

Table 4: Continued.

Age		%	Crude OR[¥] (95% CI)	*P* value	Age-adjusted *OR (95% CI)	*P* value
Health insurance ($n = 241$)						
	None	85.1	1		1	
	Yes	14.9	1.07 (0.38–3.0)	0.90	0.76 (0.20–2.43)	0.79
Regular place for healthcare						
	None	50.4	1		1	
	Yes	49.6	1.02 (0.48–2.15)	0.96	1.06 (0.44–2.56)	1
Inflammation						
	Absent	54.1	1			
	Present	45.9	0.78 (0.37–1.67)	0.52	0.96 (0.38–2.41)	1
Any STI**						
	Absent	94.6	1		1	
	Present	5.4	1.21 (0.26–5.7)	0.82	0.68 (0.12–3.69)	0.72
Vaginal infection‡						
	T. Vaginalis	9.5	0.98 (0.28–3.52)	0.98	0.87 (0.14–3.74)	1
	Gardnerella	20.2	1.66 (0.72–3.87)	0.24	1.40 (0.50–3.65)	0.6
	Candida spp.	7.4	0.81 (0.18–3.69)	0.78	0.77 (0.08–4.1)	1
	Multiple Vag. Infection	4.1	0.72 (0.09–5.9)	0.72	0.89 (0.2–7.9)	1
	Any Vag. Infection	33.1	1.46 (0.68–3.13)	0.33	1.18 (0.47–2.85)	0.84

[¥] Likelihood ratio test *P*-value; bolded values indicate a significant difference comparing response to reference level at $P < 0.05$.
*OR (95% CI): Age-adjusted Odds ratio and 95% confidence interval; reported *P*-value from exact method and Score test.
**Includes HIV/AIDS ($n = 0$), Gonorrhea ($n = 1$), and Chlamydia ($n = 13$).
‡Same woman can be counted more than once due to multiple infections; OR is odds HR HPV infection for women with specific vaginal infection compared to those without.

immunity in a population (through immunocompromise or cervical inflammation, for instance) could thus lead to higher relative prevalence of HR types other than HPV 16 [24]. Haitian women routinely practice feminine hygiene with a wide-variety of solutions containing natural and commercial products. The very high frequency of cleansing, 2-3 times daily for most women, may be causing inflammation and disrupting the immune function of cervical cells. We note that the high level of cervical inflammation in our study (45.9%) may contribute to the high prevalence of non-16 HR types.

Age patterns associated with HPV prevalence vary by population. The prevalence of HPV among Haitian women living in Miami was highest in young women (44.5%), decreasing with age until rising to a second minor peak in women over 60 years (26.1%). A recent meta-analysis suggests that HPV prevalence in the USA declines steadily with age [23]. Data from Latin America and Central America, on the other hand, indicate a U-shaped curve, with prevalence declining through middle age but increasing again in older women [32–34]. The high prevalence of HR HPV among women over 60 years in our study population (17.4%) is cause for concern, particularly in light of low screening uptake. Prevalently detected infections in older women tend to represent persistent infections with an elevated risk of progression to cervical intraepithelial neoplasia grade 2 and higher (CIN2+) [35]. Our findings highlight the importance of improving screening coverage among older women in Little Haiti.

We found slightly elevated levels of cytological abnormalities (8.7%) compared to approximately 6% in the general USA population [36]. Still, few studies have examined the validity of cytological results obtained from samples self-collected with the Fournier device [13], so we interpret this finding with caution.

There are several limitations to this study. Our original study's planned sample size (300) was calculated based on the 95% exact confidence interval approach and good precision (i.e., less than 6%). In our study the observed prevalence of HPV for 242 evaluable women is 20.7% with 95% exact confidence interval of 15.7% and 26.3%. Therefore, our study established with high confidence (97.5%) that the true HPV prevalence in the target population is not less than 15.7%. Our study size is sufficiently large to ensure good precision, that is, a 5.3% semiwidth confidence interval, for estimating the HPV prevalence in this population. The sample size was reduced as a result of budgetary restraints and input from key members of the community advisory board. As a part of an ongoing CBPR initiative, it was necessary to balance the achievement of recruitment goals against the logistical constraints imposed by utilizing CHWs, who held roles in both this research study and as employees of various other community-based organizations.

Our sample size was small and when we stratified by age group we lost power to detect significant predictors of HR HPV infection. While age and exposure to tobacco smoke at home were significantly associated with HR HPV infection among our sample, the significance of other predictors was difficult to discern after adjusting for age. Furthermore, this was not a population-based sample, and results may not be fully generalizable to women living in Little Haiti. Nearly 80% of participants in this study reported receiving a Pap smear in the past three years, which is considerably higher than the 44% indicated by our previous work with women in Little Haiti [3]. We attribute the higher utilization of screening in the current study population to the community partners' unwillingness to limit inclusion to women who had never been screened in their lifetime. Participant selection may be a limitation of the study; CBPR relies upon community participation to define the focus of research and identify culturally appropriate recruitment strategies and methods of data collection. Our partnership with the community-based organization, and the affiliated CHWs, was critical to the high participation rate achieved. We encountered few barriers to study implementation and were able to build organizational capacity to support future research and intervention. Importantly, we were also able to ensure that data could be generated by the community and for the community's benefit.

Due to cultural considerations raised by our partner community-based organization, we could only collect data on broad age groups, and were limited in our ability to collect data on sexual behavior. Both age and sexual behavior are demonstrated predictors of HPV infection, and our inability to adjust for these in multivariate analysis is a limitation. We also rely on validity assessments of the self-sampling method from other studies, as we were not able to compare self-collected specimens to physician-collected specimens here. We cannot rule out the possibility that women may not have self-sampled correctly.

This cross-sectional study is a preliminary investigation into the prevalence and distribution of HPV types among women in Little Haiti, Miami, Florida. Among participants, the burden of vaccine types 6, 11, 16, and 18 was low, but further study will be needed to determine the prevalence of HPV 16 and 18 in Haitian immigrants with cervical cancer. Although the prevalence of HPV and HR HPV does not appear to be elevated among women in Little Haiti relative to the general USA population, we note the unusually high prevalence of HR HPV among women over 60 years, which may indicate a substantial number of persistent infections at high risk of progression to CIN2+. Underutilization of screening is undoubtedly a contributing factor to the high incidence of cervical cancer, but further study is needed to explain the excess cervical cancer burden in this population relative to others in South Florida. We are presently examining common feminine hygiene practices in this community of Haitian immigrants that may physiologically alter the cervix. Changes induced by exposure to particular compounds may reduce a woman's ability to clear infections, placing women at a greater risk of persistent HR HPV infection.

Self-collection of HPV specimens has been demonstrated to be acceptable and feasible among women in Little Haiti [2], and the present study points to the need to address screening disparities in this population, particularly among older women. We hope that the findings we present here catalyze further research and the movement of resources toward implementation and scale up of screening programs tailored to underserved populations in the United States.

Conflict of Interests

The authors declare no conflict of interest.

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References

[1] FCDS, Florida Cancer Data System, 2007.

[2] L. Barbee, E. Kobetz, J. Menard et al., "Assessing the acceptability of self-sampling for HPV among haitian immigrant women: CBPR in action," *Cancer Causes & Control*, vol. 21, no. 3, pp. 421–431, 2009.

[3] E. Kobetz, J. Menard, J. Diem et al., "Community-based participatory research in Little Haiti: challenges and lessons learned," *Progress in Community Health Partnerships: Research, Education, and Action*, vol. 3, no. 2, pp. 133–137, 2009.

[4] L. Gustafsson, J. Pontén, M. Zack, and H. O. Adami, "International incidence rates of invasive cervical cancer after introduction of cytological screening," *Cancer Causes and Control*, vol. 8, no. 5, pp. 755–763, 1997.

[5] D. Saslow, C. D. Runowicz, D. Solomon et al., "American Cancer Society guideline for the early detection of cervical neoplasia and cancer," *Ca-A Cancer Journal for Clinicians*, vol. 52, no. 6, pp. 342–362, 2002.

[6] G. Saint-Jean and L. A. Crandall, "Sources and barriers to health care coverage for Haitian immigrants in Miami-Dade county, Florida," *Journal of Health Care for the Poor and Underserved*, vol. 16, no. 1, pp. 29–41, 2005.

[7] S. E. Echeverria and O. Carrasquillo, "The roles of citizenship status, acculturation, and health insurance in breast and cervical cancer screening among immigrant women," *Medical Care*, vol. 44, no. 8, pp. 788–792, 2006.

[8] F. X. Bosch, A. Lorincz, N. Muñoz, C. J. Meijer, and K. V. Shah, "The causal relation between human papillomavirus and cervical cancer," *Journal of Clinical Pathology*, vol. 55, no. 4, pp. 244–265, 2002.

[9] J. Menard, E. Kobetz, J. C. Maldonado, B. Barton, J. Blanco, and J. Diem, "Barriers to cervical cancer screening among haitian immigrant women in Little Haiti, Miami," *Journal of Cancer Education*, vol. 25, no. 4, pp. 602–608, 2010.

[10] B. Israel, A. Schulz, E. Parker, A. Becker, A. Allen, and R. Guzman, *Critical Issues in Developing and Following Community Based Research Principles*, Jossey-Bass, San Francisco, Calif, USA, 2003.

[11] M. Minkler and N. Wallerstein, *Community-Based Participatory Research for Health*, Jossey-Bass, San Francisco, Calif, USA, 2003.

[12] K. Edgren, E. Parker, B. Israel et al., "Community involvement in the conduct of a health education intervention and research project: community action against asthma," *Health Promotion Practice*, vol. 6, no. 3, pp. 263–269, 2005.

[13] B. W. Knesel, J. C. Dry, C. Wald-Scott, and A. Aftab, "Preliminary evaluation of a cervical self-sampling device with liquid-based cytology and multiparameter molecular testing," *Journal of Reproductive Medicine*, vol. 50, no. 4, pp. 256–260, 2005.

[14] P. E. Castle, A. Aftab, G. Saint-Jean, and L. Mendez, "Detection of carcinogenic human papillomavirus in specimens collected with a novel self-sampling device," *Journal of Clinical Microbiology*, vol. 44, no. 6, pp. 2158–2159, 2006.

[15] C. Awen, S. Hathway, W. Eddy, R. Voskuil, and C. Janes, "Efficacy of Thinprep® preparation of cervical smears: a 1,000-case, investigator-sponsored study," *Diagnostic Cytopathology*, vol. 11, no. 1, pp. 33–36, 1994.

[16] V. Bouvard, R. Baan, K. Straif et al., "A review of human carcinogens—part B: biological agents," *The Lancet Oncology*, vol. 10, no. 4, pp. 321–322, 2009.

[17] N. Muñoz, F. X. Bosch, S. de Sanjosé et al., "Epidemiologic classification of human papillomavirus types associated with cervical cancer," *New England Journal of Medicine*, vol. 348, no. 6, pp. 518–527, 2003.

[18] E. F. Dunne, E. R. Unger, M. Sternberg et al., "Prevalence of HPV infection among females in the United States," *Journal of the American Medical Association*, vol. 297, no. 8, pp. 813–819, 2007.

[19] A. K. Chaturvedi, J. Dumestre, A. M. Gaffga et al., "Prevalence of human papillomavirus genotypes in women from three clinical settings," *Journal of Medical Virology*, vol. 75, no. 1, pp. 105–113, 2005.

[20] N. Khanna, S. I. Mishra, G. Tian et al., "Human papillomavirus detection in self-collected vaginal specimens and matched clinician-collected cervical specimens," *International Journal of Gynecological Cancer*, vol. 17, no. 3, pp. 615–622, 2007.

[21] S. D. Datta, L. A. Koutsky, S. Ratelle et al., "Human papillomavirus infection and cervical cytology in women screened for cervical cancer in the United States, 2003–2005," *Annals of Internal Medicine*, vol. 148, no. 7, pp. 493–500, 2008.

[22] M. T. Goodman, Y. B. Shvetsov, K. McDuffie et al., "Prevalence, acquisition, and clearance of cervical human papillomavirus infection among women with normal cytology: Hawaii Human Papillomavirus Cohort Study," *Cancer Research*, vol. 68, no. 21, pp. 8813–8824, 2008.

[23] WHO/ICO Information Centre on HPV and Cervical Cancer (HPV Information Centre), "Human Papillomavirus and Related Cancers in the United States of America. Summary Report 2010," http://www.who.int/hpvcentre/, 2011.

[24] G. M. Clifford, S. Gallus, R. Herrero et al., "Worldwide distribution of human papillomavirus types in cytologically normal women in the International Agency for Research on Cancer HPV prevalence surveys: a pooled analysis," *The Lancet*, vol. 366, no. 9490, pp. 991–998, 2005.

[25] P. E. Castle, A. C. Rodriguez, C. Porras et al., "A comparison of cervical and vaginal human papillomavirus," *Sexually Transmitted Diseases*, vol. 34, no. 11, pp. 849–855, 2007.

[26] WHO/ICO Information Centre on HPV and Cervical Cancer (HPV Information Centre), "Human Papillomavirus and Related Cancers in Haiti. Summary Report 2010," http://www.who.int/hpvcentre/, 2011.

[27] C. C. R. Ragin, V. W. Wheeler, J. B. Wilson et al., "Distinct distribution of HPV types among cancer-free Afro-Caribbean women from Tobago," *Biomarkers*, vol. 12, no. 5, pp. 510–522, 2007.

[28] A. Watt, D. Garwood, M. Jackson et al., "High-risk and multiple human papillomavirus (HPV) infections in cancer-free Jamaican women," *Infectious Agents and Cancer*, vol. 4, supplement 1, pp. S1–S11, 2009.

[29] G. M. Andall-Brereton, F. Hosein, R. A. Salas et al., "Human papillomavirus genotypes and their prevalence in a cohort of women in Trinidad," *Revista Panamericana de Salud Pública*, vol. 29, no. 4, pp. 220–226, 2011.

[30] S. S. Wang, C. M. Wheeler, A. Hildesheim et al., "Human leukocyte antigen class I and II alleles and risk of cervical neoplasia: results from a population-based study in Costa Rica," *Journal of Infectious Diseases*, vol. 184, no. 10, pp. 1310–1314, 2001.

[31] H. D. Strickler, J. M. Palefsky, K. V. Shah et al., "Human papillomavirus type 16 and immune status in human immunodeficiency virus-seropositive women," *Journal of the National Cancer Institute*, vol. 95, no. 14, pp. 1062–1071, 2003.

[32] WHO/ICO Information Centre on HPV and Cervical Cancer (HPV Information Centre), "Human Papillomavirus and Related Cancers in the Americas. Summary Report 2010," http://www.who.int/hpvcentre/, 2011.

[33] S. Franceschi, R. Herrero, G. M. Clifford et al., "Variations in the age-specific curves of human papillomavirus prevalence in women worldwide," *International Journal of Cancer*, vol. 119, no. 11, pp. 2677–2684, 2006.

[34] R. Herrero, P. E. Castle, M. Schiffman et al., "Epidemiologic profile of type-specific human papillomavirus infection and cervical neoplasia in Guanacaste, Costa Rica," *Journal of Infectious Diseases*, vol. 191, no. 11, pp. 1796–1807, 2005.

[35] A. C. Rodríguez, M. Schiffman, R. Herrero et al., "Rapid clearance of human papillomavirus and implications for clinical focus on persistent infections," *Journal of the National Cancer Institute*, vol. 100, no. 7, pp. 513–517, 2008.

[36] National Cancer Institute Fact Sheet: pap test, http://www.cancer.gov/cancertopics/factsheet/detection/Pap-test, 2011.

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Review Article

Role of Protein Biomarkers in the Detection of High-Grade Disease in Cervical Cancer Screening Programs

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Since the Pap test was introduced in the 1940s, there has been an approximately 70% reduction in the incidence of squamous cell cervical cancers in many developed countries by the application of organized and opportunistic screening programs. The efficacy of the Pap test, however, is hampered by high interobserver variability and high false-negative and false-positive rates. The use of biomarkers has demonstrated the ability to overcome these issues, leading to improved positive predictive value of cervical screening results. In addition, the introduction of HPV primary screening programs will necessitate the use of a follow-up test with high specificity to triage the high number of HPV-positive tests. This paper will focus on protein biomarkers currently available for use in cervical cancer screening, which appear to improve the detection of women at greatest risk for developing cervical cancer, including Ki-67, $p16^{INK4a}$, BD ProEx C, and Cytoactiv HPV L1.

1. Introduction

Cervical cancer is the second most common cancer in women worldwide and remains a major cause of morbidity and mortality. Since the Pap test was introduced in the 1940s, there has been an approximately 70% reduction in the incidence of squamous cell cervical cancers in many developed countries by the application of organized and opportunistic screening programs. The efficacy of the Pap test, however, is hampered by high interobserver variability and high false-negative and false-positive rates [1–3]. However, as cervical cancer evolves through well-defined noninvasive intraepithelial stages, which can be distinguished morphologically, repeated screening at frequent intervals can maintain high levels of protection.

Investigators have attempted by various means to enhance the sensitivity of the Pap test. First by the introduction of liquid-based methods to address issues of specimen collection and preparation, and later by the use of computer-assisted screening systems to address screening errors and to improve screening efficiency and disease detection. Testing for oncogenic, high-risk human papillomavirus (hrHPV) DNA has been accepted as an adjunct to borderline/ASC-US cytology in primary screening. The utility of HPV as a reflex test within the ASC-US patient population is largely reflected in its negative predictive value (NPV), where an HPV negative result indicates a low likelihood of finding CIN2+ lesions upon colposcopy [4]. This triage application, however, has a very low specificity and correspondingly low positive predictive value for finding CIN2+ disease. Another testing algorithm involving cytology and HPV cotesting was reported to lead to earlier detection of CIN3+ lesions, supporting a lengthening of the screening interval [5]. In this cotesting algorithm, the combined use of cytology plus HPV testing has a very high NPV. As such, it is useful to screen a presumed normal and healthy population for

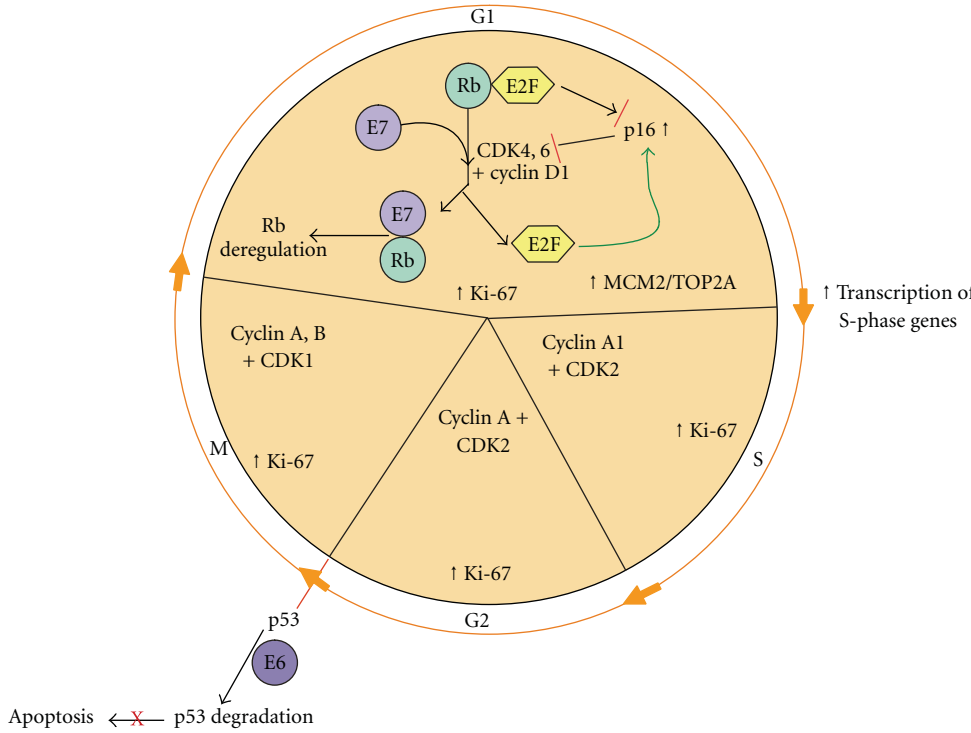

Figure 1: Cell cycle alterations induced by HPV E6 and E7 oncogenes in cervical neoplasia, adapted from Malinowski [11]. The presence of E7 disrupts G1-S phase regulation through the interference with E2F-Rb binding. P16INK40 is strongly expressed due to the loss of Rb/E2F repression and the strong activation by free E2F. The release of E2F results in the increased transcription of S-phase genes, including MCM2 and TOP2A. The interaction of E6 with p53 results in p53 ubiquitination and subsequent degradation, resulting in the abrogation of apoptosis. The proliferation marker Ki-67 is increased in the presence of cell cycle dysregulation caused by the E6 and E7 oncogenes.

the presence of CIN2+ disease and to stratify patients into two groups: (i) HPV negative, cytology normal patients who are at low risk for developing CIN2+ disease and thus qualify for an extended screening interval and (ii) patients who are either cytology abnormal or HPV positive and who are at increased risk for developing CIN2+ and require more active surveillance. Most recently, the use of hrHPV DNA detection as a primary screening test for cervical disease has been investigated [6–10]. The majority of hrHPV infections, however, induce low-grade precursor lesions, which are cleared spontaneously within one to two years of exposure, with less than 10% eventually progressing to high-grade lesions or invasive cancer. Thus, while hrHPV DNA testing has a very high sensitivity for the detection of high-grade cervical disease, it has a very low specificity and positive predictive value, thus would always require a follow-up test prior to treatment to avoid unnecessarily raising patient anxiety levels and referrals to invasive colposcopic procedures.

The use of biomarkers in both cervical cytology and histology has demonstrated the ability to overcome issues with both false-positive and false-negative results, leading to improved positive predictive value of cervical screening results. Numerous protein biomarkers for the detection of cervical disease have been identified. Many of these proteins are involved in cell cycle regulation, signal transduction, DNA replication, and cellular proliferation (reviewed in [11–13]). The altered expression of these proteins is a consequence of the binding of the high-risk HPV E6 and E7 oncogenes to host regulatory proteins, resulting in the degradation of the p53 tumor suppressor gene product and the inactivation of the retinoblastoma protein leading to dysregulation of the cell cycle (Figure 1).

In contrast to the cellular markers, the HPV L1 capsid protein is a virus-specific protein and a major stimulus of the immune system used within the HPV vaccines.

This paper will focus on protein biomarkers currently under investigation for use in cervical cancer screening that appear to improve the detection of women at greatest risk for developing cervical cancer, including Ki-67, $p16^{INK4a}$, BD ProEx C, and Cytoactiv HPV L1. These biomarkers are reported to have a role in the triage of indeterminate cytology cases, discrimination of true high-grade cervical dysplasia from mimics in histology, and may serve as predictive markers to identify lesions most likely to progress to high-grade cervical disease and cancer. The staining characteristics and reported usage of the various biomarkers in cervical cancer screening are summarized in Table 1.

2. Biomarkers Used in Cervical Screening

2.1. Ki-67. Ki-67 is a nuclear and nucleolar protein expressed during the G1, S, G2, and M phase of the cell cycle, while not being present in resting cells (G0 phase), and can, therefore,

Table 1: Biomarkers used in cervical cancer screening and diagnosis.

Biomarker	Staining *Pattern*	Cellular process detected	Reported use of biomarker
Ki-67	Nuclear	Increased Ki-67 staining reflects increased epithelial cell proliferation found in HPV-infected tissues.	(i) Measure of cell proliferative capacity. (ii) Recognizes tissues involved by HPV and extent of Ki-67 immunostaining generally parallels increasing grades of dysplasia. (iii) Predominantly used in histology applications.
$p16^{INK4a}$	Nuclear and cytoplasmic	p16 levels increased in response to irregular cell cycle inactivation resulting from the disruption of interaction of pRb with transcription factor E2F in the presence by the HPV E7 oncogene.	(i) Detection can serve as a surrogate biomarker for persistent infection with high-risk HPV. (ii) Triage of equivocal cytology findings can facilitate identification of abnormal cells in cytology preparations. (iii) Aid in interpretation of histological material. Limited evidence for use as a predictor of disease progression in histology specimens.
BD ProEx C	Nuclear	Increased cellular levels of MCM2 and TOP2A due to aberrant transcription of S-phase proteins resulting from the interaction of HPV E6 and E7 oncoproteins with cell cycle proteins p53 and Rb.	(i) Marker of cells with proliferative capacity. (ii) Triage from abnormal cytology to increase PPV over cytology alone or HPV triage for detection of CIN2+ disease. Can also facilitate identification of abnormal cells in cytology preparations. (iii) Use in histology to distinguish true dysplasia from mimics such as reactive/reparative changes, immature squamous metaplasia, and atrophy.
Cytoactiv HPV L1	Nuclear	HPV L1 capsid protein found in mild-to-moderate dysplasias, but lost in higher-grade intraepithelial neoplasias.	(i) Possible prognostic marker to identify early dysplastic lesions most likely to progress to high-grade disease.

provide an index of the cell growth fraction. While the function of the Ki-67 protein remains unclear, its expression appears to be an absolute requirement for progression through the cell-division cycle [14, 15]. Since HPV infection leads to increased epithelial cell proliferation in infected tissues, increased Ki-67 staining can be an indicator of HPV infection. In normal human cervical squamous mucosa, expression of Ki-67 is limited to the proliferating basal and parabasal cells. In dysplasia and carcinoma, however, expression extends above the basal one third of the epithelium

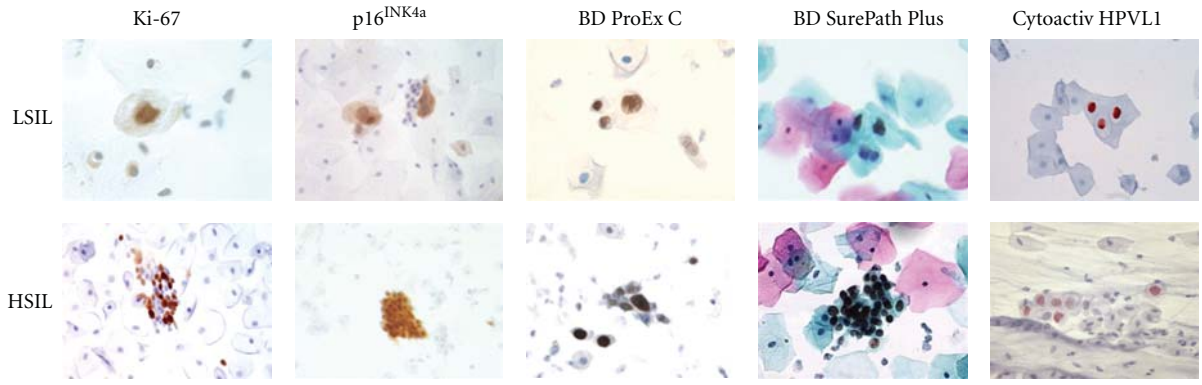

Figure 2: Biomarker expression in low-grade squamous intraepithelial lesions and high-grade squamous intraepithelial lesions in cervical cytology specimens. Ki-67, p16^{INK4a}, BD ProEx C, and BD SurePath Plus expression detected in liquid-based cytology samples and Cytoactiv HPV L1 staining performed on conventional Pap smears (L1 images courtesy of Dr. Ralf Hilfrich).

and the number of positive cells increase, with a significant positive correlation between ascending grade of squamous intraepithelial lesion and labeling index (Figure 3) [16]. The most commonly used antibody for immunohistochemical detection of the Ki-67 antigen is clone MIB-1.

2.2. p16^{INK4a}. The protein p16^{INK4a} is a cell-cycle regulator, with its expression tightly controlled in normal cells. This tumor suppressor protein inhibits cycle-dependant kinases 4 and 6, which phosphorylate the retinoblastoma (Rb) protein [17, 18]. Usually, binding of Rb to E2F blocks E2F-driven cell cycle activation and entry into the S-phase of the cell cycle. In a transforming HPV infection, however, the viral oncogene E7 disrupts the binding of the Rb protein to the E2F transcription factor, resulting in drastically increased levels of p16^{INK4a} [19, 20], the detection of which can serve as a surrogate biomarker for persistent infection with high-risk HPV (Figure 1). It is widely accepted that p16^{INK4a} is a sensitive and specific marker of dysplastic cells of the cervix and is a useful biomarker in cervical cancer lesion diagnosis and cervical screening [21–25]. Multiple antibodies to p16 have been utilized in research studies; however, the E6H4 clone (CINtec, mtm laboratories AG, Heidelberg, Germany) appears to be most commonly used. A dual p16/Ki-67 immunocytochemistry assay is now also available for use as an adjunctive test in cervical cancer screening (CINtec Plus, mtm laboratories AG, Heidelberg, Germany).

2.3. BD ProEx C. BD ProEx C is a protein-based biomarker reagent (BD Diagnostics, Burlington, NC, USA) containing antibodies to the nuclear proteins minichromosome maintenance protein 2 (MCM 2) and topoisomerase II alpha (TOP2A), proteins that have been shown to accumulate in HPV-transformed cells. BD ProEx C staining is limited to the basal proliferating layer of normal cervical epithelium and is absent in differentiated and quiescent cells. In contrast, in cervical glandular and squamous dysplasia, BD ProEx C expression is dramatically increased, due to the increased transcription of S-phase genes (aberrant S-phase induction) resulting from the action of the oncogenic HPV E7 protein.

The minichromosome maintenance (MCM) proteins function in the early stages of DNA replication through loading of the prereplication complex onto DNA and functioning as a helicase to help unwind the duplex DNA during de novo synthesis of the duplicate DNA strand [26, 27]. Origin licensing, which occurs before S phase in late mitosis and early G1, involves the stable loading of the minichromosome maintenance (MCM) complex comprising six replication proteins—MCM2, MCM3, MCM4, MCM5, MCM6, and MCM7 (termed MCM2-7)—onto DNA at replication origins. Expression of all six MCMs is seen throughout all phases of the cell cycle and is downregulated following exit from the cell cycle into quiescence, differentiation, or senescence, thus they are a unique marker of cells with proliferative capacity. Deregulation of MCM2-7 appears to be an early event in multistep tumorigenesis, and many studies have now shown that there is inappropriate expression of MCM2-7 in a wide variety of premalignant dysplasias and cancers [28–31].

Similar to the MCM2-7 markers, topoisomerase II-α (TOP2A) has been shown to be overexpressed in cervical neoplasia at both the mRNA and protein levels [32–35]. TOP2A is a nuclear enzyme that is responsible for relaxing supercoiled DNA during DNA replication and during chromosome condensation and mitosis and is required for the segregation of daughter chromosomes at the end of replication. Evaluation of TOP2A expression has shown that TOP2A overexpression is associated with the progression from CIN2 to advanced cervical neoplasia [32].

The BD ProEx C test was designed as a reflex test to identify CIN2+ disease in women with ASC-US and LSIL cytology results. The next generation test designed for primary screening applications, BD SurePath Plus, combines on one slide traditional Pap staining for cellular morphology, in combination with immunocytochemical detection of the overexpression of two biomarkers: MCM2 and MCM7 (Figure 2).

2.4. Cytoactiv HPV L1 Capsid Protein. The Cytoactiv Screening antibody detects the L1 capsid protein of all known HPV

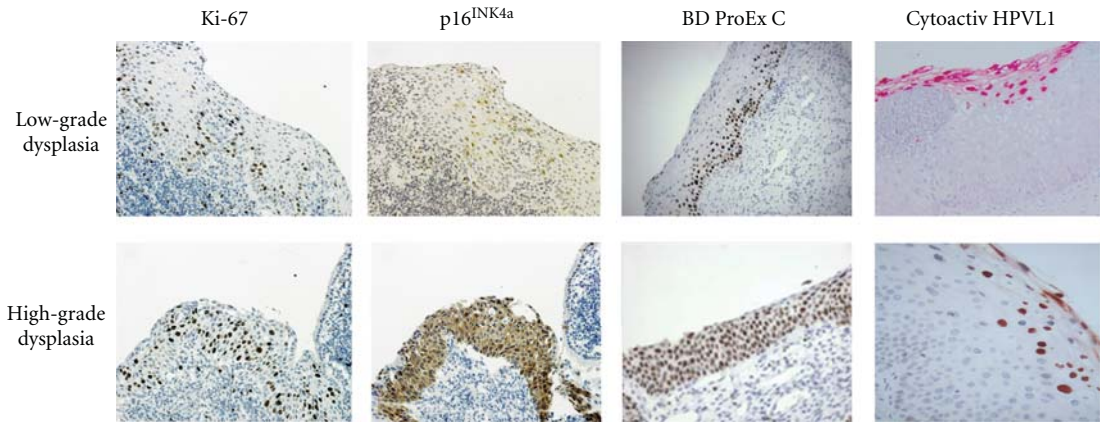

Figure 3: Biomarker expression in cervical intraepithelial neoplasia. The proliferative compartment progressively expands with histological grade, and this is paralleled by the appearance of immunostaining for Ki-67, $p16^{INK4a}$, and BD ProEx C in more superficial epithelial layers, with the majority of the epithelium staining in high-grade squamous intraepithelial lesions. For Cytoactiv HPV L1, higher expression is found in low-grade lesions, with a loss of L1 protein often observed in high-grade lesions (L1 images courtesy of Dr. Ralf Hilfrich).

types. Together with the L2 protein, the L1 capsid protein forms a protective cover for the viral genetic material. In addition, it is a ligand for a surface receptor of the host cell in the basal/parabasal cell layer of the epithelium, gaining access to the basal epithelial layer as a result of epithelial erosions or mucosal ulcerations in the transformation zone susceptible to inflammation at the cervical/endocervical junction. HPV L1 expression is found in the early, productive phase of HPV infection but is progressively lost during cervical carcinogenesis. This loss of L1 expression may result from the integration of viral DNA into the human genome, which may disrupt the L1 gene or cause loss of L1 expression by segregating the viral promoter from the L1 gene, or may reflect an abnormality in transcription factor pathways or in control of L1 protein translation [36, 37].

As the L1 capsid protein is one of main targets for T cell-mediated immune response, cells with a lack of L1 protein synthesis may escape immune system recognition, allowing disease progression. Promising data using the Cytoactiv test (Cytoimmun, Pirmasens, Germany) supports this notion, with progressive disease more frequently detected in L1-negative intraepithelial lesions [38–41].

3. Role of Biomarkers to Improve Cervical Cancer Screening

The use of biomarkers such as Ki-67, $p16^{INK4a}$, and BD ProEx C has been reported to facilitate the detection of abnormal cells within a Pap cytology sample based upon simple immunocytochemistry assay formats (Figure 2). The published reports on the use of these biomarkers in Pap cytology samples have indicated their ability to triage mildly abnormal and indeterminate cytology cases, with those found to have increased levels of biomarkers staining more likely to represent cases with true high-grade cervical disease. In addition, the biomarkers can be utilized to highlight potentially abnormal cells on a background of normal, reactive or other nonmalignant cells, through colorimetric staining, directing the attention of slide screeners to cells of interest.

The utility of Ki-67 immunocytochemistry has both been shown in conventional Pap smears [42–44] and liquid-based cervical cytology [45, 46]. In patients with ASC-US and LSIL, Ki-67 immunocytochemistry demonstrated 96% sensitivity, 67% specificity, 49% PPV, and 98% NPV for detection of high-grade CIN [42]. Sahebali et al. [45] reported that receiver operating characteristic curves indicated a test accuracy (AUC) of 0.68, 0.72, and 0.86 for ASC-US, LSIL, and HSIL, respectively.

A recent meta-analysis analyzed 27 studies evaluating the use of $p16^{INK4a}$ immunostaining in cytological specimens from the uterine cervix [24]. The proportion of cervical smears overexpressing $p16^{INK4a}$ increased with the severity of cytological abnormality, with 12% of normal smears positive for the biomarker compared to 45% of ASC-US and LSIL, and 89% of HSIL smears. In order to improve the specificity of p16 cytology, an interpretation algorithm was developed which incorporates components of both staining and nuclear score in order to facilitate the assessment of the biomarker [47]. In an ASC-US/LSIL triage study, this scoring system resulted in 95% sensitivity and 84% specificity for ASC-US and 100% sensitivity and 82% specificity in LSIL for the detection of biopsy-proven high-grade CIN [48].

In a subset of patients from the Technologies for Cervical Cancer screening (NTCC) randomized controlled trial in Italy, the performance of p16 triage of HPV primary screen-positive women was examined [49]. Sensitivity and specificity for CIN2+ and CIN3+ of p16 immunostaining was 88% (95% CI 80–94) and 61% (633/1045; 57–64), respectively, with CIN2+ as the endpoint, and 61% (95% CI 77–97) and 59% (95% CI 55–63) for the CIN3+ endpoint, respectively. This screening algorithm was reported to produce a significant increase in sensitivity compared with

conventional cytology, with no substantial increase in referral to colposcopy [39].

The CINTec Plus biomarker cocktail, composed of antibodies against $p16^{INK4a}$ and Ki-67, has been evaluated as a reflex test from borderline cytology results and in cytology negative, high-risk HPV positive cases [50–52]. In the European Equivocal or Mildly Abnormal Pap Cytology Study (EEMAPS), the sensitivity of the dual stain cytology for biopsy-confirmed CIN2+ was 92.2% for ASC-US cases and 94.2% for LSIL, with specificity of 80.6% and 68.0%, respectively [50]. As a triage for HPV-positive, cytology-negative cases in women ≥ 30, Petry et. al. [52] observed a 91.9% sensitivity and 82.1% specificity for CIN2+ on biopsy.

The performance of BD ProEx C in the detection of CIN2+ disease in ASC-US+ BD SurePath liquid-based cytology specimens has been reported in five studies, with sensitivity, specificity, PPV, and NPV for detection of CIN2+ disease ranging from 56.5–98%, 72-97.6%, 27.4–97%, and 95–99.3%, respectively [53–57]. A few studies have directly compared the performance of $p16^{INK4a}$ [21, 23, 58] and BD ProEx C [55, 56] immunocytochemistry to HPV DNA detection by Hybrid Capture 2 (Qiagen, Venlo, The Netherlands) for the detection of CIN2+ disease. The PPV of both biomarkers was found to be superior to HC2 in these studies, which analyzed ASC-US, LSIL, and ASC-H liquid-based cytology specimens. Depuydt et al. [57] evaluated the efficacy of eight cervical cancer screening strategies relative to cytology with emphasis on the use of the BD ProEx C biomarker as a tool of triage following primary cytology or hrHPV testing. In the context of a reflex application from a high-risk HPV primary screen to identify CIN2+ disease, BD ProEx C was found to increase the specificity (98.3% versus 85.0%) and PPV (41.7% versus 9.3%) of screening compared to hrHPV alone, resulting in an 82% decrease in colposcopy procedures.

4. Improvement in the Histological Classification of Cervical Biopsies

While the cytology-based Pap test is used as the screening test for cervical cancer, histopathological evaluation of a cervical biopsy from a woman with an abnormal Pap test is the "gold standard" for the diagnosis of cervical neoplasia. However, diagnosis variability has been documented among observers and depends, in part, on the grade of the abnormality [59]. Previous studies have demonstrated that the histologic detection and grading of HPV-induced CIN, especially the low-grade categories such as atypical squamous metaplasia, HPV koilocytosis, and CIN1, have poor reproducibility and are limited by interobserver variability [60, 61]. As reactive/reparative epithelial changes, immature squamous metaplasia and atrophy are well-recognized mimics of high-grade disease and frequently cause problems in histological interpretation, there is a need in pathology practice for a biomarker reagent that will help in distinguishing true dysplasia from dysplasia mimics. The ability to make this distinction will ensure that invasive procedures, such as LEEP and cone biopsy, which can result in pregnancy complications in future pregnancies, are only performed on women with true high-grade cervical disease.

It has been shown that Ki-67, $p16^{INK4a}$, and BD ProEx C immunostains are helpful in the assessment of cervical biopsies by decreasing interreader variability, assisting in the discrimination of true dysplasia from mimics, and identifying regions of focal disease that can be missed with H & E staining alone. Representative staining patterns of the biomarkers in cervical dysplasia are shown in Figure 3. While a nuclear staining pattern is observed with the Ki-67 and BD ProEx C biomarkers, the $p16^{INK4a}$ staining pattern is more variable, with staining found in the nucleus, cytoplasm, or both, the significance of which is not fully understood at the present time.

The performance of Ki-67 [16, 42, 67], $p16^{INK4a}$ (reviewed in [24]), and BD ProEx C [62–64, 66, 68–72] in histological applications has been evaluated in many studies. The comparison of biomarker performance in the detection of high-grade cervical disease is difficult due to the variability in specimens, study design, antibodies, and scoring algorithms utilized. A limited number of studies, however, have analyzed the performance of Ki-67, $p16^{INK4a}$, and BD ProEx C immunohistochemistry on the same sample sets, allowing a preliminary comparison to be made, although scoring algorithms are inconsistent [62–66] (Table 2). The performance of the three biomarkers was fairly comparable, with BD ProEx C and $p16^{INK4a}$ tending to have better diagnostic value than Ki-67. Two studies suggest that BD ProEx C has improved efficacy in the detection of low-grade lesions [62, 63]. Of the three studies using histological tissue [62–64], each concludes that the BD ProEx C/$p16^{INK4a}$ biomarker combination appears to have the best overall performance for triaging diagnostically difficult atypias in which the differential diagnosis includes HSIL, and for the detection of clinically relevant disease compared with the single biomarkers or other biomarker combinations.

Negri et al. [68] evaluated the utility of $p16^{INK4a}$, ProEx C, and Ki-67 for the diagnosis of endocervical adenocarcinoma and its precursors. $p16^{INK4a}$ was at least focally expressed in 93% (14/15) of invasive adenocarcinomas, 100% of AIS (29/29), and 32% (7/22) negative samples. ProEx C and Ki-67 both scored positive in all adenocarcinomas (15/15) and AIS (29/29), and in 36% (8/22) and 27% (6/22) of negative samples, respectively. p16 and Ki-67 individually stained positive in 94% (15/16) of glandular dysplasia cases, with 87.5% positivity (14/16) detected with ProEx C. The score differences between neoplastic and nonneoplastic samples were highly significant for each marker ($P < 0.001$), and each biomarker was shown to be useful for the diagnosis of neoplastic lesions of the glandular epithelial of the cervix uteri.

In a study examining cell block preparations [65], BD ProEx C was found to have a higher PPV for high-grade dysplasia/carcinoma (89%) than p16 (50%), with a NPV value of 93% for ProEx C compared with 100% for $p16^{INK4a}$. The study concluded that BD ProEx C had a better overall performance in differentiating NILM versus HSIL/SCC compared with $p16^{INK4a}$ with the added benefit of having clean nuclear staining.

Table 2: Biomarker positivity by histological grade of cervical intraepithelial lesion.

Study	*Biomarker*	*WNL*	*CIN1*	*CIN2+*
Shi 2007 et al. [62]	Ki-67	2/14 (14%)	29/34 (85%)	14/14 (100%)
	$p16^{INK4a}$	0/14 (0%)	26/34 (77%)	14/14 (100%)
	BD ProEx C	0/14 (0%)	32/34 (94%)	11/14 (79%)
	BD ProEx C/p16	0/14 (0%)	34/34 (100%)	14/14 (100%)
Badr 2008 et al. [63]	Ki-67	0/37 (0%)	6/22 (27%)	34/37 (91%)
	$p16^{INK4a}$	2/38 (5%)*	8/23 (35%)**	35/37 (93%)
	BD ProEx C	1/38 (3%)	11/23 (48%)	34/37 (92%)
	BD ProEx C/p16	3/35 (9%)	15/23 (65%)	37/37 (100%)
Pinto 2008 et al. [64]	Ki-67	11/23 (48%)	ND	34/36 (94%)
	$p16^{INK4a}$	13/35 (37%)	ND	51/61 (84%)
	BD ProEx C	10/35 (29%)	ND	52/60 (87%)
	BD ProEx C/p16	9/23 (39%)	ND	33/36 (92%)
Halloush 2008 et al. [65]	Ki-67	14/29 (48%)[a]	22/27 (82%)	15/16 (94%)
	$p16^{INK4a}$	19/29 (66%)[b]	25/27 (92%)	19/19 (100%)
	BD ProEx C	2/29 (7%)[c]	2/27 (7%)	16/19 (84%)
Conesa-Zamora 2009 et al. [66]	Ki-67	6/26 (23%)	10/21 (48%)	76/85 (89%)
	$p16^{INK4a}$	4/28 (14%)	12/19 (63%)	74/85 (87%)
	BD ProEx C	3/25 (12%)	10/19 (53%)	70/80 (88%)

WNL: within normal limits; CIN: cervical intraepitelial neoplasia; ND: not determined; *Spotty staining; **5/23 spotty staining; [a]12/29 <1% staining, 2/29 ≥1% staining; [b]19/29 <10% staining; [c]1/29 <10%.

5. Evaluation of Progression Risk

One major issue in the management of cervical cancer is the evaluation of progression risk of dysplastic lesions. Patients with CIN1 must be periodically followed due to the risk of progression to high-grade lesions or carcinoma. As 70–80% of low-grade lesions spontaneously regress and not all high-grade lesions progress [73], some women may undergo unnecessary treatment or have a delay in receiving treatment. Thus, the identification of biomarkers to select women truly at risk of lesion progression and in need of treatment could lead to tremendous cost savings and eliminate patient anxiety.

A limited number of studies have reported that the application of Ki-67 immunoquantitative analyses of CIN1 and CIN2 can predict disease progression, with the best features to predict progression being the 90th percentile of the stratification index and the percentage of Ki-67 positive cells in the middle third layer of the epithelium [74, 75].

Recent prospective end point studies have shown that $p16^{INK4a}$ positive low-grade lesions have a higher risk of progression than negative lesions, although this correlation was certainly not absolute [76–79]. Additional prospective data are necessary, however, to confirm this association. Ozaki et al. [80] examined expression of the $p16^{INK4a}$ and ProEx C biomarkers in premalignant lesions to determine which markers could help in prediction of the progression of CIN1. Expression of both markers was significantly higher in the progression group compared to the regression group, being sensitive (86%) and moderately specific (60% and 61%, resp.) in predicting CIN1 progression. Hariri and Hansen [81] compared the prognostic value of $p16^{INK4a}$ to BD ProEx C and HPV ISH in CIN1 cases and found BD ProEx C to be a reliable marker for prediction of 6-year outcome, with an NPV of 95.3% compared with 88.6% for p16 and 87.5% for HPV ISH and a PPV of 51% compared with 40.4% and 67.9%, for p16 and HPV ISH, respectively.

Interestingly, CIN2/3 cervical histology specimens have been described with very strong $p16^{INK4a}$ positivity, but only very focal Ki-67 staining, indicating a presence of a small subset of HSILs with low proliferative activity [82, 83]. Cases like this may represent the first stages of HSIL regression.

HPV L1 is a capsidic protein that is expressed in the early, productive phase of HPV infection, but progressively lost during cervical carcinogenesis. An analysis of thin-layer preparations showed that the L1 capsid protein is produced in about 80% of mild-to-moderate dysplasias, whereas it could only be detected in about 25% of higher-grade dysplasias using immunological methods [28], due, in part, to HPV integration that accompanies the development of cervical neoplasia. The detection of the HPV L1 capsid protein in combination with $p16^{INK4a}$, to confirm the association of the lesion with HPV, has been reported to serve as a prognostic marker that can differentiate between patients who will undergo a transition from a precursor lesion to cancer and those whose lesions will regress [39, 84]. While the data is still preliminary, in cases where the grade of lesion is morphologically difficult to assess, the L1 pattern may be helpful for deciding the appropriate management of women. L1-negative HPV high-risk positive mild and moderate lesions have an extremely low probability to regress

spontaneously (5%) in contrast to the L1 positive cases showing a low malignant potential [41].

6. Summary

A number of protein biomarkers are currently available to assist in improving the clinical performance of cervical cancer screening. The recent introduction of prophylactic HPV vaccines will eventually reduce the incidence of cervical cancers and its malignant precursors, therefore, increase the importance of biomarkers in future cervical cancer screening programs to identify for treatment only those women truly at high risk for developing cervical cancer. It is anticipated that the use of these biomarkers can be applied both as a reflex test from an atypical Pap specimen but also as a primary screen to improve the overall accuracy of the Pap test. The introduction of HPV primary screening programs will necessitate the use of a reflex test with high specificity to triage the high number of HPV positive tests. It is believed that biomarkers will also serve an important role in the optimization of this alternative screening algorithm. Current translational research investigations are continuing to discover, characterize, and validate such biomarkers for these anticipated applications.

Disclosure

D. P. Malinowski and C. A. Brown are employees of BD Diagnostics, Women's Health in North Carolina, USA.

References

[1] J. Cuzick, C. Clavel, K. U. Petry et al., "Overview of the European and North American studies on HPV testing in primary cervical cancer screening," *International Journal of Cancer*, vol. 119, no. 5, pp. 1095–1101, 2006.

[2] M. T. Fahey, L. Irwig, and P. Macaskill, "Meta-analysis of Pap test accuracy," *American Journal of Epidemiology*, vol. 141, no. 7, pp. 680–689, 1995.

[3] M. E. Sherman, M. H. Schiffman, A. T. Lorincz et al., "Toward objective quality assurance in cervical cytopathology: correlation of cytopathologic diagnoses with detection of high-risk human papillomavirus types," *American Journal of Clinical Pathology*, vol. 102, no. 2, pp. 182–187, 1994.

[4] T. C. Wright Jr., J. Thomas Cox, L. Stewart Massad, L. B. Twiggs, and E. J. Wilkinson, "2001 consensus guidelines for the management of women with cervical cytological abnormalities," *Journal of the American Medical Association*, vol. 287, no. 16, pp. 2120–2129, 2002.

[5] N. Bulkmans, J. Berkhof, L. Rozendaal et al., "Human papillomavirus DNA testing for the detection of cervical intraepithelial neoplasia grade 3 and cancer: 5-year follow-up of a randomised controlled implementation trial," *The Lancet*, vol. 370, no. 9601, pp. 1764–1772, 2007.

[6] M. H. Mayrand, E. Duarte-Franco, I. Rodrigues et al., "Human papillomavirus DNA versus Papanicolaou screening tests for cervical cancer," *The New England Journal of Medicine*, vol. 357, no. 16, pp. 1579–1588, 2007.

[7] P. Naucler, W. Ryd, S. Tornberg et al., "Human papillomavirus and Papanicolaou tests to screen for cervical cancer," *The New England Journal of Medicine*, vol. 357, no. 16, pp. 1589–1597, 2007.

[8] G. Ronco, P. Giorgi-Rossi, F. Carozzi et al., "Results at recruitment from a randomized controlled trial comparing human papillomavirus testing alone with conventional cytology as the primary cervical cancer screening test," *Journal of the National Cancer Institute*, vol. 100, no. 7, pp. 492–501, 2008.

[9] G. Ronco, P. Giorgi-Rossi, F. Carozzi et al., "Human papillomavirus testing and liquid-based cytology in primary screening of women younger than 35 years: results at recruitment for a randomised controlled trial," *The Lancet Oncology*, vol. 7, no. 7, pp. 547–555, 2006.

[10] R. Sankaranarayanan, B. M. Nene, S. S. Shastri et al., "HPV screening for cervical cancer in rural India," *The New England Journal of Medicine*, vol. 360, no. 14, pp. 1385–1394, 2009.

[11] D. P. Malinowski, "Molecular diagnostic assays for cervical neoplasia: emerging markers for the detection of high-grade cervical disease," *BioTechniques*, supplement, pp. 17–23, 2005.

[12] D. P. Malinowski, "Multiple biomarkers in molecular oncology. I. Molecular diagnostics applications in cervical cancer detection," *Expert Review of Molecular Diagnostics*, vol. 7, no. 2, pp. 117–131, 2007.

[13] N. Wentzensen and M. von Knebel Doeberitz, "Biomarkers in cervical cancer screening," *Disease Markers*, vol. 23, no. 4, pp. 315–330, 2007.

[14] E. Endl and J. Gerdes, "The Ki-67 protein: fascinating forms and an unknown function," *Experimental Cell Research*, vol. 257, no. 2, pp. 231–237, 2000.

[15] T. Scholzen and J. Gerdes, "The Ki-67 protein: from the known and the unknown," *Journal of Cellular Physiology*, vol. 182, no. 3, pp. 311–322, 2000.

[16] J. T. Keating, A. Cviko, S. Riethdorf et al., "Ki-67, cyclin E, and $p16^{INK4a}$ are complimentary surrogate biomarkers for human papilloma virus-related cervical neoplasia," *American Journal of Surgical Pathology*, vol. 25, no. 7, pp. 884–891, 2001.

[17] J. Koh, G. H. Enders, B. D. Dynlacht, and E. Harlow, "Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition," *Nature*, vol. 375, no. 6531, pp. 506–510, 1995.

[18] M. Serrano, G. J. Hannon, and D. Beach, "A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4," *Nature*, vol. 366, no. 6456, pp. 704–707, 1993.

[19] S. N. Khleif, J. Degregori, C. L. Yee et al., "Inhibition of cyclin D-CDK4/CDK6 activity is associated with an E2F-mediated induction of cyclin kinase inhibitor activity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 9, pp. 4350–4354, 1996.

[20] T. Sano, T. Oyama, K. Kashiwabara, T. Fukuda, and T. Nakajima, "Expression status of p16 protein is associated with human papillomavirus oncogenic potential in cervical and genital lesions," *American Journal of Pathology*, vol. 153, no. 6, pp. 1741–1748, 1998.

[21] M. Guo, L. Hu, M. Baliga, Z. He, and M. D. Hughson, "The predictive value of $p16^{INK4a}$ and hybrid capture 2 human papillomavirus testing for high-grade cervical intraepithelial neoplasia," *American Journal of Clinical Pathology*, vol. 122, no. 6, pp. 894–901, 2004.

[22] S. Nieh, S. F. Chen, T. Y. Chu, H. C. Lai, and E. Fu, "Expression of $p16^{INK4A}$ in Papanicolaou smears containing atypical squamous cells of undetermined significance from the uterine cervix," *Gynecologic Oncology*, vol. 91, no. 1, pp. 201–208, 2003.

[23] S. Nieh, S. F. Chen, T. Y. Chu et al., "Is $p16^{INK4A}$ expression more useful than human papillomavirus test to determine the outcome of atypical squamous cells of undetermined

significance-categorized Pap smear? A comparative analysis using abnormal cervical smears with follow-up biopsies," *Gynecologic Oncology*, vol. 97, no. 1, pp. 35–40, 2005.

[24] I. Tsoumpou, M. Arbyn, M. Kyrgiou et al., "P16^{INK4a} immunostaining in cytological and histological specimens from the uterine cervix: a systematic review and meta-analysis," *Cancer Treatment Reviews*, vol. 35, no. 3, pp. 210–220, 2009.

[25] T. Yoshida, T. Fukuda, T. Sano, T. Kanuma, N. Owada, and T. Nakajima, "Usefulness of liquid-based cytology specimens for the immunocytochemical study of p16 expression and human papillomavirus testing: a comparative study using simultaneously sampled histology materials," *Cancer*, vol. 102, no. 2, pp. 100–108, 2004.

[26] K. Labib and J. F. Diffley, "Is the MCM2-7 complex the eukaryotic DNA replication fork helicase?" *Current Opinion in Genetics and Development*, vol. 11, no. 1, pp. 64–70, 2001.

[27] S. E. Moyer, P. W. Lewis, and M. R. Botchan, "Isolation of the Cdc45/Mcm2-7/GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 27, pp. 10236–10241, 2006.

[28] A. Freeman, L. S. Morris, A. D. Mills et al., "Minichromosome maintenance proteins as biological markers of dysplasia and malignancy," *Clinical Cancer Research*, vol. 5, no. 8, pp. 2121–2132, 1999.

[29] M. A. Gonzalez, K. E. K. Tachibana, R. A. Laskey, and N. Coleman, "Control of DNA replication and its potential clinical exploitation," *Nature Reviews Cancer*, vol. 5, no. 2, pp. 135–141, 2005.

[30] S. S. Hook, J. J. Lin, and A. Dutta, "Mechanisms to control rereplication and implications for cancer," *Current Opinion in Cell Biology*, vol. 19, no. 6, pp. 663–671, 2007.

[31] G. H. Williams and K. Stoeber, "Cell cycle markers in clinical oncology," *Current Opinion in Cell Biology*, vol. 19, no. 6, pp. 672–679, 2007.

[32] M. Branca, C. Giorgi, M. Ciotti et al., "Over-expression of topoisomerase IIalpha is related to the grade of cervical intraepithelial neoplasia (CIN) and high-risk human papillomavirus (HPV), but does not predict prognosis in cervical cancer or HPV clearance after cone treatment," *International Journal of Gynecological Pathology*, vol. 25, no. 4, pp. 383–392, 2006.

[33] Y. Chen, C. Miller, R. Mosher et al., "Identification of cervical cancer markers by cDNA and tissue microarrays," *Cancer Research*, vol. 63, no. 8, pp. 1927–1935, 2003.

[34] C. Rosty, M. Sheffer, D. Tsafrir et al., "Identification of a proliferation gene cluster associated with HPV E6/E7 expression level and viral DNA load in invasive cervical carcinoma," *Oncogene*, vol. 24, no. 47, pp. 7094–7104, 2005.

[35] A. D. Santin, F. Zhan, E. Bignotti et al., "Gene expression profiles of primary HPV16- and HPV18-infected early stage cervical cancers and normal cervical epithelium: identification of novel candidate molecular markers for cervical cancer diagnosis and therapy," *Virology*, vol. 331, no. 2, pp. 269–291, 2005.

[36] L. Sherman, N. Alloul, I. Golan, M. Durst, and A. Baram, "Expression and splicing patterns of human papillomavirus type-16 mRNAs in pre-cancerous lesions and carcinomas of the cervix, in human keratinocytes immortalized by HPV 16, and in cell lines established from cervical cancers," *International Journal of Cancer*, vol. 50, no. 3, pp. 356–364, 1992.

[37] H. R. Mcmurray, D. Nguyen, T. F. Westbrook, and D. J. Mcance, "Biology of human papillomaviruses," *International Journal of Experimental Pathology*, vol. 82, no. 1, pp. 15–33, 2001.

[38] H. Griesser et al., "HPV vaccine protein L1 paredicts disease outcome of HPV high risk positive early squamous dysplastic lesions," *Amercian Journal of Clinical Pathology*, vol. 132, no. 6, pp. 840–845, 2009.

[39] R. Hilfrich and J. Hariri, "Prognostic relevance of human papillomavirus L1 capsid protein detection within mild and moderate dysplastic lesions of the cervix uteri in combination with p16 biomarker," *Analytical and Quantitative Cytology and Histology*, vol. 30, no. 2, pp. 78–82, 2008.

[40] H. Griesser, H. Sander, R. Hilfrich, B. Moser, and U. Schenck, "Correlation of immunochemical detection of HPV L1 capsid protein in pap smears with regression of high-risk HPV positive mild/moderate dysplasia," *Analytical and Quantitative Cytology and Histology*, vol. 26, no. 5, pp. 241–245, 2004.

[41] D. Rauber, G. Mehlhorn, P. A. Fasching, M. W. Beckmann, and S. Ackermann, "Prognostic significance of the detection of human papilloma virus L1 protein in smears of mild to moderate cervical intraepithelial lesions," *European Journal of Obstetrics Gynecology and Reproductive Biology*, vol. 140, no. 2, pp. 258–262, 2008.

[42] C. J. Dunton, K. H. Van Hoeven, A. J. Kovatich et al., "Ki-67 antigen staining as an adjunct to identifying cervical intraepithelial neoplasia," *Gynecologic Oncology*, vol. 64, no. 3, pp. 451–455, 1997.

[43] M. M. Goel, A. Mehrotra, U. Singh, H. P. Gupta, and J. S. Misra, "MIB-1 and PCNA immunostaining as a diagnostic adjunct to cervical Pap smear," *Diagnostic Cytopathology*, vol. 33, no. 1, pp. 15–19, 2005.

[44] Z. Zeng, G. Del Priore, J. M. Cohen, and K. Mittal, "MIB-1 expression in cervical Papanicolaou tests correlates with dysplasia in subsequent cervical biopsies," *Applied Immunohistochemistry and Molecular Morphology*, vol. 10, no. 1, pp. 15–19, 2002.

[45] S. Sahebali, C. E. Depuydt, K. Segers, A. J. Vereecken, E. Van Marck, and J. J. Bogers, "Ki-67 immunocytochemistry in liquid based cervical cytology: useful as an adjunctive tool?" *Journal of Clinical Pathology*, vol. 56, no. 9, pp. 681–686, 2003.

[46] A. Longatto Filho, M. L. Utagawa, N. Kasumi Shirata et al., "Immunocytochemical expression of p16^{INK4A} and Ki-67 in cytologically negative and equivocal pap smears positive for oncogenic human papillomavirus," *International Journal of Gynecological Pathology*, vol. 24, no. 2, pp. 118–124, 2005.

[47] N. Wentzensen, C. Bergeron, F. Cas, D. Eschenbach, S. Vinokurova, and M. Von Knebel Doeberitz, "Evaluation of a nuclear score for p16^{INK4a}-stained cervical squamous cells in liquid-based cytology samples," *Cancer*, vol. 105, no. 6, pp. 461–467, 2005.

[48] N. Wentzensen, C. Bergeron, F. Cas, S. Vinokurova, and M. Von Knebel Doeberitz, "Triage of women with ASCUS and LSIL cytology: use of qualitative assessment of p16^{INK4a} positive cells to identify patients with high-grade cervical intraepithelial neoplasia," *Cancer*, vol. 111, no. 1, pp. 58–66, 2007.

[49] F. Carozzi, M. Confortini, P. D. Palma et al., "Use of p16^{INK4A} overexpression to increase the specificity of human papillomavirus testing: a nested substudy of the NTCC randomised controlled trial," *The Lancet Oncology*, vol. 9, no. 10, pp. 937–945, 2008.

[50] D. Schmidt, C. Bergeron, K. J. Denton, R. Ridder, and European CINtec Cytology Study Group, "p16/ki-67 dual-stain cytology in the triage of ASCUS and LSIL papanicolaou cytology: results from the European equivocal or mildly abnormal Papanicolaou cytology study," *Cancer Cytopathology*, vol. 119, no. 3, pp. 158–166, 2011.

[51] N. Edgerton, C. Cohen, and M. T. Siddiqui, "Evaluation of CINtec PLUS(R) testing as an adjunctive test in ASC-US diagnosed SurePath(R) preparations," *Diagnostic Cytopathology*. In press.

[52] K. U. Petry, D. Schmidt, S. Scherbring et al., "Triaging Pap cytology negative, HPV positive cervical cancer screening results with p16/Ki-67 Dual-stained cytology," *Gynecologic Oncology*, vol. 121, no. 3, pp. 505–509, 2011.

[53] D. Kelly, E. Kincaid, Z. Fansler, D. L. Rosenthal, and D. P. Clark, "Detection of cervical high-grade squamous intraepithelial lesions from cytologic samples using a novel immunocytochemical assay (ProExtrade mark C)," *Cancer*, vol. 108, no. 6, pp. 494–500, 2006.

[54] R. H. Tambouret, J. Misdraji, and D. C. Wilbur, "Longitudinal clinical evaluation of a novel antibody cocktail for detection of high-grade squamous intraepithelial lesions on cervical cytology specimens," *Archives of Pathology and Laboratory Medicine*, vol. 132, no. 6, pp. 918–925, 2008.

[55] M. T. Siddiqui, C. Cohen, and A. Nassar, "Detecting high-grade cervical disease on ASC-H cytology: role of BD ProEx C and digene hybrid capture II HPV DNA testing," *American Journal of Clinical Pathology*, vol. 130, no. 5, pp. 765–770, 2008.

[56] M. T. Siddiqui, K. Hornaman, C. Cohen, and A. Nassar, "ProEx C immunocytochemistry and high-risk human papillomavirus DNA testing in papanicolaou tests with atypical squamous cell (ASC-US) cytology: correlation study with histologic biopsy," *Archives of Pathology and Laboratory Medicine*, vol. 132, no. 10, pp. 1648–1652, 2008.

[57] C. E. Depuydt, A. P. Makar, M. J. Ruymbeke, I. H. Benoy, A. J. Vereecken, and J. J. Bogers, "BD-ProExC as adjunct molecular marker for improved detection of CIN2 + after HPV primary screening," *Cancer Epidemiology Biomarkers and Prevention*, vol. 20, no. 4, pp. 628–637, 2011.

[58] J. L. Meyer, D. W. Hanlon, B. T. Andersen, O. F. Rasmussen, and K. Bisgaard, "Evaluation of $p16^{INK4a}$ expression in ThinPrep cervical specimens with the CINtec $p16^{INK4a}$ assay: correlation with biopsy follow-up results," *Cancer*, vol. 111, no. 2, pp. 83–92, 2007.

[59] M. H. Stoler and M. Schiffman, "Interobserver reproducibility of cervical cytologic and histologic interpretations: realistic estimates from the ASCUS-LSIL triage study," *Journal of the American Medical Association*, vol. 285, no. 11, pp. 1500–1505, 2001.

[60] S. M. Ismail, A. B. Colclough, J. S. Dinnen et al., "Observer variation in histopathological diagnosis and grading of cervical intraepithelial neoplasia," *British Medical Journal*, vol. 298, no. 6675, pp. 707–710, 1989.

[61] S. M. Ismail, A. B. Colclough, J. S. Dinnen et al., "Reporting cervical intra-epithelial neoplasia (CIN): intra- and interpathologist variation and factors associated with disagreement," *Histopathology*, vol. 16, no. 4, pp. 371–376, 1990.

[62] J. Shi, H. Liu, M. Wilkerson et al., "Evaluation of $pp16^{INK4a}$, minichromosome maintenance protein 2, DNA topoisomerase IIalpha, ProEX C, and $p16^{INK4a}$/ProEX C in cervical squamous intraepithelial lesions," *Human Pathology*, vol. 38, no. 9, pp. 1335–1344, 2007.

[63] R. E. Badr, A. E. Walts, F. Chung, and S. Bose, "BD ProEx C: a sensitive and specific marker of HPV-associated squamous lesions of the cervix," *American Journal of Surgical Pathology*, vol. 32, no. 6, pp. 899–906, 2008.

[64] A. P. Pinto, N. F. Schlecht, T. Y C Woo, C. P. Crum, and E. S. Cibas, "Biomarker (ProEx C, $p16^{INK4A}$, and MiB-1) distinction of high-grade squamous intraepithelial lesion from its mimics," *Modern Pathology*, vol. 21, no. 9, pp. 1067–1074, 2008.

[65] R. A. Halloush, I. Akpolat, Q. Jim Zhai, M. R. Schwartz, and D. R. Mody, "Comparison of ProEx C with $p16^{INK4a}$ and Ki-67 immunohistochemical staining of cell blocks prepared from residual liquid-based cervicovaginal material: a pilot study," *Cancer*, vol. 114, no. 6, pp. 474–480, 2008.

[66] P. Conesa-Zamora, A. Doménech-Peris, F. J. Orantes-Casado et al., "Effect of human papillomavirus on cell cycle-related proteins p16, Ki-67, cyclin D1, p53, and ProEx C in precursor lesions of cervical carcinoma: a tissue microarray study," *American Journal of Clinical Pathology*, vol. 132, no. 3, pp. 378–390, 2009.

[67] D. Van Niekerk, M. Guillaud, J. Matisic et al., "p16 and MIB1 improve the sensitivity and specificity of the diagnosis of high grade squamous intraepithelial lesions: methodological issues in a report of 447 biopsies with consensus diagnosis and HPV HCII testing," *Gynecologic Oncology*, vol. 107, no. 1, supplement 1, pp. S233–S240, 2007.

[68] G. Negri, G. Bellisano, E. Carico et al., "Usefulness of $p16^{INK4a}$, ProEX C, and Ki-67 for the diagnosis of glandular dysplasia and adenocarcinoma of the cervix uteri," *International Journal of Gynecological Pathology*, vol. 30, no. 4, pp. 407–413, 2011.

[69] D. Aximu, A. Azad, R. Ni, T. Colgan, and S. Nanji, "A pilot evaluation of a novel immunohistochemical assay for topoisomerase II-α and minichromosome maintenance protein 2 expression (ProEx C) in cervical adenocarcinoma in situ, adenocarcinoma, and benign glandular mimics," *International Journal of Gynecological Pathology*, vol. 28, no. 2, pp. 114–119, 2009.

[70] P. Conesa-Zamora, A. Doménech-Peris, S. Ortiz-Reina et al., "Immunohistochemical evaluation of ProEx C in human papillomavirus-induced lesions of the cervix," *Journal of Clinical Pathology*, vol. 62, no. 2, pp. 159–162, 2009.

[71] S. Sanati, P. Huettner, and L. R. Ylagan, "Role of ProExC: a novel immunoperoxidase marker in the evaluation of dysplastic squamous and glandular lesions in cervical specimens," *International Journal of Gynecological Pathology*, vol. 29, no. 1, pp. 79–87, 2010.

[72] M. Guo, A. C. Baruch, E. G. Silva et al., "Efficacy of p16 and ProExC immunostaining in the detection of high-grade cervical intraepithelial neoplasia and cervical carcinoma," *American Journal of Clinical Pathology*, vol. 135, no. 2, pp. 212–220, 2011.

[73] M. Follen and R. Richards-Kortum, "Emerging technologies and cervical cancer," *Journal of the National Cancer Institute*, vol. 92, no. 5, pp. 363–365, 2000.

[74] A. J. Kruse, J. P. A. Baak, E. A. Janssen et al., "Low- and high-risk CIN 1 and 2 lesions: prospective predictive value of grade, HPV, and Ki-67 immuno-quantitative variables," *Journal of Pathology*, vol. 199, no. 4, pp. 462–470, 2003.

[75] A. J. Kruse, J. P. A. Baak, E. A. Janssen et al., "Ki67 predicts progression in early CIN: validation of a multivariate progression-risk model," *Cellular Oncology*, vol. 26, no. 1-2, pp. 13–20, 2004.

[76] G. Negri, F. Vittadello, F. Romano et al., "$P16^{INK4a}$ expression and progression risk of low-grade intraepithelial neoplasia of the cervix uteri," *Virchows Archiv*, vol. 445, no. 6, pp. 616–620, 2004.

[77] J. L. Wang, B. Y. Zheng, X. D. Li, T. Ångstrom, M. S. Lindstrom, and K. L. Wallin, "Predictive significance of the alterations of $p16^{INK4A}$, p14ARF, p53, and proliferating cell nuclear antigen expression in the progression of cervical cancer," *Clinical Cancer Research*, vol. 10, no. 7, pp. 2407–2414, 2004.

[78] S. S. Wang, M. Trunk, M. Schiffman et al., "Validation of $p16^{INK4a}$ as a marker of oncogenic human papillomavirus infection in cervical biopsies from a population-based cohort in Costa Rica," *Cancer Epidemiology Biomarkers and Prevention*, vol. 13, no. 8, pp. 1355–1360, 2004.

[79] J. Hariri and A. Oster, "The negative predictive value of $p16^{INK4a}$ to assess the outcome of cervical intraepithelial neoplasia 1 in the uterine cervix," *International Journal of Gynecological Pathology*, vol. 26, no. 3, pp. 223–228, 2007.

[80] S. Ozaki, Y. Zen, and M. Inoue, "Biomarker expression in cervical intraepithelial neoplasia: potential progression predictive factors for low-grade lesions," *Human Pathology*, vol. 42, no. 7, pp. 1007–1012, 2011.

[81] J. Hariri and T. Hansen, "The prognostic value of BD ProEx C in CIN1 compared with p16 and HPV In Situ Hybridization," *Cytopathology*, vol. 19, supplement 1, p. 217, 2008.

[82] L. Iaconis, E. Hyjek, L. H. Ellenson, and E. C. Pirog, "p16 and Ki-67 immunostaining in atypical immature squamous metaplasia of the uterine cervix: correlation with human papillomavirus detection," *Archives of Pathology and Laboratory Medicine*, vol. 131, no. 9, pp. 1343–1349, 2007.

[83] X. Qiao, T. A. Bhuiya, and M. Spitzer, "Differentiating high-grade cervical intraepithelial lesion from atrophy in postmenopausal women using Ki-67, cyclin E, and p16 immunohistochemical analysis," *Journal of Lower Genital Tract Disease*, vol. 9, no. 2, pp. 100–107, 2005.

[84] G. Negri, G. Bellisano, G. F. Zannoni et al., "$P16^{INK4a}$ and HPV L1 immunohistochemistry is helpful for estimating the behavior of low-grade dysplastic lesions of the cervix uteri," *American Journal of Surgical Pathology*, vol. 32, no. 11, pp. 1715–1720, 2008.

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Review Article

Lm-LLO-Based Immunotherapies and HPV-Associated Disease

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HPV infection is a direct cause of neoplasia and malignancy. Cellular immunologic activity against cells expressing HPV E6 and E7 is sufficient to eliminate the presence of dysplastic or neoplastic tissue driven by HPV infection. Live attenuated *Listeria monocytogenes*- (*Lm*-) based immunotherapy (ADXS11-001) has been developed for the treatment of HPV-associated diseases. ADXS11-001 secretes an antigen-adjuvant fusion (*Lm*-LLO) protein consisting of a truncated fragment of the *Lm* protein listeriolysin O (LLO) fused to HPV-16 E7. In preclinical models, this construct has been found to stimulate immune responses and affect therapeutic outcome. ADXS11-001 is currently being evaluated in Phase 2 clinical trials for cervical intraepithelial neoplasia, cervical cancer, and HPV-positive head and neck cancer. The use of a live attenuated bacterium is a more complex and complete method of cancer immunotherapy, as over millennia *Lm* has evolved to infect humans and humans have evolved to prevent and reject this infection over millennia. This evolution has resulted in profound pathogen-associated immune mechanisms which are genetically conserved, highly efficacious, resistant to tolerance, and can be uniquely invoked using this novel platform technology.

1. Introduction

It has been estimated that HPV infection accounts for approximately 5 percent of all cancers worldwide [1]. Persistent HPV infections are now recognized as the cause of essentially all cervical cancers. In 2010, it was estimated that about 12,000 women in the United States would be diagnosed with this type of cancer and more than 4,000 would die from it. Cervical cancer is diagnosed in nearly half a million women each year worldwide, claiming a quarter of a million lives annually. HPV infection also causes some cancers of the anus, vulva, vagina, and penis [1]. Sexually transmitted HPV infections are very common and have peak prevalence between the ages of 18 and 30. Most of these infections resolve spontaneously, but, in 10–20% of women, these infections remain persistent and are at risk of progression to Grade 2/3 cervical intraepithelial neoplasm (CIN) and eventually to invasive cancer of the cervix (ICC). CINs are genetically unstable lesions with a 30–40% risk of progression to ICC. If left untreated, they form a spectrum of increasing cytological atypia, ranging from low-grade CIN 1 to high-grade CIN 3; the latter are caused almost exclusively by high-risk HPVs, namely, HPV 16 and 18.

Oral HPV infection causes some cancers of the oropharynx (the middle part of the throat, including the soft palate, the base of the tongue, and the tonsils) [1–5]. HPV is associated with 20–50% of oral squamous cell carcinomas [6], and the incidence is dramatically increasing. HPV has also been implicated as having a role in certain colorectal cancers and lung cancers although the association is somewhat controversial.

2. HPV as a Target for Immunotherapy

HPV is a double-stranded, circular DNA virus devoid of an envelope. Depending upon the strain, its genome contains either six or seven early proteins (E1, E2, E4, E5, E6, E7, and E8) and 2 structural proteins which appear later (L1 and L2). Infection with the virus occurs in replicating, differentiating basal epithelial cells. As keratinocytes differentiate and mature, the expression of viral genes results in viral protein production until terminally differentiated surface cells express the late proteins, the viral capsid is assembled, and the virus is shed. HPV-induced cancer can occur when viral DNA integrates into the genome of the host, typically with the deletion of the genes E2, E4, E5, L1, and L2. The

Table 1: Modification of *Lm*-LLO-based immunotherapy for HPV-associated cervical cancer.

Vaccine name	Design	Strain modification	Antigen	Ref.
ADXS11-001 (*Lm*-LLO-E7)	Plasmid	*prfA*$^-$	E7, HPV-16	[7]
Lm-PEST-E7	Plasmid	*prfA*$^-$	E7, HPV-16	[8]
Lm-ActA-E7	Plasmid	*prfA*$^-$	E7, HPV-16	[8]
Lm-dd-TV	Plasmid	*dal*$^-$ *dat*$^-$	E7, HPV-16	[9]

loss of the viral E2 gene, which is a transcriptional inhibitor, leads to the upregulation of two oncoproteins from genes E6 and E7. The viral oncoprotein E6 complexes with the tumor inhibitor gene *p53* and the oncoprotein E7 complexes with the tumor suppressor protein retinoblastoma (pRb) [10], disrupting cell cycle regulation and leading to genomic instability and subsequent neoplasia [11].

HPV-associated neoplasia is one of the most clear-cut situations in medicine where infection with an exogenous agent (a virus) is a direct cause of neoplasia and malignancy. Cellular immunologic activity against cells expressing HPV E6 and E7 is sufficient to eliminate the presence of dysplastic or neoplastic tissue driven by HPV infection. The variable but significant rate of spontaneous remission is felt to be due to immunologic recognition of the HPV proteins expressed in transformed cells and higher numbers of $CD8^+$ cells and a higher ratio of $CD8^+$/FOXp3 cells infiltrating the dysplastic tissue [4, 12–14]. An immunologic stimulus may be required to overcome tolerance that has developed to the HPV-transformed dysplastic cells.

3. Use of *Lm*-LLO Immunotherapy for HPV-Associated Disease

A therapeutic change in the ratio of $CD8^+$ TIL to Tregs has been observed as a result of the administration of *Lm*-LLO immunotherapies in a variety of models [15–18]. *Lm*-LLO-E7 (ADXS11-001) has been found in a variety of preclinical models to stimulate immune responses and affect therapeutic outcomes and is currently in clinical trials.

Just such a live attenuated *Listeria monocytogenes*- (*Lm*-) based immunotherapy (ADXS11-001) has been developed for the treatment of HPV-associated diseases by Advaxis, Inc. ADXS11-001 secretes an antigen-adjuvant fusion (*Lm*-LLO) protein consisting of a truncated fragment of the *Lm* protein listeriolysin O (LLO) fused to HPV16-E7. A Phase 1 study has been completed with ADXS11-001 [19] and 4 Phase 2 clinical trials are active or about to be initiated.

Lm-LLO immunotherapies have multiple simultaneous mechanisms of action that can summate in a therapeutic response [20]. *Lm* stimulates innate immunity and infects APC where it naturally cross-presents to stimulate both arms of the adaptive immune system resulting in activated $CD4^+$ and $CD8^+$. These agents reduce intratumoral Tregs and MDSC, but not those in spleen or lymph nodes. They can stimulate the maturation of immature immune cells to terminally differentiated effector cells and shift the kinetics of bone marrow to produce increased numbers of myeloid cells. Effects have been observed in vascular endothelial cells to facilitate chemotaxis and the extravasation of activated immune cells. *Lm* is an entirely cellular immune stimulating agent, and antibody formation of the type that can inactivate viruses does not occur with *Lm*. Interestingly, consolidated immune memory responses to *Lm* antigens have been observed to occur rapidly, with correlates of immune memory to *Lm* occurring as early as 5 hours after exposure [21].

The use of a live attenuated bacterium is a different way to approach cancer immunotherapy than those based upon synthetic chemistry or antibody-based agents. It is more complex, as *Lm* has evolved to infect humans and humans have evolved to prevent and reject this infection over millennia. This evolution has resulted in profound pathogen-associated immune mechanisms which are genetically conserved, highly efficacious, and can be uniquely invoked using this novel platform technology.

4. *Listeria monocytogenes (Lm)*: A Potent Vector for Immunotherapy for Neoplastic and Infectious Disease

Previous studies have shown that bioengineered *Lm* is a potent vector not only for immunotherapy of cancer but also for infectious diseases [20, 22]. This makes HPV infections and consequently HPV-associated cancers a prime target for therapy. With *Lm*-LLO-based immunotherapy, it is possible to (a) eradicate tumors induced by HPV and (b) prevent reoccurrence of the tumor. Advaxis in collaboration with Yvonne Paterson's Lab (University of Pennsylvania School of Medicine) has developed various vectors expressing the tumor-specific antigens (TSAs) that target HPV-induced cancer as described in Table 1. Preclinical studies using different plasmid backbones for delivering E7 show similar antitumor therapeutic efficacy in all the vectors described in Table 1. The ADXS11-001 (*Lm*-LLO-E7) was selected for human studies as it was extensively studied and tested in preclinical settings. Furthermore, ADXS11-001 pathogenicity was attenuated by 10^4 to 10^5 logs, compared to the wild-type *Lm* parent strain 10403S, thus increasing its safety for clinical use.

Various methods of bioengineering allow *Lm* to express TSA on the plasmid or in the genome via chromosomal insertion [23, 24]. At Advaxis, two complementation mechanisms have been designed for the *in vivo* retention of plasmids in attenuated bacterial strains. One strain is a *prfA* deletion mutant which is avirulent due to the absence of the master virulence regulating protein P*rfA*, rendering it unable to escape the phagolysosome, but the intracellular growth

ability is restored through the complementation of P*rfA* on a plasmid. This complementation ensures *in vivo* retention of the plasmid, but for *in vitro* manipulation antibiotic resistance markers such as chloramphenicol resistance gene were used [7, 8]. Another backbone is a mutant strain defective for D-alanine synthesis, which is essential for bacterial cell wall synthesis. Survival of *Lm* strain deficient in *dal* and *dat* genes depends upon the plasmid-based complementation of the *dal* gene. To eliminate the possibility of recombination of *dal* gene present in the plasmid and the *Lm* genome, *Bacillus subtillis dal* gene was used for complementation of *in vivo* and *in vitro* growth. This complementation not only creates an antibiotic-marker-free plasmid delivery system but also attenuates the vector by 0.5 to 1 log [9]. ADXS11-001 with the *prfA* deletion was found to be cleared by SCID mice using innate immunity alone [20], and the clearance kinetics of the *dal dat* demonstrated clearance within 72 hours. A highly attenuated Lm dal^- dat^- $actA^-$ (*Lm*ddA) backbone was created at Advaxis [24], which is cleared rapidly *in vivo* and contains an antibiotic-marker-free plasmid for expression of TSA, which is strong candidate for immunotherapy in the clinic. Similar *in vivo* clearance of *Lm*ddA strain in both normal and interferon-gamma knockout mice demonstrates that this strain is highly attenuated and safe for clinical use.

5. LLO: An Adjuvant for Immunotherapy

Listeriolysin O is a hemolytic, thiol-activated, cholesterol-dependent pore-forming protein which is essential for intracellular escape of *Lm* from the phagolysosome [25]. Recent advances in immunology have resulted in a number of potential adjuvant candidates that are able to modulate the immune response in a more controlled and specific manner [26]. These adjuvants modulate and target specific immune components, such as activation of different cells, receptors, or signaling pathways. [26]. Advaxis studies show that nonhemolytic LLO also harbors unique properties of an adjuvant: (a) augments the effects of "non-self-foreign" antigens as do classical adjuvants, (b) breaks tolerance of "self-/tumor-associated antigens," (c) specifically activates or augments functions involved in antitumor activity, (d) regulates complex soluble mediators and their receptors to optimize the antitumor activity, and (e) modulates signals to activate different arms of the immune systems for antitumor activity. Gunn et al. engineered an LLO molecule truncated at the C-terminal of the protein, which rendered the LLO nonhemolytic [20]. Neeson et al. [27] independently reported that LLO has adjuvant properties when used in the form of a recombinant protein vaccine. Fusion of LLO to tumor antigens delivered by other vaccine modalities, such as viral vectors [28] and DNA vaccines [29], also enhances their therapeutic efficacy. These properties of recombinant LLO positions it as an attractive adjuvant not only for breaking local and peripheral immunological tolerance of tumors and associated antigens but also for mounting an antigen-specific and antigen-coordinated anticancer immune response as described followingly in mouse models for HPV-related cancer.

6. Intracellular Events and Antigen Presentation of *Lm*-LLO-Ag (HPV) Fusion Protein

As shown in Figure 1, attenuated *Lm* carrying the HPV antigen fused to LLO can be phagocytized by antigen-presenting cells, macrophages, and other cells [20, 30]. The attenuated bacterial cells are taken up into the endosome where they evoke a conserved pathogenic assault [31] and redirect the tumor antigen [23]. The PEST-like sequence of LLO is important as it has been shown to increase antitumor efficacy of *Lm*-based vectors expressing the fusion protein LLO-PEST-E7 in HPV-16 immortalized tumors [8]. This process stimulates cell-mediated immune response generating $CD4^+$ cells and $CD8^+$ T cells [20]. The fusion of antigens to LLO facilitates the secretion of the antigen [32] and increases antigen presentation [8] with a profound influence on $CD8^+$ T-cell activation [20, 33].

7. *In Vivo* Response to *Lm*-LLO-Ag (HPV) Fusion Protein and Cellular Events in the Tumor Environment

An *in vivo* response to *Lm*-LLO-Ag (HPV) fusion protein induces several immune functions which are well coordinated to break the local and peripheral tolerance to tumor-specific antigens and to initiate a chain of antitumor activities utilizing various soluble mediators and cells as shown in Figure 2. LLO is a potent inducer of inflammatory cytokines, such as IL-6, IL-8, IL-12, IL-18, TNF-α, IFN-γ, and GM-CSF; nitric oxide, chemokines, as well as costimulatory molecules that are important for innate and adaptive immune responses [20, 35–37]. One example of the high Th-1 cytokine-inducing activity of LLO is that protective immunity to *Lm* can be induced with killed or avirulent *Lm* when administered together with LLO, but not in the absence of LLO [38]. Cytokines induced in macrophages in the presence of LLO [39] in turn activate NK cells to release IFN-γ [40].

8. Generation of Tumor-Antigen-Specific Cytotoxic T Cells and Regression of HPV-Associated Tumors

Preclinical studies using a genetically engineered attenuated strain of *Lm* expressing HPV-16 E7 demonstrated therapeutic activity against E7-expressing tumors in animal models [7]. Two *Lm*-LLO-based immunotherapy vectors, one of which expresses the antigen HPV-16 E7 alone and one which expresses E7 fused to a truncated form of LLO, showed regression of the E7-expressing tumor, TC-1, established in syngeneic C57BL/6 mice [7]. A lung epithelial cell line (TC-1) immortalized with HPV-16 E6 and E7 and transformed with the c-ras oncogene was used in these studies. Paterson et al. have recently utilized a new recombinant strain of *L* that uses a multicopy episomal expression system (*Lm*-ActA-E7)

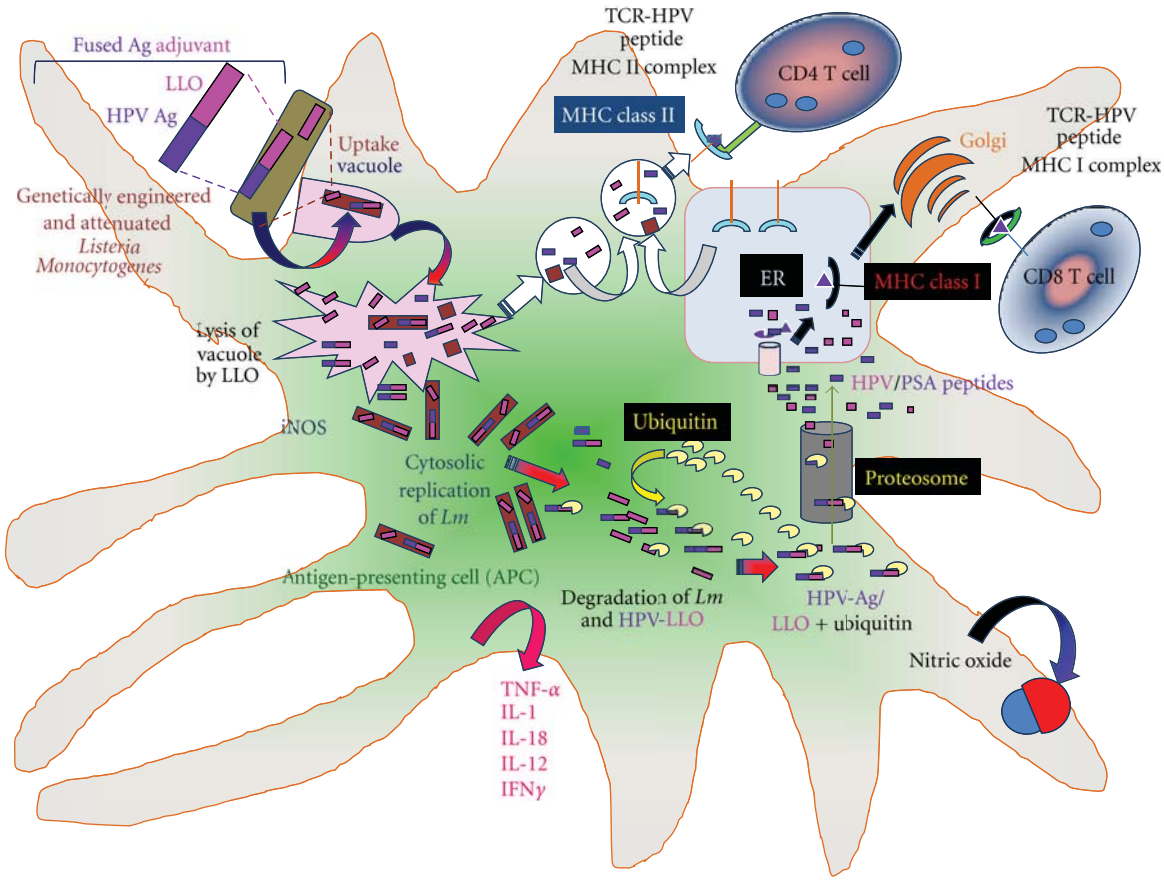

Figure 1: Schematic presentation of LLO-Ag (HPV) fusion protein processing and presentation in antigen-presenting cell (APC) by *Listeria monocytogenes*. Advaxis in collaboration with Paterson et al. has developed human papilloma virus (HPV) and listeriolysin (LLO) fusion proteins in *Lm* for immunotherapy [17, 22–24]. Upon injection *in vivo*, these *Lm* are sequestered and engulfed by antigen-presenting cells (APCs) such as dendritic cells [23, 24, 31]. The bacteria are engulfed by vacuoles where most of the *Listeria* are killed [18, 32]. The bacterium while processing the tumor-associated antigen (HPV) and listeriolysin O (LLO) stimulates both arms of the adaptive immune system [20, 34]. Part of the antigen from the vacuole is processed via the MHC class II molecules which generate $CD4^+$ T cells. Five to ten percent of these *Lm* escape into the cytosol with the assistance of the LLO where *Listeria* can undergo replication. The cytosolic HPV-LLO fusion protein behaves as endogenous antigens. The HPV-LLO fusion protein undergoes ubiquitination, and it is processed via the proteasome [20]. The resulting peptides are presented via the MHC class I molecules to generate $CD8^+$ T cells [34]. These cells generate strong cell-mediated immune responses. *Lm* also evokes a strong innate immune response which leads to generation of numerous mediators such as nitric oxide which is involved in killing of the bacteria in the vacuoles and cytokines (such as TNF-β, IL-1, IL-18, IL-12, and IFNγ) which impart several types of bystander effects [20, 33, 35–37].

to secrete the HPV protein E7 fused to the *Lm* protein ActA as shown in Figure 3.

The *Lm*-ActA-E7-based immunotherapy (but not *Lm*-ActA-NP treated—used as nonspecific—controls) or untreated controls caused 75% regression of the HPV-positive tumors on day 20 when compared to the established tumor on day 7. However, more than 90% regression of tumors was observed when *Lm*-ActA-E7-induced tumor reduction as compared to controls on day 28 (Figure 4).

Sewell et al. showed that antitumor activity of *Lm*-LLO-based immunotherapy against E7 could also be seen in solid tumors implanted in transgenic mice [8, 49]. This model system also revealed the enhanced antitumor efficacy of *Lm-LLO*-based vectors expressing the fusion protein LLO-PEST-E7 in HPV-16 immortalized tumors in syngeneic mice. It should be noted that this immunotherapy has the potential not only to cause tumor regression but also to prevent the recurrence of tumors. A cytotoxic T-lymphocyte assay revealed that administration of *Lm*-LLO-based vector caused the generation of cytotoxic T cells specific for E7 (Figure 5).

9. Ability of *Lm*-LLO-E7 to Induce $CD8^+$ T-Cell Memory and Regression of Established Tumors after Antibiotic Administration

It should be noted that although *Lm*-LLO-based immunotherapy required a live attenuated bacteria as a carrier of the fusion antigen, the bacteria may be killed shortly after administration by antibiotic treatment and the immunotherapy will continue to demonstrate antitumor activity [20].

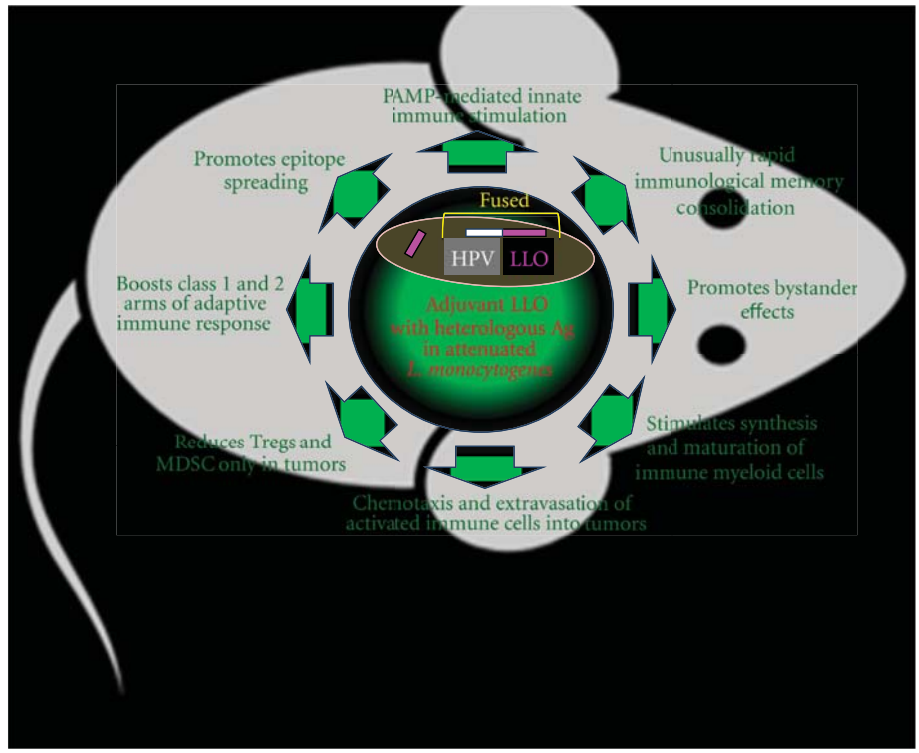

Figure 2: Hypothetical representation of *in vivo* effects of *Lm*-LLO-based immunotherapy. *Lm*-LLO-based immunotherapy evokes a cascade of events *in vivo* which involves multiple cell types that may (a) regress existing tumors and (b) block tumor reoccurrence. The physiological events associated with these potent therapeutic and prophylactic events include the following: (1) unusually rapid immunological memory consolidation is generated with five-hour post-*Listeria*-based immunotherapy [41, 42]; (2) promotes bystander effects via activation of cytokines, chemokines, and/or their receptors regulate functions such as leukocytosis, memory, and listeriosis [20]; (3) stimulates synthesis and maturation of immune myeloid cells by stimulating formation of myeloid cells and maturation of dendritic cells [39, 43, 44]; (4) guides heterologous Ag (HPV) processing to generate antigen-specific $CD4^+$ and $CD8^+$ cells, via MHC class II and I pathways, respectively [34, 43]; (5) reduces Tregs and MDSC only in tumors and diminishes the tumor's resistance to immune attack by antigen-specific cells [4, 14, 20, 45]; (6) boosts class 1 and 2 arms of adaptive immune response which generates strong cell-based antitumor immunity [9, 24, 30]; (7) chemotaxis and extravasation of activated immune cells is part of an innate immune response, involving the recruitment of nonspecific leukocytes into tumors [34, 46, 47]; (8) PAMP-mediated innate immune stimulation facilitates processing of live *Listeria* which evokes the essential activity of inflammasomes and innate immunity [48].

Experiments in mice by Bajénoff et al. showed that *Lm*-specific and *Lm*-nonspecific memory $CD8^+$ T cells could be observed within 6 hours of infection and with *Lm* burden [41]. The *Lm*-specific and *Lm*-nonspecific memory $CD8^+$ T cells were localized in red pulp of the spleen which formed clusters around *Lm*-infected cells. Memory $CD8^+$ T cells produced inflammatory cytokines such as IFN-γ and CCL3 nearby infected myeloid cells which are known to be crucial for *Lm* killing. Corbin and Harty [42] reported that *Lm*-infected mice treated with antibiotics at 24 hours postinfection showed a robust increase in antigen-specific $CD4^+$ and $CD8^+$ T cells similar to the response in controls that did not receive the antibiotics. Furthermore, antibiotic treatment did not alter secondary antigen-specific T-cell expansion or protection with or without the antibiotics [42]. These experiments demonstrate that development of early $CD4^+$ and $CD8^+$ T cells show functional memory, independent of prolonged infection or antigen display on day 28. Figure 6 shows that administration of antibiotics on day 3 posttreatment with ADXS11-001 has no effect on efficacy as more than 90% tumor regression occurred in mice.

10. Intracellular Milieu in Tumors

The presence of a complex immune suppressive network in the tumor microenvironment includes, but is not limited to, (a) Tregs, (b) myeloid-derived suppressor cells (MDSCs) along with their mediators (i.e., IL-10, TGF-β, GM-CSF, PGE_2, B7-H1, PD-1, and Arginase I), (c) functionally impaired immune cells, and (d) tumor-associated macrophages (TAMs) and their mediators such as nitric oxide which effectively halts the antitumor immunity [45]. The intracellular milieu is a challenging aspect for any immunotherapy including *Lm*-LLO-based immunotherapy. Figure 2 summarizes some of the *in vivo* events manifested by *Lm*-LLO-based immunotherapy which have the ability to neutralize and/or reverse cell functions and mediator release involved in tumor immunity. Much of these events are also

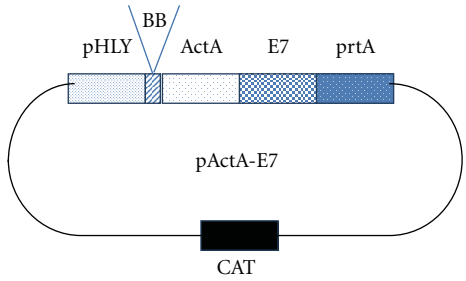

Figure 3: A schematic representation of the plasmid pActA-E7. The recombinant plasmid was used to transform the *Lm* strain XFL-7 to create *Lm*-ActA-E7. The vector includes a promoter (*pHly*) and signal sequence (ss) from the *hly* gene, the *actA* gene, the human papillomavirus 16 E7 gene, and the transcription factor *prfA*. XFL-7 is a *prfA*-deleted strain of *Lm*. Thus, only bacteria that retain the plasmid will replicate *in vivo*. Adopted and modified from [49].

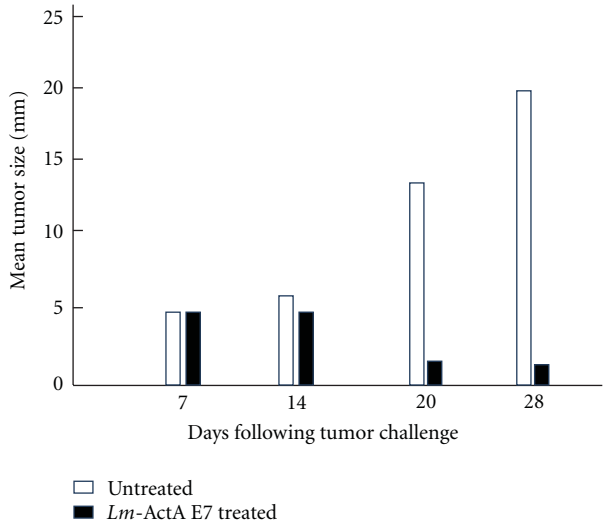

Figure 4: *Lm*-ActA-E7 causes regression of established TC1 tumors. C57BL/6 mice received 2×10^5 TC1 cells subcutaneously on the left flank. Tumors grew to 5 mm after 7 days. The mice were then treated with 0.1 median lethal dose of *Lm*-ActA-E7 or *Lm*-LLO-NP (data not shown) as a negative control on day 7, and a booster dose was given on day 14. The third and final group was left untreated. The average tumor diameter was measured with calipers and is shown for each mouse. The difference in tumor sizes between the *Lm*-ActA-E7 group and either control group at days 20 and 28 is statistically significant ($P \leq .005$ and $P \leq .001$, resp.). Depicted is 1 experiment representative of 4. The figure and legend were adopted and modified from [49].

induced in the animal model of E7-induced tumors during *Lm*-LLO-HPV-induced immunotherapy. For example, studies by Advaxis and Paterson Lab showed a correlation between $CD8^+$ T-cell induction, tumor homing, and the antitumor efficacy of the *Lm-LLO*-based immunotherapy [20].

The effect on different T-cell populations in tumor microenvironment after treatment with *Lm*-E7 and *Lm*-LLO-E7 in mice harboring TC1 tumors is shown in Table 2. There was an increase in TILs and a decrease in $CD25^+$

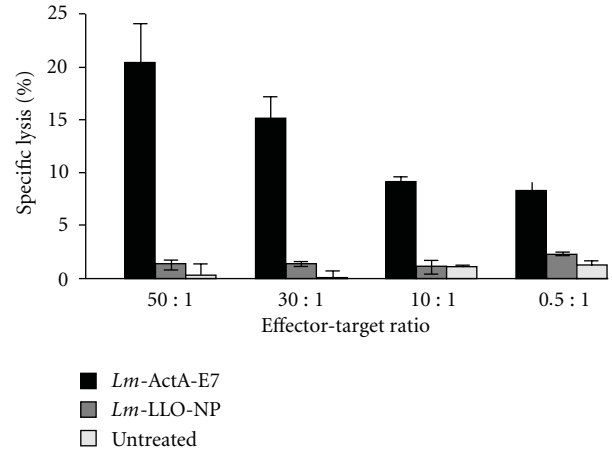

Figure 5: *Lm*-ActA-E7 induces E7-specific cytotoxic T-lymphocyte (CTL) activity. C57BL/6 mice were immunized with 0.1 median lethal dose of *Lm*-ActA-E7 or Lm-LLO-NP. A separate group of mice was left untreated. A booster immunization was administered 7 days later. Splenocytes were harvested 7 days after the booster and established in primary culture with irradiated TC1 cells for 7 days. Following the primary culture, CTL activity was assayed via chromium 51 (^{51}Cr) release from EL4-E7 cells. The CTL activity was significantly higher in those mice that were vaccinated with *Lm*-ActA-E7 than in controls ($P \leq 0.05$). Results are expressed as the mean of triplicate cultures. These results are representative of 3 experiments. The figure and legend are adopted from [49].

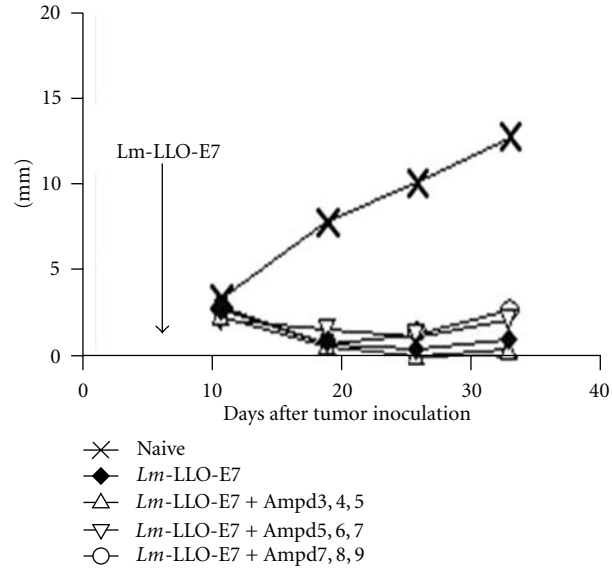

Figure 6: Effect of ampicillin treatment on therapy of TC1 by ADXS11-001. C57BL/6 mice were injected with 1×10^5 TC1 tumor cells. Seven days later, the mice were treated with $0.1 \times LD_{50}$ of ADXS11-001. Beginning 3, 5, or 7 days after ADXS11-001 treatment, some of the mice received daily injections of 10 mg of Ampicillin, delivered for three consecutive days; the mice were then maintained on drinking water supplemented with Ampicillin at a concentration of 0.5 mg/mL. The data is adopted from [17].

Table 2: *Lm*-LLO-based immunotherapy increases $CD8^+$ T cells (TILs) and decreases $CD25^+CD4^+FoxP3^+$ Tregs in tumor. Comparison of $CD8^+$ T cells (TILs) and $CD25^+CD4^+FoxP3^+$ Tregs in TC1 mouse tumor after treatment with *Lm*-E7 or *Lm*-LLO-E7. The data has been adopted and modified from Shahabi et al. [17].

Immunotherapy group	E7/Db tetramer positive activated $CD8^+$ T cells in tumors (TILs)	$CD25^+CD4^+FoxP3^+$ Tregs in the tumor	$CD8^+$ TIL : Tregs ratio
Lm-E7	9.4%	11.8%	0.80
Lm-LLO-E7	36.8%	1.7%	21.65

Table 3: Percent of intratumoral Tregs in a TC1 model following treatment with *Lm*-E7 and *Lm*-LLO-E7 in mouse model of cervical cancer [20].

Percent intratumoral Tregs by vector type			
Lm-E7		*Lm*-LLO-E7	
Spleen	Tumor	Spleen	Tumor
6.4	12.1	4.5	2.3
7.0	12.2	3.9	2.0
6.9	14.9	4.5	1.1
6.5	8.9	3.9	1.3

$CD4^+FoxP3^+$ Tregs in tumors of mice immunized with *Lm*-LLO-E7 suggesting that LLO-E7 fusion not only increases T-cell infiltration but also reduces suppressive cells intratumorally. In order to determine if similar effect on Tregs was observed in the periphery, the distribution of these cells was monitored in the spleen. As shown in Table 3 treatment with *Lm*-LLO-E7 vaccine causes a preferential decrease in the Tregs intratumorally and has no effect on the periphery such as spleen. These studies show that *Lm*-LLO-based immunotherapies cause specific reduction of Tregs within the tumor to stimulate antitumor immunity.

The fusion of antigens to LLO also appears to facilitate the secretion of the antigen [7, 32] and increased antigen presentation with a profound influence on the $CD8^+$ T-cell activation [50]. *Lm*-LLO-Ag reduces the percentage of immunesuppressive Tregs infiltrating the tumor and helps to stimulate the maturation of DCs and other myeloid cells [44]. Singh et al. have shown a decrease in MDSC to play a critical role in tumor regression with *Lm*-LLO-based immunotherapy in mouse cancer models (unpublished data. Advaxis, Inc.). Previous studies have reported accumulation of *Lm* within the tumor during immunotherapy [20]. *Lm*-based vaccines have been reported to infect the primary tumor and metastases tumor *in vivo* [51]. Kim et al. [51] suggested that *Lm* vaccines could kill tumors (a) by directly infecting the tumor and increasing the levels of ROS and (b) by directing CTL responses against cells expressing specific antigens.

Preclinical studies demonstrate that *Lm*-LLO-based immunotherapy encompasses a coordinated and comprehensive cellular reaction towards tumor destruction in E7-induced tumors in mouse models. These preclinical data show that *Lm*-LLO-based immunotherapy is pleotropic in nature and has many of the traits required for overcoming the central and peripheral immunological tolerance that is exerted in the tumor microenvironment described above. Furthermore, *Lm*-LLO-based immunotherapy is antigen, tissue specific, and unlike chemotherapy, once the tumor is eradicated, it persistently blocks its reoccurrence in mouse models of cancer due to the development of immunological memory. These experiments also demonstrated the efficacy of *Lm*-LLO-based immunotherapy to a tumor that is induced by a viral oncogene.

11. Clinical Development Plan for ADXS11-001

The most likely diseases to evaluate the safety and efficacy of ADXS11-001 are cervical intraepithelial neoplasia (CIN) and cervical cancer, HPV-positive head and neck cancer, and perhaps other HPV-associated diseases like vulvar intraepithelial neoplasia (VIN), and even lung and colorectal cancer where an HPV link can be identified. To date, a Phase 1 study has been completed, two Phase 2 trials are ongoing, and 2 additional Phase 2 trials currently await institutional approval to begin.

11.1. Phase 1 Study. A Phase 1 trial of ADXS11-001 was conducted in 15 patients with previously treated metastatic, refractory, or recurrent cervical cancer who had failed previous cytotoxic therapy [19] and in a population where no therapeutic regimen had been shown to extend survival. ADXS11-001 was administered by intravenous infusion at three (3) dose levels (1×10^9 CFU, 3.3×10^9 CFU, and 1×10^{10} CFU) using a dose escalation design across cohorts with each patient in a cohort receiving only two administrations of the same dose. The infusion was administered to each study participant over 30 minutes and occurred once every 21 days for a total of two treatments on days 1 and 22, respectively. Overall, 15 (100%) of patients experienced cytokine-mediated adverse events (AEs). The most commonly reported AEs were pyrexia, chills, anemia, headache, vomiting, nausea, tachycardia, and musculoskeletal pain. Drug-related AEs were mild to moderate, transient in nature, and consisted of "flu-like" symptoms such as pyrexia, vomiting, chills, headache, tachycardia, and nausea and which responded to standard nonprescription agents. Infusion of 1×10^{10} or more CFU without premedication resulted in a dose limiting toxicity (DLT) of Grade 2 diastolic hypotension occurring within hours after the ADXS11-001 infusion that required therapeutic intervention. In all patients, the hypotension was successfully controlled with IV fluids and supporting medication. Similar DLT have been observed at 1×10^{10} for other live *Lm*-based vectors in trials conducted by other sponsors [19]; therefore, doses of 1×10^9 CFU or less were selected for subsequent clinical evaluation.

Historically, the median survival of these patients is approximately 6 months with a one-year survival of 5% (unpublished data (GOG 127 series Phase 2 studies)). In the Phase 1 study of ADXS11-001, 4 of 13 evaluable patients experienced a reduction of their tumor burden; median survival was 347 days, and one-year survival was 53%. 11/15 patients (73%) had a performance status ECOG 2–4. The clinical benefit of increased survival and tumor shrinkage observed in this advanced malignancy setting merited further investigation.

11.2. Phase 2 Studies. Most immunotherapies seem to work best in earlier stages of disease where the tumor burden is lower and there has been less prior therapy. In the case of HPV-associated cervical cancer, there is a clear and slowly progressing maturation of dysplasia toward cervical cancer known as CIN. Frequent Pap smears and colposcopic examination can identify subjects with CIN. The standard of care for high-grade CIN (CIN 2/3) is a surgical resection of the dysplastic tissue in the cervix. While this is typically an outpatient procedure, it can compromise future fertility of the woman and recurrence can occur. There is also a significant spontaneous remission rate in women with CIN which is inversely proportional to the grade of their CIN. An agent that can induce immunologic remission of high-grade cervical dysplasia could eliminate the risks associated with surgery and provide immunologic memory that could in theory protect against recurrence.

Lm-LLO-E7-07 is a randomized, single blind, placebo-controlled, dose escalation Phase 2 trial being conducted in the US in 120 women with CIN 2/3. The initial 40 subject cohort has been completed with 31 subjects receiving 80 doses. Each subject received 3 doses each of dose 5×10^7 CFU or placebo (3 : 1 randomization). Enrollment of the second cohort is ongoing.

Lm-LLO-E7-015 is a randomized Phase 2 trial being conducted in India in women with progressive cervical cancer who have failed cytotoxic therapy. Patients are randomized to 3 doses of ADXS-011 at 1×10^9 CFU or 4 doses of 1×10^9 CFU with cisplatin chemotherapy between doses 1 and 2. As of 8/1/11, 54 patients have received 117 doses.

In both studies, Naprosyn and oral promethazine are given as premedications to ameliorate potential side effects, and a course of ampicillin is given 3 days after infusion as a precautionary measure. From this clinical experience, a clear pattern of treatment-related adverse events has emerged consisting of fever, chills, nausea, and vomiting which are consistent with the release of immunologic cytokines commonly associated with immune activation. Between 15 and 23% of the doses administered have been associated with a drug-related adverse event; typically a transient Grade 1 or 2 (mild-moderate) flu-like symptom, which appears within a few hours to 3 days after infusion. Symptoms either self-resolve or respond quickly to symptomatic treatment. Thus far, there have been no serious adverse events associated with ADXS11-001 in 171 doses, no evidence of listeriosis, no persistent symptoms, no delayed symptoms, and no evidence of cumulative toxicity in subsequent doses.

A GOG Phase 2 trial in the US in patients with recurrent/refractory cervical cancer and a Phase 1/2 safety and efficacy of ADXS11-001 in HPV-positive oropharyngeal head and neck cancer were funded by Cancer Research UK (CRUK).

12. Conclusion

ADXS-11-001 immunotherapy can be safely administered to healthy young subjects as well as patients with advanced cancer and presents a predictable and manageable safety profile. This agent has the capability of inducing the type of immunologic response that has been observed in cases of spontaneous remission and responding HPV-transformed lesions. ADXS-11-001 can generate a Th-1 type immunologic response generating $CD8^+$ T cells that target HPV-E7-transformed cells while simultaneously suppressing the Treg- and MDSC-driven immunologic tolerance within the lesions, increasing the CD8/Treg(FOX P3+) ratio, and causing clinical remission. Clinical trials are ongoing to evaluate the activity of this agent across the spectrum of diseases caused by HPV transformation from cervical intraepithelial neoplasia (CIN 2/3) through locally advanced cervical cancer to advanced recurrent cervical cancer. Other HPV-associated malignancies are also being investigated or are of interest including HPV-positive head and neck cancer and types of lung and colorectal cancer where an HPV link can be identified.

References

[1] D. M. Parkin, "The global health burden of infection-associated cancers in the year 2002," *International Journal of Cancer*, vol. 118, no. 12, pp. 3030–3044, 2006.

[2] G. D'Souza, A. R. Kreimer, R. Viscidi et al., "Case-control study of human papillomavirus and oropharyngeal cancer," *The New England Journal of Medicine*, vol. 356, no. 19, pp. 1944–1956, 2007.

[3] J. L. Brandsma and A. L. Abramson, "Association of papillomavirus with cancers of the head and neck," *Archives of Otolaryngology—Head and Neck Surgery*, vol. 115, no. 5, pp. 621–625, 1989.

[4] C. Loddenkemper, C. Hoffmann, J. Stanke et al., "Regulatory (FOXP3+) T cells as target for immune therapy of cervical intraepithelial neoplasia and cervical cancer," *Cancer Science*, vol. 100, no. 6, pp. 1112–1117, 2009.

[5] P. Lohavanichbutr, J. Houck, W. Fan et al., "Genomewide gene expression profiles of HPV-positive and HPV-negative oropharyngeal cancer potential implications for treatment choices," *Archives of Otolaryngology—Head and Neck Surgery*, vol. 135, no. 2, pp. 180–188, 2009.

[6] N. Chocolatewala and P. Chaturvedi, "Role of human papilloma virus in the oral carcinogenesis: an Indian perspective," *Journal of Cancer Research and Therapeutics*, vol. 5, no. 2, pp. 71–77, 2009.

[7] G. R. Gunn, A. Zubair, C. Peters, Z. K. Pan, T. C. Wu, and Y. Paterson, "Two Listeria monocytogenes vaccine vectors that express different molecular forms of human papilloma virus-16 (HPV-16) E7 induce qualitatively different T cell immunity that correlates with their ability to induce regression of established tumors immortalized by HPV-16," *Journal of Immunology*, vol. 167, no. 11, pp. 6471–6479, 2001.

[8] D. A. Sewell, V. Shahabi, G. R. Gunn III, Z. K. Pan, M. E. Dominiecki, and Y. Paterson, "Recombinant Listeria vaccines containing PEST sequences are potent immune adjuvants for the tumor-associated antigen human papillomavirus-16 E7," *Cancer Research*, vol. 64, no. 24, pp. 8821–8825, 2004.

[9] T. Verch, Z. K. Pan, and Y. Paterson, "Listeria monocytogenes-based antibiotic resistance gene-free antigen delivery system applicable to other bacterial vectors and DNA vaccines," *Infection and Immunity*, vol. 72, no. 11, pp. 6418–6425, 2004.

[10] S. F. Jabbar, L. Abrams, A. Glick, and P. F. Lambert, "Persistence of high-grade cervical dysplasia and cervical cancer requires the continuous expression of the human papillomavirus type 16 E7 oncogene," *Cancer Research*, vol. 69, no. 10, pp. 4407–4414, 2009.

[11] H. Zur Hausen, "Papillomaviruses and cancer: from basic studies to clinical application," *Nature Reviews Cancer*, vol. 2, no. 5, pp. 342–350, 2002.

[12] I. T. Øvestad, E. Gudlaugsson, I. Skaland et al., "Local immune response in the microenvironment of CIN2-3 with and without spontaneous regression," *Modern Pathology*, vol. 23, no. 9, pp. 1231–1240, 2010.

[13] S. Adurthi, S. Krishna, G. Mukherjee, U. D. Bafna, U. Devi, and R. S. Jayshree, "Regulatory T cells in a spectrum of HPV-induced cervical lesions: cervicitis, cervical intraepithelial neoplasia and squamous cell carcinoma," *The American Journal of Reproductive Immunology*, vol. 60, no. 1, pp. 55–65, 2008.

[14] S. Patel and S. Chiplunkar, "Host immune responses to cervical cancer," *Current Opinion in Obstetrics and Gynecology*, vol. 21, no. 1, pp. 54–59, 2009.

[15] S. F. Hussain and Y. Paterson, "Regulatory T cells that secreast TGFβ and IL-10 are preferentially induced by a vaccine vector," *Journal of Immunotherapy*, vol. 27, no. 5, pp. 339–346, 2004.

[16] J. Nitcheu-Tefit, M. S. Dai, R. J. Critchley-Thorne et al., "Listeriolysin O expressed in a bacterial vaccine suppresses $CD4^+CD25^{high}$ regulatory T cell function in vivo," *Journal of Immunology*, vol. 179, no. 3, pp. 1532–1541, 2007.

[17] V. Shahabi, M. Reyes-Reyes, A. Wallecha, S. Rivera, Y. Paterson, and P. Maciag, "Development of a Listeria monocytogenes based vaccine against prostate cancer," *Cancer Immunology, Immunotherapy*, vol. 57, no. 9, pp. 1301–1313, 2008.

[18] V. Shahabi, M. M. Seavey, P. C. MacIag, S. Rivera, and A. Wallecha, "Development of a live and highly attenuated Listeria monocytogenes-based vaccine for the treatment of Her2/neu-overexpressing cancers in human," *Cancer Gene Therapy*, vol. 18, no. 1, pp. 1–10, 2011.

[19] P. C. Maciag, S. Radulovic, and J. Rothman, "The first clinical use of a live-attenuated *Listeria monocytogenes* vaccine: a Phase I safety study of *Lm*-LLO-E7 in patients with advanced carcinoma of the cervix," *Vaccine*, vol. 27, no. 30, pp. 3975–3983, 2009.

[20] J. Rothman, A. Wallecha, P. C. Maciag, S. Rivera, V. Shahabi, and Y., Paterson, "The use of a living Listeria monocytogenes as an active immunotherapy for the treatment of cancer," in *Emerging Cancer Therapy: Microbial Approaches and Biotechnological Tools*, A. M. Fialho and A. M. Chakrabarty, Eds., John Wiley & Sons, New York, NY, USA, 2010.

[21] L. Campisi, S. M. Soudja, J. Cazareth et al., "Splenic $CD8\alpha^+$ dendritic cells undergo rapid programming by cytosolic bacteria and inflammation to induce protective $CD8^+$ T-cell memory," *European Journal of Immunology*, vol. 41, pp. 1594–1605, 2011.

[22] L. M. Weiskirch and Y. Paterson, "Listeria monocytogenes: a potent vaccine vector for neoplastic and infectious disease," *Immunological Reviews*, vol. 158, pp. 159–169, 1997.

[23] R. Singh and A. Wallecha, "Cancer immunotherapy using recombinant Listeria monocytogenes: transition from bench to clinic," *Human Vaccines*, vol. 7, no. 5, pp. 497–505, 2011.

[24] A. Wallecha, P. C. Maciag, S. Rivera, Y. Paterson, and V. Shahabi, "Construction and characterization of an attenuated Listeria monocytogenes strain for clinical use in cancer immunotherapy," *Clinical and Vaccine Immunology*, vol. 16, no. 1, pp. 96–103, 2009.

[25] S. Dramsi and P. Cossart, "Listeriolysin O: a genuine cytolysin optimized for an intracellular parasite," *Journal of Cell Biology*, vol. 156, no. 6, pp. 943–946, 2002.

[26] C. S. Schmidt, W. J. W. Morrow, and N. A. Sheikh, "Smart adjuvants," *Expert Review of Vaccines*, vol. 6, no. 3, pp. 391–400, 2007.

[27] P. Neeson, Z. K. Pan, and Y. Paterson, "Listeriolysin O is an improved protein carrier for lymphoma immunoglobulin idiotype and provides systemic protection against 38C13 lymphoma," *Cancer Immunology, Immunotherapy*, vol. 57, no. 4, pp. 493–505, 2008.

[28] Y. Paterson, "Rational approaches to immune regulation," *Immunologic Research*, vol. 27, no. 2-3, pp. 451–462, 2003.

[29] X. Peng, J. Treml, and Y. Paterson, "Adjuvant properties of listeriolysin O protein in a DNA vaccination strategy," *Cancer Immunology, Immunotherapy*, vol. 56, no. 6, pp. 797–806, 2007.

[30] V. Shahabi, P. C. Maciag, S. Rivera, and A. Wallecha, "Live, attenuated strains of Listeria and Salmonella as vaccine vectors in cancer treatment," *Bioengineered Bugs*, vol. 1, no. 4, pp. 235–243, 2010.

[31] J. A. Vázquez-Boland, M. Kuhn, P. Berche et al., "Listeria pathogenesis and molecular virulence determinants," *Clinical Microbiology Reviews*, vol. 14, no. 3, pp. 584–640, 2001.

[32] R. Singh, M. E. Dominiecki, E. M. Jaffee, and Y. Paterson, "Fusion to Listeriolysin O and delivery by Listeria monocytogenes enhances the immunogenicity of HER-2/neu and reveals subdominant epitopes in the FVB/N mouse," *Journal of Immunology*, vol. 175, no. 6, pp. 3663–3673, 2005.

[33] R. E. Berg, E. Crossley, S. Murray, and J. Forman, "Relative contributions of NK and CD8 T cells to IFN-γ mediated innate immune protection against Listeria monocytogenes," *Journal of Immunology*, vol. 175, no. 3, pp. 1751–1757, 2005.

[34] G. Ikonomidis, Y. Paterson, F. J. Kos, and D. A. Portnoy, "Delivery of a viral antigen to the class I processing and presentation pathway by Listeria monocytogenes," *Journal of Experimental Medicine*, vol. 180, no. 6, pp. 2209–2218, 1994.

[35] A. S. MacFarlane, D. Huang, M. G. Schwacha, J. J. Meissler, J. P. Gaughan, and T. K. Eisenstein, "Nitric oxide mediates immunosuppression induced by Listeria monocytogenes infection: quantitative studies," *Microbial Pathogenesis*, vol. 25, no. 5, pp. 267–277, 1998.

[36] S. A. dos Santos, D. R. de Andrade Júnior, and D. R. de Andrade, "TNF-α production and apoptosis in hepatocytes after Listeria monocytogenes and Salmonella Typhimurium invasion," *Revista do Instituto de Medicina Tropical de Sao Paulo*, vol. 53, no. 2, pp. 107–112, 2011.

[37] Y. Iizawa, J. F. Brown, and C. J. Czuprynski, "Early expression of cytokine mRNA in mice infected with Listeria monocytogenes," *Infection and Immunity*, vol. 60, no. 10, pp. 4068–4073, 1992.

[38] M. M. Freeman and H. K. Ziegler, "Simultaneous Th1-type cytokine expression is a signature of peritoneal CD4+ lymphocytes responding to infection with *Listeria monocytogenes*," *Journal of Immunology*, vol. 175, no. 1, pp. 394–403, 2005.

[39] A. Demuth, W. Goebel, H. Ulrich Beuscher, and M. Kuhn, "Differential regulation of cytokine and cytokine receptor mRNA expression upon infection of bone marrow-derived macrophages with Listeria monocytogenes," *Infection and Immunity*, vol. 64, no. 9, pp. 3475–3483, 1996.

[40] T. Nishibori, H. Xiong, I. Kawamura, M. Arakawa, and M. Mitsuyama, "Induction of cytokine gene expression by listeriolysin O and roles of macrophages and NK cells," *Infection and Immunity*, vol. 64, no. 8, pp. 3188–3195, 1996.

[41] M. Bajénoff, E. Narni-Mancinelli, F. Brau, and G. Lauvau, "Visualizing early splenic memory CD8+ T cells reactivation against intracellular bacteria in the mouse," *PLoS One*, vol. 5, no. 7, Article ID e11524, 2010.

[42] G. A. Corbin and J. T. Harty, "Duration of infection and antigen display have minimal influence on the kinetics of the CD4+ T cell response to *Listeria monocytogenes* infection," *Journal of Immunology*, vol. 173, no. 9, pp. 5679–5687, 2004.

[43] M. Hamon, H. Bierne, and P. Cossart, "Listeria monocytogenes: a multifaceted model," *Nature Reviews Microbiology*, vol. 4, no. 6, pp. 423–434, 2006.

[44] A. Kolb-Mäurer, F. Weissinger, O. Kurzai, M. Mäurer, M. Wilhelm, and W. Goebel, "Bacterial infection of human hematopoietic stem cells induces monocytic differentiation," *FEMS Immunology and Medical Microbiology*, vol. 40, no. 2, pp. 147–153, 2004.

[45] T. L. Whiteside, "Immune suppression in cancer: effects on immune cells, mechanisms and future therapeutic intervention," *Seminars in Cancer Biology*, vol. 16, no. 1, pp. 3–15, 2006.

[46] D. A. Drevets, "*Listeria monocytogenes* infection of cultured endothelial cells stimulates neutrophil adhesion and adhesion molecule expression," *Journal of Immunology*, vol. 158, no. 11, pp. 5305–5313, 1997.

[47] D. A. Drevets, "*Listeria monocytogenes* virulence factors that stimulate endothelial cells," *Infection and Immunity*, vol. 66, no. 1, pp. 232–238, 1998.

[48] S. Kayal, A. Lilienbaum, C. Poyart, S. Memet, A. Israel, and P. Berche, "Listeriolysin O-dependent activation of endothelial cells during infection with *Listeria monocytogenes*: activation of NF-κB and upregulation of adhesion molecules and chemokines," *Molecular Microbiology*, vol. 31, no. 6, pp. 1709–1722, 1999.

[49] D. A. Sewell, D. Douven, Z. K. Pan, A. Rodriguez, and Y. Paterson, "Regression of HPV-positive tumors treated with a new Listeria monocytogenes vaccine," *Archives of Otolaryngology—Head and Neck Surgery*, vol. 130, no. 1, pp. 92–97, 2004.

[50] A. Lamikanra, Z. K. Pan, S. N. Isaacs, T. C. Wu, and Y. Paterson, "Regression of established human papillomavirus type 16 (HPV-16) immortalized tumors in vivo by vaccinia viruses expressing different forms of HPV-16 E7 correlates with enhanced CD8+ T-cell responses that home to the tumor site," *Journal of Virology*, vol. 75, no. 20, pp. 9654–9664, 2001.

[51] S. H. Kim, F. Castro, Y. Paterson, and C. Gravekamp, "High efficacy of a Listeria-based vaccine against metastatic breast cancer reveals a dual mode of action," *Cancer Research*, vol. 69, no. 14, pp. 5860–5866, 2009.

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Review Article

Lung Cancer and Human Papilloma Viruses (HPVs): Examining the Molecular Evidence

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Human papilloma virus (HPV), known to be an etiological agent for genital cancers, has been suggested also to be a possible contributory agent for lung cancer. Alternatively, lung cancer, formerly considered to be solely a smoker's disease, may now be more appropriately categorised into never smoker's and smoker's lung cancer. Through this paper we attempt to bring forth the current knowledge regarding mechanisms of HPV gaining access into the lung tissue, various strategies involved in HPV-associated tumorigenesis in lung tissue.

1. Introduction

The World Health Organization (WHO) states lung cancer to be the most common cause of cancer-related deaths worldwide (1.3 million/year). The most important and common cause of lung cancer is the long-term exposure to tobacco smoke. Nevertheless, the global burden of 15% of men and 53% of women with lung cancer, which is not attributable to tobacco smoking, amounts up to 25% of all lung cancer cases worldwide [1]. That would position lung cancer in never smokers among the top ten of the causes of cancer-related deaths worldwide. Suggested risk factors for the disease in never smokers include environmental tobacco smoke, radon gas, cooking oil vapours, indoor coal and wood burning, asbestos, genetic factors, and viral agents.

Syrjänen in 1979 first suggested the possibility of human papilloma virus (HPV) involvement in bronchial squamous cell carcinoma [2]. Several studies after that, put together, further suggest strongly a role for HPV as an aetiological agent of lung cancer. Starting from the preliminary step of HPV gaining access to the lung tissue, discrete molecular and genetic changes have to occur for tumor initiation and progression. However, studies on HPV and lung cancer are very limited, of which epidemiological reports outnumber molecular mechanisms. There are several questions to be answered to get a complete picture of any possible mechanism for HPV-induced lung cancer. This paper tries to summarize work carried out in this field and dwells into possible mechanisms of molecular pathogenesis of HPV-induced lung cancer.

2. HPV and Carcinogenesis

Human papilloma viruses, belonging to the Papillomaviridae family, are DNA viruses and are strictly host specific and exquisitely tissue tropic, having a preference to infect cutaneous or internal mucosal surfaces. Nearly 200 subtypes of HPV have been identified which are categorized as high risk (HR) and low risk (LR) based on their oncogenic potential. HPV infects the keratinocytes found in the basal layer of the skin (stratum germinativum). Several investigations have convincingly proved the presence of human papilloma virus in the lesions of upper aerodigestive tract (UADT). We have previously described the distribution of HPV types in oral cancer [3] as well as in the larynx [4]. The process of HPV-induced cell transformation is a combined manifestation of several discrete cellular, genetic, and molecular alterations accumulated in the mucosal tissue, termed "condemned mucosa syndrome," which later progresses onto invasive

cancer [5]. The viral proteins E6 and E7 contribute predominantly to the process of carcinogenesis and further tumor progression. These oncoproteins interact with critical cell cycle regulators to hamper their activity ensuing deregulation of the cell cycle machinery leading to uncontrolled cell proliferation. The virally encoded E6 binds to a cellular ubiquitin/protein ligase, E6-AP, and to p53 resulting in ubiquitination of p53 leading to its proteolytic degradation [6]. On the other hand, the E7 oncoprotein binds to the pRb dissociating the transcription factor E2F from the pRb/E2F complex, resulting in the transcriptional activation of several genes which facilitate cell proliferation. Rb/E7 complex formation is important for E7-induced cell transformation [7].

3. HPV and the Lung

3.1. Why to Study HPV in relation to Lung Cancer? The Epidemiology. Roglić et al. in 1975 and Rubel and Reynolds in 1979 provided first evidence for involvement of HPV in benign bronchial lesions, through the observations of koilocytosis in sputum samples characteristic of HPV infections [8, 9]. Syrjänen described that the epithelial changes seen in bronchial carcinoma closely resembled HPV-induced genital lesions [2].

Klein et al., in 2008, have beautifully concised worldwide epidemiological data available in the HPV incidence in lung cancer [10]. The study suggests an incidence of 24.5% of HPV-associated lung cancers. The distribution pattern ranges from a prevalence of 15% in the American continent and 17% in Europe to a mean incidence of 35.7% in Asia. In majority of the studies, the high-risk subtypes of HPV detected in the lung cancer tissues were 16, 18, 31, and 33, and the most prevalent low-risk subtypes were HPV 6 and 11. The alarmingly high percentages of incidences reported in Greece (69%), Taiwan (78.5%), and Japan (79%) suggest HPV to be the second most important possible etiological agent of lung cancer after cigarette smoking and advocate the need for further research in this issue.

3.2. The Histological Fondness. The human respiratory tract has a divergent histology of the mucous lining with columnar epithelium throughout the respiratory tract and stratified columnar epithelium covering the mucosa of the pharynx and larynx. The resulting squamocolumnar junctions (SCJs) can be compared to a similar junction in the cervix and could favour establishment of HPV infections. Lung cancer can be histologically categorised, based on the biology, therapy, and prognosis as small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC). NSCLCs are further classified into squamous cell carcinoma (SCC), adenocarcinoma (ADC), and large-cell lung carcinoma (LCLC). Lung cancer in never smokers when considered as a different disease is more frequent in the NSCLCs than in SCLCs [11].

3.3. Mode of Transmission. HPV cannot bind to the live tissue, instead infects the epithelial tissue through the exposed basal keratinocytes following microabrasions of skin as would occur after a sexual intercourse. However, how a well-protected organ like lung deeply positioned in the thoracic cavity, where probability of acquiring abrasions and thereby getting exposed to HPV, is not clear. Multiple sex partners, oral-genital sex, and oral-anal sex could be some of the factors favouring transmission of HPV to the oral cavity and oropharyngeal cancers. Prevalence of oncogenic mucosal HPV is higher in younger age oral cavity or oropharynx cancer cases whose sexual practices are typically associated with sexual transmission of the virus [12].

The study results from two different cancer registries in the United States, Connecticut, and the Surveillance, Epidemiology, and End Results (SEER) program tumor registries report evidence of shared etiological factors of various second primary cancers following anal and cervical cancer. There were 275 cases of lung or bronchial cancer following cervical cancer recorded in these registries as compared with 91.8 expected cases, a relative risk of 3.0 (95% confidence interval 2.7–3.4) [13]. Studies conducted by Hennig et al. found that 49% of bronchopulmonary carcinomas were detected to be HPV DNA positive in the women who had a clinical history of CIN III lesions [14].

HPV 16 and 18 E6 mRNA detected in the peripheral blood of cervical cancer patients [15] suggests a possibility that HPV infection in the lung tissue may originate in the cervix and later infect lung via a hematogenous spread (Figure 1) [16]. It has also been suggested that the presence of circulating HPV 16/18 DNA may act as a risk marker for lung cancer [17]. Peripheral blood mononuclear cells (PBMCs) can function as HPV carriers and might spread the virus through blood. In all the samples tested positive, the HPV genome was found to be in the episomal form, although in a low copy number [18].

All these reports put together designate oncogenic HPV DNA as a possible risk factor in developing second primary cancers after HPV-related primary neoplasias. Whether or not persistent HPV infection or HPV-induced cervical cancer is a prerequisite for the development of HPV-associated lung cancer needs further investigation.

3.4. Entry of Virus into the Cells. The entry of HPV into the cells *in vitro* is initiated by binding to a cell surface receptor in contrast to the *in vivo* situation where the basement membrane is a primary site of virus binding [19]. Widely distributed and evolutionary conserved cell surface receptors, heparin sulphate, and stabilizing proteoglycans (HSPGs) are presumed to be epithelial cell receptors for HPV [20]. The existence of HSPGs on the cell surface, [21] as well as extracellular matrix [22] of lung fibroblasts, can make them suitable receptors for viral entry into lung tissue (Figure 1). As ubiquitous members of extracellular membrane (ECM) microenvironment and hence the cancer stem cell niche, HSPGs are major factors responsible for the microenvironment changes, involved in the tumor initiation, progression, and malignant conversion [23]. HSPG functions as an attachment factor in HPV infection, and the resulting interaction promotes essential conformational changes in viral capsid, but HSPGs are obviously not the cell

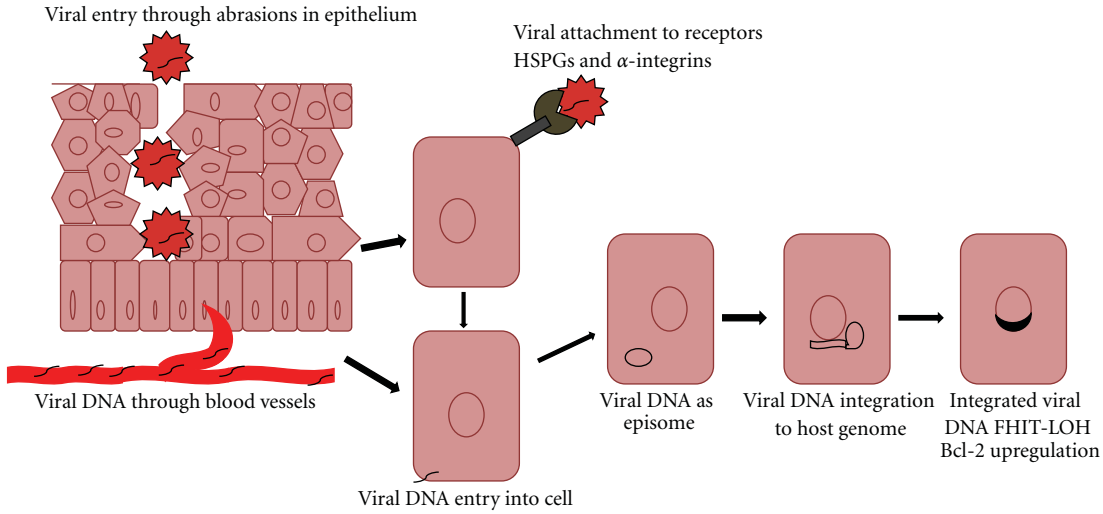

Figure 1: Viral entry and integration. The diagram shows the entry of viral DNA into the lung tissue either through the microabrasions in the membrane or through the haematogenous route from a different entry point as cervix or anus. Molecules such as HSPGs and α-integrins serve as surface receptors for the viral attachment and entry. Viral DNA exists and episome and later on integrates into the host genome losing an FHIT gene allele resulting in heterozygosity and subsequently upregulates the expression of proapoptotic protein Bcl-2.

surface receptors that arbitrate virion internalization and the subsequent events in infection. The cell adhesion receptors $\alpha 6$ integrins have close association with HSPGs as matrix components, and act as secondary receptors for HPV after its interaction with HSPG [19]. The drug surviving side population cells from human lung tumor tissue express the $\alpha 6$ integrin otherwise termed as very late antigen-6 (VLA-6) [24]. The presence of CD49f receptors (another term for $\alpha 6$ integrins) in the adult mouse lung stem cells have also been demonstrated [25] all suggestive of the possible involvement of lung cancer stem cells in the HPV entry leading to infection and cancer. The integrins, heterodimeric glycoproteins comprising of α and β subunits, are expressed in a variety of cell types, primarily involved in cell-matrix and cell-cell interactions, act as virus receptors, and aid in initial binding and/or internalization of viruses into the host tissue. Of the various α and β subunits known, $\alpha 6$, $\beta 4$, and $\beta 1$ play a major role in the HPV binding and internalization. The preferential expression of $\alpha 6$ subunit with the $\beta 4$ subunit forming the $\alpha 6\beta 4$ complex expressed exclusively in the stratified squamous epithelium [26], the primary sites of HPV infection, makes $\alpha 6\beta 4$-integrin the key receptor for HPV binding and possibly accounts for the predominance of HPV in squamous cell carcinoma than in other histologic subtypes of lung cancer.

3.5. Presence of HPV DNA in the Lung Cancer Tissue: Proof for a Causal Association. Although the presence of oncogenic HPV in the lung tumor tissue has been reported in several studies, the causal association of the HPV and lung cancer needs to be authenticated with evidence. An argument supporting the causative role of HPV in the tumorigenesis of lung tissue is that HPV DNA is indeed integrated into the host genome, which is the initial key step in the tumor initiation [27]. Furthermore, the viral oncoproteins E6 and E7 are in fact found to be expressed in the lung tumors, down regulating tumor suppressor genes such as p53 [28]. Taiwanese nonsmokers had significantly high prevalence of HPV16/18, suggesting HPV infection as a possible etiological agent of lung cancer in nonsmokers. Moreover, the nonsmoking females are more prone to HPV positivity than their male counterparts. Also, compared to the male smokers, the female nonsmokers are at a higher risk of having HPV in their lung tumor tissue [16]. The reason for this gender-based prevalence is yet unidentified. Though a few studies reported HPV absence in nonsmokers with lung cancer, this can be attributed to the technical variations in the experimental conditions such as specificity of the primers and probes used, disparity in the study subjects, and above all the overall prevalence of HPV in the study population.

4. HPV-Induced Lung Tissue Carcinogenesis: Possible Mechanisms

The process of HPV-mediated carcinogenesis is the consequence of an integrated process of defective apoptosis, neovascularisation, and cellular immortality [3]. Once the pathogen gains entry into its tissue of interest, it takes up the cellular machinery, replicates its genome, evades cell apoptosis, and initiates tumor formation. A few possible molecular events manifested upon HPV infection in addition to the already reported ones in the HPV-induced lung tumor tissue are summarised here.

4.1. FHIT LOH. Once inside the cells, HPV genome integrates into the host genome. The fragile site FRA3B, adjacent to the fragile histidine triad (FHIT) is a site of integration of HPV genome. The FHIT gene located in chromosome 3p14.2 undergoes frequent allele loss of heterogeneity (LOH) or homozygous deletions in several cancers including lung

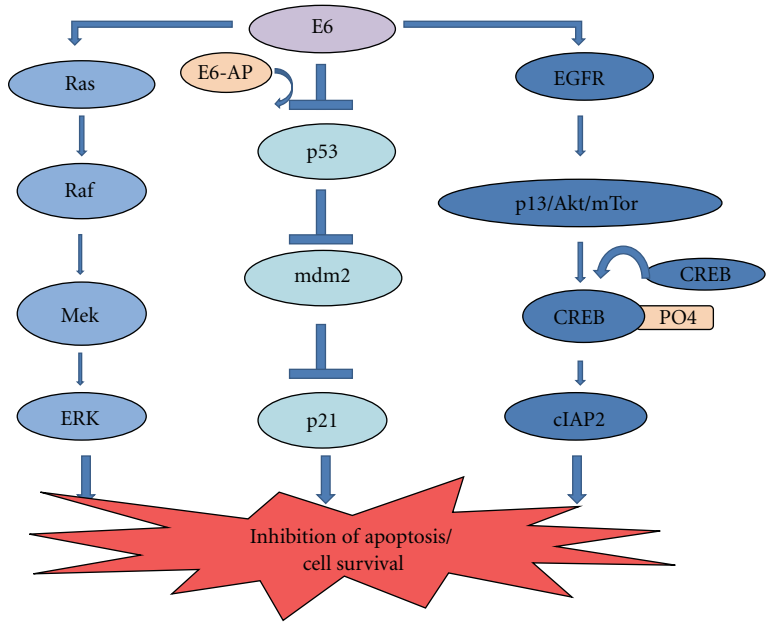

Figure 2: E6-mediated survival signaling. The figure summarizes the different survival signalling pathways activated by the viral protein E6. While it activates the mitogenic signals in the Ras/Raf/Mek pathway, E6 blocks the p53 activation in turn inhibiting the action of p21, all ultimately inducing cell survival. E6 also upregulates the expression of epidermal growth factor receptor (EGFR) and inhibits apoptosis by the activation of an inhibitor-of-apoptosis protein (cIAP2) thereby obstructing apoptosis.

cancer. Upon integration of HPV to this site, allele loss can occur to the FHIT gene (Figure 1). A study conducted among HPV infected, nonsmoking, female lung cancer patients from Taiwan suggested that tumorigenesis may be in part due to the increase in frequency of the FHIT LOH, reducing the expression of FHIT [29].

4.2. Bcl-2 Up-Regulation. The status of HPV in the tissue may determine expression level of the proapoptotic protein Bcl-2, the levels of which being significantly higher in integrated HPV positive patients than those that were negative or with episomal forms (Figure 1) [30].

4.3. Ras Gene Mutations. One of the most important molecular changes documented in lung cancer progression is activation of dominant oncogenes such as ras family genes. Of various genes in the ras family, k-ras gene is found to have point mutations in codon 12, in most of the lung carcinomas. Also, in 50% of HPV positive lung tumors, k-ras mutation was seen to coexist, constitutively activating the Ras/Raf/Mek pathway (Figure 2). This suggests that HPV infection is not sufficient by itself for malignant transformation but requires cooperation of the activated ras gene [31].

4.4. Does the E6 and E7 Genes Have the Same Role and Importance in Lung Cancer Pathogenesis as They Have in Cervical Cancer?

4.4.1. E6-p53. The E6 protein has a well-recognised signature in pathogenesis of HPV-induced cervical and oral cancer. E6 is the major viral protein which can activate two independent pathways to prevent apoptosis-p53-dependent and p53-independent pathway [32]. Relationship between E6 and p53 has been conclusively shown in HPV-infected cells [33]. E6 protein binds to the E6-AP, a host cell ubiquitin ligase, and p53 tumor suppressor protein simultaneously and induces accelerated proteosomal degradation of p53 (Figure 2) [6].

Kinoshita et al. in 1995 reported expression of E6 mRNA in the HPV 18 DNA-positive lung tumor tissues [34]. In this study, they also put forward the possibility of cellular targets of HPV other than p53. HPV E6 proteins are indeed expressed in lung tumor tissues, which are negatively associated with the p53 expression. The corresponding downregulation of mdm2 and p21 (Waf1/Sdi1/Cip1), which are the downstream targets of p53, leads to the conclusion that HPV E6 protein can inactivate the expression of p53 in lung tumor tissues (Figure 2) [27]. The human Dead-box RNA helicase (DDX3), which plays a role in regulation of gene expression via RNA metabolism, transcription, splicing, mRNA export, and translation, has been associated with development of viral associated cancers. DDX3 transcription is predominantly regulated by p53, and p21 transcription is being synergistically suppressed by the alteration of the p53-DDX3 pathway via E6, in lung tumors. Reduction of p21 make lung cancer patients vulnerable to tumor reversion and thereby bring about poor relapse-free survival [35].

4.4.2. E6-cIAP. The viral oncoprotein E6 can also promote carcinogenesis by upregulating expression of inhibitors of antiapoptosis proteins (IAPs). Inhibitors of antiapoptosis proteins (IAPs) directly inhibit caspases to block cell

apoptosis. HPV E6 upregulates the expression of cIAP2 via epidermal growth factor receptor (EGFR)/phosphatidyl inositol 3-kinase (PI3K)/AKT cascade. CREB, which is a regulatory targeting molecule of AKT, and is phosphorylated via the EGFR/PI3K/AKT pathway, which plays a crucial role in the upregulation of cIAP2 by E6 protein (Figure 2). This upregulation has been shown to confer resistance to cisplatin in HPV 16/18 infected lung cancer [36].

4.4.3. E6-Bak. The Bcl-2 homologous antagonist/killer (Bak) protein is a proapoptotic member of the Bcl-2 gene family which is involved in apoptosis. In the p53-independent pathway, E6 protein binds the Bak protein and degrades it. The proapoptotic effect of Bak is a main target of HPV E6 protein. E6 from high-risk HPV subtypes (16,18) and low-risk type (11) can bind to Bak, *in vitro*, and can stimulate its degradation, *in vivo*. Bak can associate with E6-associated protein in the absence of E6 in contrast to p53, and the subsequent inhibits the Bak-induced apoptosis through this E6-AP-dependent process [37]. Though the status of Bak expression in HPV-induced lung cancer is yet to be studied, we strongly suspect this mechanism to be active in promoting the tumorigenesis of lung tissue.

4.4.4. E7-pRb. The coexpression of E7 along with the E6 is indispensable for the increased oncogenic activity of the HPV. The E7 protein of the high-risk HPV has a strong affinity to bind to pRb, abrogating the pRb signalling pathway. Kinoshita et al. in 1995 reported the coexpression of E7 mRNA along with E6 mRNA in the HPV 18 DNA-positive lung tumor tissue [34]. The cell cycle regulatory gene CDKN2A regulated by pRb is found to be altered in the HPV associated tumors. Of the two proteins encoded by CDKN2A, $p16^{INK4a}$ and $p14^{ARF}$, the former prevents the cells from S-phase entry by inhibiting CDK4/6-mediated phosphorylation of Rb and the $p14^{ARF}$ is a p53 stabilizer. $p16^{INK4a}$ gene is epigenetically modified by CpG island promoter hypermethylation, thus silencing it. HPV E7 inactivates the pRb, releasing the histone deacetylases (HDAC) from the E2F-Rb-HDAC complex to enhance $p16^{INK4a}$ hypermethylation through chromatin remodelling by HDAC (Figure 3). Enzymes named DNA methyl transferases (DNMTs) mainly of three types (DNMT1, DNMT3a, and DNMT3b) by their complex interplay establish the cytosine methylation pattern. The $p16^{INK4a}$ promoter hypermethylation was analysed using methylation-specific PCR (MSP), the results of which showed $p16^{INK4a}$ hypermethylation in 59.7% of smoking males, 36.6% of nonsmoking males, and 60.3% of nonsmoking females lung tumors among Taiwanese population [38]. $p16^{INK4a}$ hypermethylation frequency in nonsmoking female lung tumors with HPV infection was as high as 70% compared to those without HPV infection, which was as low as 30% [38]. A statistically significant correlation was observed only in the nonsmoking female lung cancer cases ($P = 0.017$), but not in male smoking or nonsmoking lung tumors. Furthermore, reports from the same research group demonstrated involvement of HPV infection in increased expression of the DNMT3b (DNA methyl transferase), to cause $p16^{INK4a}$ promoter hypermethylation among the nonsmoking female lung tumor (Figure 3) [39].

4.5. Role of E5 Protein of HPV in the Transforming Activity. The HPV E5 viral protein has been shown to have weak transforming activity. Once the HPV genome is integrated into the host genome, the E5 gene is frequently deleted, and hence the E5 gene expression is terminated during the progression of disease from the low grade to malignant stage [40, 41]. So if at all E5 protein has an effect on the process of carcinogenesis, it has to act as an early-stage protein, that is, before the HPV genome integration. The key activity of HPV E5 seems to be the upregulation of EGF receptors in a ligand-dependent manner. The E5 protein controls mitogenic signalling pathways by forming complex with EGFR [42, 43]. This complex formation enhances the activation by EGF [43]. HPV E5 exerts its action by upregulation of the EGF receptor in a ligand-dependent manner. The mechanism by which the HPV E5 does this appears to be due to (i) a decrease in the rate of receptor degradation mediated by binding of E5 to the 16 kDa subunit of the endosomal proton pump ATPase and (ii) a net increase in the receptor phosphorylation without decreased downregulation [44, 45]. E5 protein can also activate MAP kinases via PKC (protein kinase C) and Ras-mediated receptor tyrosine kinase (RTK) pathway. The E5 protein can activate the nuclear oncogenes, c-jun and jun-B, through the activation protein-1 (AP-1) binding site [46]. The activity of tumor-suppressor gene p21 (Waf1/Sdi1/Cip1) is being repressed through the activation of c-jun by the HPV E5 protein, which promotes cell proliferation [47]. Suppression of p21 gene by HPV E5 activates the CDK4-cyclin D complexes, which in turn phosphorylates pRb and inactivates pRb checkpoint control leading to uncontrolled cell replication (Figure 4).

The fact that the EGFRs are often upregulated in the lung tumor tissues highlights the possibility of the E5 protein playing a role in the early stages of the transformation process. On the contrary, Crusius et al. reported that the HPV 16 E5 protein modulates the stress-dependent activation of ERK1/2 and p38 MAP kinase activation in human keratinocytes by an EGF-independent mechanism [48]. No studies are reported yet, indicating the presence of HPV E5 protein in the lung tumor tissues. Studies towards this aspect would probably suggest new vaccine candidates or therapeutic targets against HPV-induced lung cancer.

4.6. Does ER-EGFR Crosstalk Favour HPV Persistence Leading to Tumorigenesis? HPV-Induced Lung Cancer Is More Prevalent in Females Than in Males. Estrogen contributes to a large extent to the onset of HPV infection and tumor progression [49]. Though the presence of estrogen receptors (ERs) in human lung tissue has been controversial for many years, it has been proved beyond doubt that some forms of both ERα and ERβ are indeed present in normal lung cells as well as in lung tumors, which are undeniably functional, as evidenced by the estrogen-induced cell proliferation in the lung *in vitro* as well as *in vivo*; activation

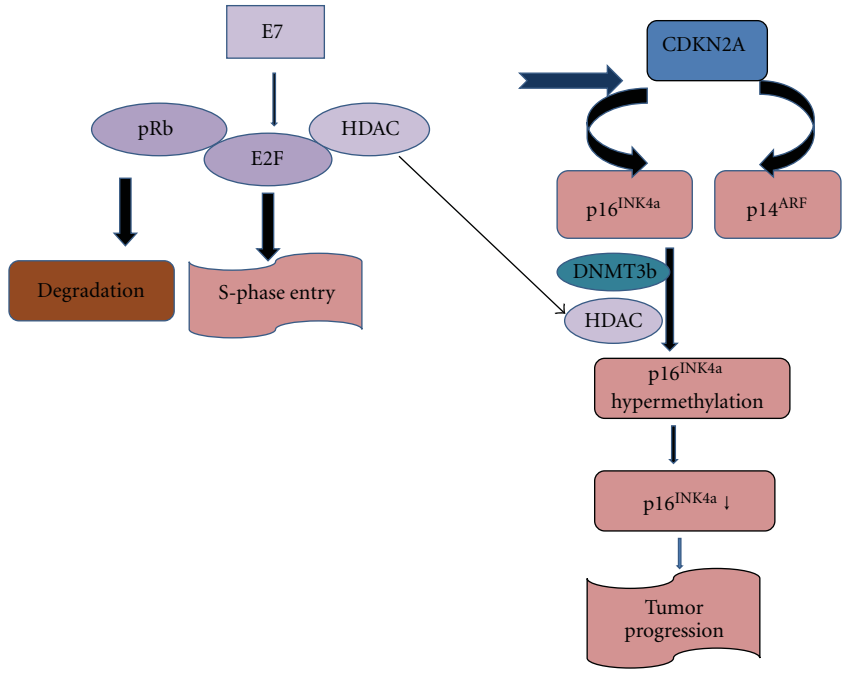

Figure 3: E7-dependent tumorigenic signaling. Diagram summing up the steps targeted by the viral protein E7 in the tumorigenesis of the lung tissue. The histone deacetylases (HDAC) gets released from the retinoblastoma protein (pRb), elongation factor (E2F), and HDAC complex when E7 binds to it and leads to the downregulation of tumor-suppressor-p16^{INK4} A protein by hypermethylation thus leading to tumorigenesis.

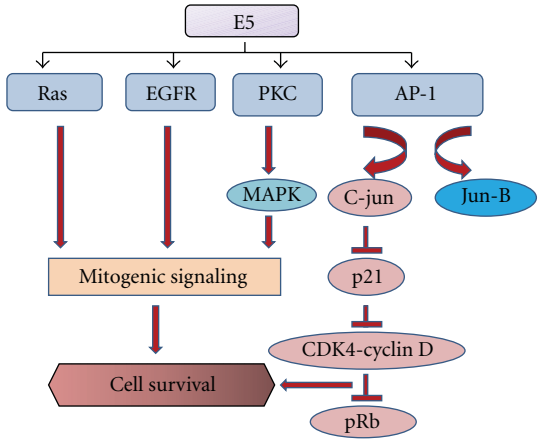

Figure 4: Possible role of E5 protein in tumor initiation. This figure condenses the multiple pathways that are suspected to be activated by viral protein E5 leading to cell survival. The mitogenic signaling downstream of Ras, epidermal growth factor receptor (EGFR) and protein kinase C (PKC) are turned on by the E5 protein. Additionally, E5 can also activate the activator protein-1 (AP-1), which through c-jun may result in cell survival.

of transcription from estrogen response elements (ERE) in lung cancer cells by estrogen and estrogen-stimulated secretion of a growth factor thought to be involved in lung tumorigenesis, collectively demonstrating that ERs play a biological role in the lungs [50]. Aromatase, the enzyme involved in catalysing the biosynthesis of estrogen, is induced in cervical carcinomas favouring the increased expression of viral oncogenes E6 and E7 [51]. This is expressed in lung tissue also which catalyses the local production of estrogen from androgen and is suggested to be a predictive biomarker for the prognosis of NSCLCs [52]. This enhanced local site production of estrogen triggers the nongenomic mechanism of estrogen action, which rapidly activates EGFR. The well-established crosstalk between ER and EGFR in head and neck cancers [53] arbitrates this action which is further validated by the colocalised membrane ER and EGFR in the lung tumors. Combined targeting of ER and EGFR in NSCLC has been proved to enhance antiproliferative effects in the treatment of lung cancer [54]. We strongly argue that this ER-EGFR crosstalk could occur in the lung tissue which can consequently favour HPV persistence and malignant transformation of the lung tissue (Figure 5). Furthermore, when this probable crosstalk fits in position, the increased vulnerability of female never smokers to develop HPV-induced lung cancer as well as the histologic affinity of HPV to NSCLC is better explained.

4.7. Estrogen—Hypoxia-Induced Factor—EGFR Crosstalk. Hypoxia-inducible factors (HIFs) are heterodimeric transcription factors that respond to changes in available oxygen in the cellular environment, specifically, to decreases in oxygen, or hypoxia by promoting the transcription of several hypoxia inducible genes. HIFs are frequently upregulated in solid tumors and are found to be expressed in three isoforms—HIF-1, HIF-2, and HIF-3— each with an α and

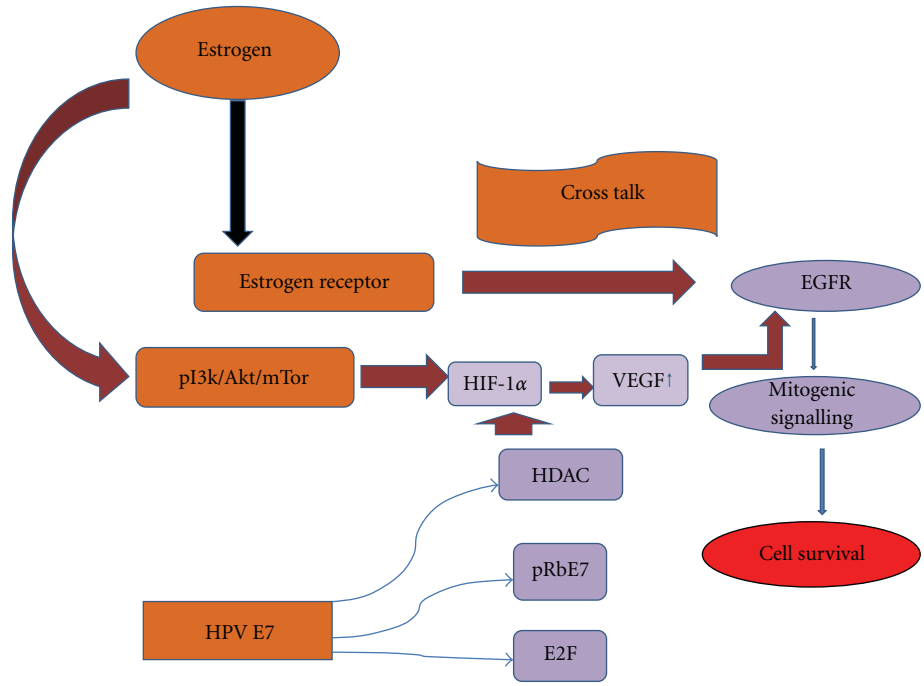

Figure 5: Possible estrogen-EGFR-HIF-1α crosstalk. Diagrammatic representation of the possible crosstalks between estrogen, epidermal growth factor receptor (EGFR), and hypoxia-inducible factor-1α (HIF-1α) activating the mitogenic signalling resulting in the cell survival.

a β subunit. Estrogen can regulate the HIF-1α expression through PI3K-Akt pathway (Figure 5) [55]. The oncogenic protein E7 of HPV props up HIF-1α-dependent transcription by blocking the interaction of HDACs with HIF-1α in a manner dependent on the HDAC binding domain of E7. The E6 protein primarily blocks the inhibitory effects of p53 on HIF-1 activity [56]. HIF-1 induces expression of the genes essential for adapting to hypoxia including those for erythropoietin, glucose transporters, glycolytic enzymes, and vascular endothelial growth factor (VEGF), eliciting successful homeostatic regulation under hypoxic conditions. In cervical cancer, VEGF upregulates EGFR and downregulates IGF-BP3, thus amplifying the cell proliferative activity of EGFR (Figure 5). This action of VEGF seems to be mediated, directly through EGFR or indirectly through HPV-E6 in the HPV-positive cancers [57]. EGFR upregulation triggers the downstream mitogenic signals supporting tumorigenesis. We strongly suspect this mechanism to be active in HPV-infected lung tissue which favours angiogenesis and metastasis.

5. Therapeutic Strategies

Would the vaccines such as Gardasil, Cervarix, and so forth, directed towards the cervical cancer ensure protection against HPV-induced lung cancer too? This would be a focal question of interest that needs to be addressed to combat the disease prophylactically. Given that the HPV subtypes infecting and most of the molecular events occurring in the cervical cancer may be extended to HPV-induced lung cancer, these vaccines can presumed to be effective against lung cancer too.

Lung cancer in nonsmokers is molecularly different disease from that seen in the smoker population. Mutation frequency as well as profile of the genes encoding EGFR, p53 and k-ras is conspicuously dissimilar in smoking and nonsmoking groups. Also, the HPV infection paves way to altering the signalling pathways leading to cancer. The fact that EGFR has a crucial role in lung carcinogenesis makes it an admirable target for therapy. Monoclonal antibodies targeted against EGF receptors, small molecule TK inhibitors, and antisense oligonucleotides can reduce the activity of EGFR thereby inactivating the mitogenic signalling pathways like Ras/Raf/Mek and PI3k/Akt/mTor pathway. One such anti-EGFR monoclonal antibody being used is cetuximab , in combination with chemotherapeutic agents such as Carboplatin and Paclitaxel, when provided concurrently or sequentially gave positive results in clinical trials [58–60]. Other than these, matuzumab which is a human monoclonal antibody, along with chemotherapy and certain other multikinase inhibitors, can be used in the treatment of advanced non-small-cell lung cancer [61]. Tyrosine kinase inhibitors such as Gefitinib interrupt the downstream signalling activated during the binding of ligands or mutational activation of EGFR [62]. Combined targeting of estrogen receptor and EGFR, using fulvestrant and gefitinib has proved to have enhanced antiproliferative effects in the *in vivo* studies [55]. Combination therapy targeting HIFs and EGFR would probably also enhance the treatment outcome.

MEK, a mitogenic signalling pathway protein activated as a result of k-ras mutations in HPV infection is another, suitable target for therapy. Ci-1040 difluorobenzamide is a MEK

inhibitor which has been clinically tested in NSCLC patients [63]. Inhibitors of mammalian target of rapamycin (mTor), a protein kinase which regulates cell growth by regulating different cell processes: Rapamycin and Temsirolimus, gave partial response in patients with NSCLC [64].

6. Conclusion and Future Perspectives

From the reported studies and suspected crosstalks, we robustly argue EGFR to be a core molecular hub in pathogenesis of HPV-induced lung cancer. It can be presumed that combination therapy targeting EGFR and other molecules having crosstalk with EGFR may be effective in treatment of the disease rather than a single target therapy. It is still not apparent whether HPV is a causal factor of lung cancer or whether it is just an opportunistic pathogen in the lung cancer tissue and the exact molecular mechanisms behind it. Almost all of the signalling pathways having a role in lung cancer are found to be altered or blocked by human papilloma viral proteins initiating tumorigenesis. Further evidence is mandatory to substantiate beyond doubt the causative role of HPV in the lung tissue tumorigenesis. Moreover, the cofactors supplementing the HPV in the transformation processes are yet to be classified. Studies directed towards these targets would give a clearer image of the disease which will eventually pave the way to designing new prophylactic or therapeutic strategies in combating the disease.

References

[1] D. M. Parkin, F. Bray, J. Ferlay, and P. Pisani, "Global cancer statistics, 2002," *Ca: A Cancer Journal for Clinicians*, vol. 55, no. 2, pp. 74–108, 2005.

[2] K. J. Syrjänen, "Condylomatous changes in neoplastic bronchial epithelium. Report of a case," *Respiration*, vol. 38, no. 5, pp. 299–304, 1979.

[3] M. R. Pillai, A. Phanidhara, A. L. Kesari, P. Nair, and M. K. Nair, "Cellular manifestations of human papillomavirus infection in the oral mucosa," *Journal of Surgical Oncology*, vol. 71, no. 1, pp. 10–15, 1999.

[4] S. E. Jacob, S. Sreevidya, E. Chacko, and M. R. Pillai, "Cellular manifestations of human papilloma virus infection in laryngeal tissues," *Journal of Surgical Oncology*, vol. 79, pp. 142–150, 2002.

[5] M. R. Pillai and M. K. Nair, "Development of a condemned mucosa syndrome and pathogenesis of human papillomavirus-associated upper aerodigestive tract and uterine cervical tumors," *Experimental and Molecular Pathology*, vol. 69, no. 3, pp. 233–241, 2000.

[6] A. Ferenczy and E. Franco, "Persistent human papillomavirus infection and cervical neoplasia," *The Lancet Oncology*, vol. 3, no. 1, pp. 11–16, 2002.

[7] S. L. Gonzalez, M. Stremlau, X. He, J. R. Basile, and K. Münger, "Degradation of the retinoblastoma tumor suppressor by the human papillomavirus type 16 E7 oncoprotein is important for functional inactivation and is separable from proteasomal degradation of E7," *Journal of Virology*, vol. 75, no. 16, pp. 7583–7591, 2001.

[8] M. Roglić, S. Jukić, and I. Damjanov, "Cytology of the solitary papilloma of the bronchus," *Acta Cytologica*, vol. 19, no. 1, pp. 11–13, 1975.

[9] L. R. Rubel and R. E. Reynolds, "Cytologic description of squamous cell papilloma of the respiratory tract," *Acta Cytologica*, vol. 23, no. 3, pp. 227–230, 1979.

[10] F. Klein, W. F. M. Amin Kotb, and I. Petersen, "Incidence of human papilloma virus in lung cancer," *Lung Cancer*, vol. 65, no. 1, pp. 13–18, 2009.

[11] S. Sun, J. H. Schiller, and A. F. Gazdar, "Lung cancer in never smokers—a different disease," *Nature Reviews Cancer*, vol. 7, no. 10, pp. 778–790, 2007.

[12] E. M. Smith, J. M. Ritchie, K. F. Summersgill et al., "Age, sexual behavior and human papillomavirus infection in oral cavity and oropharyngeal cancers," *International Journal of Cancer*, vol. 108, no. 5, pp. 766–772, 2004.

[13] C. S. Rabkin, R. J. Biggar, M. Melbye, and R. E. Curtis, "Second primary cancers following anal and cervical carcinoma: evidence of shared etiologic factors," *American Journal of Epidemiology*, vol. 136, no. 1, pp. 54–58, 1992.

[14] E. M. Hennig, Z. Sou, F. Karlsen, R. Holm, S. Thoresen, and J. M. Nesland, "HPV positive bronchopulmonary carcinomas in women with previous high-grade cervical intraepithelial neoplasia (CIN III)," *Acta Oncologica*, vol. 38, no. 5, pp. 639–647, 1999.

[15] C. J. Tseng, C. C. Pao, J. D. Lin, Y. K. Soong, J. H. Hong, and S. Hsueh, "Detection of human papillomavirus types 16 and 18 mRNA in peripheral blood of advanced cervical cancer patients and its association with prognosis," *Journal of Clinical Oncology*, vol. 17, no. 5, pp. 1391–1396, 1999.

[16] Y. W. Cheng, H. L. Chiou, G. T. Sheu et al., "The association of human papillomavirus 16/18 infection with lung cancer among nonsmoking Taiwanese women," *Cancer Research*, vol. 61, no. 7, pp. 2799–2803, 2001.

[17] H. L. Chiou, M. F. Wu, Y. C. Liaw et al., "The presence of human papillomavirus type 16/18 DNA in blood circulation may act as a risk marker of lung cancer in Taiwan," *Cancer*, vol. 97, no. 6, pp. 1558–1563, 2003.

[18] S. Bodaghi, L. V. Wood, G. Roby, C. Ryder, S. M. Steinberg, and Z. M. Zheng, "Could human papillomaviruses be spread through blood?" *Journal of Clinical Microbiology*, vol. 43, no. 11, pp. 5428–5434, 2005.

[19] C. A. Horvath, G. A. Boulet, V. M. Renoux, P. O. Delvenne, and J. P. J. Bogers, "Mechanisms of cell entry by human papillomaviruses: an overview," *Virology Journal*, vol. 7, article 11, 2010.

[20] T. Giroglou, L. Florin, F. Schäfer, R. E. Streeck, and M. Sapp, "Human papillomavirus infection requires cell surface heparan sulfate," *Journal of Virology*, vol. 75, no. 3, pp. 1565–1570, 2001.

[21] V. Lories, J. J. Cassiman, H. Van den Berghe, and G. David, "Differential expression of cell surface heparan sulfate proteoglycans in human mammary epithelial cells and lung fibroblasts," *Journal of Biological Chemistry*, vol. 267, no. 2, pp. 1116–1122, 1992.

[22] A. Heremans, J. J. Cassiman, H. van den Berghe, and G. David, "Heparan sulfate proteoglycan from the extracellular matrix of human lung fibroblasts. Isolation, purification, and core protein characterization," *Journal of Biological Chemistry*, vol. 263, no. 10, pp. 4731–4739, 1988.

[23] L. M. Haupt and L. R. Griffiths, "Heparan sulfate proteoglycans, tumour progression and the cancer stem cell niche," *Current Cancer Therapy Reviews*, vol. 5, no. 4, pp. 256–260, 2009.

[24] V. Levina, A. M. Marrangoni, R. DeMarco, E. Gorelik, and A. E. Lokshin, "Drug-selected human lung cancer stem cells: cytokine network, tumorigenic and metastatic properties," *PLoS One*, vol. 3, no. 8, Article ID e3077, 2008.

[25] J. L. McQualter, K. Yuen, B. Williams, and I. Bertoncello, "Evidence of an epithelial stem/progenitor cell hierarchy in the adult mouse lung," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 4, pp. 1414–1419, 2010.

[26] T. Letian and Z. Tianyu, "Cellular receptor binding and entry of human papillomavirus," *Virology Journal*, vol. 7, article 2, 2010.

[27] F. Aguayo, A. Castillo, C. Koriyama et al., "Human papillomavirus-16 is integrated in lung carcinomas: a study in Chile," *British Journal of Cancer*, vol. 97, no. 1, pp. 85–91, 2007.

[28] Y. W. Cheng, M. F. Wu, J. Wang et al., "Human papillomavirus 16/18 E6 oncoprotein is expressed in lung cancer and related with p53 inactivation," *Cancer Research*, vol. 67, no. 22, pp. 10686–10693, 2007.

[29] J. Wang, Y. W. Cheng, D. W. Wu et al., "Frequent FHIT gene loss of heterozygosity in human papillomavirus-infected non-smoking female lung cancer in Taiwan," *Cancer Letters*, vol. 235, no. 1, pp. 18–25, 2006.

[30] Y. Xu, B. Cheng, H. Pan, A. Wu, and L. Zhang, "The relationship between the status of human papillomavirus 16/18 infection and the expression of Bcl-2 and Bax in squamous cell carcinomas of the lung," *Chinese Journal of Lung Cancer*, vol. 12, no. 8, pp. 849–852, 2009.

[31] A. Noutsou, M. Koffa, M. Ergasaki, N. M. Siafakas, and D. A. Spandidos, "Detection of human papilloma virus and K-ras mutations in lung carcinomas," *International Journal of Oncology*, vol. 8, pp. 1089–1093, 1996.

[32] H. Pan and A. E. Griep, "Temporally distinct patterns of p53-dependent and p53-independent apoptosis during mouse lens development," *Genes and Development*, vol. 9, no. 17, pp. 2157–2169, 1995.

[33] M. R. Pillai, S. Halabi, A. McKalip et al., "The presence of human papillomavirus-16/-18 E6, p53, and bcl-2 protein in cervicovaginal smears from patients with invasive cervical cancer," *Cancer Epidemiology Biomarkers and Prevention*, vol. 5, no. 5, pp. 329–335, 1996.

[34] I. Kinoshita, H. Dosaka-Akita, M. Shindoh et al., "Human papillomavirus type 18 DNA and E6-E7 mRNA are detected in squamous cell carcinoma and adenocarcinoma of the lung," *British Journal of Cancer*, vol. 71, no. 2, pp. 344–349, 1995.

[35] D.-W. Wu, W.-S. Liu, J. Wang, C.-Y. Chen, Y.-W. Cheng, and H. Lee, "Reduced p21WAF1/CIP1 via alteration of p53-DDX3 pathway is associated with poor relapse-free survival in early-stage human papillomavirus-associated lung cancer," *Clinical Cancer Research*, vol. 17, no. 7, pp. 1895–1905, 2011.

[36] H. H. Wu, J. Y. Wu, Y. W. Cheng et al., "cIAP2 upregulated by E6 oncoprotein via epidermal growth factor receptor/phosphatidylinositol 3-kinase/AKT pathway confers resistance to cisplatin in human papillomavirus 16/18-infected lung cancer," *Clinical Cancer Research*, vol. 16, no. 21, pp. 5200–5210, 2010.

[37] M. Thomas and L. Banks, "Inhibition of Bak-induced apoptosis by HPV-18 E6," *Oncogene*, vol. 17, no. 23, pp. 2943–2954, 1998.

[38] M. F. Wu, Y. W. Cheng, J. C. Lai et al., "Frequent p16INK4a promoter hypermethylation in human papillomavirus-infected female lung cancer in Taiwan," *International Journal of Cancer*, vol. 113, no. 3, pp. 440–445, 2005.

[39] T. S. Lin, H. Lee, R. A. Chen et al., "An association of DNMT3b protein expression with P16INK4a promoter hypermethylation in non-smoking female lung cancer with human papillomavirus infection," *Cancer Letters*, vol. 226, no. 1, pp. 77–84, 2005.

[40] D. DiMaio and D. Mattoon, "Mechanisms of cell transformation by papillomavirus E5 proteins," *Oncogene*, vol. 20, no. 54, pp. 7866–7873, 2001.

[41] H. Zur Hausen and E. M. De Villiers, "Human papillomaviruses," *Annual Review of Microbiology*, vol. 48, pp. 427–447, 1994.

[42] M. Conrad, D. Goldstein, T. Andresson, and R. Schlegel, "The E5 protein of HPV-6, but not HPV-16, associates efficiently with cellular growth factor receptors," *Virology*, vol. 200, no. 2, pp. 796–800, 1994.

[43] E. S. Hwang, T. Nottoli, and D. Dimaio, "The HPV16 E5 protein: expression, detection, and stable complex formation with transmembrane proteins in COS cells," *Virology*, vol. 211, no. 1, pp. 227–233, 1995.

[44] S. W. Straight, P. M. Hinkle, R. J. Jewers, and D. J. McCance, "The E5 oncoprotein of human papilloma virus type 16 transforms fibroblasts and effects the down regulation of the epidermal growth factor receptor in keratinocytes," *Journal of Virology*, vol. 67, pp. 4521–4532, 1993.

[45] K. Crusius, E. Auvinen, B. Steuer, H. Gaissert, and A. Alonso, "The human papillomavirus type 16 E5-protein modulates ligand-dependent activation of the EGF receptor family in the human epithelial cell line HaCaT," *Experimental Cell Research*, vol. 241, no. 1, pp. 76–83, 1998.

[46] S.-L. Chen, C. H. Huang, T. C. Tsai, K. Y. Lu, and Y. P. Tsao, "The regulation mechanism of c-jun and junB by human papillomavirus type 16 E5 oncoprotein," *Archives of Virology*, vol. 141, no. 5, pp. 791–800, 1996.

[47] Y. P. Tsao, L. Y. Li, T. C. Tsai, and S. L. Chen, "Human papillomavirus type 11 and 16 E5 represses $p21^{Wafl/SdiI/CipI}$ gene expression in fibroblasts and keratinocytes," *Journal of Virology*, vol. 70, no. 11, pp. 7535–7539, 1996.

[48] K. Crusius, I. Rodriguez, and A. Alonso, "The human papillomavirus type 16 E5 protein modulates ERK1/2 and p38 MAP kinase activation by an EGFR-independent process in stressed human keratinocytes," *Virus Genes*, vol. 20, no. 1, pp. 65–69, 2000.

[49] T. Brake and P. F. Lambert, "Estrogen contributes to the onset, persistence, and malignant progression of cervical cancer in a human papillomavirus-transgenic mouse model," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 7, pp. 2490–2495, 2005.

[50] L. P. Stabile, A. L. Gaither Davis, C. T. Gubish et al., "Human non-small cell lung tumors and cells derived from normal lung express both estrogen receptor α and β and show biological responses to estrogen," *Cancer Research*, vol. 62, no. 7, pp. 2141–2150, 2002.

[51] H. B. Nair, R. Luthra, N. Kirma et al., "Induction of aromatase expression in cervical carcinomas: effects of endogenous estrogen on cervical cancer cell proliferation," *Cancer Research*, vol. 65, no. 23, pp. 11164–11173, 2005.

[52] D. C. Márquez-Garbán, H. W. Chen, L. Goodglick, M. C. Fishbein, and R. J. Pietras, "Targeting aromatase and estrogen signaling in human non-small cell lung cancer," *Annals of the New York Academy of Sciences*, vol. 1155, pp. 194–205, 2009.

[53] A. M. Egloff, M. E. Rothstein, R. Seethala, J. M. Siegfried, J. R. Grandis, and L. P. Stabile, "Cross-talk between estrogen receptor and epidermal growth factor receptor in head and

neck squamous cell carcinoma," *Clinical Cancer Research*, vol. 15, no. 21, pp. 6529–6540, 2009.

[54] L. P. Stabile, J. S. Lyker, C. T. Gubish, W. Zhang, J. R. Grandis, and J. M. Siegfried, "Combined targeting of the estrogen receptor and the epidermal growth factor receptor in non-small cell lung cancer shows enhanced antiproliferative effects," *Cancer Research*, vol. 65, no. 4, pp. 1459–1470, 2005.

[55] K. Hua, J. Din, Q. Cao et al., "Estrogen and progestin regulate HIF-1α expression in ovarian cancer cell lines via the activation of Akt signaling transduction pathway," *Oncology Reports*, vol. 21, no. 4, pp. 893–898, 2009.

[56] J. M. Bodily, K. P.M. Mehta, and L. A. Laimins, "Human papillomavirus E7 enhances hypoxia-inducible factor 1-mediated transcription by inhibiting binding of histone deacetylases," *Cancer Research*, vol. 71, no. 3, pp. 1187–1195, 2011.

[57] R. S. Mathur and S. P. Mathur, "Vascular endothelial growth factor (VEGF) up-regulates epidermal growth factor receptor (EGF-R) in cervical cancer in vitro: this action is mediated through HPV-E6 in HPV-positive cancers," *Gynecologic Oncology*, vol. 97, no. 1, pp. 206–213, 2005.

[58] F. Robert, G. Blumenschein, R. S. Herbst et al., "Phase I/IIa study of cetuximab with gemcitabine plus carboplatin in patients with chemotherapy-naïve advanced non-small-cell lung cancer," *Journal of Clinical Oncology*, vol. 23, no. 36, pp. 9089–9096, 2005.

[59] C. D. Thienelt, P. A. Bunn, N. Hanna et al., "Multicenter phase I/II study of cetuximab with paclitaxel and carboplatin in untreated patients with stage IV non-small-cell lung cancer," *Journal of Clinical Oncology*, vol. 23, no. 34, pp. 8786–8793, 2005.

[60] K. Kelly, R. S. Herbst, J. J. Crowley et al., "Concurrent chemotherapy plus cetuximab or chemotherapy followed by cetuximab in advanced non-small cell lung cancer (NSCLC): a randomized phase II selectional trial SWOG 0342," *Journal of Clinical Oncology*, vol. l24, no. 18S, p. 7015, 2006.

[61] C. Kollmannsberger, M. Schittenhelm, F. Honecker et al., "A phase I study of the humanized monoclonal anti-epidermal growth factor receptor (EGFR) antibody EMD 72000 (matuzumab) in combination with paclitaxel in patients with EGFR-positive advanced non-small-cell lung cancer (NSCLC)," *Annals of Oncology*, vol. 17, no. 6, pp. 1007–1013, 2006.

[62] N. Thatcher, A. Chang, P. Parikh et al., "Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small-cell lung cancer: results from a randomised, placebo-controlled, multicentre study," *The Lancet*, vol. 366, no. 9496, pp. 1527–1537, 2005.

[63] J. Rinehart, A. A. Adjei, P. M. LoRusso et al., "Multicenter phase II study of the oral MEK inhibitor, CI-1040, in patients with advanced non-small-cell lung, breast, colon, and pancreatic cancer," *Journal of Clinical Oncology*, vol. 22, no. 22, pp. 4456–4462, 2004.

[64] M. Hidalgo, J. C. Buckner, C. Erlichman et al., "A phase I and pharmacokinetic study of temsirolimus (CCI-779) administered intravenously daily for 5 days every 2 weeks to patients with advanced cancer," *Clinical Cancer Research*, vol. 12, no. 19, pp. 5755–5763, 2006.

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Research Article

Complex Etiology Underlies Risk and Survival in Head and Neck Cancer Human Papillomavirus, Tobacco, and Alcohol: A Case for Multifactor Disease

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Findings are inconsistent about whether tobacco, alcohol, and human papillomavirus (HPV) are two independent HNC risk factor groups that distinguish an infection-associated cancer from a tobacco/alcohol-associated HNC. We found that cancer in the oral cavity risk was greater in HPV-E6/E7 seropositive/heavy tobacco users (adjusted OR = 3.5) than in HPV-seronegative/heavy tobacco users (adjusted OR = 1.4); and HPV-seropositive/heavy alcohol users (adjusted OR = 9.8) had greater risk than HPV-seronegative/heavy alcohol users (adjusted OR = 3.1). In contrast, the risk of oropharyngeal cancer was greater in the HPV-seronegative/heavy tobacco (adjusted OR = 11.0) than in HPV-seropositive/heavy tobacco (adjusted OR = 4.7) users and greater in HPV-seronegative/heavy alcohol users (adjusted OR = 24.3) compared to HPV-seropositive/heavy alcohol users (adjusted OR = 8.5). Disease-specific and recurrence-free adjusted survival were significantly worse in oropharyngeal HPV-seronegative cases with no survival differences by HPV status seen in oral cavity cases. The association between tobacco/alcohol, HPV, and tumor site is complex. There appear to be distinct tumor site differences in the combined exposure risks, suggesting that different molecular pathways are involved.

1. Introduction

Human papillomavirus (HPV) is now an established risk factor for head and neck cancers (HNC) [1, 2]. Whether HPV is an independent risk factor of tobacco and alcohol, the other two major causes of tumors at these sites, has not been well clarified [1, 3–5]. It is known that those who are infected with HPV have significantly better survival [6, 7], thus it would be important to clarify the role of these risk factors. Many case-control studies traditionally have employed the anti-VLP-HPV antibody ELISA test to detect a history of HPV infection. Fewer studies have assessed the risk of HNC associated with antibodies against HPV E6/E7 oncoproteins. E6/E7 antibodies are biomarkers of HPV-associated cancer and possibly precancerous lesions and as such should provide a more sensitive measure of HPV-related disease in individuals and in evaluating the association with or independence from tobacco and alcohol. The purpose of this study was to examine HPV E6/E7 antibody status in patients with oral cavity and oropharyngeal HNC, and in healthy controls, for differences in HPV status associated with tobacco and alcohol use. The study also evaluated site-specific HNC survival and recurrence by HPV E6/E7 antibody status compared to HPV tumor tissue status associated with tobacco and alcohol.

2. Materials and Methods

2.1. Patient Data Collection. Patient characteristics and exclusions have been described previously [1]. Cases with a

primary HNC ages 18 and older diagnosed between 2001 and 2004 at the University of Iowa Hospitals, Department of Otolaryngology and the Iowa City Veterans Administration Hospital, were eligible for enrollment. All oral cavity ($N = 170$) and oropharyngeal ($N = 74$) sites were included. There were 244 eligible cases enrolled after exclusions. Gender and 5-year age group frequency matched controls ($n = 428$) were recruited from clinics in Family Medicine and in Internal Medicine at these hospitals who were seeking routine medical care.

Patients signed an institutionally approved informed consent form prior to completing a self-administered risk factor questionnaire that included information about sociodemographics, medical history, tobacco and alcohol use, sexual practices, and history of HPV-related diseases and oral lesions. Prior cancer history, head and neck tumor site, treatments, and staging were collected from medical records and pathology reports. All tobacco and alcoholic products were included and equivalents were used [1].

2.2. HPV Laboratory Methods. A blood specimen was drawn from participants to detect HPV at the time of diagnosis prior to cancer treatment for cases or during the interview for controls. Detection of HPV-specific antibodies has been described previously [3–8]. The Luminex procedure detected the presence of HPV E6 and E7 and HPV types were identified with multiplex serology [9, 10]. Multiplex serology uses viral L1 proteins expressed in bacteria as glutathione S-transferase (GST) fusion proteins as antigens [10, 11].

Paraffin-embedded tumor tissue was available from 204 cases to evaluate for HPV. Sample preparation, PCR analyses, DNA hybridization, and HPV typing procedures for assessment of tumor tissue were based on a standard protocol [12]. Each PCR reaction included primers to amplify the β-globin gene [13] and verified sufficient DNA and adequacy of the PCR amplification. Extracted DNA was PCR-amplified with MY09/MY11 primers [14] to detect HPV and with primer HMB01 to better amplify HPV-51 [15]. Positive samples underwent heminested PCR-amplification with MY09 and GP5+ primers [16]. DNA sequencing was used to determine the HPV types in each specimen, and sequences were compared to GenBank sequences using the BLAST program [17]. High-risk, oncogenic HPV types (HR-HPV) detected in tumor tissue included HPV-16 and 33.

2.3. Statistics. Odds ratios (OR), 95% confidence intervals (CI) and P values for risk factors, and pathologic characteristics were adjusted for continuous age, gender, tobacco pack-years, and average drinks per week. Categorical variables included age, education, number of sexual partners, tobacco pack-years, and average number of alcoholic drinks per week. Cut points for moderate and heavy tobacco or alcohol users were based on the overall distributions or median values among HPV-negative cases and controls. ORs and CIs were calculated using multivariate logistic regression analyses. For variables with zero cells, ORs and CIs were generated using logit methods, adjusting for categorical age, tobacco, and alcohol except when limited by small cell sizes.

Individual and joint effects of tobacco and alcohol stratified by HPV status were examined by tumor site and never users of tobacco or alcohol (reference groups) were compared to moderate or heavy tobacco or alcohol users among cases and controls. Additive joints effects and 95% CIs were assessed using the synergy index [18] while multiplicative interactions were examined by including the appropriate interaction term in the multivariate logistic regression models [19]. Survival curves were generated using Kaplan-Meier methods while Cox proportional hazards models were used to generate hazard ratios (HR) and 95% confidence intervals (CI), adjusted for continuous age and stage of disease (I/II versus III/IV). Nodal involvement, tumor site, and grade were excluded from models due to their high correlation with disease stage. Gender, pack-years, and drinks per week were not associated with survival or recurrence and thus were not included in the models. Analyses comparing HPV E6/E7 assays and tumor HPV DNA included only those patients with results from both assays ($N = 204$ cases). Sensitivity was defined as the percent of HPV DNA positive who were E6/E7 positive; specificity as the percent of HPV DNA negative who were E6/E7 negative; and, concordance as the percent positive for both tests and negative for both tests. Magnitude of agreement was measure by the Kappa statistic. All P values were two sided, and values ≤ 0.05 were considered statistically significant. Statistical analyses were performed using SAS version 9.2.

3. Results

3.1. Risk Factors. The prevalence of HPV-16/18/33 E6 and/or E7 was greater in HNC cases than in controls: 26% versus 7%; it was higher in oropharyngeal cases (64%) compared to oral cavity cases (9%) or controls (7%; Table 1). HPV-16 was the most frequent type detected in cases and controls (25%/5%), followed by HPV-33 (15%/2%), and HPV-18 (0.8/0.9%). Almost all cases with HPV-33 also were detected with HPV-16 (97%) whereas only a third (33%) of controls were. Those detected with HPV-18 were not detected with any other HPV type. The frequency of HPV-16 E6 or E7 only was similar in both cases (20%/18%) and controls (1%/4%). Compared to HPV-seronegative cases, HPV-seropositive cases were significantly more likely to be male. Those with tumors in the oropharynx who were HPV E6/E7 seropositive were more likely to be younger, more educated, and perform oral-genital sex (Table 1). They also showed higher grade and positive nodal status than did tumors in the oral cavity. No differences among controls were identified.

3.2. HPV E6/E7, Tobacco, and Alcohol Status Associated with Site-Specific HNC Risk. We next examined the relationship between tobacco, alcohol, and HPV E6/E7 status by tumor site (Table 2). Compared to controls, those with cancer of the oral cavity had a minimal increased risk among heavy tobacco users whereas the risk of oropharyngeal cancer was increased in both tobacco dose-duration groups and higher in heavy users. Parallel findings were seen for alcohol use although the risks were twice those seen for

Table 1: Association between HPV[1] E6 and/or E7 status with head and neck cancer risk factors.

Risk Factors	Oral cavity cases ($N = 170$)			Oropharynx cases ($N = 74$)			Controls ($N = 428$)		
	HPV E6/E7+ *N* (%)	HPV E6/E7− *N* (%)	Adjusted *P* value	HPV E6/E7+ *N* (%)	HPV E6/E7− *N* (%)	Adjusted *P* value	HPV E6/E+ *N* (%)	HPV E6/E7− *N* (%)	Adjusted *P* value
HPV prevalence	16 (9.4)	154 (90.6)		47 (63.5)	27 (36.5)		31 (7.2)	397 (92.8)	
Age[3]	Oral cavity positive versus oropharyngeal positive: *P* value = 0.01; oral cavity negative versus oropharyngeal negative: *P* value = 0.33								
Mean	64.7 (3.6)	61.2 (1.5)	0.35	54.5 (2.1)	58.2 (2.7)	0.28	56.8 (2.7)	58.1 (0.7)	0.63
Range	45–86	21–93		36–82	29–85		18–77	22–93	
≤ 55	6 (37.5)	55 (35.7)	0.96	29 (61.7)	11 (40.7)	0.05	14 (45.2)	166 (41.8)	0.83
>55	10 (62.5)	99 (64.3)		18 (38.3)	16 (59.3)		17 (54.8)	231 (58.2)	
Gender									
Male	14 (87.5)	79 (51.3)	0.03	40 (85.1)	19 (70.4)	0.04	21 (67.7)	247 (62.2)	0.10
Female	2 (12.5)	75 (48.7)		7 (14.9)	8 (29.6)		10 (32.3)	150 (37.8)	
Education[3]									
<12	5 (31.3)	34 (22.4)	0.58	5 (10.9)	11 (40.7)	0.07	2 (6.5)	52 (13.1)	0.87
12–15	9 (56.3)	87 (57.2)	0.50	32 (69.6)	10 (37.0)	0.51	18 (58.1)	226 (57.1)	0.61
≥16	2 (12.5)	31 (20.4)	Ref	9 (19.6)	6 (22.2)	Ref	11 (35.5)	118 (29.8)	Ref
Tobacco[4]									
Never	3 (18.8)	54 (35.3)	Ref	8 (17.4)	2 (7.4)	Ref	10 (34.5)	150 (37.9)	Ref
>0.0–30	3 (18.8)	41 (26.8)	0.59	19 (41.3)	6 (22.2)	0.85	15 (51.7)	127 (32.1)	0.24
>30	10 (62.5)	58 (37.9)	9.94	19 (41.3)	19 (70.4)	0.37	4 (13.8)	119 (30.1)	0.24
Alcohol[5]									
Never	3 (18.8)	57 (37.3)	Ref	6 (13.0)	2 (7.4)	Ref	14 (45.2)	170 (43.0)	Ref
1–21	5 (31.3)	54 (35.3)	0.92	27 (58.7)	10 (37.0)	0.67	15 (48.4)	166 (42.0)	0.95
>21	8 (50.0)	42 (27.5)	0.64	13 (28.3)	15 (55.6)	0.14	2 (6.5)	59 (14.9)	0.30
Number of Partners									
0-1	6 (42.9)	60 (42.9)	Ref	3 (7.1)	4 (16.7)	Ref	10 (34.5)	122 (32.3)	Ref
2–10	5 (35.7)	59 (42.1)	0.56	23 (54.8)	13 (54.2)	0.55	16 (55.2)	186 (49.2)	0.64
≥11	3 (21.4)	21 (15.0)	0.66	16 (38.1)	7 (29.2)	0.70	3 (10.3)	70 (18.5)	0.21
Oral-genital sex									
Yes	3 (37.5)	39 (49.4)	0.83	24 (92.3)	8 (50.0)	0.02	16 (51.6)	184 (53.8)	0.27
No	5 (62.5)	40 (50.6)		2 (7.7)	8 (50.0)		15 (48.4)	158 (46.2)	
Stage									
I/II	8 (50.0)	58 (39.5)	Ref	3 (6.8)	5 (19.2)	Ref			
III/IV	8 (50.0)	89 (60.5)	0.30	41 (93.2)	21 (80.8)	0.19			
Grade									
Poor/undifferentiated	5 (33.3)	23 (16.2)	0.13	25 (55.6)	3 (12.5)	0.004			
well/moderate	10 (66.7)	119 (83.8)	Ref	20 (44.4)	21 (87.5)	Ref			
Nodal status									
Yes	7 (43.7)	54 (36.0)	0.87	42 (89.4)	20 (74.1)	0.07			
No	9 (56.3)	96 (64.0)	Ref	5 (10.6)	7 (25.9)	Ref			

[1] Positive versus negative for HPV-16 or HPV-18 or HPV-33 E6 and/or E7 status; [2]adjusted for age, gender, tobacco, and alcohol; [3]years; [4]pack-years; [5]drinks/week.

Table 2: Site-specific head and neck cancer associated with tobacco, alcohol, and HPV E6/E7 status.[1]

Characteristic	Cases: Oral cavity (N = 170) N (%)	Cases: Oropharynx (N = 74) N (%)	Controls (N = 428) N (%)	Oral Cavity versus controls OR[2] (95% CI)	Oropharynx versus controls OR[2] (95% CI)
Tobacco					
Never	57 (33.7)	10 (13.7)	160 (37.7)	1.0	1.0
≤30	44 (26.0)	25 (34.2)	142 (33.4)	0.9 (0.5–1.4)	2.1 (0.9–5.2)
>30	68 (40.2)	38 (52.1)	123 (28.9)	1.5 (0.9–2.4)	5.6 (2.3–13.7)
Alcohol					
Never	60 (35.5)	8 (11.0)	184 (43.2)	1.0	1.0
≤21	59 (34.9)	37 (50.7)	181 (42.5)	1.1 (0.7–1.8)	4.3 (1.7–10.4)
>21	50 (29.6)	28 (38.4)	61 (14.3)	3.6 (2.1–6.2)	11.7 (4.2–32.7)
Tobacco/alcohol					
Never/never	42 (25.0)	3 (4.1)	100 (23.6)	1.0	1.0
≤30/≤21	23 (13.7)	14 (19.2)	72 (17.0)	1.0 (0.5–1.8)	5.2 (1.2–21.7)
≤30/>21	10 (6.0)	8 (11.0)	26 (6.1)	1.4 (0.6–3.5)	15.9 (3.2–80.2)
>30/≤21	22 (13.1)	17 (23.3)	55 (13.0)	1.3 (0.6–2.4)	16.6 (3.9–71.4)
>30/>21	39 (23.2)	19 (26.0)	29 (6.9)	5.2 (2.6–10.5)	34.6 (7.5–158.8)
HPV E6/E7 status[1]					
Positive	16 (9.4)	47 (63.5)	31 (7.2)	1.7 (0.9–3.3)	24.3 (12.9–45.8)
Negative[3]	154 (90.6)	27 (36.5)	397 (92.8)	1.0	1.0

[1] Percentages based on available data; [2]odds ratio adjusted by age (continuous), gender, HPV status, tobacco, and alcohol; [3]HPV E6/E7 negative status for all types, E6/E7 positive status for HPV-16, -18, and/or -33.

tobacco exposures, regardless of tumor site. The adjusted risk of tumors in the oral cavity did not increase in the joint heavy tobacco/alcohol users (>30/>21, OR = 5.2) over heavy tobacco users and heavy alcohol users. There was no multiplicative effect associated with tobacco/alcohol among tumors in the oropharynx for heavy users of both, but there was a significant additive effect (OR = 34.6). The independent risk was significantly greater among those with HPV-seropositive status after controlling for tobacco, alcohol, and other risk factors but only in tumors of the oropharynx.

3.3. Site-Specific HNC Risk Stratified by HPV, Tobacco, and Alcohol Status . Based on the distinct findings by tumor site for tobacco and alcohol risk, data were next stratified by HPV E6/E7 status (Table 3). Compared to controls, cases with oral cavity tumors who were heavy tobacco users had an elevated risk but only among those who were HPV-seropositive (OR = 3.5) but not HPV-seronegative. Those with oropharyngeal cancer had elevated risks at the higher tobacco dose-duration level as well but in both the HPV-seropositive and HPV-seronegative groups, with the odds much higher for the HPV-seronegative group (OR = 11.0 versus 4.7). HPV-seropositive risk was higher in the oropharynx than found in the oral cavity. The risk associated with alcohol use was elevated only in the heaviest users (>21) in oral cavity cases regardless of HPV E6/E7 status, but was higher in the HPV-seropositive group (OR = 9.8 versus 3.1). The ORs for the oropharynx were significantly elevated for both alcohol levels regardless of HPV E6/E7 status although, in contrast to the oral cavity, they were higher in the HPV-seronegative cases. Again there was little difference in risk between the two tumor sites among HPV-seropositive heavy users.

When both tobacco and alcohol were examined in stratified analyses associated with HPV status, only the heavy tobacco/alcohol (>30/>21) group was elevated for cancer of the oral cavity (Table 3). Consistent with the individual tobacco and alcohol groups, higher risk was particularly seen in those who were HPV E6/E7 seropositive. Again, a different picture was seen for oropharyngeal cancers. Increased risk was shown for almost all tobacco/alcohol groups compared to never users of both. Risks were greater among those who were HPV-seronegative/heavy tobacco/heavy alcohol users than among those who were HPV-seropositive/heavy users of both. CIs were wide due to the expected small rate of HPV E6/E7 positivity in controls.

3.4. Comparison between HPV E6/E7 Serology and HPV Tumor DNA . There were 145 oral cavity and 59 oropharynx cases with both serology and tumor tissue assessed for HPV status. The prevalence of HPV-seropositive in the oral cavity was 10% and 64% in the oropharynx, and 13%/58% for oral cavity/oropharynx tumor HPV DNA-positive status (Table 4). Type-specific concordance between HPV-positive tumors (HPV-16 and/or -33) and HPV-seropositivity was 93% (K = 0.9, 0.7–0.99) for the oropharynx and 84% (K = 0.2, 0.003–0.4) in the oral cavity. The sensitivity of HPV E6/E7 antibodies to DNA HPV-positive tumors in the oral cavity was 26% and 100% in the oropharynx.

Table 3: Site-specific head and neck cancer stratified by HPV E6/E7, tobacco, and alcohol status.[1]

		Cases		Controls	Oral cavity versus controls	Oropharynx versus controls
HPV E6/E7[2]	Risk factor	Oral cavity $N = 170$ (%)	Oropharynx $N = 74$ (%)	$N = 428$ (%)	OR (95% CI)	OR (95% CI)
	Tobacco[3]					
Negative	Never	54 (32.0)	2 (2.7)	150 (35.3)	1.0	1.0
	≤30	41 (24.3)	6 (8.2)	127 (29.9)	0.9 (0.6–1.5)	3.2 (0.6–16.6)
	>30	58 (34.3)	19 (26.0)	119 (28.0)	1.4 (0.8–2.3)	11.0 (2.4–51.7)
	test for trend *P* value				*0.19*	*<0.0001*
Positive	Never	3 (1.8)	8 (11.0)	10 (2.4)	1.0	1.0
	≤30	3 (1.8)	19 (26.0)	15 (3.5)	0.3 (0.03–2.4)	1.4 (0.4–4.9)
	>30	10 (5.9)	19 (26.0)	4 (0.9)	3.5 (0.5–26.9)	4.7 (1.03–21.7)
	test for trend *P* value				*0.008*	*0.01*
	Alcohol[4]					
Negative	Never	57 (33.7)	2 (2.7)	170 (39.9)	1.0	1.0
	≤21	54 (32.0)	10 (13.7)	166 (39.0)	1.1 (0.7–1.8)	5.4 (1.1–25.7)
	>21	42 (24.8)	15 (20.6)	59 (13.9)	3.1 (1.7–5.5)	24.3 (4.9–121.3)
	test for trend *P* value				*0.009*	*<0.0001*
Positive	Never	3 (1.8)	6 (8.2)	14 (3.3)	1.0	1.0
	≤21	5 (3.0)	27 (37.0)	15 (3.5)	1.2 (0.2–7.3)	4.1 (1.2–14.7)
	>21	8 (4.7)	13 (17.8)	2 (0.5)	9.8 (0.9–106.9)	8.5 (1.2–60.2)
	test for trend *P* value				*0.002*	*0.001*
	Tobacco/alcohol[5]					
Negative	Never/never	40 (23.8)	1 (1.4)	93 (22.0)	1.0	1.0
	≤30/≤21	21 (12.5)	1 (1.4)	63 (14.9)	1.0 (0.5–1.9)	1.9 (0.1–31.1)
	≤30/>21	10 (6.0)	4 (5.5)	24 (5.7)	1.5 (0.6–3.6)	22.8 (2.2-235.2)
	>30/≤21	20 (11.9)	8 (11.0)	53 (12.5)	1.2 (0.6–2.3)	18.7 (2.2–160.8)
	>30/>21	31 (18.4)	11 (15.1)	29 (6.7)	4.0 (2.0–8.3)	56.3 (6.2–512.7)
	test for trend *P* value				*0.07*	*<0.0001*
Positive	Never/never	2 (1.2)	2 (2.7)	7 (1.7)	1.0	1.0
	≤30/≤21	2 (1.2)	13 (17.8)	9 (2.1)	0.7 (0.04–12.0)	8.9 (0.96–82.9)[7]
	≤30/>21	0 (0.0)	4 (5.5)	2 (0.5)	0.6 (0.02–17.2)[6]	13.7 (0.8–227.9)
	>30/≤21	2 (1.2)	9 (12.3)	2 (0.5)	2.6 (0.1–67.4)	29.3 (2.1–408.7)[7]
	>30/>21	8 (4.8)	8 (11.0)	0 (0.0)	55.0 (0.8–3651.0)[6,7]	18.3 (0.5–627.2)[6,7]
	test for trend *P*-value				*0.008*	*0.001*

[1] Percentages based on available data; [2]HPV E6/E7 seronegative for HPV-16, -18, and/or -33; HPV E6/E7 seropositive for HPV-16, -18, and/or -33; [3]adjusted for age (continuous), gender, and average drinks/week (continuous); [4]adjusted for age (continuous), gender, and tobacco pack-years (continuous); [5]adjusted for age (continuous) and gender, all never groups combined with never/never as reference group; [6]Logit estimator, 0.5 is added to zero cells; [7]CMH *P*-value < 0.049.

Moderate smokers (compared to never or to heavy smokers) had elevated ORs for both HPV-seropositive and tumor HPV DNA-positive compared to HPV-negative status. Both moderate and heavy alcohol users had elevated ORs compared to never drinkers for either HPV assessment. For the joint effects of alcohol and tobacco, moderate smokers/drinkers (≤30/≤21) and moderate smokers/heavy drinkers (≤30/≥21) had the greatest odds of being HPV-positive; other risks were similar for serology and tumor tissue. Sample sizes were insufficient to evaluate risk factors for HPV serology and DNA by tumor site.

3.5. Survival and Recurrence Associated with HPV E6/E7 and Tumor DNA Status. Table 5 displays the HRs for disease-specific survival (DSS) and recurrence-free survival (RFS) for newly diagnosed patients with survival information (DSS = 144; RFS = 127). Also shown is a comparison of DSS and RFS between HPV tumor and serology among newly diagnosed cases with HPV results available for both outcomes ($N = 157$). Among those who did not survive, 56% died from HNC, 28% from other causes and 16% had an unknown cause. A first recurrence occurred in 30% of all patients. Median follow-up time for DSS was 5.3 years (range: 1

Table 4: Tobacco and alcohol status in head and neck cancer cases by HPV E6/E7[1,2] and tumor HPV status[1] ($N = 204$).

Risk factor	E6/E7 positive N (%)	E6/E7 negative N (%)	OR (95% CI)	Tumor positive[3] N (%)	Tumor negative[3] N (%)	OR (95% CI)
Prevalence	52 (25.5)	152 (74.5)		53 (26.0)	151 (74.0)	
Tumor site						
Oral cavity	14 (26.9)	131 (86.2)	1.0	19 (35.9)	126 (83.4)	1.0
Oropharynx	38 (73.1)	21 (13.8)	17.3 (7.4–40.5)	34 (64.2)	25 (16.6)	8.8 (4.1–19.0)
Tobacco[4]						
Never	8 (15.7)	49 (32.5)	1.0	9 (17.3)	48 (32.0)	1.0
≤30	20 (39.2)	37 (24.5)	2.1 (0.8–5.7)	23 (44.2)	34 (22.7)	3.0 (1.2–7.5)
>30	23 (45.1)	65 (43.0)	1.1 (0.4–3.1)	20 (38.5)	68 (45.3)	1.2 (0.5–3.4)
≤30 versus *>30*			*1.9 (0.8–4.2)*			2.3 (1.1–5.1)
Alcohol[5]						
Never	7 (13.7)	51 (33.6)	1.0	8 (15.4)	50 (33.1)	1.0
≤21	24 (47.1)	56 (36.8)	2.3 (0.8–6.2)	27 (51.9)	53 (35.1)	3.0 (1.2–7.8)
>21	20 (39.2)	45 (29.6)	2.1 (0.7–6.4)	17 (32.7)	48 (31.8)	2.2 (0.7–6.8)
≤21 versus *>21*			*1.1 (0.5–2.5)*	3 (5.8)	10 (6.7)	1.3 (0.6–3.0)
Tobacco/alcohol[6]						
Never//never	4 (7.8)	38 (25.2)	1.0			1.0
≤30/≤21	13 (25.5)	21 (13.9)	3.2 (0.9–12.0)	15 (28.9)	19 (12.7)	4.2 (1.2–14.0)
≤30/>21	4 (7.8)	10 (6.6)	1.8 (0.3–9.3)	5 (9.6)	9 (6.0)	2.6 (0.6–11.8)
>30/≤21	8 (15.7)	24 (15.9)	1.6 (0.4–6.7)	9 (17.3)	23 (15.3)	1.9 (0.5–6.8)
>30/>21	15 (29.4)	34 (22.5)	1.6 (0.4–5.9)	11 (21.2)	38 (25.3)	1.2 (0.3–4.2)

[1]Percentages based on available data; [2]HPV E6/E7 seronegative for HPV-16, and -33; HPV E6/E7 seropositive for HPV-16 and/or -33; [3]DNA negative for all HPV DNA types; DNA positive for HPV-16 and/or -33; [4]adjusted for age (continuous), gender, tumor site (oral cavity, oropharynx), and average drinks/week (continuous); [5]adjusted for age (continuous), gender, tumor site (oral cavity, oropharynx), and tobacco pack-years (continuous); [6]adjusted for age (continuous) and gender.

Table 5: Adjusted[1] hazard ratios for disease-specific and recurrence free survival.[2]

Risk Factors	Oral cavity				Oropharynx			
	Disease-specific Survival[1] HR (95% CI)		Recurrence-free survival[1] HR (95% CI)		Disease-specific survival[1] HR (95% CI)		Recurrence-free survival[1] HR (95% CI)	
	Patients with HPV serology ($N = 182$)							
		$N = 116$		$N = 96$		$N = 52$		$N = 43$
E6 and/or E7	n		n		n		n	
Negative	103 (88.8)	1.3 (0.4–4.2)	86 (89.6)	1.1 (0.3–3.6)	16 (30.8)	5.7 (1.7–20.0)	13 (30.2)	6.7 (1.7–26.1)
Positive	13 (11.2)	1.0	10 (10.4)	1.0	36 (69.2)	1.0	30 (69.8)	1.0
Age[2]		1.02 (1.0–1.1)		1.02 (1.0–1.04)		1.04 (0.96–1.1)		1.03 (0.95–1.1)
Stage								
0/I/II		1.0		1.0		1.0		1.0
III/IV		7.9 (2.8–22.5)		1.5 (0.7–3.1)		1.5 (0.2–11.4)[3]		1.3 (0.03–58.8)
	Patients with both HPV tumor and serology ($N = 157$)							
		$N = 100$		$N = 88$		$N = 44$		$N = 39$
E6 and/or E7	n		n		n		n	
Negative	89 (89.0)	1.4 (0.4–4.5)	80 (90.9)	1.1 (0.3–3.5)	14 (31.8)	4.2 (1.2–15.1)	12 (30.8)	10.4 (2.4–52.4)
Positive	11 (11.0)	1.0	8 (9.1)	1.0	30 (68.2)	1.0	27 (69.2)	1.0
HPV tumor status								
Negative	88 (88.0)	0.8 (0.3–2.0)	78 (88.6)	0.5 (0.2–1.3)	17 (38.6)	3.0 (0.9–10.7)	15 (38.5)	7.3 (1.5–36.4)
High Risk	12 (12.0)	1.0	10 (11.4)	1.0	27 (61.4)	1.0	24 (61.5)	1.0

[1]Adjusted for stage and continuous age; [2]in years; [3]odds ratio Logit estimator adjusted for stage and categorical age.

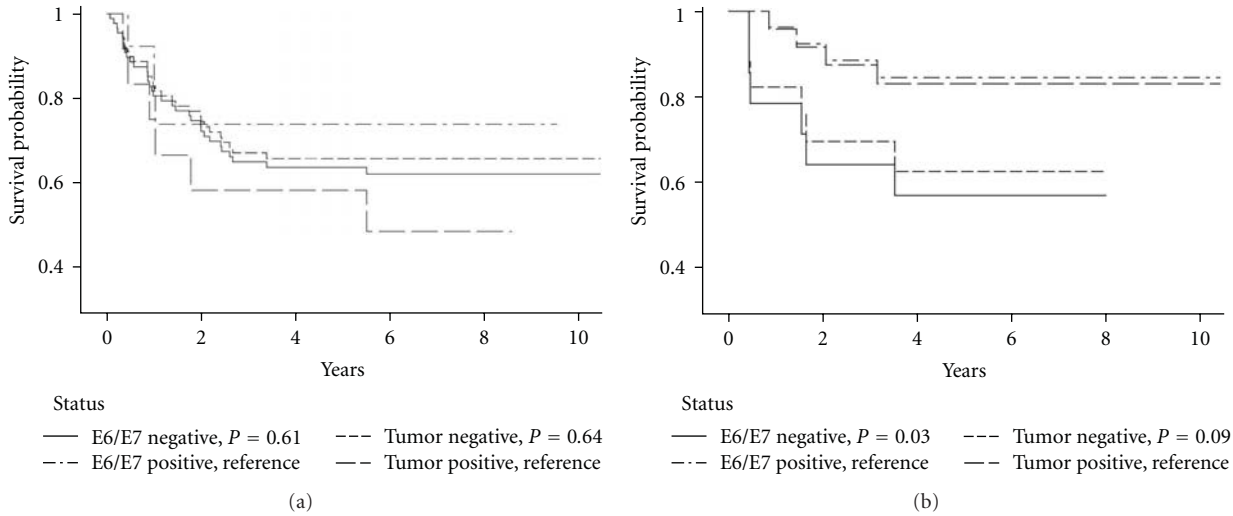

Figure 1: (a) Oral cavity disease-specific Kaplan-Meier survival curves by E6/E7 and tumor DNA status. (b) Oropharynx disease-specific Kaplan-Meier survival curves by E6/E7 and tumor DNA status.

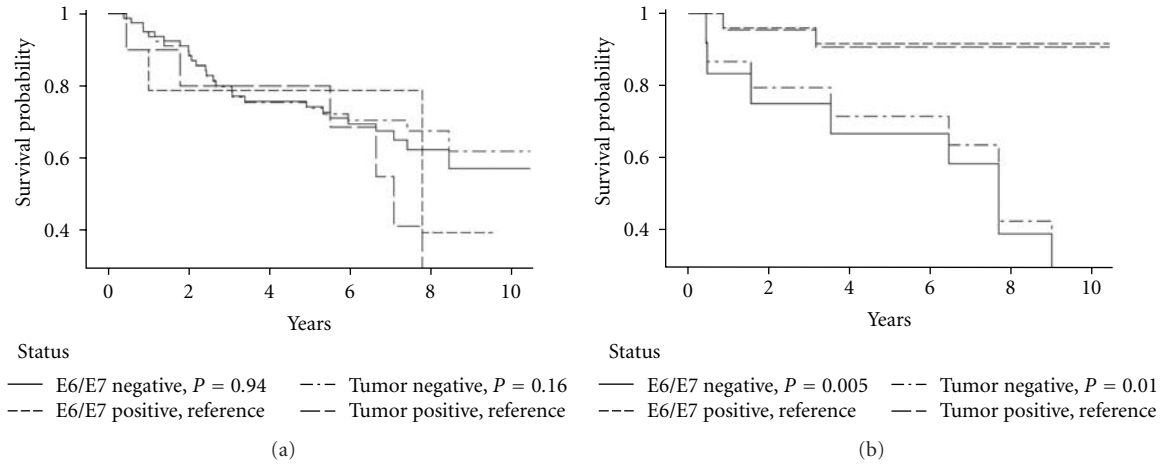

Figure 2: (a) Oral cavity recurrence-free specific Kaplan-Meier survival curves by E6/E7 and tumor DNA status. (b) Oropharynx recurrence-free specific Kaplan-Meier survival curves by E6/E7 and tumor DNA status.

month–10.4 years). Among those who had a DS death, 76% died within 2 years of diagnosis. The median follow-up time for RFS was 5.6 years (range: 2 months–10.4 years). For patients with a first recurrence, 76% occurred within 2 years of diagnosis. Time to recurrence did not significantly vary by HPV E6/E7 status for either site.

HPV status was not significantly related to DSS or RFS for oral cavity cancers (Table 5, Figures 1(a) and 2(a)). In contrast, oropharyngeal cancer cases who were HPV-seronegative or DNA-negative reported significantly lower DSS and RFS compared to HPV-positive cases (Table 5, Figures 1(b) and 2(b)). In a DSS model with tumor site, age, stage, alcohol, and tobacco, only age, higher stage, and tumor site were significant predictors of higher mortality risk (oral cavity versus oropharynx: HR = 2.1, 1.03–4.4).

Concordance between the HPV tests for both sites combined was high for DSS and RFS (88%/88%, $K = 0.7$ both). An examination of sensitivity and specificity for HPV E6/E7 antibodies for HPV DNA status and survival outcomes showed that HPV E6/E7 seronegative status was a strong indicator of DSS and RFS in tumor DNA HPV-negative tumors of the oral cavity (DSS/RFS censored: 90%/94%; death/recurrence: 97%/85%). However, there was weaker sensitivity between HPV-seropositive and HPV DNA-positive outcomes (DSS/RFS censored: 33%/20%; death/recurrence: 33%/67%). In the oropharynx, serology was a much stronger overall indicator and demonstrated strong sensitivity and specificity for tumor tissue status and survival (HPV-negative DSS/RFS censored: 73%/100%; death/recurrence: 100%/100%; HPV-positive DSS/RFS censored: 100%/88%; death/recurrence: 100%/100%; Figures 1 and 2).

4. Discussion

The results of this investigation demonstrate that HPV, tobacco, and alcohol represent three independent risk factors for HNC in both the oral cavity and oropharynx. Some

previous studies have suggested [3, 4] that HNC would fall into two distinct tumor groups with separate risk factors, HPV or tobacco/alcohol use. Here we show that each risk factor exerted a significant and independent effect by tumor site, although the risks associated with oropharyngeal cancers were consistently higher than that for the oral cavity. This finding is similar to our previous study based on HPV VLP antibody assessment [1], a lifetime indicator of HPV infection. In this study, we evaluated HPV E6/E7 antibodies, a measure of HPV-related current or possibly, precursor cancer lesions. Other investigations have assessed HNC or oropharyngeal tumors and also have shown an independent effect of each of these exposures [5, 20] based on HPV VLP antibodies.

The current study is one of the few to also examine these risk factors separately for cancers of the oral cavity. In the oral cavity, the risk was more likely to be elevated among HPV-positive/higher tobacco and alcohol level users compared to controls whereas in the oropharynx the opposite was found with HPV-negative/higher tobacco and alcohol level users having a greater risk than the seropositive cases. Although these trends are consistent with our previous investigation based on VLPs, the risks are higher and more evident with the HPV E6/E7 antibodies. Unlike Hafkamp et al. [21] but consistent with Fakhry et al. [22], we found that HPV-positive tumors in never and ever smokers to be unrelated to DSS or RFS (data not shown). Additionally, we found no relationship in HPV-positive or -negative cases and alcohol use. Although the INHANCE analyses did not examine HPV, greater dose and duration of the independent effects of tobacco and alcohol use were associated with higher risk in the oropharynx than in the oral cavity [23].

This also is one of the first studies to examine concordance and sensitivity between E6/E7 serology and tumor DNA for both tumor subsites. We found that they were higher in the oropharynx than in the oral cavity (concordance: 93%/84%; sensitivity: 89%/36%). Previously, Herrero et al. [5] reported better although somewhat lower concordance between HPV E6/E7 serology and tumor HPV DNA in the oropharynx than in the oral cavity: 65% versus 13%. Their corresponding sensitivity was better in the oropharynx than oral cavity as well: 64% versus 14% ($K = 0.6/K = 0.08$). The higher percentages in our study associated with the oral cavity may be due to our use of laser microdissection which is more critical for tumors at this subsite.

It is unclear why site-specific HPV E6/E7 serology/DNA tumor concordance was consistently high for survival outcomes with the exception of the HPV E6/E7-seropositive/DNA-positive cancers in the oral cavity. Other studies of HPV status in association with HNC survival have focused on oropharyngeal cancer and none has reported on the sensitivity of HPV serology associated with tumor DNA and clinical outcomes [3]. This assessment showed that not only is DS survival better in HPV-positive oropharyngeal cases than in HPV-positive oral cavity cases, but also that they have better RFS than in the oral cavity. Rotnáglová et al. [24] also have shown that DSS is significantly better in HPV E6/E7 seropositive or tumor DNA-positive tonsillar cases followed an average four years, although they did not compare cases with both measures of HPV status.

This investigation suggests that while risk of HNC by tumor site is both different between oral cavity and oropharynx, both sites are nonetheless associated with independent effects for each of the three major HNC risk factors. The combined effects alter HNC risk differently in a comparison of HPV-negative versus HPV-positive cases in the oral cavity and the oropharynx. Further, patients with oropharyngeal tumors are different from oral cavity cases not only for the three risk factors but also because they are younger in age, have higher prevalence of heavy tobacco and alcohol use, and are diagnosed at a later stage and with nodal involvement. It is not clear how these differences explain the distinctly different survival and recurrence outcomes found. Additional studies are need that examine HPV in the presence or absence of tobacco and alcohol to evaluate differences in chromosomal alterations and molecular pathways to clarify these risk and survival differences by tumor site.

Acknowledgments

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References

[1] E. M. Smith, L. M. Rubenstein, T. H. Haugen, E. Hamsikova, and L. P. Turek, "Tobacco and alcohol use increases the risk of both HPV-associated and HPV-independent head and neck cancers," *Cancer Causes and Control*, vol. 21, no. 9, pp. 1369–1378, 2010.

[2] A. R. Kreimer, G. M. Clifford, P. Boyle, and S. Franceschi, "Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systemic review," *Cancer Epidemiology Biomarkers and Prevention*, vol. 14, no. 2, pp. 467–475, 2005.

[3] M. L. Gillison, G. D'Souza, W. Westra et al., "Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers," *Journal of the National Cancer Institute*, vol. 100, no. 6, pp. 407–420, 2008.

[4] K. M. Applebaum, C. S. Furniss, A. Zeka et al., "Lack of association of alcohol and tobacco with HPV16-associated head and neck cancer," *Journal of the National Cancer Institute*, vol. 99, no. 23, pp. 1801–1810, 2007.

[5] R. Herrero, X. Castellsagué, M. Pawlita et al., "Human papillomavirus and oral cancer: the international agency for research on cancer multicenter study," *Journal of the National Cancer Institute*, vol. 95, no. 23, pp. 1772–1783, 2003.

[6] E. M. Smith, M. Pawlita, L. M. Rubenstein, T. H. Haugen, E. Hamsikova, and L. P. Turek, "Risk factors and survival by HPV-16 E6 and E7 antibody status in human papillomavirus positive head and neck cancer," *International Journal of Cancer*, vol. 127, no. 1, pp. 111–117, 2010.

[7] J. P. Klussmann, E. Gültekin, S. J. Weissenborn et al., "Expression of p16 protein identifies a distinct entity of tonsillar carcinomas associated with human papillomavirus," *American Journal of Pathology*, vol. 162, no. 3, pp. 747–753, 2003.

[8] E. M. Smith, L. M. Rubenstein, J. M. Ritchie et al., "Does pretreatment seropositivity to human papillomavirus have prognostic significance for head and neck cancers?" *Cancer Epidemiology Biomarkers and Prevention*, vol. 17, no. 8, pp. 2087–2096, 2008.

[9] T. Waterboer, P. Sehr, K. M. Michael et al., "Multiplex human papillomavirus serology based on in situ-purified glutathione S-transferase fusion proteins," *Clinical Chemistry*, vol. 51, no. 10, pp. 1845–1853, 2005.

[10] T. Waterboer, P. Sehr, and M. Pawlita, "Suppression of nonspecific binding in serological Luminex assays," *Journal of Immunological Methods*, vol. 309, no. 1-2, pp. 200–204, 2006.

[11] P. Sehr, M. Müller, R. Höpfl, A. Widschwendter, and M. Pawlita, "HPV antibody detection by ELISA with capsid protein L1 fused to glutathione S-transferase," *Journal of Virological Methods*, vol. 106, no. 1, pp. 61–70, 2002.

[12] K. F. Summersgill, E. M. Smith, H. L. Kirchner, T. H. Haugen, and L. P. Turek, "p53 polymorphism, human papillomavirus infection in the oral cavity, and oral cancer," *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics*, vol. 90, no. 3, pp. 334–339, 2000.

[13] F. F. Chehab, M. Doherty, S. Cai, Y. W. Kan, S. Cooper, and E. M. Rubin, "Detection of sickle cell anaemia and thalassaemias," *Nature*, vol. 329, no. 6137, pp. 293–294, 1987.

[14] Y. Ting and M. M. Manos, "Detection and typing of genital human papillomaviruses," in *PCR Protocols, A Guide to Methods and Applications*, M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, Eds., pp. 356–367, Academic Press, Berkeley, Calif, USA, 1990.

[15] A. Hildesheim, M. H. Schiffman, P. E. Gravitt et al., "Persistence of type-specific human papillomavirus infection among cytologically normal women," *Journal of Infectious Diseases*, vol. 169, no. 2, pp. 235–240, 1994.

[16] A. M. de Roda Husman, J. M. M. Walboomers, A. J. C. Van den Brule, C. J. L. M. Meijer, and P. J. F. Snijders, "The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR," *Journal of General Virology*, vol. 76, part 4, pp. 1057–1062, 1995.

[17] S. F. Altschul, T. L. Madden, A. A. Schäffer et al., "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs," *Nucleic Acids Research*, vol. 25, no. 17, pp. 3389–3402, 1997.

[18] D. W. Hosmer and S. Lemeshow, "Confidence interval estimation of interaction," *Epidemiology*, vol. 3, no. 5, pp. 452–456, 1992.

[19] L. Kalilani and J. Atashili, "Measuring additive interaction using odds ratios," *Epidemiologic Perspectives and Innovations*, vol. 3, article 5, pp. 1–10, 2006.

[20] K. R. Dahlstrom, J. A. Little, M. E. Zafereo, M. Lung, Q. Wei, and E. M. Sturgis, "Squamous cell carcinoma of the head and neck in never smoker-never drinkers: a descriptive epidemiologic study," *Head and Neck*, vol. 30, no. 1, pp. 75–84, 2008.

[21] H. C. Hafkamp, J. J. Manni, A. Haesevoets et al., "Marked differences in survival rate between smokers and nonsmokers with HPV 16-associated tonsillar carcinomas," *International Journal of Cancer*, vol. 122, no. 12, pp. 2656–2664, 2008.

[22] C. Fakhry, W. H. Westra, S. Li et al., "Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial," *Journal of the National Cancer Institute*, vol. 100, no. 4, pp. 261–269, 2008.

[23] M. Hashibe, P. Brennan, S. Benhamou et al., "Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: pooled analysis in the international head and neck cancer epidemiology consortium," *Journal of the National Cancer Institute*, vol. 99, no. 10, pp. 777–789, 2007.

[24] E. Rotnáglová, R. Tachezy, M. Saláková et al., "HPV involvement in tonsillar cancer: prognostic significance and clinically relevant markers," *International Journal of Cancer*, vol. 129, pp. 101–110, 2010.

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Research Article

Towards a "Sample-In, Answer-Out" Point-of-Care Platform for Nucleic Acid Extraction and Amplification: Using an HPV E6/E7 mRNA Model System

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The paper presents the development of a "proof-of-principle" hands-free and self-contained diagnostic platform for detection of human papillomavirus (HPV) E6/E7 mRNA in clinical specimens. The automated platform performs chip-based sample preconcentration, nucleic acid extraction, amplification, and real-time fluorescent detection with minimal user interfacing. It consists of two modular prototypes, one for sample preparation and one for amplification and detection; however, a common interface is available to facilitate later integration into one single module. Nucleic acid extracts ($n = 28$) from cervical cytology specimens extracted on the sample preparation chip were tested using the PreTect HPV-Proofer and achieved an overall detection rate for HPV across all dilutions of 50%–85.7%. A subset of 6 clinical samples extracted on the sample preparation chip module was chosen for complete validation on the NASBA chip module. For 4 of the samples, a 100% amplification for HPV 16 or 33 was obtained at the 1 : 10 dilution for microfluidic channels that filled correctly. The modules of a "sample-in, answer-out" diagnostic platform have been demonstrated from clinical sample input through sample preparation, amplification and final detection.

1. Introduction

Over the past decade, in a drive towards automation of laboratory testing, there has been considerable interest in developing more sensitive and specific molecular diagnostics with reduced time to result, in a way that is cost effective, requires minimal manual handling, and can be performed in a low-resource setting. In a recent review of "point-of-care" (POC) technologies, it was concluded that the most promising technology which fulfils this need is based on microfluidics, as it has the potential to control both the complex flu idic handling required during sample processing and the rea

gent mixing for nucleic acid amplification, in areas which are separated spatially and temporally, while being compatible with inexpensive materials and fabrication methods [1]. Microfluidic approaches in diagnostics achieve significant reagent volume reduction and thus cost-drive innovation, potentially achieving widespread penetration in non-hospital, non-specialised environments. However, microfluidic approaches are not without their challenges [2]. The field of "lab-on-a-chip" (LOC) diagnostics has grown rapidly from this basic need, and it is fast accelerating towards a "sample-in answer-out" platform for molecular diagnostics. A number of reviews have explored the potential use of LOC technology in areas such as clinical diagnostics, personalized medicine, global health, and forensics [1, 3–8].

While the level of complexity of LOC devices varies, the development of "sample-in and answer-out", multifunctional, integrated LOC platforms is not beyond reach. Such a device which would facilitate the transfer of POC diagnostics to the near patient setting would have multiple positive out comes for patient care. The potential introduction of POC-LOC technology to the doctor's office could dramatically reduce the time-to-result, facilitating early disease intervention and reduced patient anxiety. As generic nucleic acid technology can be adapted to a wide range of tests, a nucleic acid-based POC-LOC approach may facilitate a more complete and accurate diagnosis: however, it is not without its challenges, in particular, it must account for the enormous variation in source clinical material to obtain a valid result while also accounting for the heterogeneity that exists within a clinical sample type cohort.

The technology platform presented herein uses human papillomavirus (HPV) mRNA detection from cervical liquid-based cytology specimens as a model system. HPV is the oncogenic viral factor in cervical cancer [9]. Published data show that the incidence of cervical cancer is much higher in low-income countries than high-income countries [10]. As much as 83% of the cervical cancers occur in developing countries, which counts for 15% of female cancers. In developed countries, cervical cancer accounts for only 3.6% of new cancers. The mortality rates for cervical cancer are considerably lower than incidence as cervical cancer is highly preventable through cytological screening programs that facilitate the detection and treatment of precancerous lesions. Well-developed screening programs, especially in high-income areas, have contributed to a substantial decline for cervical cancer incidence and mortality. Introducing a platform as presented in this work for screening of precancerous lesions combined with effective treatment (test and treat possibilities) in low-income countries has enormous potential to reduce the mortality rate in these areas in the world [10, 11].

The LOC modules described in this study comprises two distinct microfluidic chips with their associated supporting technologies, one for sample preparation and the other for nucleic acid amplification. At present, the modules may be used in tandem and will eventually be integrated together, achieving the "sample-in, answer-out" approach (Figure 1). Nucleic acid sequence-based amplification (NASBA) [12] technology with real-time fluorescence measurement was adapted to detect HPV mRNA in cervical specimens and cervical cell lines. The isothermal nature of NASBA greatly simplifies amplification strategies for nucleic acid detection on chip. This platform has huge potential within POC diagnostics as up to 16 different targets can be detected simultaneously for each clinical sample analysed. We present detailed information in relation to NASBA chip construction, microfluidics and results on analysis of cell line and clinical samples with the devices. We adopted an iterative approach to the chip development and optimisation of chemistries and assays and have used an industry gold standard (PreTect HPV-Proofer, NorChip AS, Norway) for HPV E6/E7 mRNA detection to compare our results. The standard PreTect HPV-Proofer was used to (a) determine the initial HPV status of the samples/cell lines and (b) to analyse the quality of the nucleic acid extract from the sample prep-aration chip. We outline some of the challenges involved in the creation of a fully integrated "sample-in and answer-out" LOC system.

2. Materials and Methods

2.1. Cell Lines and Clinical Specimens. For optimisation of both the sample preparation and the NASBA chip, total nucleic acid (TNA) extracted using the NucliSENS easyMAG instrument (bioMeriéux, France), from CaSki and SiHa cervical cancer cell lines obtained from the American Type Culture Collection (ATCC) were used. Nucleic acids were extracted from cell pellets containing 50,000–5 cell/mL [13]. For clinical evaluation, cervical smear specimens collected in PreservCyt, (Hologic Inc. Bedford, Mass, USA) and PreTect TM (NorChip AS, Norway) were used. Specimens with high-grade cervical intraepithelial neoplasia were chosen for "proof of principle" experiments. For gold standard HPV tests, total nucleic acid were extracted from 5 mL clinical PreservCyt specimen on Qiagen M48 BioRobot and eluted in 50 μL. The baseline HPV mRNA status of specimens was determined for the 5 high-risk HPV types (HPV16, 18, 31, 33, and 45) and internal housekeeping control (U1A) using PreTect HPV-Proofer [11, 14–19]. Ethical approval for the study was obtained from the Research Ethics Committee at the Coombe Women and Infants University Hospital, Dublin, Ireland and the South African Ethics Committee at Pretoria Academic Hospital, Pretoria, South Africa.

2.2. Sample Preparation Chip. Cell lines and clinical specimens were extracted on a sample preparation platform which has been previously described elsewhere [13]. The sample preparation system is capable of performing total sample preparation and automated extraction of nucleic acids from human clinical specimens fixed in a methanol-based solution.

All necessary reagents for cell lysis, washing, and elution are stored on chip and the extraction is performed in 2 filter stages: one for cell preconcentration and the other for nucleic acid capture. The chip consists of COC (cyclic olefin copolymer; Ticona COC-5013) sealed with COP (cyclic olefin poly mer; Zeon, Zeonor 1420R). All chips were fabricated by mill ing into blank injection moulded chips of size 64 mm $\times$ 43 mm $\times$ 3 mm. After mounting of the filters, the chips were solvent bonded with a 100 μm COP foil and three turning

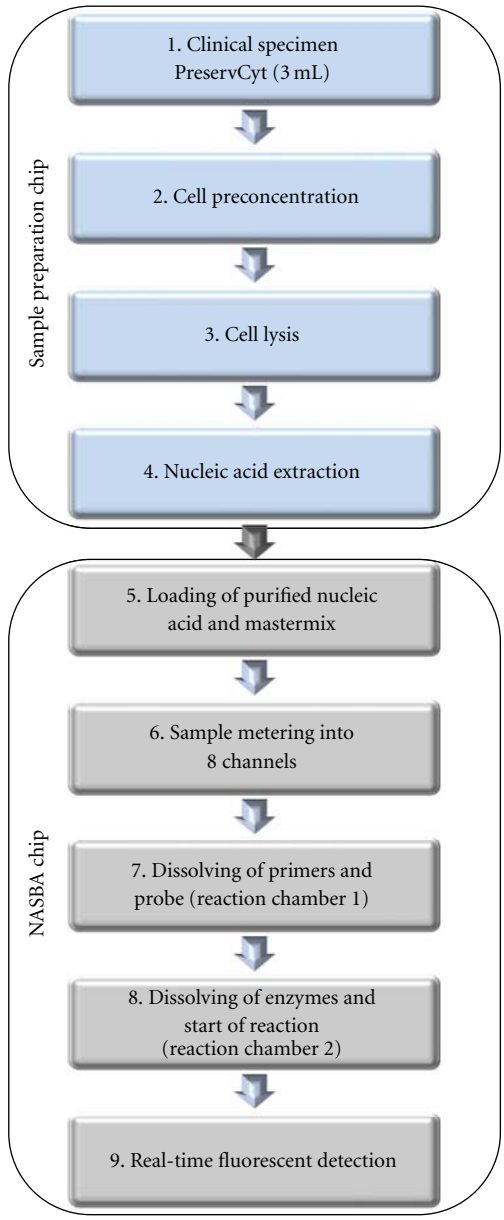

Figure 1: Schematic approach to the development of an integrated LOC device for the detection of HPV nucleic acid (involving a nucleic acid extraction chip and NASBA amplification chip).

valves were mounted on the chip surface. A customised design for the valve seals of the turning valves allows for the selective connection of channels on the chip.

The sample preparation chip (Figure 2(a)) consists of a sample inlet (1), cell filter (2), a silica phase extraction filter (SPE) (3), nucleic acid extraction reagent storage chamber (4A), storage chamber for DMSO and sorbitol (4B) turning valves (5), waste outlet (6), sample outlet (7), and a pressure sensor (8). For nucleic acid extraction, cells were collected and concentrated on a filter (Buckmann GmbH & Co. KG, Germany: Nylon, mesh width 10 μm), where they were subsequently chemically lysed [13]. The released nucleic acid was captured downstream onto a silica filter (Genomed GmbH, Germany), in the presence of a chaotropic salt and extracted by solid-phase extraction, using a modified Boom's extraction method. Following extraction, downstream washing steps were performed to remove cellular debris. Subsequent air-drying was carried out before nucleic acid elution. Details of the reagents for lysis, wash, and elution have been previously published [13].

2.3. Sample Preparation Instrument. The sample preparation instrument (Figure 2(b)) has been described previously [13]. Briefly, it holds two modified syringe pumps containing two syringes: one to pump 3 mL sample through the cell capture filter and a second one for fluid actuation and drying by pressurised air. The latter is connected to an external 3-port/2way valve to allow for syringe reloading so that several

Figure 2: Automated LOC system for sample preconcentration, nucleic acid extraction, amplification, and real-time fluorescent detection. (a) Sample preparation chip: sample inlet [1], cell filter [2], a silica phase extraction filter [3], nucleic acid extraction reagent storage chamber [4A], storage chamber for DMSO and sorbitol [4B] turning valves [5], waste outlet [6], sample outlet [7], and a pressure sensor [8] (b) Sample preparation instrument (c) NASBA chip: sample inlet [1], supply channel [2], metering channels [3], valve 1 [4A], valve 2 [4B], valve 3 [4C], chamber 1 [5A], chamber 2 [5B], waste chamber [6], and instrument connection interface [7] (d) NASBA instrument.

piston strokes are possible, thus avoiding the need to draw air through the chip. Fluid control is achieved through 3 turning valves which are connected to motors below the chip holder table. A heater below the chip table elevates the temperature during lysis and for drying of the SPE filter before elution of nucleic acid. Custom built electronics addressed by a LabVIEW program automatically control all steps of operation from sample load to expulsion of the purified nucleic acid.

2.4. NASBA Chip. The NASBA chip consists of a disposable microfluidic cartridge composed of injection moulded COC (Topas 5013S-04 from Topas Advanced Polymers, Germany). The chips were manufactured by injection moulding using a Battenfeld, EM50/120 machine. The chip consists of a sample inlet (1), a supply channel (cross-section of 400 μm $\times$ 200 μm) (2), eight parallel reaction channels (3), 3 types of hydrophobic valves (4A, 4B, 4C), reaction chamber 1 (5A), reaction chamber 2 (5B), and a waste chamber (6) containing a highly absorbent filter paper (Figure 2(c)). Each of the parallel reaction channels consist of three parts; a metering channel (400 μm $\times$ 120 μm, volume of 740 nL) and two mixing/reaction chambers (volume approximately 800 nL). The two chambers and the metering channel are separated by hydrophobically coated capillary valves of increasing strength; the valve dimensions for the three valves 4A, 4B, and 4C are 200 μm $\times$ 80 μm, 125 μm $\times$ 80 μm, and 50 μm $\times$ 50 μm, respectively. Reaction chamber 2 serves as the real-time fluorescent detection chamber. The overall chip size is 75 mm $\times$ 44 mm $\times$ 1.5 mm. The chip surface was coated with a hydrophilic surface coating using 0.5% PEG (P2263, Sigma Aldrich, Norway) in methanol. The hydrophobic valves were coated with a mixture of 0.5% Teflon (Teflon AF 1600, Du-Pont, Germany) and 0.25% carbon black (type 901, Degussa, Germany) dissolved in perfluorinated solvent (Fluorinert FC-77, 3 M, Germany) and spotted using a PipeJet

P9 dispenser (BioFluidix, Germany) [20]. After surface modification, a cotton linter filter (Schleicher & Schuell BioScience GmbH, Germany) was placed in the waste chamber, and the chips were manually sealed with polyolefin sealing foil containing microencapsulated glue on the contact side (HJ Bioanalytik GmbH, Germany).

2.5. The NASBA Reagents. The reagents used for NASBA chip reactions were obtained from the PreTect HPV- Proofer assay with the addition of 0.013 μg/μL BSA (Sigma Aldrich, Germany) for NASBA amplification. Premixed NASBA reaction mixtures were mixed according to the manufacturer's instructions. In order to proceed to the incubation temperature of 41°C, a prepreparation step was required. For the premixed reaction mixtures this involved incubating the master mix and purified nucleic acid sample together at 65°C for 2 minutes off chip. Subsequently, the temperature was reduced to 41°C and the enzyme was added to the reaction mixture prior to loading onto the NABSA chip.

For some analyses performed on the NASBA chip, rea-gent spotting on chip was conducted using the BioSpot liq-uid handling platform (BioFluidix GmbH, Germany) and the PipeJet dispenser [21]. The aluminium chip holder was cooled down to −80°C prior to the spotting to ensure freezing of the NASBA reagents upon impact within the chambers on chip. A volume of 125 nL of enzyme solution including 2% PEG was dispensed in reaction chamber 2 and 250 nL of the primer/probe mix was dispensed into reaction chamber 1. The freeze-drying procedure was performed in a commercial freeze-dryer (Triad, Labconco, USA). (The parameters for the freeze-drying procedure is described in the supplementary information available online at doi: 10.1155/2012/905024.) In the prepreparation step for the NASBA chips containing freeze-dried enzyme and primer/probe mixes, only the remaining master mix reagents and the purified nucleic acid were mixed and incubated off chip at 65°C for 2 minutes. The temperature was reduced to 41°C prior to loading the reaction mixture on chip.

The reaction volume for PreTect HPV-Proofer was 20 μL, while the reaction volume for NASBA on chip was 740 nL per reaction chamber.

2.6. The NASBA Instrument. Two versions of the NASBA instrument were developed for this study. The Uniplex Detector Version 1 of the instrument (Figure 2(d)) contains an optical unit, which has one excitation wavelength and one emission wavelength with the ability to detect HPV 16, 31, and 33. The instrument comprises two major units: a system for fluidic actuation and control and an optical detection unit.

The fluidic activation mechanism is operated by a pressure pulse generation system, consisting of a 500 mL pressure reservoir, three SMLD-5B valves from TechElan, a 0-1 PSID pressure sensor (Honeywell, Norway) for system feedback and a custom made syringe pump integrated into the instrument for pressure level adjustment. Sample heating and temperature control of 41°C and 65°C are achieved by a Peltier element (Marlow Industries, Sweden) and a thermistor (Elfa, Norway) for feedback is located beneath the chip surface. The accuracy of the heating system has been determined to be better than 0.1°C.

The optical detection unit consists of a fluorescence excitation detection module and a scanner. The scanner is a linear actuator (NEMA 17 linear actuator stepper motor from Ultra motion) which scans across the chip, halting above each reaction chamber to perform fluorescent measurements. The illumination fibre is a 600 μm core diameter multimode fibre with numerical aperture of 0.22 (Thorlabs, Sweden). The detection fibre is a 1000 μm core diameter multimode fibre with numerical aperture of 0.48 (Edmund Optics, UK). In the first generation instrument, (Uniplex Detector Version 1), a 1 W blue LED from Luxeon was used for excitation (excitation maximum wavelength 470 nm), and a band pass filter (Semrock, USA) was used to suppress unwanted wavelengths. The instrument has since been modified to include an additional amber LED (excitation maximum wavelength 597 nm) assessor (Luxeon) to facilitate multiple colour detection in the Multiplex Detector Version 2. During operation, the LED is modulated with a 310 Hz square pulse train with 50% duty cycle. The signal is demodulated by a digital lock-in amplifier, and integrated for 1 second. The amount of light emitted from the fibre end is close to 10 mW. At the detector side, two lenses (Edmund Optics, UK) were used to limit the angular cone through the emission filter (Semrock, USA). The modified instrument has additional emission filters. A Multi-Pixel Photon Counter (MPPC from Hamamatsu, Sweden) was chosen to serve as detector of the fluorescent signal. Excitation maximum wavelengths for the two fluorescent colours used, FAM and ROX, are 494 nm and 587 nm, with emission maximum wavelengths of 518 nm and 607 nm, respectively.

In addition to the above-described components, the instrument contains custom made electronics. The control of all instrument functions is performed *via* in-house software developed in LabVIEW (National Instruments).

3. Results

The overall objective of the system is to develop a hands-free diagnostic platform for detection of target nucleic acid in clinical specimens. The automated platform presented here performs chip-based sample preconcentration, nucleic acid extraction, amplification and real-time fluorescent detection with minimal user interfacing. It consists of two modular prototypes, one for sample preparation and one for amplification and detection. The prototype instruments with their associated disposable microfabricated chips (Figures 2(a) and 2(c)) have been tested individually; however, a common interface is available to facilitate later integration into one single setup.

The sample preparation system is capable of performing total sample preparation and automated extraction of nucleic acids from human clinical specimens fixed in a methanol-based solution. The automated sample preparation module [13] and early work on the amplification and detection module [20, 22–24], are presented elsewhere. In this paper, we present the combined sample preparation and detection

platform in a "proof of principle" study with particular reference to testing from biological specimens and cell lines.

3.1. The Sample Preparation Chip and Instrument. This system has been described previously and tested on cell lines [13]. In this paper, we demonstrate its utility in clinical samples and its integration with the NASBA chip. Briefly, the cervical smear specimen was collected and concentrated on a filter, where it was subsequently chemically lysed. The released nucleic acid was then captured further downstream onto a silica filter in the presence of a chaotrophic salt solution and extracted by solid phase extraction using a variant of Boom's method [25]. Several washing steps were performed to remove the cell debris from the solid phase extraction matrix and after air drying the purified nucleic acid is eluted. The nucleic acid extracted is then transferred manually to the NASBA chip for amplification and detection.

3.2. The NASBA Chip and Instrument. Nucleic acid extracted on the sample preparation chip is mixed with master mix and incubated at 65°C off chip, as described above. Initial experiments were performed on the NASBA chip using PreTect HPV-Proofer reagents premixed and added onto the chip with the nucleic acid. A further development of the NASBA chip involved freeze-drying primer/probes and enzyme onto the chip. Data on this is presented below. The mixture is loaded onto the chip inlet (Figure 2(c)), where it spontaneously is drawn into the supply channel by capillary forces. While the sample liquid fills the supply channel towards the waste chamber, the parallel metering channels are sequentially filled up to the position of the first capillary valve. When the sample reaches the waste chamber, it comes into contact with the filter paper which starts to absorb the liquid and hence acts as a capillary pump. The supply channel is effectively drained, leaving a precisely metered sample aliquot inside each of the metering channels. The pressure pulse system of the instrument is then actuated, transferring these sample plugs in parallel to the first reaction chamber. After a prescribed time, the pressure pulse system is again actuated transferring the sample plugs to the second reaction chamber, where the real-time fluorescence detection is initiated. The shape of the amplification curve generated by real-time increases in fluorescence determines whether the reaction is positive or not (Figure 3).

Sample metering was evaluated in terms of the number of channels per chip successfully filled with sample during the biochemical and fluidic evaluations. The data is gathered from clinical tests using premixed reagents described below. In total, 16 chips were tested, corresponding to 128 metering channels. Of these channels, 102 were successfully filled with sample during the sample loading process, that is, a yield of 79.7%. Of the 16 chips tested, 6 were completely filled in all 8 channels.

Additionally, 9 NASBA chips were tested with freeze-dried enzymes deposited in reaction chamber 2 or the combination of freeze dried primer/probe mix deposited in reaction chamber 1 and enzymes in reaction chamber 2. In this case, 83.3% of the channels filled completely. Two chips filled all 8 channels. The unsuccessful filling of 20.3% and

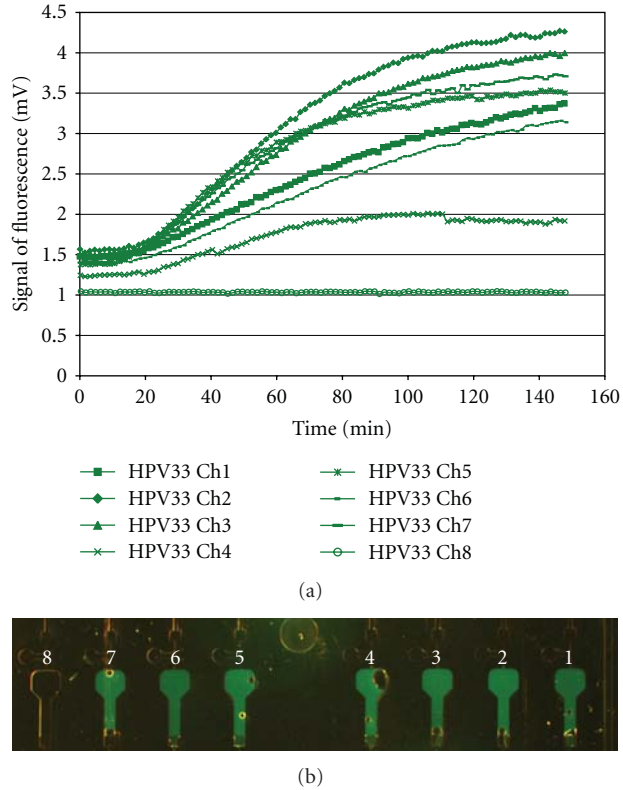

Figure 3: (a) The amplification plot of the clinical sample 508 (HPV 33, 1 : 10 dilution). All the 7 reaction chambers filled with sample are classified as positive using our inhouse data analysis code also used in the conventional PreTect Analyser instrument. Reaction chamber number 8 was not filled with sample; hence, no increase of fluorescence was observed. The offset of the curve is also lower than the filled chambers, as the autofluorescence of the reaction mixture was not present. (b) Fluorescent micrograph of reaction chambers (8–1) after amplification of clinical sample 508 (HPV 33, 1 : 10 dilution). The air bubble located in reaction chamber number 4 contributes to the lower fluorescent signal detected by the NASBA instrument. No sample has entered reaction chamber 8.

16.7% of the metering channels may be due to clogging of the reaction channel, poor surface coating with PEG or Teflon overflow into the metering channel from valve 1 during spotting.

All on-chip flow control is performed *via* a single chip-to-world pressure interface. The chip and fluidic control system are designed such that the sample liquid is at no time in contact with the instrument. All on-chip fluidic control is achieved by pressurized air *via* the fluidic interface. In this way, the risk of cross-contamination between samples is avoided.

Flow actuation is achieved by generation of negative-amplitude pressure pulses delivered to the downstream end of the reaction channels *via* the connector block. During operation, the syringe pump and the feedback pressure sensor are used to set the desired pressure level in the reservoir. Once the correct pressure is reached, the sample plugs are transferred via hydrophobic valves on chip.

Table 1: NASBA chip results on clinical specimens. Six clinical specimens extracted on the sample preparation platform were tested on the NASBA chip platform for the dilutions 1 : 5 and 1 : 10. Positive amplification of target is denoted with "+", while a negative result is denoted "−". The table also shows the number of indeterminate reactions on chip, the number of channels filled and the overall amplification efficiency.

Sample			NASBA chip				
Clinical specimen ID	HPV type	Dilution of eluate	+	−	Indeterminate	No. channels filled	Overall [% positive] channels filled
478	HPV16	1 : 5	1	6	0	7	14
478	HPV16	1 : 10	0	7	0	7	0
479	HPV33	1 : 5	0	0	8	8	0
479	HPV33	1 : 10	8	0	0	8	100
508	HPV16	1 : 5	1	7	0	8	12.5
508	HPV16	1 : 10	0	0	0	0	0
508	HPV33	1 : 5	7	0	0	7	100
508	HPV33	1 : 10	7	0	0	7	100
511	HPV16	1 : 5	4	4	0	8	50
511	HPV16	1 : 10	6	2	0	8	75
520	HPV16	1 : 5	4	0	0	4	100
520	HPV16	1 : 10	5	0	0	5	100
522	HPV16	1 : 5	3	1	1	5	60
522	HPV16	1 : 10	8	0	0	8	100
522	HPV33	1 : 5	0	6	0	6	0
522	HPV33	1 : 10	0	6	0	6	0

In order to detect the reactions in eight parallel chambers of the chip, the optical detection system consists of a fluorescence excitation/detection module and a scanner. The scanning function is realized mechanically by introducing an optical probe connected *via* flexible fibres to the illumination and detection source of the optical system. The probe is attached to a linear actuator and scanned across the chip, halting above each reaction chamber to perform the necessary fluorescence measurements. Two prototypes of the NASBA instrument were produced. The main difference of the two instruments is the optical features. The Uniplex Detector Version 1 detects one fluorescent colour, while the Multiplex Detector Version 2 detects two fluorescent colours.

3.3. "Proof of Principle": The Sample Preparation Platform. The preliminary sensitivity and specificity data for cell line samples purified on the sample preparation platform was presented in Baier et al. 2009 [13]. The new data we present here refers specifically to the testing of clinical samples on the platform.

For evaluation of the sample preparation and nucleic acid extraction chip, cervical smear specimens collected in PreservCyt solution from 20 different patients were analysed. The HPV status of these specimens was as follows; HPV16 ($n = 13$), HPV33 ($n = 3$), coinfection HPV 16/33 and or 18 ($n = 2$), and negative ($n = 2$). From these 20 specimens a total of 28 extractions were performed to confirm the reproducibility of the system. Nucleic acid extracts from the sample preparation chip were then tested using the PreTect HPV-Proofer. We tested a range of nucleic acid extract dilutions, (1 : 1, 1 : 5 and 1 : 10). Overall, the detection rate for HPV obtained across all dilutions ranged from 50 to 85.7%. We did not observe a consistent pattern of amplification at any one dilution.

A subset of samples extracted on the sample preparation chip platform ($n = 6$) were chosen for complete validation on the NASBA chip platform using the criteria described in Section 2.

3.4. "Proof of Principle": The NASBA Chip Platform. The preliminary sensitivity data generated on an earlier version of the NASBA chip platform showed detection limit of 20 cells/μL for the SiHa cell line, which is comparable to the performance of PreTect HPV-Proofer [23]. The data we present here refers specifically to the testing of clinical samples on the NASBA chip platform which were processed for nucleic acids using the sample preparation platform.

In total, 16 NASBA chips were run using 6 clinical samples to evaluate reproducibility and to compare with PreTect HPV-Proofer as described above. All of the clinical specimens which amplified on PreTect HPV-Proofer did show positive amplification on NASBA chip (Table 1) in one or more dilutions of the TNA from the sample preparation platform.

Dilution of the nucleic acid extract 1 : 10 yielded a greater number of positive results on the NASBA chip than the undiluted extract and of the channels which filled correctly with the 1 : 10 dilution of extract, 69% were positive on the NASBA chip, compared with 37.7% of the channels correctly filled with the 1 : 5 dilution of extract. In 4 of the 6 samples tested, 100% amplification for HPV 16 or 33 was obtained at the 1 : 10 dilution for channels that filled correctly. An example of a positive amplification plot and fluorescent micrograph can be seen in Figure 3.

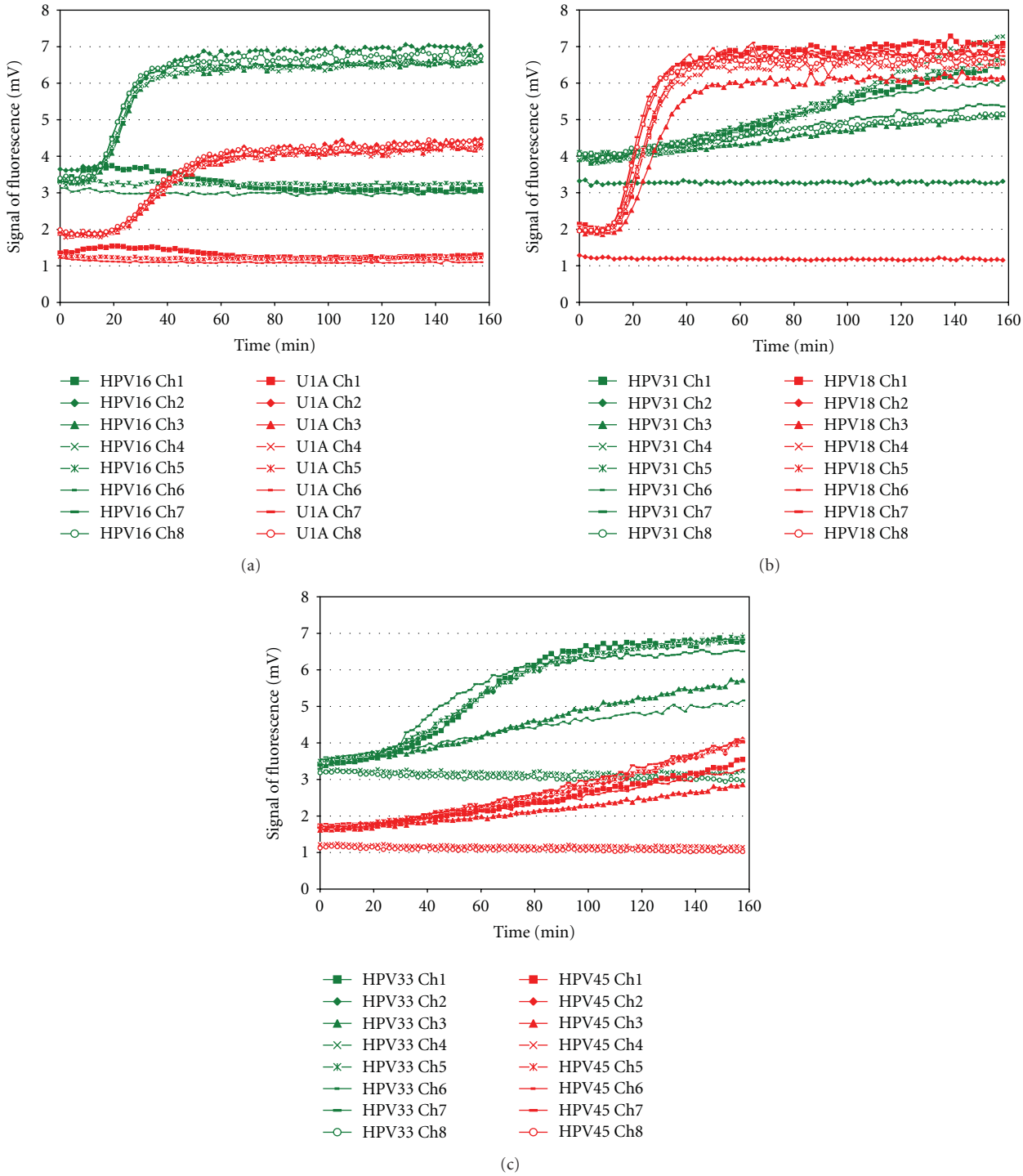

Figure 4: Two colour detection of fluorescence from NASBA amplified products using the Multiplex Detector Version 2 (a) Detection of HPV 16-FAM and U1A-ROX, Channels 1, 5, and 6 were empty. (b) Detection of HPV 18-ROX and HPV 31-FAM, Channel 2 empty. (c) Detection of HPV 33-FAM and HPV 45-ROX, Channels 4 and 8 empty.

3.5. Further Developments towards a "Sample-In Answer-Out" Platform for Nucleic Acid Extraction and Amplification Using Freeze-Dried Reagents and Duplex Fluorescent Detection. The fluorescent reader used in this study has been developed further since the establishment of proof of principle on clinical specimens. Firstly, the fluorescent reader has been improved to detect multiple colours (Multiplex Detector Version 2). Detection of a second fluorescent colour doubles the number targets which can be identified on a single sample. Its performance was evaluated using the PreTect

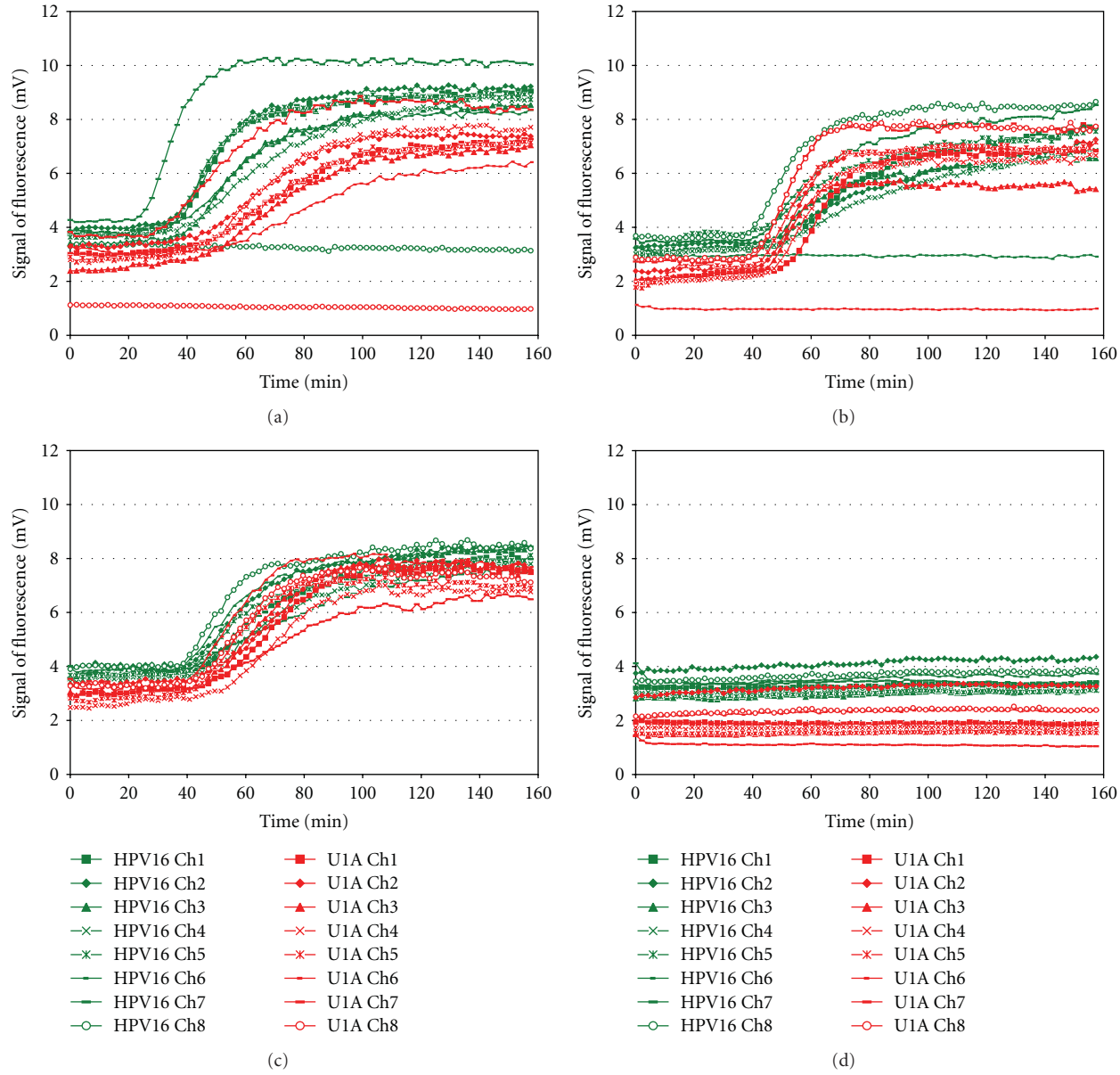

Figure 5: U1A and HPV16 amplification plots from NASBA chips containing freeze-dried reagents. Freeze-dried primers and probes (U1A/HPV16) were deposited in reaction chamber 1 (250 nL) and enzymes were deposited in reaction chamber 2 (125 nL). Incubation time in reaction chamber 1 was 5 minutes. The chips were stored at room temperature for 8 and 9 days. (a) Positive control U1A/HPV 16. Channel 8 was empty. (b) CaSki (1 : 10 dilution, equivalent to 370 cell/reaction chamber on chip). Channel 6 was empty. (c) HPV 16 positive clinical specimen (Pretoria). (d) No template control. Channel 7 was empty.

HPV-Proofer positive controls (Primer/Probe Mix: U1A-ROX, HPV16-FAM), (Primer/Probe Mix: HPV18-ROX, 31-FAM), (Primer/Probe Mix: HPV 33-FAM, 45-ROX), Figure 4. Acceptable sensitivity of the duplex detection was achieved and it is comparable with uniplex amplification.

In a second further development, some of the NASBA reagents were integrated into the NASBA chip. This was achieved by freeze drying of primer and probes and enzyme mix onto the chip as shortly described in materials and methods, a comprehensive description is published elsewhere [26]. For this experiment, we tested 4 chips including an HPV16/U1A positive control, CaSki cell mRNA, mRNA extracted from an HPV16 positive clinical specimen (from Pretoria Hospital) and a no template control (Figure 5). All channels which filled with reaction mix were positive for U1A and HPV16. The no template control was negative (Figure 5). Initial experiments have demonstrated that freeze dried enzymes can be stored on chip at room temperature with no loss of stability for at least 1 month (Figure 6).

4. Discussion

This paper demonstrates the utility of a sample preparation microfluidic LOC device and associated instrument and a NASBA-based amplification chip with optical readers for

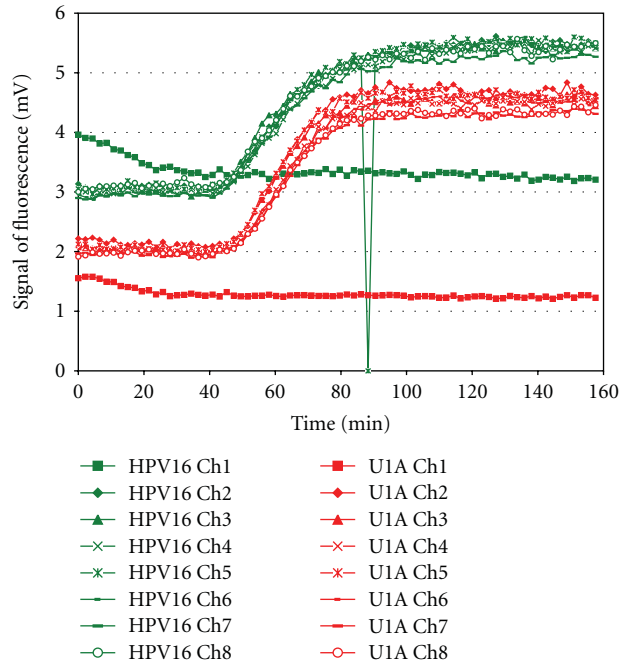

Figure 6: Amplification plot of HPV16 and U1A performed on the NASBA chip with freeze-dried enzymes in reaction chamber 2 stored at room temperature for 32 days. Channel 1 was empty. An instrumental error resulted in an outlier measurement for HPV16 in channel 5 after 88 minutes.

sequential nucleic acid extraction and NASBA amplification of clinical material. The sample preparation device and a detailed description of the NASBA chip and reader has been described previously by the group [13, 24]. However, this paper describes further progress towards a "sample-in, answer-out" integrated microfluidic system tested on clinical samples. The proof of principle test identifies the challenges that must be overcome to achieve a fully integrated walk-away LOC system and demonstrates its potential utility with viscous and multicellular clinical specimens in a methanol-based cytology medium (PreservCyt).

This development of a "sample-in, answer-out" chip achieves many of the goals of miniaturised LOC approaches and has the potential to offer a minimal handling, walk-away lab environment at an affordable price. In terms of the LOC environment, our approach to developing a "sample-in, answer-out" system has several potential commercial outcomes: including separate extraction and amplification on disposable chips, unified extraction and amplification, and an adaptable NASBA amplification platform for use in a range of disease analyses.

The development of a fully functional integrated LOC microfluidic system that can perform cell lysis, nucleic acid extraction amplification, and detection with on-chip reagents is still a great challenge [2]; however, discrete examples of subfunctions of a complete system have been demonstrated in chip format over the last number of years and tested predominantly with cell line material, bacterial cultures, whole blood, or saliva specimens [27–30].

We have previously demonstrated successful amplification of the HPV targets of CaSki, HeLa, and MS751 HPV positive cell line mRNA prepared by the sample preparation platform to a detection limit of 5 cells, 5 cells and 50 cells, respectively, by PreTect HPV-Proofer [13]. In the present study, 20 clinical PreservCyt specimens were processed by the sample preparation platform and the performance of the combined sample preparation and NASBA amplification system was observed. The heterogeneity and varied cellular component of the test set of cervical PreservCyt specimens posed a serious challenge. To overcome this, an algorithm was developed in the LabView software to allow sequential loading of the cellular material to the cell capture filter membrane for cell lysis. For successful amplification of the nucleic acid extracted by the sample preparation device, dilution was necessary. The extraction chip produced nucleic acid of adequate quality for amplification in the NASBA reaction, with a detection rate of between 50–85%, depending on dilution of the extract from the LOC device. The requirement to dilute the eluate for successful NASBA following removal from the chip would indicate impurities in the nucleic acid preparation most likely related to incomplete evaporation of ethanol containing buffer from the SPE matrix and/or the retention of chaotropic salts from the lysis buffer on the membrane. Examples of this were samples 479 (HPV33+) and 522 (HPV16+) which achieved amplification in all channels at the 1 : 10 dilution but at the 1 : 5 gave 8 indeterminate results for HPV33 and 60% successful amplification for HPV16 at the 1 : 5 dilution, respectively. The requirement to dilute the nucleic acid was completely dependent on the quality of the individual sample extract, with one sample amplifying equally well at the 1 : 5 as at the 1 : 10 dilution (Sample 508: HPV33) and another sample giving consistently bad results at either dilution. Many studies including those describing LOC devices have highlighted the amplification problems associated with contaminating ethanol and salts [27, 28].

In this study, 16 NASBA chips were used to amplify nucleic acid from 6 specimens processed by the sample preparation platform which achieved positive amplification by PreTect HPV-Proofer and 9 NASBA chips were used in the optimisation of the freeze-dried reagents on control oligos. We have described previously the development of the NASBA platform [24] but have not demonstrated its capacity to amplify nucleic acids from either the sample preparation platform or clinical specimens until now. Overall, amplification efficiency for the NASBA chip was 69% (based on the number of positive reactions per channels filled on the chip), while the number of channels on the chips that filled correctly was 79.7% (premixed reagents). It is important to note that positive amplification of HPV mRNA was observed for each of the 6 clinical specimens tested. Amplification failure on chip, in channels that filled correctly, can be due to several reasons including: transfer of NASBA inhibitors from extraction chip elute and surface coating issues that result in a nonhydrophilic surface for NASBA reaction.

Incomplete filling of amplification channels can result from failure of fluid progression through the microfluidic channel and clogging of the reaction channel at a number of critical stages in the chip assembly, including the bonding manufacturing process, bubble formation, the valve

spotting procedure resulting in Teflon overflow, during the current manual sealing process or during the manual chip preparation process which involves application of a thin layer of grease to the chip to create an airtight seal between the chip and the instrument. The issue of Teflon overflow was a serious challenge and has since been eliminated by improving the spotting procedures, spotting accuracy, using more favourable valve geometry, and by optimizing concentrations of the hydrophobic valve solution [20]. It is not unlikely that the manual chip preparation procedures as well as the prototype nature of the instruments contribute to the observed malfunctions, and it should be possible to eliminate this by improving the chip fabrication and the fluidic interface between the instrument and the chip. It is important to note that the microfluidic yield of 79.7% (NASBA chips with premixed reaction mixture) and 83.3% (NASBA chips containing freeze dried reagents) is a highly encouraging result, especially taking into account the current manual procedures of lamination foil preparation, sealing and chip preparation prior to insertion into the instrument. This is the first study to comment on the performance of the microfluidic device that was developed therein on a number of clinical specimens.

This paper presents predominantly a uniplex amplification detection approach. However, to demonstrate multiplexing feasibility, two spectrally resolvable dyes FAM and ROX have been successfully applied for duplex detection (Figures 4 and 5). Introduction of the duplex detection on-chip is an important improvement of the platform as it doubles the number of potential targets to be detected simultaneously on a single sample. The sensitivity of the duplex detection of different HPV types and testing amplification of freeze-dried reagents on-chip are acceptable and comparable with uniplex amplification. Testing for an increased number of targets on one and the same sample also reduces analysis costs. The use of specifically selected filters in the detection device allows separate detection spectra for the reporter molecules. The optical scanning device is spatially calibrated, allowing precise localisation of the detection chamber. Several detectors were originally evaluated and a detector based on best signal to noise characteristics and price was chosen.

During the development of the NASBA LOC device, issues in relation to preloading of reagents (NASBA master mix, primers, probes, and enzyme) on chip became apparent. In order to achieve preloading of primers, probes, and enzyme a freeze-drying approach was adopted. This resulted in successful amplification of HPV16 and U1A in all channels which achieved sample filling (Figure 5). We are currently modifying the formulation of the NASBA master mix to achieve successful preloading of all reagents on chip. NASBA reagents such as DMSO and sorbitol not suitable for freeze drying will be stored on the sample preparation module as liquid (Figure 2(a)). Additionally, the next step involves preloading of all primers and probes mixes for the 5 HPV types and the U1A house keeping gene of the PreTect HPV-Proofer in the parallel reaction chambers on chip to achieve a complete "sample-in answer-out" point-of-care diagnostic platform for HPV.

We have recently adapted the extraction and amplification chip for sequential integration. Test structures for chip to chip interface have been evaluated with promising results. The operating microfluidic principles of the two chips are different; in the sample preparation chip, the sample is pushed through the chip by pressure driven flow, while for the NASBA chip, capillary forces and pneumatic pressure are the actuation principles respectively. To ensure a leak-proof bond between the two chips, the connection point requires a gasket and air venting to remove the overpressure generated in the sample preparation chip before the eluate reaches the NASBA chip where capillary forces take over the actuation.

5. Conclusion

The current paper describes a substantial advance on the state of the art for point-of-care HPV diagnostics technology. We presented a combined extraction and amplification in a microfluidic device consisting of extraction and NASBA chips. In addition, the adopted NASBA method offers the unique characteristics of isothermal amplification, greatly simplifying the thermocycling requirements for the system: effectively allowing a "blackbox" technology to be developed encompassing amplification and detection simultaneously in a real-time format. The amplification efficiency of the prototype NASBA platform has been compared to an industry gold standard for HPV detection with encouraging results. By this, we have demonstrated the subcomponents of a complete integrated in vitro diagnostic system: from clinical sample input to sample preparation to amplification to detection, thus advancing towards a "sample-in, answer-out" diagnostic platform.

Finally, the technology platform is not limited to diagnostics of cervical precancer and cancer as studied in this work, but it has enormous potential in the monitoring and diagnosis of gene activity in areas such as infectious disease, oncology, immune response to allergens, immunotherapies, and chemotherapies.

Acknowledgments

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References

[1] P. Yager, G. J. Domingo, and J. Gerdes, "Point-of-care diagnostics for global health," *Annual Review of Biomedical Engineering*, vol. 10, pp. 107–144, 2008.

[2] C. Zhang and D. Xing, "Miniaturized PCR chips for nucleic acid amplification and analysis: latest advances and future

trends," *Nucleic Acids Research*, vol. 35, no. 13, pp. 4223–4237, 2007.

[3] D. Mark, S. Haeberle, G. Roth, F. Von Stetten, and R. Zengerle, "Microfluidic lab-on-a-chip platforms: requirements, characteristics and applications," *Chemical Society Reviews*, vol. 39, no. 3, pp. 1153–1182, 2010.

[4] T. H. Schulte, R. L. Bardell, and B. H. Weigl, "Microfluidic technologies in clinical diagnostics," *Clinica Chimica Acta*, vol. 321, no. 1-2, pp. 1–10, 2002.

[5] A. J. Tüdos, G. A. J. Besselink, and R. B. M. Schasfoort, "Trends in miniaturized total analysis systems for point-of-care testing in clinical chemistry," *Lab on a Chip*, vol. 1, no. 2, pp. 83–95, 2001.

[6] P. Yager, T. Edwards, E. Fu et al., "Microfluidic diagnostic technologies for global public health," *Nature*, vol. 442, no. 7101, pp. 412–418, 2006.

[7] C. D. Chin, V. Linder, and S. K. Sia, "Lab-on-a-chip devices for global health: past studies and future opportunities," *Lab on a Chip*, vol. 7, no. 1, pp. 41–57, 2007.

[8] F. Su, K. Chakrabarty, and R. B. Fair, "Microfluidics-based biochips: technology issues, implementation platforms, and design-automation challenges," *IEEE Transactions on Computer-Aided Design of Integrated Circuits and Systems*, vol. 25, no. 2, pp. 211–223, 2006.

[9] H. zur Hausen, "Papillomaviruses and cancer: from basic studies to clinical application," *Nature Reviews Cancer*, vol. 2, no. 5, pp. 342–350, 2002.

[10] D. M. Parkin, F. Bray, J. Ferlay, and P. Pisani, "Global cancer statistics, 2002," *Ca-A Cancer Journal for Clinicians*, vol. 55, no. 2, pp. 74–108, 2005.

[11] S. Hovland, M. Arbyn, A. K. Lie et al., "A comprehensive evaluation of the accuracy of cervical pre-cancer detection methods in a high-risk area in East Congo," *British Journal of Cancer*, vol. 102, no. 6, pp. 957–965, 2010.

[12] J. Compton, "Nucleic acid sequence-based amplification," *Nature*, vol. 350, no. 6313, pp. 91–92, 1991.

[13] T. Baier, T. E. Hansen-Hagge, R. Gransee et al., "Hands-free sample preparation platform for nucleic acid analysis," *Lab on a Chip*, vol. 9, no. 23, pp. 3399–3405, 2009.

[14] T. Molden, J. F. Nygård, I. Kraus et al., "Predicting CIN2+ when detecting HPV mRNA and DNA by PreTect HPV-proofer and consensus PCR: a 2-year follow-up of women with ASCUS or LSIL Pap smear," *International Journal of Cancer*, vol. 114, no. 6, pp. 973–976, 2005.

[15] H. Keegan, J. McInerney, L. Pilkington et al., "Comparison of HPV detection technologies: hybrid capture 2, PreTect HPV-Proofer and analysis of HPV DNA viral load in HPV16, HPV18 and HPV33 E6/E7 mRNA positive specimens," *Journal of Virological Methods*, vol. 155, no. 1, pp. 61–66, 2009.

[16] S. W. Sørbye, S. Fismen, T. J. Gutteberg, and E. S. Mortensen, "HPV mRNA test in women with minor cervical lesions: experience of the University Hospital of North Norway," *Journal of Virological Methods*, vol. 169, no. 1, pp. 219–222, 2010.

[17] S. W. Sørbye, S. Fismen, T. Gutteberg, and E. S. Mortensen, "Triage of women with minor cervical lesions: data suggesting a "test and treat" approach for HPV E6/E7 mRNA testing," *PLoS ONE*, vol. 5, no. 9, Article ID e12724, 2010.

[18] K. S. Cuschieri, M. J. Whitley, and H. A. Cubie, "Human papillomavirus type specific DNA and RNA persistence—implications for cervical disease progression and monitoring," *Journal of Medical Virology*, vol. 73, no. 1, pp. 65–70, 2004.

[19] I. Kraus, T. Molden, R. Holm et al., "Presence of E6 and E7 mRNA from human papillomavirus types 16, 18, 31, 33, and 45 in the majority of cervical carcinomas," *Journal of Clinical Microbiology*, vol. 44, no. 4, pp. 1310–1317, 2006.

[20] L. Riegger, M. M. Mielnik, A. Gulliksen et al., "Dye-based coatings for hydrophobic valves and their application to polymer labs-on-a-chip," *Journal of Micromechanics and Microengineering*, vol. 20, no. 4, Article ID 045021, 2010.

[21] W. Streule, T. Lindemann, G. Birkle, R. Zengerle, and P. Koltay, "PipeJet: a simple disposable dispenser for the nano- and microliter range," *Journal of the Association for Laboratory Automation*, vol. 9, no. 5, pp. 300–306, 2004.

[22] A. Gulliksen, L. Solli, F. Karlsen et al., "Real-time nucleic acid sequence-based amplification in nanoliter volumes," *Analytical Chemistry*, vol. 76, no. 1, pp. 9–14, 2004.

[23] A. Gulliksen, L. A. Solli, K. S. Drese et al., "Parallel nanoliter detection of cancer markers using polymer microchips," *Lab on a Chip*, vol. 5, no. 4, pp. 416–420, 2005.

[24] L. Furuberg, M. Mielnik, A. Gulliksen et al., "RNA amplification chip with parallel microchannels and droplet positioning using capillary valves," *Microsystem Technologies*, vol. 14, no. 4-5, pp. 673–681, 2008.

[25] R. Boom, C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-Van Dillen, and J. Van Der Noordaa, "Rapid and simple method for purification of nucleic acids," *Journal of Clinical Microbiology*, vol. 28, no. 3, pp. 495–503, 1990.

[26] L. Riegger, *Back-End Processing in Lab-on-a-Chip Fabrication*, Suedwestdeutscher Verlag fuer Hochschulschriften, 2011.

[27] J. Kim, M. Mauk, D. Chen et al., "A PCR reactor with an integrated alumina membrane for nucleic acid isolation," *Analyst*, vol. 135, no. 9, pp. 2408–2414, 2010.

[28] Z. Chen, M. G. Mauk, J. Wang et al., "A microfluidic system for saliva-based detection of infectious diseases," *Annals of the New York Academy of Sciences*, vol. 1098, pp. 429–436, 2007.

[29] I. K. Dimov, J. L. Garcia-Cordero, J. O'Grady et al., "Integrated microfluidic tmRNA purification and real-time NASBA device for molecular diagnostics," *Lab on a Chip*, vol. 8, no. 12, pp. 2071–2078, 2008.

[30] Y. Zhang, S. Park, S. Yang, and T. H. Wang, "An all-in-one microfluidic device for parallel DNA extraction and gene analysis," *Biomedical Microdevices*, vol. 12, no. 6, pp. 1043–1049, 2010.

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Review Article

Molecular Mechanism and Potential Targets for Blocking HPV-Induced Lesion Development

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Persistent infection with high-risk HPV is the etiologic agent associated with the development of cervical cancer (CC) development. However, environmental, social, epidemiological, genetic, and host factors may have a joint influence on the risk of disease progression. Cervical lesions caused by HPV infection can be removed naturally by the host immune response and only a small percentage may progress to cancer; thus, the immune response is essential for the control of precursor lesions and CC. We present a review of recent research on the molecular mechanisms that allow HPV-infected cells to evade immune surveillance and potential targets of molecular therapy to inhibit tumor immune escape.

1. Introduction

Infection with oncogenic types of human papillomavirus (HPV) is the main etiologic factor in cervical cancer (CC) and in its precursor, neoplasia. CC can be a model system for studying the interactions between cells transformed by an oncogenic agent and the immune system, during the progression of the squamous intraepithelial lesion (SIL) [1].

The majority of women clear HPV infection spontaneously by the host immune response, but persistence of HPV infection has been suggested to be associated with development of SIL [2]. The fact that only a small proportion of HPV-infected individuals will eventually develop cancer of the cervix and the long latency period between primary infections and cancer emergence suggest that additional factors are involved in the progression. Other factors such as genetic susceptibility or alteration of the immune response increase the incidence of HPV-associated lesions. A substantial majority of SILs and cancers develop within a specific region of the cervix, the transformation zone, implying that other exogenous or endogenous factors specific to the anatomical milieu may be conducive to SIL and cancer development [3].

A great number of tumors have been identified in humans, most of them growing after the reproductive age. Somatic mutations allow some antigenic tumors to evade the immune response, to grow successfully and persist in our organism in spite of a functionally adequate immune system. The immunology of tumors associated with infectious agents is a remnant of immune response against external pathogens, and low levels of infectious agents can coexist with T-cell-mediated immunity; as a result, the immune system is unable to eliminate all infected cells [4].

In this paper, we review the mechanisms that allow CC cells to evade immune surveillance and the molecular therapy to inhibit tumor immune escape. The tumor immune escape refers to the mechanism by which the body maximizes immune tolerance, through the production of soluble immunosuppressive factors (Interleukin (IL)-10, transforming growth factor-beta1 (TGF-β1), tumor-infiltrating cells such as macrophages and granulocytes), and the recruitment of suppressive cells of the adaptive and innate immune system.

2. Cervical Cancer Molecular Mechanism

2.1. Cellular Immune Response in Cervical Cancer. Tumor immunity in CC is activated by helper T cell type 1 (Th1) cytokines and inhibited by Th2 cytokines. Several cytokines have been shown to contribute to the initiation or suppression of cellular immune responses, such as IL-4, IL-12, IL-10, and/or TGF-β1, produced by various cell types, including macrophages, dendritic cells, and keratinocytes [5].

As an approach to understanding the factors involved in the generation and maintenance of an efficient antitumor response in CC, several research groups have examined the local expression profile of Th1, Th2, and Th3 cytokines in HPV-positive CC biopsies. The data indicate that more than 80% of the tumors expressed low levels of CD4 mRNA, with all of them expressing higher CD8 mRNA levels. Most tumors expressed IL-4 and IL-10 messenger RNA (mRNAs) and, most importantly, all of them expressed TGF-β1 and interferon γ (IFN-γ) mRNA. None of the studied tumors expressed IL-12, IL-6, or tumor necrosis factor (TNF) mRNA [5–15].

There are more tumor infiltrating T lymphocytes (TIL) in the stroma than in epithelium, in biopsies from women with SIL (including a carcinoma *in situ* and a normal region), and in advanced stages of the disease where CD8+ T cells prevailed [7]. Consistent with other reports [8], it was found that CD8+ T cells are predominant, compared to CD4+ T cells, in women with CC. However, what are the mechanisms behind this distribution as well as behind the inability of these CD8+ T cells to eliminate the tumor in CC remains unclear [7].

Immunohistochemical analysis identified IL-10 only in tumor cells and koilocytic cells, but not in tumor-infiltrating lymphocytes, suggesting that IL-10-producing cells are those transformed by HPV. It was found a correlation between immunostaining for IL-10 protein and the level of IL-10 mRNA expression and supernatants from HPV-transformed cell lines containing IL-10 and TGF-β1. These findings show a predominant expression of immunosuppressive cytokines, which help to downregulate tumor-specific immune responses in the tumor microenvironment [6, 10]. Furthermore, using an experimental murine model, it has been demonstrated that HPV16 tumors are not only infiltrated by large numbers of M2-like macrophages (TAM), but there is an expansion of myeloid cells in the spleen and altered T/B lymphocyte ratio in the peripheral lymph nodes of tumor bearing mice, indicating systemic effects initiated by the tumors. It has been shown that TAM and myeloid populations in the spleen of HPV16 tumor-bearing mice are important for tumor growth via stimulation of specific regulatory T cells, in a mechanism partially dependent on IL-10 expression by TAM [16, 17]. Furthermore, in C3 tumor-bearing mice, Gr-1(+) cells completely blocked T-cell response to a peptide presented by major histocompatibility complex class I (MHC class I) *in vitro* and *in vivo*. Blocking of the specific MHC class I molecules on the surface of Gr-1(+) cells completely abrogated the observed effects of these cells. Thus, immature myeloid cells specifically inhibited CD8-mediated Ag-specific T-cell response, but not CD4-mediated T-cell response [18]. This represents a mechanism of cellular immune response inhibition, as a potential therapeutic alternative.

Previous studies have suggested that a reduced T cell function can be associated with alterations in CD3ζ protein expression [8]. Thus, the CD3ζ mRNA expression by T cells has been examined as an indicator of possibly decreased T-cell function in CC patients. CC progression has been associated with lower CD3ζ mRNA, which was even lower in TIL. Studies were done to determine whether decreased CD3ζ mRNA expression correlated with low T-cell proliferation in CC. As expected, there was a significant correlation between low T-cell proliferation and decreased CD3ζ mRNA expression by anti-CD3 stimulated T cells. Thus, decreased T-cell function appears to correlate with CC progression, which is in agreement with a decreased T-cell proliferation in CC patients [7].

To establish the possible association of cytokines with levels of CD3ζ expression, we evaluated the relationship between a number of cytokines and CD3ζ expression by PBL and PBL versus TIL. As expected, there were significant positive associations between CD3ζ/IL-2 and CD3ζ/IFN-γ mRNA expression. Moreover, there was an inverse association for IL-10/CD3ζ mRNA expression in PBL. These results show that an optimal expression of CD3ζ is associated with expression of IL-2 and IFN-γ [7]. Furthermore, it has been demonstrated that *in vivo* suppression of CD3ζ chains in patients with CIN can be the result of a circulating factor [15]. We believe that this circulating factor is composed of IL-10 and TGF-β1 that reduce CD3ζ expression [7].

2.2. Tumor Immune Evasion in Cervical Cancer. Persistent infection is a prerequisite, but may not be sufficient for progressing to CC. HPV "stealth" and immune evasive mechanisms enable infection to persist [19]. Several conditions are required to establish HPV infection among others: the viral lifecycle occurs within the epithelium, there is no viremia, no cell death, and no inflammation, and a local immunosuppression caused by HPV proteins is present. This immunosuppressive state is characterized by repression of TLR9 signal pathways by E6/E7, influence on IFN-γ expression by E6/E7, influence on interferon-dependent signal pathways by E6/E7, induction of TGF-β1 expression by E6/E7, induction of IL-10 expression by E2 protein, and reduction of migration of Langerhans cells (reduction of E-cadherin by E6) [20–22]. It has been suggested that to optimize immunotherapy strategies, correction of immune-activating signals, eradication of inhibitory factors, and the evasion of newly developed immunoresistant tumor phenotypes need to be simultaneously considered [23].

3. Potential Targets for Blocking HPV-Induced Lesion Development

3.1. HPV E2 Protein as a Potential Therapeutic Target. The E2 protein of papillomavirus is a regulatory protein playing crucial roles during the vegetative viral cycle [24]. In HPV-infected cells, the binding to the LCR is thought to repress

HPV gene expression, and E2 contributes to the control of cell proliferation by regulating the expression of E6/E7. However, in cervical carcinomas, the HPV genome often becomes integrated into the host genome, resulting in loss of E2 expression [25]. This leads to increased levels of E6/E7 and, as a consequence, increased cell proliferation and, presumably, increased tumourigenesis. Moreover, HPV E2 protein possesses antiproliferative effects when the HPV E2 gene is re-introduced experimentally into HPV-transformed cells [26–29]. Ectopic expression of E2 from HPV 16, 18, and bovine papillomavirus type 1 induces cell cycle arrest, increases cell senescence, and strongly inhibits cell proliferation and increased apoptosis [26, 30–32]. The induction of G1 growth arrest by HPV 18 E2 protein in HeLa cells it has been associated with the E6/E7 oncogenes repression, which induces stabilization of p53 [29]. In addition, the induction of apoptosis by HPV E2 is shown in many HPV-negative carcinoma cell lines, such as C33 A (cervical cancer), MCF7 (breast cancer), Saos-2 (osteosarcoma). Furthermore, E2-mediated apoptosis is not specific to transformed cells as it also occurs in primary epithelial cells [31, 33, 34].

These results show that the proapoptotic activity of E2 is independent of other viral functions, and more specifically of the transcriptional repression of the E6/E7 viral oncogenes. This activity appears as an autonomous function by which the E2 protein can directly modify cell physiology. So the HPV E2 will be a potential therapeutic toll to repress the E6/E7 oncogenes, inducing inhibition of cell proliferation and cell death by apoptosis in cervical cells.

In addition to its proapoptotic activity, HPV 16 E2 protein possesses antitumor effects in nude mice bearing tumors generated by inoculation of human HPV-transformed cell lines [35, 36]. Immunization of animals with a recombinant vaccinia virus containing the papillomavirus E2 protein promotes tumor regression and decreases the number of new papilloma that are formed [37]. On the other hand, the administration of an adenovirus HPV 16 E2 recombinant had antitumor effects on an experimental tumor in immunocompetent mice inoculated with the BMK-16/myc (murine cell line transformed with HPV16), indicating antitumor effects of the HPV 16 E2 [38]. These results highlight the clinically relevant therapeutic targets derived from the possible use of the HPV E2 protein for the prevention and treatment of HPV-associated cancer.

3.2. Cytokine-Based Therapies in an Immunosuppressive State in Cervical Cancer. The study of immune conditions permissive to tumor regression is a component of a broad strategy aimed at the identification of more effective therapeutic strategies. The cytokines that are produced in the tumour microenvironment have an important role in cancer pathogenesis. Cytokines are released in response to infection; inflammation and immunity can function to inhibit tumour development and progression. Alternatively, cancer cells can respond to host-derived cytokines that promote growth, attenuate apoptosis and facilitate invasion and metastasis. A more detailed understanding of cytokine tumour cell interactions provides new opportunities for improving cancer immunotherapy [39].

In cervical tissue with HPV infection, anti-inflammatory and immunosuppressive cytokines are expressed in the cervical microenvironment, determining the persistence of HPV and tumor progression by subverting cellular immune surveillance mechanisms the shift is a secondary effect induced by the tumor cells, or may be due to the persistence of the viral infection itself [12, 40]. Certain cytokines (IL-4, IL-10, and TGF-β) are highly expressed locally in biopsies from patients with premalignant lesions and CC, and may have induced a local immunosuppression state. In particular, IL-10 is highly expressed in tumor cells and its expression is directly proportional to the development of HPV-positive CC, suggesting an important role of HPV proteins in the expression of IL-10 [6, 11]. The elevated expression of IL-10 may allow for virus persistency, the transformation of cervical epithelial cells, and consequently cancer development. These findings may probably point toward the potential usefulness of cytokine assays for determining prognosis or this lack of immune stimulation may be overcome by enhancing the presentation of the tumor antigens to T cells and by delivering immunostimulatory cytokines [41].

Modification of the immune response against cancer, using specific cytokines, may prove effective against cancers such as CC [41]. Of all cytokines tested in several experimental tumor models, IL-2 and IL-12 seem to have the strongest antitumor activity [42]. IL-2 is a stronger stimulator of proliferation and cytolytic activity that induces Th1-type immune responses through inducing the maturation of Th1 cells from uncommitted T-cell population. IL-12 is a stronger inducer of IFN-γ from natural killer (NK) cells. Furthermore, IL-12 was able to inhibit angiogenesis through the IFN-inducible protein-10 [43]. Several studies have reported that as a protein, IL-12 has a critical role in inducing antiviral and antitumor effects *in vivo*. Direct administration by gene therapy cDNA expressing IL-12 or IL-12 protein can affect tumor progression and metastasis in animal models [44–46]. In particular in CC, the direct intratumoral injection of adenovirus expressing IL-12 (AdIL-12) resulted in a significant suppression of tumor growth in a CC animal model system. The injection of AdIL-12 with E7 antigen into either a tumor site or the distance site, along with AdIL-12, further enhanced antitumor effects significantly, more than AdIL-12 or E7 protein injection alone [47]. The antitumor effect of IL-12 was associated with enhancement of IFN-γ levels and induction of antigen-specific CD8+ T-cell response. In addition, the treatment with IL-12 gene has been employed using nonviral gene therapy (naked DNA), viral gene therapy [48] with the use of adenovirus [47], *ex vivo* gene therapy [49], and in combination with the E6/E7 oncogenes [48–50], as well as genes of immunomodulatory molecules of the cellular immune response such as B7 [49]. Suppression of tumor growth was observed in all cases. IL-12 is able to inhibit experimental metastasis formation and is considered a good candidate for gene therapy against CC [47]. Additionally, IL-12 gene therapy against CC has been used in conjunction with other cytokines such as GM-CSF and IL-2, resulting in an increase of the protective effect against tumor growth [51].

Additionally, a contribution to tumour progression in CC, by immunosuppressive cytokines such as IL-10, has been previously suggested [6, 11]. IL-10 is a Th2-type pleiotropic cytokine that is produced at the tumour site and is increased in sera of patients suffering from different cancer types [52]. IL-10 has been shown to hinder a number of immune functions, for example T-lymphocyte proliferation, Th1-type cytokine production, antigen presentation, and lymphokine-activated killer cell cytotoxicity [10, 53]. One of the main actions of this cytokine is its ability to inhibit the production of proinflammatory cytokines, such as TNF-α, IL-1, and IL-12, which are synthesized by macrophages in response to bacterial components, such as lipopolysaccharides (LPS) [14]. This activity results in decreased IFN-γ production by macrophages and Th1 lymphocytes and inhibition of cell-mediated immune responses, while concomitantly enhancing humoral immunity [50, 54]. Furthermore, IL-10 strongly reduces antigen-specific T-cell proliferation by inhibiting the antigen-presenting capability of monocytes by down-regulation of the expression of their major histocompatibility complex class II (MHC-II) [55]. IL-10 is endowed with multiple positive regulatory activities: it is a growth factor for mature and immature T cells, it enhances the growth and differentiation of CD28+ cytotoxic T lymphocytes (CTLs), and it induces MHC-II expression in resting B cells, sustaining their viability *in vitro* [56–58].

Since IL-10 has potent immunosuppressive and antiinflammatory properties and is produced by some cancers, it has been hypothesized that its production by tumor cells may contribute to the escape from immune surveillance [11]; however, the results obtained in *in vivo* models are controversial.

The increase of tumour growth by IL-10 could be induced by at least three different simultaneous mechanisms: direct stimulation of cell proliferation through an autocrine mechanism, induction of angiogenesis, and the suppression of the local immune system.

Particularly in CC, immunosuppression is the main mechanism proposed by which IL-10 could promote tumour growth in human tumours and murine models [11, 59]. It has been shown that IL-10 can be an autocrine growth factor in culture cells [60]. A previous study reported the effect of IL-10 on tumour growth in a mouse melanoma model and the induction of cell proliferation, either through autocrine stimulation of tumour cells or just through depression of the immune system. To confirm that the enhanced tumour growth was exerted by IL-10 secretion, the tumour growth of the highest IL-10-producer cells (B16-10) was explored in mice treated with an IL-10-neutralizing antibody. In these mice, the tumours grew slower than in control mice and behaved in a similar way to those induced by non-transfected tumour cells, confirming that IL-10 secreted by transfected cells is actually promoting the tumour growth in a melanoma-B16 model. All the effects induced by IL-10 were prevented in mice treated with a neutralizing anti-IL-10 monoclonal antibody. In several *in vitro* models, IL-10 may inhibit different immune mechanisms involved in the antitumour response. However, in mouse models, the effect of IL-10 on the anti-tumour immune response is controversial. In some models, IL-10 inhibits tumoral growth by stimulation of the immune system, mostly of CTLs and NK cells. In other models, the IL-10 promotes the tumoral growth through a local immunosuppression, inhibiting APC functions, CTLs, and the Th1 response. This contradiction might be explained by differences in IL-10-mediated outcomes due to concentration-dependent effects. In CC tumour models where IL-10 suppresses the immune response and increases tumour growth, the production of IL-10 is much lower [61]. It is important to mention that inhibition of IL-10 production by T cells or malignant cells, using low-dose cyclophosphamide [62], anti-IL-10/IL-10R-blocking antibodies [63, 64], or anti-IL-10 antisense oligonucleotides [65], improves cancer-specific immune responses in some preclinical tumor models, which leads the authors to advocate the use of IL-10-neutralizing agents as immunological adjuvants in the design of anticancer vaccines [53].

3.3. MicroRNAs and Cervical Cancer. Knowledge about the RNA interference (RNAi) mechanism has progressed considerably and now we know that microRNAs are a new family of small endogenous RNA that have diverse sequences, have independent tissue-specific and time-specific expression patterns, are evolutionarily conserved, and are implicated in posttranscriptional regulatory mechanisms for the silencing of the expression of sequence-specific genes [66–68]. We know that most eukaryotic organisms have a great number of genes that are transcribed as small RNA called microRNA, which are natural effector molecules of the RNAi mechanism in eukaryotic cells [69]. MicroRNAs induce their effects at the mRNA level, by arresting the translation or inducing the cleavage of target mRNA. The level at which the specific microRNA and mRNA are complementary defines which process will be carried out. The pathway in which the nucleotides in microRNA and mRNA are perfectly complementary induces cleavage of transcripts, while mismatches between several unpaired bases produce an arrest of translation [69]. Therefore, for the human species, the relevance of gene expression silencing by RNAi will be better understood when we know the molecular components and the regulatory mechanisms of this process, in normal physiological conditions as well as during the development of pathologies that have gene expression disruption, such as in the carcinogenesis process. Many efforts have been made to design new drugs and develop gene therapy to treat CC [41]. Alternatively, it has been demonstrated that the RNAi mechanism represses the expression of viral oncogenes at the posttranscriptional level, by several orders of magnitude and more efficiently than another treatments [70, 71]. Therefore, knowledge of molecular events in gene expression silencing by RNAi, and their applications during CC development, are a real and efficient gene therapy strategy against the development of this neoplasia.

3.4. siRNAs for HPV E6 and E7 Oncogenes, as Potential Gene Therapy for Cervical Cancer. RNAi may silence the expression of genes that encode for tumoral antigens or viral

oncogenes, in order to repress the specific proliferation of cancerous cells. As a consequence, the silencing of genes by RNAi is a potential mechanism to inactivate foreign DNA sequences and a successful strategy to silence the expression of HPV oncogenes in CC. The findings reported by several groups in this kind of studies are summarized in Table 1.

The first studies carried out with synthetic siRNA in order to induce the silencing of HPV16 E6 and E7 oncogenes expression were developed by Jiang and Milner in 2002 [72]. In this study, the authors showed the biological effect of siRNAs in human cells from cervical carcinoma. The administration of siRNAs led to mRNA cleavage and the specific silencing of HPV16 E6 and E7 oncogenes expression. Besides this, E6 silencing induced the expression of gene p53, transactivation of the inhibiting gene of p21-CIP1/WAF1 cyclin-kinase, and decrease of cellular proliferation, whereas silencing of E7 induced cellular death by apoptosis. Thus, the findings reported by this group demonstrated, for the first time, that the expression of HPV E6 and E7 oncogenes may be specifically silenced by siRNAs in human tumoral cervical cells that have been transformed by HPV.

3.5. Silencing of HPV E6 and E7 Bicistron with siRNAs. Attention has been focused on an aspect of the use of siRNAs for HPV E6/E7 oncogenes, which is their ability to silence the HPV E6-E7 bicistron. The effect of synthetic siRNA for HPV16 E6 oncogene on SiHa cells (HPV16+) has been reported and the silencing of both E6 and E7 oncogenes has been observed [73]. In addition, observations have been made as to the inhibition of cellular proliferation, p53 protein expression, the induction of p21-CIP1/WAF1 gene, and the identification of the cell cycle arrest mediator—the hypophosphorylated form of pRb associates—as well as the inactivation of E2F transcription factor. Furthermore, when the SiHa cells were inoculated in immune deficient (SCID) mice and treated with siRNAs for E6 oncogene, a decrease in the ability of cancerous cells to induce tumor formation in the animals was detected. Therefore, the findings reported by this group show that siRNAs for HPV16 E6 oncogene have an effect on the E6/E7 bicistron *in vitro* as well as *in vivo*. Another group has reported the use of synthetic siRNAs for HPV18 E6 oncogene [74]. In this study, the induction of apoptosis of the CaSki cells (HPV16+), the increase of p53 and p21-CIP1/WAF1 expression, and the expression of the hypophosphorylated isoform of pRb were demonstrated. A finding that was noteworthy in this study was that siRNAs for HPV18 E6 did not affect HPV18 E7 expression. Initially, this observation seems to contradict what was reported by Jiang et al., who demonstrated that siRNAs for E6 oncogen have an effect on both E6 and E7 oncogenes [72]. Nevertheless, these data are not totally contradictory since they can be explained by the design of siRNA nucleotidic sequences. When we did a more in-depth analysis of both studies, we found that the design of siRNAs is directed to different sequences of E6 oncogene. This suggests a silencing effect that is dependent on the complementary position of the bases between siRNAs and mRNA. Therefore, the silencing of the HPV E6-E7 bicistronic transcript is dependent on the design of siRNA sequences for HPV E6 and E7 oncogenes. In addition, when analyzing the effect of HPV18 E6-E7 bicistron silencing, it was seen that administration of siRNAs specific for E7 induces silencing of both E6/E7 oncogenes, whereas siRNAs for E6 only inhibit E6 expression but do not have effects on E7 expression [75]. In the analysis of the design of siRNA sequences for E7, we observed that the complementarity of bases with the corresponding mRNA occurs in a position that affects the expression of the E6-E7 bicistron; nevertheless, siRNAs for E6 are complementary with mRNA in a sequence that does not influence the silencing of the E6-E7 bicistronic transcript. Again, this evidence supports the fact that the silencing of HPV E6-E7 bicistron expression is dependent on the design of the siRNA sequences and suggests that the alternative splicing of HPV E6/E7 oncogenes precedes the silencing by siRNAs. Additionally, this same study analyzed the functionality of siRNAs for E6/E7 and demonstrated the induction of expression in p53, p16, p21, p27, and in the hypophosphorylated isoform of pRb, the silencing of cyclin A gene, and the induction of apoptosis in human cancer cells. This evidence supports the fact that viral oncoproteins can have antiapoptotic properties by their influence on p53 protein function and when these oncogenes are silenced with siRNAs, p53 recovers the cellular cycle control functions and apoptosis [76]. Thus, E6 oncoprotein may have an important impact on other components of the apoptosis regulatory machinery. The high risk HPV E6 oncoproteins induce the proteolytic inactivation of certain proapoptotic proteins such as p53 [77], Bak [78], FADD [79], procaspase-8 [80], or c-Myc [81, 82]. In human keratinocytes immortalized by E6, low levels of apoptosis as compared to the non-immortalized control cells were observed after CD95 (Fas) agonist treatment [79]. Interestingly, in addition to p53 and p21, protein levels of antiapoptotic proteins Bcl-2 and Flip were reduced. Proteosomal inhibition increased the susceptibility of E6 expressing cells to CD95-mediated apoptosis. In addition, several studies have examined the sensitivity of cells expressing E6 to TNF. HPV-16 E6 was shown to bind to the C-terminal end TNF receptor 1 (TNF R1) and protect cells from TNF-induced apoptosis in mouse fibroblasts and human histiocyte/monocyte and osteosarcoma cells [83, 84]. E6 binding to TNF R1 interfered with the Fas pathway. Furthermore, use of an inducible E6 expression system demonstrated that this protection is dose dependent, with higher levels of E6 leading to greater protection. Although E6 suppresses activation of both caspase 3 and caspase 8, it does not affect apoptotic signaling through the mitochondrial pathway. Mammalian two hybrid are demonstrated that E6 binds directly to the death effectors domain of Fas-associated death domain (FADD) and to protect cells from Fas-induced apoptosis. In addition, binding to E6 leads to degradation of FADD, with the loss of cellular FADD proportional to the amount of E6 expressed. These results support a model in which E6-mediated degradation of FADD prevents transmission of apoptotic signals via the Fas pathway [85].

3.6. Chemotherapeutical Agents and siRNAs for HPV E6 and E7. Although we know the effect of different chemotherapeutical drugs on the expression of p53 protein in human

Table 1

HPV oncogenes	siRNA design	Biological effects	References
HPV16 E6 and E7	Synthetic siRNA	Silencing of HPV16 E6 and E7, p53, p21 and pRb expression. Apoptosis induction *in vitro*.	[72]
HPV16 E6	Synthetic siRNA	Silencing of HPV16 E and E7, p53, p21 and pRb expression. Cellular proliferation inhibition *in vitro*. Tumor growth inhibition *in vivo*.	[73]
HPV18 E6	Synthetic siRNA	Silencing of HPV16 E6, p53, p21, and pRb expression. Apoptosis induction *in vitro*.	[74]
HPV16 E7	Synthetic siRNA in bio-adhesive gels	Silencing of HPV16 E7. Apoptosis induction *in vitro*.	[87]
HPV18 E6 and E7	Synthetic siRNA with chemotherapy	Silencing of HPV18 E6 and E7, as well as p53 expression. Cytotoxicity decrease *in vitro*.	[94]
HPV18 E6	siRNA in lentivirus with chemotherapy	Silencing of HPV18 E6 and E7, as well as p53 expression. Cytotoxicity decrease *in vitro*.	[86]
HPV16 E6 and E7	Synthetic siRNA in liposomes	Silencing of HPV16 E6 and E7. Apoptosis induction *in vitro*. Tumor growth inhibition *in vivo*.	[88]
HPV18 E6 and E7	siRNA with atelocollagen	Silencing of HPV18 E6 and E7, as well as p53 and pRb expression. Cellular senescence induction *in vitro*. Tumor growth inhibition *in vivo*.	[89]
HPV18 E6 and E7	Synthetic siRNA	Silencing of HPV18 E6 and E7 and cyclin A gene, as well as p53, pRb, p16, p21, and p27 expression. Apoptosis induction *in vitro*.	[75]
HPV18 E6 and E7	siRNA in pSUPER plasmid	Silencing of HPV16 E6 and E7, as well as p53 and pRb expression. Transcriptome expression analysis of cancer cells.	[91]
HPV16 E6 and E7	siRNA in psiCheck 2 plasmid	Silencing of HPV16 E6 and E7, as well as p53 and p21 expression. Cellular senescence induction *in vitro*. Tumor growth inhibition *in vivo*.	[95]
HPV16 E7	siRNA in pSIRE-DNR plasmid	Silencing of HPV E6 and E7, as well as p53, p21 and pRb expression. Apoptosis induction *in vitro*.	[96]
High risk HPV E6 and E7	siRNA in lentivirus	Silencing of HPV16 E6 and E7. p53 and p21 expression. Apoptosis induction *in vitro* and *in vivo*. High efficiency of infection of proliferation cells and quiescent cells.	[90]
HPV16 E6 and E7	siRNA in pSilencer 1.0U6-plasmid	Silencing of HPV16 E6 and E7. p53 and pRb expression. Cellular proliferation inhibition. Autophagic and apoptosis induction of tumor cells *in vivo*.	[97]

tumoral cervical cells, we do not know if there is an association between the activation of gene p53, the cytotoxic effect of drugs, and the silencing of HPV oncogenes with siRNAs. Thus, different groups have analyzed the expression of p53 protein in HeLa cells (HPV18+) by the administration of siRNAs for the HPV18 E6 oncogene, combined with treatment by carboplatin, cisplatin, doxorubicin, etoposide, gemcitabine, mitomicine, mitoxantrone, oxaliplatin, paclitaxel, and topotecan [76]. In this study, the silencing of HPV18 E6/E7 oncogenes was observed, as well as an increase in p53 protein expression and changes in cytotoxicity that were dependent on the nature of each chemotherapeutical compound. Also, another group demonstrated, using HeLa cells, that the administration of specific siRNAs for HPV18 E6 generated in lentivirus, combined with cisplatin, the drug most frequently used in the treatment of advanced CC, produces the silencing of HPV18 E6 and E7 oncogenes, an increase in p53 expression and death of cancer cells by cellular senescence [86]. This evidence suggests that the silencing of HPV E6/E7 oncogenes by siRNAs can increase the cells sensitivity to the cytotoxic effects of drugs and that the combined treatment may have a synergistic effect in decreasing the resistance to chemotherapeutical drugs, which is an advantage for treatment.

3.7. siRNAs Transporting Molecules for HPV E6/E7. In the evaluation of the biological effects of siRNAs, *in vitro* as well as *in vivo,* protocols for the administration of synthetic siRNAs have been developed, for HPV E6/E7 oncogenes, where these molecules are coupled with liposomes as transport vehicles [87]. In these studies, it has been shown in CaSki (HPV16+) cells that when siRNAs for HPV16 E6/E7 are administered, silencing of both oncogenes is induced and the cancer cells die by apoptosis. With this same system, and through the development of a murine tumor model with CaSki cells, the effects of siRNAs for E6 have been evaluated and the silencing of the viral oncogene has been determined, as well as the induction of apoptosis of tumor cells and a significant inhibition of the growth of the tumoral mass *in vivo* [87]. Although these findings are significant, when synthetic siRNAs are used and administered by lipofection

to mammalian cells, a potential problem that appears is the cleavage of siRNAs by the action of endogenous cellular endonucleases. An alternative design to protect the siRNAs from this cleavage is the synthesis of siRNAs with chemical modifications; however, this may induce undesirable collateral effects. Another problem that arises in the systemic administration of siRNAs is that we do not have a dose dependent effect on target organs. Thus, in order to overcome these methodological inconveniences, it has been reported that the specific siRNAs for HPV oncogenes may be administered in a liposome based system contained in biogels [88]. The combination of adhesive biogels and liposomes containing siRNAs for the HPV16 E7 oncogene has resulted in the specific silencing of E7 and the induction of apoptosis of cancerous cells *in vitro.* In addition, the use of atelocollagen as a vehicle to administer the siRNAs for HPV18 E6 and E7, *in vitro* as well as *in vivo,* has also been reported [89]. In this study, it has been observed that siRNAs silence E6/E7 oncogenes expression, inhibit cellular proliferation, induce the expression of the hypophosphorylated isoform of pRB, and induce death of cancerous cells by cellular senescence. In this same system, it was also demonstrated that the administration of siRNAs for HPV18 E6/E7, attached to atelocollagen, inhibits the growth of the tumor mass in a murine tumoral model [89]. Although the silencing effects of synthetic siRNAs are evident, the half life of these molecules after their administration is relatively short, even when they are attached to transporting molecules; this limits their application in preclinical or clinical trials. Besides this, the real application of siRNAs for high-risk HPV oncogenes in clinical phase studies, requires a better understanding of the development of highly specific siRNAs and greater efficiency in the *in vivo* liberation systems. In this sense, protocols have been developed for the generation of lentiviruses as molecular liberation vectors for specific siRNAs for high-risk HPV E6 and E7 oncogenes, as well as for their stable transfection and transduction in human CC cells. The biological effects of silencing with siRNAs, *in vitro* as well as *in vivo* models, are being evaluated [90].

3.8. Transcriptome Regulation by the Effect of siRNAs for HPV E6/E7. Another aspect of the silencing of HPV E6/E7 oncogenes that has been studied, using siRNAs, is the effect it has on transcriptome regulation of human tumor cervical cells. In 2007, Kuner et al. [91] analyzed the transcriptome of HeLa cells and cervical cells from patient biopsies, inducing the silencing of HPV 18 E6 and E7 with siRNAs generated in the silencing plasmid of pSUPER. In this study, 360 cellular genes were identified which had a negative regulation and 288 genes with positive regulation due to the effect of siRNAs for E6/E7. Most of these genes are involved in relevant biological processes during the development of the tumor cell, such as: apoptosis control, regulation of the cell cycle, formation of the mitotic spindle, processing of mRNA by splicing, metabolism, DNA replication and repair, nuclear transport, cell proliferation, and gene regulation by c-Myc. These findings complement previous studies where the expression of HPV E2 protein has been analyzed. This protein inhibits HPV E6/E7 expression, and the expression of the transcriptome in human tumor cervical cells. The potential of this type of studies lies in the fact that the basic cell pathways for viral transformation may be identified, which may be targets for the development of therapeutical strategies. Also, new molecular biomarkers may be found for diagnosis and prognosis of CC. An example of these biomarkers is the enhancer of zeste homolog 2 (EZH2), which is repressed by the inhibition of E6/E7 by siRNAs. These data suggest that this biomarker is active in CC cells transformed by HPV [91]. Thus, the information generated by the study of the transcriptome of CC cells, using siRNAs for HPV oncogenes, may contribute to the diagnosis prognosis and treatment of this neoplasia. It has been previously demonstrated that silencing E6 and E7 has effects on several cellular targets. For example, a connection between oncogenic HPVs and C-MYC during the transformation process has been previously discussed. For example, insertion of HPVs close to the MYC locus is observed in about 10% of HPV positive genital cancers, and it has been speculated that the HPV transcriptional control region may induce overexpression of the nearby MYC gene [92]. Ectopically expressed E6 has been reported to stimulate the C-MYC promoter, and E6 and/or E7 can increase C-MYC expression under certain experimental conditions [93]. The data on transcriptome regulation by siRNA, for E6 and E7, would be consistent with the idea that the viral E6/E7 genes can either directly or indirectly activate C-MYC expression; this may be an alternative mechanism for inducing downstream C-MYC targets during HPV-associated carcinogenesis.

4. Conclusions

Cervical lesions caused by HPV persistent infection can be removed naturally by the host immune response, and only a small percentage may progress to cancer, thus, the immune response is essential for the control of precursor lesions and CC. Therefore, to know the molecular mechanisms and potential targets for blocking HPV-induced, lesion development is of high relevance, because 80% percent of CC cases are in developing countries. HPV prophylactic vaccines and construction of the sanitary structure are very expensive; therefore, to develop techniques base on genomic approach will be very useful for CC therapy. The relevance of this approach will be better appreciated once it is applied in clinical protocols.

The correction of immune-activating signals, eradication of inhibitory factors, and the evasion from newly developed immunoresistant tumor phenotypes need to be simultaneously considered.

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References

[1] C. Mougin, V. Dalstein, J. L. Prétet, C. Gay, J. P. Schaal, and D. Riethmuller, "Epidemiology of HPV cervical infections: recent knowledge," *Presse Medicale*, vol. 30, no. 20, pp. 1017–1023, 2001.

[2] C. Mougin, L. Mo, and V. Dalstein, "Natural history of papillomavirus infections," *Revue du Praticien*, vol. 56, no. 17, pp. 1883–1889, 2006.

[3] B. C. Sheu, W. C. Chang, H. H. Lin, S. N. Chow, and S. C. Huang, "Immune concept of human papillomaviruses and related antigens in local cancer milieu of human cervical neoplasia," *Journal of Obstetrics and Gynaecology Research*, vol. 33, no. 2, pp. 103–113, 2007.

[4] L. J. Old, "Tumor immunology: the first century," *Current Opinion in Immunology*, vol. 4, no. 5, pp. 603–607, 1992.

[5] S. L. Giannini, W. Al-Saleh, H. Piron et al., "Cytokine expression in squamous intraepithelial lesions of the uterine cervix: implications for the generation of local immunosuppression," *Clinical and Experimental Immunology*, vol. 113, no. 2, pp. 183–189, 1998.

[6] J. M. Alcocer-González, J. Berumen, R. Taméz-Guerra et al., "In vivo expression of immunosuppressive cytokines in human papillomavirus-transfonned cervical cancer cells," *Viral Immunology*, vol. 19, no. 3, pp. 481–491, 2006.

[7] C. E. Díaz-Benítez, K. R. Navarro-Fuentes, J. A. Flores-Sosa et al., "CD3ζ expression and T cell proliferation are inhibited by TGF-β1 and IL-10 in cervical cancer patients," *Journal of Clinical Immunology*, vol. 29, no. 4, pp. 532–544, 2009.

[8] T. D. De Gruijl, H. J. Bontkes, F. Peccatori et al., "Expression of CD3-ζ on T-cells in primary cervical carcinoma and in metastasis-positive and -negative pelvic lymph nodes," *British Journal of Cancer*, vol. 79, no. 7-8, pp. 1127–1132, 1999.

[9] A. D. Santin, A. Ravaggi, S. Bellone et al., "Tumor-infiltrating lymphocytes contain higher numbers of type 1 cytokine expressors and DR+ T cells compared with lymphocytes from tumor draining lymph nodes and peripheral blood in patients with cancer of the uterine cervix," *Gynecologic Oncology*, vol. 81, no. 3, pp. 424–432, 2001.

[10] B. C. Sheu, R. H. Lin, H. C. Lien, H. N. Ho, S. M. Hsu, and S. C. Huang, "Predominant Th2/Tc2 polarity of tumor-infiltrating lymphocytes in human cervical cancer," *Journal of Immunology*, vol. 167, no. 5, pp. 2972–2978, 2001.

[11] V. H. Bermudez-Morales, L. X. Gutiérrez, J. M. Alcocer-González, A. Burguete, and V. Madrid-Marina, "Correlation between IL-10 gene expression and HPV infection in cervical cancer: a mechanism for immune response escape," *Cancer Investigation*, vol. 26, no. 10, pp. 1037–1043, 2008.

[12] A. G. Bais, I. Beckmann, J. Lindemans et al., "A shift to a peripheral Th2-type cytokine pattern during the carcinogenesis of cervical cancer becomes manifest in CIN III lesions," *Journal of Clinical Pathology*, vol. 58, no. 10, pp. 1096–1100, 2005.

[13] T. D. De Gruijl, H. J. Bontkes, A. J. C. Van den Muysenberg et al., "Differences in cytokine mRNA profiles between premalignant and malignant lesions of the uterine cervix," *European Journal of Cancer*, vol. 35, no. 3, pp. 490–497, 1999.

[14] D. F. Fiorentino, A. Zlotnik, T. R. Mosmann, M. Howard, and A. O'Garra, "IL-10 inhibits cytokine production by activated macrophages," *Journal of Immunology*, vol. 147, no. 11, pp. 3815–3822, 1991.

[15] S. M. Shondel, C. W. Helm, C. Gercel-Taylor, and D. D. Taylor, "Differential expression of T-cell CD3-zeta chains in patients with cervical dysplasia before and after treatment," *International Journal of Gynecological Cancer*, vol. 17, no. 6, pp. 1278–1282, 2007.

[16] A. P. Lepique, K. R. P. Daghastanli, I. Cuccovia, and L. L. Villa, "HPV16 tumor associated macrophages suppress antitumor T cell responses," *Clinical Cancer Research*, vol. 15, no. 13, pp. 4391–4400, 2009.

[17] A. Bolpetti, J. S. Silva, L. L. Villa, and A. P. Lepique, "Interleukin-10 production by tumor infiltrating macrophages plays a role in Human Papillomavirus 16 tumor growth," *BMC Immunology*, vol. 11, article 27, 2010.

[18] D. I. Gabrilovich, M. P. Velders, E. M. Sotomayor, and W. M. Kast, "Mechanism of immune dysfunction in cancer mediated by immature Gr-1+ myeloid cells," *Journal of Immunology*, vol. 166, no. 9, pp. 5398–5406, 2001.

[19] H. Trottier and E. L. Franco, "Human papillomavirus and cervical cancer: burden of illness and basis for prevention," *American Journal of Managed Care*, vol. 12, no. 17, pp. S462–S472, 2006.

[20] M. Stanley, "Immune responses to human papillomavirus," *Vaccine*, vol. 24, no. 1, pp. S16–S22, 2006.

[21] O. Peralta-Zaragoza, V. Bermúdez-Morales, L. Gutiérrez-Xicotencatl, J. Alcocer-González, F. Recillas-Targa, and V. Madrid-Marina, "E6 and E7 oncoproteins from human papillomavirus type 16 induce activation of human transforming growth factor β1 promoter throughout Sp1 recognition sequence," *Viral Immunology*, vol. 19, no. 3, pp. 468–480, 2006.

[22] V. H. Bermúdez-Morales, O. Peralta-Zaragoza, J. M. Alcocer-González, J. Moreno, and V. Madrid-Marina, "IL-10 expression is regulated by HPV E2 protein in cervical cancer cells," *Molecular Medicine Reports*, vol. 4, no. 2, pp. 369–375, 2011.

[23] M. Ahmad, R. C. Rees, and S. A. Ali, "Escape from immunotherapy: possible mechanisms that influence tumor regression/progression," *Cancer Immunology, Immunotherapy*, vol. 53, no. 10, pp. 844–854, 2004.

[24] M. C. Lai, B. H. Teh, and W. Y. Tarn, "A human papillomavirus E2 transcriptional activator: the interactions with cellular splicing factors and potential function in pre-mRNA processing," *The Journal of Biological Chemistry*, vol. 274, no. 17, pp. 11832–11841, 1999.

[25] C. C. Baker, W. C. Phelps, V. Lindgren, M. J. Braun, M. A. Gonda, and P. M. Howley, "Structural and transcriptional analysis of human papilloma- virus type 16 sequences in cervical carcinoma cell lines," *Journal of Virology*, vol. 19, pp. 962–971, 1987.

[26] F. Thierry and M. Yaniv, "The BPV1-E2 trans-acting protein can be either an activator or a repressor of the HPV18 regulatory region," *The EMBO Journal*, vol. 6, no. 11, pp. 3391–3397, 1987.

[27] E. S. Hwang, L. K. Naegerm, and D. DiMario, "Acti- vation of the endogenous p53 growth in- hibitory pathway in HeLa cervical carci- noma cells by expression of the bovine papillomavirus E2 gene," *Oncogene*, vol. 12, pp. 795–803, 1996.

[28] E. S. Hwang, D. J. Riese, J. Settleman et al., "Inhibition of cervical carcinoma cell line proliferation by the introduction of a bovine papillomavirus regulatory gene," *Journal of Virology*, vol. 67, no. 7, pp. 3720–3729, 1993.

[29] C. Desaintes, C. Demeret, S. Goyat, M. Yaniv, and F. Thierry, "Expression of the papillomavirus E2 protein in HeLa cells leads to apoptosis," *The EMBO Journal*, vol. 16, no. 3, pp. 504–514, 1997.

[30] J. J. Dowhanick, A. A. McBride, and P. M. Howley, "Suppression of cellular proliferation by the papillomavirus E2 protein," *Journal of Virology*, vol. 69, no. 12, pp. 7791–7799, 1995.

[31] C. Demeret, A. Garcia-Carranca, and F. Thierry, "Transcription-independent triggering of the extrinsic pathway of apoptosis by human papillomavirus 18 E2 protein," *Oncogene*, vol. 22, no. 2, pp. 168–175, 2003.

[32] S. Blachon and C. Demeret, "The regulatory E2 proteins of human genital papillomaviruses are pro-apoptotic," *Biochimie*, vol. 85, no. 8, pp. 813–819, 2003.

[33] C. Desaintes, S. Goyat, S. Garbay, M. Yaniv, and F. Thierry, "Papillomavirus E2 induces p53-independent apoptosis in HeLa cells," *Oncogene*, vol. 18, no. 32, pp. 4538–4545, 1999.

[34] K. Webster, J. Parish, M. Pandya, P. L. Stern, A. R. Clarke, and K. Gaston, "The human papillomavirus (HPV) 16 E2 protein induces apoptosis in the absence of other HPV proteins and via a p53-dependent pathway," *The Journal of Biological Chemistry*, vol. 275, no. 1, pp. 87–94, 2000.

[35] C. Rosales, V. Valadez Graham, G. A. Rosas, H. Merchant, and R. Rosales, "A recombinant vaccinia virus containing the papilloma E2 protein promotes tumor regression by stimulating macrophage antibody-dependent cytotoxicity," *Cancer Immunology Immunotherapy*, vol. 49, no. 7, pp. 347–360, 2000.

[36] V. V. Graham, G. Sutter, M. V. José et al., "Human tumor growth is inhibited by a vaccinia virus carrying the E2 gene of bovine papillomavirus," *Cancer*, vol. 88, no. 7, pp. 1650–1662, 2000.

[37] J. Berumen, L. Casas, E. Segura, J. L. Amezcua, and A. Garcia-Carranca, "Genome amplification of human papillomavirus types 16 and 18 in cervical carcinomas is related to the retention of E1/E2 genes," *International Journal of Cancer*, vol. 56, no. 5, pp. 640–645, 1994.

[38] V. H. Bermúdez-Morales, O. Peralta-Zaragoza, E. Guzmán-Olea et al., "HPV 16 E2 protein induces apoptosis in human and murine HPV 16 transformed epithelial cells and has antitumoral effects in vivo," *Tumor Biology*, vol. 30, no. 2, pp. 61–72, 2009.

[39] G. Dranoff, "Cytokines in cancer pathogenesis and cancer therapy," *Nature Reviews Cancer*, vol. 4, no. 1, pp. 11–22, 2004.

[40] M. Clerici, M. Merola, E. Ferrario et al., "Cytokine production patterns in cervical intraepithelial neoplasia: association with human papillomavirus infection," *Journal of the National Cancer Institute*, vol. 89, no. 3, pp. 245–250, 1997.

[41] V. H. Bermúdez-Morales, O. Peralta-Zaragoza, and V. Madrid-Marina, "Gene therapy with cytokines against cervical cancer," *Salud Publica de Mexico*, vol. 47, no. 6, pp. 458–468, 2005.

[42] M. Del Vecchio, E. Bajetta, S. Canova et al., "Interleukin-12: biological properties and clinical application," *Clinical Cancer Research*, vol. 13, no. 16, pp. 4677–4685, 2007.

[43] X. Yin, X. Yan, Q. Yang, H. Cao, and H. Liang, "AntiTumor mechanism of recombinant murine interleukin-12 vaccine," *Cancer Biotherapy and Radiopharmaceuticals*, vol. 25, no. 3, pp. 263–268, 2010.

[44] C. E. Steding, S.-T. Wu, Y. Zhang, M.-H. Jeng, B. D. Elzey, and C. Kao, "The role of interleukin-12 on modulating myeloid-derived suppressor cells, increasing overall survival and reducing metastasis," *Immunology*, vol. 133, no. 2, pp. 221–238, 2011.

[45] L. H. L. Marchi, T. Paschoalin, L. R. Travassos, and E. G. Rodrigues, "Gene therapy with interleukin-10 receptor and interleukin-12 induces a protective interferon-γ-dependent response against B16F10-Nex2 melanoma," *Cancer Gene Therapy*, vol. 18, no. 2, pp. 110–122, 2010.

[46] H. Komita, X. Zhao, A. K. Katakam et al., "Conditional interleukin-12 gene therapy promotes safe and effective antitumor immunity," *Cancer Gene Therapy*, vol. 16, no. 12, pp. 883–891, 2009.

[47] W. S. Ahn, S. M. Bae, T. Y. Kim et al., "A therapy modality using recombinant IL-12 adenovirus plus E7 protein in a human papillomavirus 16 E6/E7-associated cervical cancer animal model," *Human Gene Therapy*, vol. 14, no. 15, pp. 1389–1399, 2003.

[48] Y. K. He, V. W. Y. Lui, J. Baar et al., "Potentiation of E7 antisense RNA-induced antitumor immunity by co-delivery of IL-12 gene in HPV16 DNA-positive mouse tumor," *Gene Therapy*, vol. 5, no. 11, pp. 1462–1471, 1998.

[49] S. Hallez, O. Detremmerie, C. Giannouli et al., "Interleukin-12-secreting human papillomavirus type 16-transformed cells provide a potent cancer vaccine that generates E7-directed immunity," *International Journal of Cancer*, vol. 81, no. 3, pp. 428–437, 1999.

[50] K. Taga, H. Mostowski, and G. Tosato, "Human interleukin-10 can directly inhibit T-cell growth," *Blood*, vol. 81, no. 11, pp. 2964–2971, 1993.

[51] M. Indrová, J. Bubeník, R. Mikysková et al., "Chemoimmunotherapy in mice carrying HPV16-associated, MHC class I+ and class I- tumours: effects of CBM-4A potentiated with IL-2, IL-12, GM-CSF and genetically modified tumour vaccines," *International Journal of Oncology*, vol. 22, no. 3, pp. 691–695, 2003.

[52] F. De vita, M. Orditura, and G. Galizia, "Serum interleukin-10 is an independent prognos-tic factor in advanced solid tumors," *Oncology Reports*, vol. 7, pp. 357–361, 2000.

[53] S. Mocellin, F. M. Marincola, and H. A. Young, "Interleukin-10 and the immune response against cancer: a counterpoint," *Journal of Leukocyte Biology*, vol. 78, no. 5, pp. 1043–1051, 2005.

[54] D. F. Fiorentino, A. Zlotnik, P. Vieira et al., "IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells," *Journal of Immunology*, vol. 146, no. 10, pp. 3444–3451, 1991.

[55] R. De Waal Malefyt, J. Haanen, H. Spits et al., "Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression," *Journal of Experimental Medicine*, vol. 174, no. 4, pp. 915–924, 1991.

[56] I. A. MacNeil, T. Suda, K. W. Moore, T. R. Mosmann, and A. Zlotnik, "IL-10, a novel growth cofactor for mature and immature T cells," *Journal of Immunology*, vol. 145, no. 12, pp. 4167–4173, 1990.

[57] W. F. Chen and A. Zlotnik, "IL-10: a novel cytotoxic T cell differentiation factor," *Journal of Immunology*, vol. 147, no. 2, pp. 528–534, 1991.

[58] N. F. Go, B. E. Castle, R. Barrett et al., "Interleukin 10, a novel B cell stimulatory factor: unresponsiveness of X chromosome-linked immunodeficiency B cells," *Journal of Experimental Medicine*, vol. 172, no. 6, pp. 1625–1631, 1990.

[59] J. Kim, R. L. Modlin, R. L. Moy et al., "IL-10 production in cutaneous basal and squamous cell carcinomas: a mechanism for evading the local T cell immune response," *Journal of Immunology*, vol. 155, no. 4, pp. 2240–2247, 1995.

[60] F. Y. Yue, R. Dummer, R. Geertsen et al., "Interleukin-10 is a growth factor for human melanoma cells and down-regulates HLA class-I, HLA class-II and ICAM-1 molecules," *International Journal of Cancer*, vol. 71, no. 4, pp. 630–637, 1997.

[61] M. L. García-Hernández, R. Hernández-Pando, P. Gariglio, and J. Berumen, "Interleukin-10 promotes B16-melanoma growth by inhibition of macrophage functions and induction of tumour and vascular cell proliferation," *Immunology*, vol. 105, no. 2, pp. 231–243, 2002.

[62] P. Matar, V. R. Rozados, S. I. Gervasoni, and O. G. Scharovsky, "Down regulation of T-cell-derived IL-10 production by low-dose cyclophosphamide treatment in tumor-bearing rats restores in vitro normal lymphoproliferative response," *International Immunopharmacology*, vol. 1, no. 2, pp. 307–319, 2001.

[63] V. M. Jovasevic, L. Gorelik, J. A. Bluestone, and M. B. Mokyr, "Importance of IL-10 for CTLA-4-Mediated Inhibition of Tumor-Eradicating Immunity," *Journal of Immunology*, vol. 172, no. 3, pp. 1449–1454, 2004.

[64] A. P. Vicari, C. Chiodoni, C. Vaure et al., "Reversal of tumor-induced dendritic cell paralysis by CpG immunostimulatory oligonucleotide and anti-interleukin 10 receptor antibody," *Journal of Experimental Medicine*, vol. 196, no. 4, pp. 541–549, 2002.

[65] B. G. Kim, H. G. Joo, I. S. Chung, H. Y. Chung, H. J. Woo, and Y. S. Yun, "Inhibition of interleukin-10 (IL-10) production from MOPC 315 tumor cells by IL-10 antisense oligodeoxynucleotides enhances cell-mediated immune responses," *Cancer Immunology Immunotherapy*, vol. 49, no. 8, pp. 433–440, 2000.

[66] M. Lagos-Quintana, R. Rauhut, W. Lendeckel, and T. Tuschl, "Identification of novel genes coding for small expressed RNAs," *Science*, vol. 294, no. 5543, pp. 853–858, 2001.

[67] N. C. Lau, L. P. Lim, E. G. Weinstein, and D. P. Bartel, "An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans," *Science*, vol. 294, no. 5543, pp. 858–862, 2001.

[68] R. C. Lee and V. Ambros, "An extensive class of small RNAs in Caenorhabditis elegans," *Science*, vol. 294, no. 5543, pp. 862–864, 2001.

[69] J. G. Doench, C. P. Petersen, and P. A. Sharp, "siRNAs can function as miRNAs," *Genes and Development*, vol. 17, no. 4, pp. 438–442, 2003.

[70] S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, and T. Tuschl, "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells," *Nature*, vol. 411, no. 6836, pp. 494–498, 2001.

[71] S. M. Elbashir, J. Martinez, A. Patkaniowska, W. Lendeckel, and T. Tuschl, "Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate," *The EMBO Journal*, vol. 20, no. 23, pp. 6877–6888, 2001.

[72] M. Jiang and J. Milner, "Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference," *Oncogene*, vol. 21, no. 39, pp. 6041–6048, 2002.

[73] M. Yoshinouchi, T. Yamada, M. Kizaki et al., "In vitro and in vivo growth suppression of human papillomavirus 16-positive cervical cancer cells by E6 siRNA," *Molecular Therapy*, vol. 8, no. 5, pp. 762–768, 2003.

[74] K. Butz, T. Ristriani, A. Hengstermann, C. Denk, M. Scheffner, and F. Hoppe-Seyler, "siRNA targeting of the viral E6 oncogene efficiently kills human papillomavirus-positive cancer cells," *Oncogene*, vol. 22, no. 38, pp. 5938–5945, 2003.

[75] J. S. Lea, N. Sunaga, M. Sato et al., "Silencing of HPV 18 oncoproteins with RNA interference causes growth inhibition of cervical cancer cells," *Reproductive Sciences*, vol. 14, no. 1, pp. 20–28, 2007.

[76] K. Butz, C. Denk, A. Ullmann, M. Scheffner, and F. Hoppe-Seyler, "Induction of apoptosis in human papillomavirus-positive cancer cells by peptide aptamers targeting the viral E6 oncoprotein," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 12, pp. 6693–6697, 2000.

[77] M. Scheffner, B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley, "The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53," *Cell*, vol. 63, no. 6, pp. 1129–1136, 1990.

[78] M. Thomas and L. Banks, "Inhibition of Bak-induced apoptosis by HPV-18 E6," *Oncogene*, vol. 17, no. 23, pp. 2943–2954, 1998.

[79] M. Filippova, L. Parkhurst, and P. J. Duerksen-Hughes, "The human papillomavirus 16 E6 protein binds to Fas-associated death domain and protects cells from Fas-triggered apoptosis," *The Journal of Biological Chemistry*, vol. 279, no. 24, pp. 25729–25744, 2004.

[80] T. O. Garnett, M. Filippova, and P. J. Duerksen-Hughes, "Accelerated degradation of FADD and procaspase 8 in cells expressing human papilloma virus 16 E6 impairs TRAIL-mediated apoptosis," *Cell Death and Differentiation*, vol. 13, no. 11, pp. 1915–1926, 2006.

[81] S. Gross-Mesilaty, E. Reinstein, B. Bercovich et al., "Basal and human papillomavirus E6 oncoprotein-induced degradation of Myc proteins by the ubiquitin pathway," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 14, pp. 8058–8063, 1998.

[82] A. Lagunas-Martínez, V. Madrid-Marina, and P. Gariglio, "Modulation of apoptosis by early human papillomavirus proteins in cervical cancer," *Biochimica et Biophysica Acta*, vol. 1805, no. 1, pp. 6–16, 2010.

[83] M. Filippova, H. Song, J. L. Connolly, T. S. Dermody, and P. J. Duerksen-Hughes, "The human papillomavirus 16 E6 protein binds to tumor necrosis factor (TNF) R1 and protects cells from TNF-induced apoptosis," *The Journal of Biological Chemistry*, vol. 277, no. 24, pp. 21730–21739, 2002.

[84] X. Luo, I. Budihardjo, H. Zou, C. Slaughter, and X. Wang, "Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors," *Cell*, vol. 94, no. 4, pp. 481–490, 1998.

[85] H. Kuwano, K. Sumiyoshi, K. Sonoda et al., "Pathogenesis of esophageal squamous cell carcinoma with lymphoid stroma," *Hepato-Gastroenterology*, vol. 48, no. 38, pp. 458–461, 2001.

[86] L. N. Putral, M. J. Bywater, W. Gu et al., "RNA interference against human papillomavirus oncogenes in cervical cancer cells results in increased sensitivity to cisplatin," *Molecular Pharmacology*, vol. 68, no. 5, pp. 1311–1319, 2005.

[87] X. Y. Niu, Z. L. Peng, W. Q. Duan, H. Wang, and P. Wang, "Inhibition of HPV 16 E6 oncogene expression by RNA interference in vitro and in vivo," *International Journal of Gynecological Cancer*, vol. 16, no. 2, pp. 743–751, 2006.

[88] M. Jiang, C. P. Rubbi, and J. Milner, "Gel-based application of siRNA to human epithelial cancer cells induces RNAi-dependent apoptosis," *Oligonucleotides*, vol. 14, no. 4, pp. 239–248, 2004.

[89] F. Takuma, S. Miyuki, I. Eri et al., "Intratumor injection of small interfering RNA-targeting human papillomavirus 18 E6 and E7 successfully inhibits the growth of cervical cancer ," *International Journal of Oncology*, vol. 29, no. 3, pp. 541–548, 2006.

[90] W. Gu, L. Putral, and N. McMillan, "SiRNA and shRNA as anticancer agents in a cervical cancer model," *Methods in Molecular Biology*, vol. 442, pp. 159–172, 2008.

[91] R. Kuner, M. Vogt, H. Sultmann et al., "Identification of cellular targets for the human papillomavirus E6 and E7 oncogenes by RNA interference and transcriptome analyses," *Journal of Molecular Medicine*, vol. 85, no. 11, pp. 1253–1262, 2007.

[92] M. Peter, C. Rosty, J. Couturier, F. Radvanyi, H. Teshima, and X. Sastre-Garau, "MYC activation associated with the integration of HPV DNA at the MYC locus in genital tumors," *Oncogene*, vol. 25, no. 44, pp. 5985–5993, 2006.

[93] R. A. DeFilippis, E. C. Goodwin, L. Wu, and D. DiMaio, "Endogenous human papillomavirus E6 and E7 proteins differentially regulate proliferation, senescence, and apoptosis in HeLa cervical carcinoma cells," *Journal of Virology*, vol. 77, no. 2, pp. 1551–1563, 2003.

[94] R. Koivusalo, E. Krausz, H. Helenius, and S. Hietanen, "Chemotherapy compounds in cervical cancer cells primed by reconstitution of p53 function after short interfering RNA-mediated degradation of human papillomavirus 18 E6 mRNA: opposite effect of siRNA in combination with different drugs," *Molecular Pharmacology*, vol. 68, no. 2, pp. 372–382, 2005.

[95] K. Yamato, T. Yamada, M. Kizaki et al., "New highly potent and specific E6 and E7 siRNAs for treatment of HPV16 positive cervical cancer," *Cancer Gene Therapy*, vol. 15, pp. 140–153, 2008.

[96] N. Sima, W. Wang, D. Kong et al., "RNA interference against HPV16 E7 oncogene leads to viral E6 and E7 suppression in cervical cancer cells and apoptosis via upregulation of Rb and p53," *Apoptosi*, vol. 13, pp. 273–281, 2008.

[97] J. Salazar-León, F. Reyes-Román, A. Meneses-Acosta et al., "Silencing of HPV16 E6 and E7 oncogenic activities by small interference RNA induces autophagy and apoptosis in human cervical cancer cells," *Journal of Nucleic Acids Investigation*, vol. 2, pp. 59–69, 2011.

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Research Article

Adolescent Understanding and Acceptance of the HPV Vaccination in an Underserved Population in New York City

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Background. HPV vaccination may prevent thousands of cases of cervical cancer. We aimed to evaluate the understanding and acceptance of the HPV vaccine among adolescents. *Methods*. A questionnaire was distributed to adolescents at health clinics affiliated with a large urban hospital system to determine knowledge pertaining to sexually transmitted diseases and acceptance of the HPV vaccine. *Results*. 223 adolescents completed the survey. 28% were male, and 70% were female. The mean age for respondents was 16 years old. Adolescents who had received the HPV vaccine were more likely to be female and to have heard of cervical cancer and Pap testing. Of the 143 adolescents who had not yet been vaccinated, only 4% believed that they were at risk of HPV infection and 52% were willing to be vaccinated. *Conclusions*. Surveyed adolescents demonstrated a marginal willingness to receive the HPV vaccine and a lack of awareness of personal risk for acquiring HPV.

1. Introduction

Approximately 20 million people in the United States are infected with genital human papillomavirus (HPV) [1]. An estimated 5.5 million people will acquire a new genital HPV infection each year, and the incidence of infection is highest among sexually active adolescent girls and young women between the age of 18 and 28 years old. It has been reported that 37% of males and 28% of females in the ninth grade have had sexual intercourse and 7% of students had sexual intercourse before the age of 13 [2]. Therefore, HPV vaccination ideally would be directed toward preadolescents and young adolescents in an effort to provide the greatest public health benefit offered by a prophylactic HPV vaccine.

It is predicted that HPV vaccination will prevent thousands of cases of cervical cancer worldwide. In addition, the emotional stress and economic burden associated with abnormal Papanicolaou test results and the treatment of preinvasive cervical lesions will be greatly reduced. The quadrivalent HPV vaccine (Gardasil, Merck & Co.) was found to have 97% efficacy at preventing HPV 16 and/or 18 related cervical intraepithelial neoplasia 2 or 3, adenocarcinoma in situ, and cervical cancer [3]. The quadrivalent HPV is approved for use in males and females between 9 and 26 years of age. On October 16, 2009, the FDA approved a recombinant HPV bivalent vaccine (Cervarix, Glaxo Smith Kline Biologicals) to prevent cervical cancer and precancerous lesions caused by HPV types 16 and 18. This HPV vaccine is approved for use in females between 10 and 25 years of age and is the second HPV vaccine licensed for use in females in the United States.

Until recently, studies of women and young adults have shown poor levels of knowledge about HPV, Papanicolaou smear testing, and cervical cancer [4, 5]. Current research

indicates that understanding has improved. However, knowledge of some relevant issues is higher than others. A review by Brewer and Fazekas reported that only 21% of respondents knew that HPV is common, 59% knew the purpose of a Papanicolaou smear, and 68% knew that HPV is a sexually transmitted infection [6]. In addition, awareness of HPV among a racially diverse sample of young adults, aged 18–26 years old, was found to be relatively high, with more than 75% of study participants indicating that they had heard of HPV from various sources [7]. However, another recent study examining the acceptability of the HPV vaccination among Latina immigrants and African American women found that 61% of Latinas and 78% of African Americans had never heard about HPV [8].

Continuing, several studies indicate that most parents, especially with proper knowledge of HPV and the vaccine, are willing to accept the HPV vaccine for their children [9–12]. Healthcare providers are an important source of information for parents and children, and studies show that they generally have a positive attitude towards recommending HPV vaccination [13, 14]. The vaccination series can be started beginning at 9 years old, and the Advisory Committee on Immunization Practices currently recommends routine vaccination of females aged 11 or 12 years with three doses of HPV vaccine. In addition, the quadrivalent HPV vaccination has been approved in males aged 9 through 26 years to reduce their likelihood of acquiring genital warts. Ideally, vaccination should be administered before potential exposure to HPV through sexual contact [15].

The purpose of this study was to evaluate the understanding and potential acceptance of the HPV vaccine by adolescents aged 13 to 18 years at adolescent health clinics affiliated with New York Presbyterian Hospital. Although previous research shows that most adolescents, parents, and health care providers are willing to accept universal vaccination for themselves, their attitudes and knowledge regarding the vaccine have yet to be evaluated. Improved knowledge of adolescent understanding and acceptance of the HPV vaccine would help practitioners provide appropriate and useful information to parents, providers, and adolescents when making the decision to vaccinate.

2. Materials and Methods

Approval for this study was received from the institutional review board. We administered a questionnaire to all adolescent patients (defined as between 13 and 18 years of age) awaiting annual well-patient medical examinations in adolescent health clinics affiliated with Weill Cornell Medical College and Columbia University Medical Center. These health clinics provide affordable, comprehensive services to a diverse patient population including underserved populations from each of the five boroughs in the New York City area. The adolescent patient was provided the questionnaire in English and/or Spanish. We could not predict the number of adolescent patients who would be interested in participating in this study, and therefore prior to study initiation we defined the survey collection period as September 1, 2007 through February 1, 2008 with the goal of collecting the maximum number of surveys possible. The research personnel emphasized that participation was voluntary, anonymous, and without incentive for questionnaire completion.

Table 1: Description of adolescent characteristics.

Variable		n (%)
Mean age		15.9 (range 13–18)
Sex		
	Male	62 (27.8)
	Female	155 (69.5)
	Blank	4 (1.8)
Race		
	African American	47 (21.1)
	Native American/Aleutian/Eskimo	1 (0.4)
	Hispanic	145 (65.0)
	Asian	3 (1.3)
	Caucasian	6 (8.5)
	Other	19 (8.5)
	Blank	2 (0.9)
Language		
	English	210 (94.2)
	Spanish	11 (4.9)
	Blank	2 (0.9)
Religion		
	Catholic	132 (59.2)
	Jewish	1 (0.4)
	Buddhist	2 (0.9)
	Protestant	3 (1.3)
	Muslim	8 (3.6)
	Jehovah's Witness	3 (1.3)
	No religion	29 (13.0)
	Other	39 (17.5)
	Blank	6 (2.7)

The questionnaire consisted of two parts; the first part elicited the adolescent's demographic information and knowledge pertaining to sexually transmitted diseases and vaccinations. When the first part was completed, the adolescent was provided with a second part that contained current facts about the HPV vaccine and cervical cancer, followed by questions specifically designed to ascertain adolescent acceptance of the vaccination. Adolescents whose primary language was neither English nor Spanish were excluded from the study since the questionnaire was self-administered and available only in English and Spanish.

Data were analyzed using commercially available software (SPSS version 16.0; SPSS, Inc., Chicago, IlL). A series of $\chi 2$ and Fisher's exact tests were used for comparison of proportions between "HPV vaccine status" and 15 other variables of interest (see Table 2) and "Willingness to receive the HPV vaccine" and 15 other variables of interest (see Table 3). A P value of < 0.05 was considered significant for all tests. For the series of 15 analyses, the sample sizes of 223 participants (HPV vaccine status) and 143 participants (willingness to be vaccinated) provided a mean power >99.9% for detecting a difference between both whether the adolescent

Table 2: HPV vaccine status.

	Have had HPV vaccine		Have not had HPV vaccine		Do not know		*P* value
	#	%	#	%	#	%	
Language							1.000
English	69	32.9%	137	65.2%	4	1.9%	
Spanish	4	36.4%	7	63.6%	0	0.0%	
Age (mean)		16.08		15.85		—	0.311
Gender							<0.001
Male	2	3.1%	60	93.8%	2	3.1%	
Female	71	45.2%	84	53.5%	2	1.3%	
Race							0.677
African American	19	40.4%	27	57.4%	1	2.1%	
Native American/Aleutian/Eskimo	0	0.0%	1	100.0%	0	0.0%	
Hispanic	47	32.4%	95	65.5%	3	2.1%	
Asian	0	0.0%	3	100.0%	0	0.0%	
White	3	50.0%	3	50.0%	0	0.0%	
Other	4	21.1%	15	78.9%	0	0.0%	
Religion							0.708
Catholic	44	33.3%	86	65.2%	2	1.5%	
Jewish	1	100.0%	0	0.0%	0	0.0%	
Buddhist	0	0.0%	2	100.0%	0	0.0%	
Protestant	0	0.0%	3	100.0%	0	0.0%	
No Religion	11	37.9%	17	58.6%	1	3.4%	
Muslim	2	25.0%	6	75.0%	0	0.0%	
Jehovah's Witness	0	0.0%	3	100.0%	0	0.0%	
Other	15	38.5%	23	59.0%	1	2.6%	
Blank	0	0.0%	4	100.0%	0	0.0%	
Mother's completed level of education							0.031
Less than high school	25	41.0%	36	59.0%	0	0.0%	
High school/GED	19	22.9%	62	74.7%	2	2.4%	
College	21	47.7%	23	52.3%	0	0.0%	
Professional degree	4	22.2%	13	72.2%	1	5.6%	
Other	1	16.7%	5	83.3%	0	0.0%	
Blank	3	33.3%	5	55.6%	1	11.1%	
Father's completed level of education							0.287
<high school	25	37.9%	41	62.1%	0	0.0%	
High school/GED	24	31.2%	50	64.9%	3	3.9%	
College	7	26.9%	18	69.2%	1	3.8%	
Professional degree	1	10.0%	9	90.0%	0	0.0%	
Other	6	54.5%	5	45.5%	0	0.0%	
Blank	10	32.3%	21	67.7%	0	0.0%	
Has an older sibling who received the HPV vaccine							1
Yes	44	34.1%	83	64.3%	2	1.6%	
No	27	34.6%	50	64.1%	1	1.3%	
Blank	0	0.0%	1	1.0%	0	0.0%	
Unclear	2	15.4%	10	76.9%	1	7.7%	
Has a younger sibling who received the HPV vaccine							1
Yes	40	33.1%	79	65.3%	2	1.7%	
No	29	34.1%	55	64.7%	1	1.2%	
Blank	0	0.0%	1	100.0%	0	0.0%	
Unclear	4	28.6%	9	64.3%	1	7.1%	
Heard of cervical cancer							<0.001
Yes	69	41.1%	97	57.7%	2	1.2%	
No	4	7.5%	47	88.7%	2	3.8%	

Table 2: Continued.

	Have had HPV vaccine		Have not had HPV vaccine		Do not know		P value
	#	%	#	%	#	%	
Heard of Pap screening							0.003
Yes	44	42.7%	56	54.4%	3	2.9%	
No	28	23.9%	88	75.2%	1	0.1%	
Understand the goal of Pap screening							0.003
Yes	29	50.9%	27	47.4%	1	1.8%	
No	43	26.7%	115	71.4%	3	1.9%	
Heard of sexually transmitted disease							0.241
Yes	69	34.2%	130	64.4%	3	1.5%	
No	4	21.1%	14	73.7%	1	5.3%	
Have had other vaccines							<0.001
Yes	35	72.9%	12	25.0%	1	2.1%	
No	33	22.6%	112	76.7%	1	0.7%	
Do not know	2	16.7%	8	66.7%	2	16.7%	
Believe at risk for abnormal Pap/cervical cancer							0.333
Yes	8	57.1%	6	42.9%	0	0.0%	
No	43	31.2%	92	66.7%	3	2.2%	
Do not know	16	28.6%	39	69.6%	1	1.8%	

had been vaccinated and whether the adolescent was willing to receive the HPV vaccine and the respective variable, using 2-tailed test with statistical significance defined as $P < 0.05$.

3. Results

Of the 223 adolescents who completed the survey, 28% were male and 70% were female (2% did not respond to the question). The mean age of respondents was 16 years old (range 13–18). The demographics of adolescents including in this study are displayed in Table 1. A majority of adolescents surveyed (168, 75%) stated that they had prior knowledge of cervical cancer. However, a smaller percentage of adolescents (103, 46%) acknowledged prior knowledge of what a "Pap smear" was, and only 57 respondents (26%) were aware that the Pap smear was used to screen for cervical cancer. 5% (11) of respondents reported knowing someone who had/has cervical cancer, and 91% (202) were familiar with the term "sexually transmitted disease" or "STD".

Of the 223 adolescent surveyed, 33% stated that they had received the HPV vaccine, 64% had not had the vaccine, and 3% where not sure. Among the 143 adolescents who had not yet been vaccinated, 52% were willing to get the HPV vaccine, 12% were not willing, and 36% were unsure. Of the 143 adolescents who had not yet been vaccinated, only 4% believed that they were at risk for being exposed to HPV.

Of the 73 respondents that stated that they had received the HPV vaccine, the mean age was 16 years old (range 13–18). Female respondents were more likely to have received the HPV vaccine than male respondents (45% versus 3%, $P < 0.001$). HPV vaccination status was not significantly associated with language, race, religion, or having younger or older siblings who had received the HPV vaccine. HPV vaccine status was associated with knowledge of cervical cancer (41% of those who had heard of cervical cancer were vaccinated), knowledge of Pap screening (43% who had heard of Pap screening were vaccinated), and having had other vaccines (73% of those who had received other vaccines were vaccinated) (Table 2).

Among the adolescents who had not yet received the HPV vaccine, 58% of females and 42% of males were willing to be vaccinated; however this difference was not statistically significant. In addition, 53% of Hispanic adolescents and 56% of African American adolescents were willing to accept vaccination. There was no association with willingness to receive the vaccine and gender, race, religion, language, having vaccinated siblings, knowledge of cervical cancer, Pap screening, sexually transmitted disease, or any other studied variable.

4. Discussion

The current study reveals only a marginal willingness of teenagers to accept the HPV vaccine. The majority of data to date suggest that young women are interested in vaccines that prevent HPV and other sexually transmitted infections. However, most of these studies have been conducted with women and men of 18 years of age or older. Holcomb et al. found that women are interested in learning about HPV, how the virus is transmitted, and how women can prevent becoming infected [16]. In 2001, a study of women recruited from community and clinical sites displayed that roughly 85% of participants indicated an intention to receive an HPV vaccine for cervical cancer prevention once it became available [17]. A study of young, Australian, men and women aged 18–23 years old showed a high vaccine acceptance despite inadequate knowledge of HPV infection [18]. Another study of male and female college students found an overall acceptance rate of the HPV vaccine of 74%. In this study, although females were more likely to have already been vaccinated, gender did not affect vaccine acceptance among unvaccinated adolescents [19]. In contrast

Table 3: Willingness to receive the HPV vaccine among adolescent who have not yet been vaccinated.

	Yes		No		Do not know		P
	#	%	#	%	#	%	
Language							0.873
English	71	52.2%	17	12.5%	48	35.3%	
Spanish	3	42.9%	1	14.3%	3	42.9%	
Age (mean)	74	1605.00%	18	16.06			0.997
Gender							0.126
Male	25	42.4%	10	16.9%	24	40.7%	
Female	49	58.3%	8	9.5%	27	32.1%	
Race							0.062
African American	15	55.6%	0	0.0%	12	44.4%	
Native American/Aleutian/Eskimo	0	0.0%	1	100.0%	0	0.0%	
Hispanic	50	52.6%	13	13.7%	32	33.7%	
Asian	0	0.0%	0	0.0%	2	100.0%	
White	1	33.3%	1	33.3%	1	33.3%	
Other	8	53.3%	3	20.0%	4	26.7%	
Religion							0.569
Catholic	48	55.8%	10	11.6%	28	32.6%	
Buddhist	0	0.0%	0	0.0%	1	100.0%	
Protestant	1	33.3%	1	33.3%	1	33.3%	
No Religion	10	58.8%	2	11.8%	5	29.4%	
Muslim	1	16.7%	1	16.7%	4	66.7%	
Jehovah's Witness	1	33.3%	1	33.3%	1	33.3%	
Other	11	47.8%	3	13.0%	9	39.1%	
Blank	2	50.0%	0	0.0%	2	50.0%	
Mother's completed level of education							0.094
<high school	21	60.0%	3	8.6%	11	31.4%	
High school/GED	35	56.5%	9	14.5%	18	29.0%	
College	10	43.5%	2	8.7%	11	47.8%	
Professional degree	7	53.8%	3	23.1%	3	23.1%	
Other	0	0.0%	0	0.0%	5	100.0%	
Blank	1	20.0%	1	20.0%	3	60.0%	
Father's completed level of education							0.204
<high school	25	62.5%	2	5.0%	13	32.5%	
High school/GED	21	42.0%	8	16.0%	21	42.0%	
College	6	33.3%	3	16.7%	9	50.0%	
Professional degree	4	44.4%	3	33.3%	2	22.2%	
Other	3	60.0%	0	0.0%	2	40.0%	
Blank	15	71.4%	2	9.5%	4	19.0%	
Has an older sibling who received the HPV vaccine							0.683
Yes	41	49.4%	13	15.7%	29	34.9%	
No	26	52.0%	5	10.0%	19	38.0%	
Blank	1	100.0%	0	0.0%	0	0.0%	
Unclear	6	66.7%	0	0.0%	3	33.3%	
Has a younger sibling who received the HPV vaccine							0.183
Yes	41	51.9%	7	8.9%	31	39.2%	
No	26	47.3%	11	20.0%	18	32.7%	
Blank	1	100.0%	0	0.0%	0	0.0%	
Unclear	6	75.0%	0	0.0%	2	25.0%	
Heard of cervical cancer							0.111
Yes	55	56.7%	13	13.4%	29	29.9%	
No	19	41.3%	5	10.9%	22	47.8%	

Table 3: Continued.

	Yes		No		Do not know		P
	#	%	#	%	#	%	
Heard of Pap screening							0.131
Yes	33	58.9%	6	10.7%	17	30.4%	
No	41	47.1%	12	13.8%	34	39.1%	
Know the use of Pap							0.451
Yes	16	59.3%	4	14.8%	7	25.9%	
No	56	49.1%	14	12.3%	44	38.6%	
Heard of sexually transmitted disease							0.447
Yes	69	53.5%	16	12.4%	44	34.1%	
No	5	35.7%	2	14.3%	7	50.0%	
Have had other vaccines							0.319
Yes	9	75.0%	1	8.3%	2	16.7%	
No	53	47.7%	17	15.3%	41	36.9%	
Do not know	6	50.0%	0	0.0%	6	50.0%	
Believe at risk for abnormal Pap/cervical cancer							0.3
Yes	3	50.0%	2	33.3%	1	16.7%	
No	46	50.0%	13	14.1%	33	35.9%	
Do not know	21	55.3%	2	5.3%	15	39.5%	

to most of the data available in the literature, this current review sampled a younger population. We found a generalized lack of awareness of HPV among adolescents. Although the majority of adolescents had heard of cervical cancer (75%), only 46% knew what a Pap smear was, and only 26% knew that the Pap smear is a screening test for cervical cancer. Furthermore, only 4% of the adolescents surveyed who had not yet received the HPV vaccine believed that they were at risk for HPV infection. The lack of awareness about cervical cancer screening and underestimated risk of HPV risk likely contributes to the limited willingness of the adolescents surveyed to accept the HPV vaccine for themselves.

Many of the adolescents stated that they would seek advice from a parent or physician when deciding whether or not to accept the HPV vaccine. Interestingly, we found no significant association between having had the HPV vaccine or willingness to receive the vaccine and the level of education of the adolescent's parents. There was also no association between having younger or older siblings who had been vaccinated and having had the HPV vaccine or willingness to receive the vaccine. Many studies have shown that most parents are willing to accept the HPV vaccine for their children. A recent study, postlicensure of the vaccine, evaluated mothers of children in primary and secondary schools in England and found that 75% of mothers would accept the HPV vaccine for their daughter. Acceptance was higher in mothers who had experienced cancer in their families, had older daughters, perceived approval from their husbands/partners, and believed vaccine acceptance would be more normative [20]. Another postlicensure study used a random-digit-dial telephone survey to examine the likelihood of parental acceptance of the HPV vaccination for young adolescent girls in California. Within this sample, 75% of parents were likely to vaccinate a daughter before age 13. Hispanic parents were more likely to accept vaccination than were non-Hispanic parents, and African American and Asian American parents were the least likely to accept vaccination [21]. Overall, parental attitude and acceptance studies suggest a great deal of parental interest in HPV vaccination for their adolescent children. Healthcare providers may be able to encourage participation in HPV vaccine programs by bolstering parents' underlying desire to protect their children, as recommendation by a healthcare provider is a crucial prompt for vaccination.

Healthcare providers are an important source of information for parents and children, and studies show that they generally have a positive attitude towards recommending an HPV vaccination. One study of 207 fellows of the American College of Obstetricians and Gynecologists found a fairly positive disposition towards recommending the HPV vaccine, with a mean rating for vaccine recommendation of 79 out of 100 [22]. Similarly, 224 nurse practitioners had a favorable attitude about HPV vaccine recommendation, with a mean rating of 72 out of 100 [23]. Our study reveals that knowledge of cervical cancer and Pap screening is associated with having had the HPV vaccine, however interestingly not with willingness to accept the vaccine among those not yet vaccinated.

Previous studies have shown that knowledge of personal risk is generally limited with regard to sexually transmitted infections. A study of sexually active heterosexual college students found that personal risk of contracting Acquires Immune Deficiency Syndrome was estimated to be significantly lower than risk for each of a set of hypothetical persons who varied in degree of similarity to respondents [24]. In a study of university students in the United Kingdom, screening for chlamydia was limited, and perception of personal risk was poor [25]. In a pilot study among Australian university students, even though the majority of students were comfortable with opportunistic testing for chlamydia by their general practitioner, the likelihood of being tested in the upcoming year for most students was low, as was

personal concern about chlamydia infection [26]. With regard to HPV, a recent study of Australian women with diverse sexual orientation, found that the majority of women had risk factors for HPV but few felt personally at risk of acquiring infection [27]. Among the 143 adolescents in our study who had not yet received the HPV vaccine, only 4% believed that they were at risk for being exposed to HPV. The very low personal risk perception for HPV suggests the need for targeted education for this group regarding HPV transmission and prevention.

Study limitations should be considered when interpreting the present findings. Participants were recruited from a hospital-based adolescent health clinic serving a racially and ethnically diverse, predominantly low-income, population. As a result, the findings may not be applicable to all adolescents in the United States. In order to include 13 and 14 years old in this study at an adolescent health clinic, we were unable to ask certain direct questions, including "Are you sexually active?". The answer to this question may have provided more information regarding age of sexual activity in the current study population and would allow direct comparison of these results with a personal risk perception question. In addition, because the time frame was not stated explicitly in the question regarding personal risk, it is unclear whether participants were rating their risk of HPV infection at present, in the immediate future, or over their lifetime, which would also affect the accuracy of their ratings.

5. Conclusion

It is clear that education will play an important role in the implementation of an HPV vaccination program. In this study, only 53% of adolescents who had not yet received the HPV vaccine were willing to be vaccinated, and lack of education likely contributes to this hesitancy. Healthcare providers must be proactive in educating and discussing benefits of vaccination. Based on factors affecting vaccine acceptability, HPV vaccine programs in the United States should emphasize high vaccine effectiveness, the high likelihood of HPV infection, physician recommendations, and address barriers to vaccination. It is important to educate patients that the vaccine is most effective prior to sexual activity and HPV exposure. However, the vaccine does not replace routine screening for cervical cancer or the need for education about continued cervical cancer screening. Thus, it is essential for healthcare providers to offer guidance about the ongoing need for screening. Future studies will be necessary to evaluate public health issues that arise once the HPV vaccination programs have been established more extensively.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

J. Blumenthal and M. K. Frey contributed equally to the work.

References

[1] H. Weinstock, S. Berman, and W. Cates Jr., "Sexually transmitted diseases among American youth: incidence and prevalence estimates, 2000," *Perspectives on Sexual and Reproductive Health*, vol. 36, no. 1, pp. 6–10, 2004.

[2] Centers for Disease Control, "Youth risk behavior surveillance—United States, 2003," *Morbidity and Mortality Weekly Report*, vol. 53, no. 2, pp. 1–96, 2004.

[3] F. E. Skjeldestad, "Prophylactic quadrivalent human papillomavirus (HPV) (types 6, 11, 16, 18) L1 virus-like particle VLP) vaccine (Gardasil) reduces cervical intraepithelial neoplasia (CIN) 2/3 risk," in *Proceedings of the 43rd Annual Meeting of the Infectious Disease Society of America*, San Francisco, Calif, USA, October 2005.

[4] D. R. Blake, B. R. Weber, and K. E. Fletcher, "Adolescent and young adult women's misunderstanding of the term Pap smear," *Archives of Pediatrics and Adolescent Medicine*, vol. 158, no. 10, pp. 966–970, 2004.

[5] M. Pitts and T. Clarke, "Human papillomavirus infections and risks of cervical cancer: what do women know?" *Health Education Research*, vol. 17, no. 6, pp. 706–714, 2002.

[6] N. T. Brewer and K. I. Fazekas, "Predictors of HPV vaccine acceptability: a theory-informed, systematic review," *Preventive Medicine*, vol. 45, no. 2-3, pp. 107–114, 2007.

[7] M. A. Gerend and Z. F. Magloire, "Awareness, knowledge, and beliefs about human papillomavirus in a racially diverse sample of young adults," *Journal of Adolescent Health*, vol. 42, no. 3, pp. 237–242, 2008.

[8] I. C. Scarinci, I. C. Garcés-Palacio, and E. E. Partridge, "An examination of acceptability of HPV vaccination among African American women and Latina immigrants," *Journal of Women's Health*, vol. 16, no. 8, pp. 1224–1233, 2007.

[9] R. M. Mays, L. A. Sturm, and G. D. Zimet, "Parental perspectives on vaccinating children against sexually transmitted infections," *Social Science and Medicine*, vol. 58, no. 7, pp. 1405–1413, 2004.

[10] E. Lazcano-Ponce, L. Rivera, E. Arillo-Santillán, J. Salmerón, M. Hernández-Avila, and N. Muñoz, "Acceptability of a human papillomavirus (HPV) trial vaccine among mothers of adolescents in Cuernavaca, Mexico," *Archives of Medical Research*, vol. 32, no. 3, pp. 243–247, 2001.

[11] B. Slomovitz, C. C. Sun, M. Frumovitz et al., "Are women ready for the HPV vaccine?" *Gynecologic Oncology*, vol. 103, no. 1, pp. 151–154, 2006.

[12] K. Davis, E. D. Dickman, D. Ferris, and J. K. Dias, "Human papillomavirus vaccine acceptability among parents of 10- to 15-year-old adolescents," *Journal of Lower Genital Tract Disease*, vol. 8, no. 3, pp. 188–194, 2004.

[13] J. C. Raley, K. A. Followwill, G. D. Zimet, and K. A. Ault, "Gynecologists' attitudes regarding human papilloma virus vaccination: a survey of Fellows of the American College of Obstetricians and Gynecologists," *Infectious Disease in Obstetrics and Gynecology*, vol. 12, no. 3-4, pp. 127–133, 2004.

[14] R. M. Mays and G. D. Zimet, "Recommending STI vaccination to parents of adolescents: the attitudes of nurse practitioners," *Sexually Transmitted Diseases*, vol. 31, no. 7, pp. 428–432, 2004.

[15] Centers for Disease Control and Prevention, "FDA licensure of bivalent human papillomavirus vaccine (HPV2, Cervarix) for use in females and updated HPV vaccination recommendations from the advisory committee on immunization practices (ACIP)," *Morbidity and Mortality Weekly Report*, vol. 59, no. 20, pp. 626–629, 2010.

[16] B. Holcomb, J. M. Bailey, K. Crawford, and M. T. Ruffin, "Adults' knowledge and behaviors related to human papillomavirus infection," *Journal of the American Board of Family Practice*, vol. 17, no. 1, pp. 26–31, 2004.

[17] J. A. Kahn, S. L. Rosenthal, T. Hamannn, and D. I. Bernstein, "Attitudes about human papillomavirus vaccine in young women," *International Journal of STD and AIDS*, vol. 14, no. 5, pp. 300–306, 2003.

[18] A. McClelland and P. Liamputtong, "Knowledge and acceptance of human papillomavirus vaccination: perspectives of young Australians living in Melbourne, Australia," *Sexual Health*, vol. 3, no. 2, pp. 95–101, 2006.

[19] C. W. Boehner, S. R. Howe, D. I. Bernstein, and S. L. Rosenthal, "Viral sexually transmitted disease vaccine acceptability among college students," *Sexually Transmitted Diseases*, vol. 30, no. 10, pp. 774–778, 2003.

[20] L. A. Marlow, J. Waller, and J. Wardle, "Parental attitudes to pre-pubertal HPV vaccination," *Vaccine*, vol. 25, no. 11, pp. 1945–1952, 2007.

[21] N. A. Constantine and P. Jerman, "Acceptance of human papillomavirus vaccination among Californian parents of daughters: a representative statewide analysis," *Journal of Adolescent Health*, vol. 40, no. 2, pp. 108–115, 2007.

[22] J. C. Raley, K. A. Followwill, G. D. Zimet, and K. A. Ault, "Gynecologists' attitudes regarding human papilloma virus vaccination: a survey of Fellows of the American College of Obstetricians and Gynecologists," *Infectious Disease in Obstetrics and Gynecology*, vol. 12, no. 3-4, pp. 127–133, 2004.

[23] R. M. Mays and G. D. Zimet, "Recommending STI vaccination to parents of adolescents: the attitudes of nurse practitioners," *Sexually Transmitted Diseases*, vol. 31, no. 7, pp. 428–432, 2004.

[24] S. E. Mickler, "Perceptions of vulnerability: impact on AIDS-preventive behavior among college adolescents," *AIDS Education and Prevention*, vol. 5, no. 1, pp. 43–53, 1993.

[25] A. Greaves, S. Lonsdale, S. Whinney, E. Hood, H. Mossop, and B. Olowokure, "University undergraduates' knowledge of chlamydia screening services and chlamydia infection following the introduction of a National Chlamydia Screening Programme," *European Journal of Contraception and Reproductive Health Care*, vol. 14, no. 1, pp. 61–68, 2009.

[26] B. Zakher and M. Kang, "Attitudes to chlamydia screening in general practice among Australian university students: a pilot study," *Sexual Health*, vol. 5, no. 4, pp. 359–363, 2008.

[27] R. McNair, J. Power, and S. Carr, "Comparing knowledge and perceived risk related to the human papilloma virus among Australian women of diverse sexual orientations," *Australian and New Zealand Journal of Public Health*, vol. 33, no. 1, pp. 87–93, 2009.

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Research Article

Human Papillomavirus Genotyping and E6/E7 mRNA Expression in Greek Women with Intraepithelial Neoplasia and Squamous Cell Carcinoma of the Vagina and Vulva

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A large proportion of vaginal and vulvar squamous cell carcinomas (SCCs) and intraepithelial neoplasias (VAIN and VIN) are associated with HPV infection, mainly type 16. The purpose of this study was to identify HPV genotypes, as well as E6/E7 mRNA expression of high-risk HPVs (16, 18, 31, 33, and 45) in 56 histology samples of VAIN, VIN, vaginal, and vulvar SCCs. HPV was identified in 56% of VAIN and 50% of vaginal SCCs, 71.4% of VIN and 50% of vulvar SCCs. E6/E7 mRNA expression was found in one-third of VAIN and in all vaginal SCCs, 42.9% of VIN and 83.3% of vulvar SCCs. Our data indicated that HPV 16 was the commonest genotype identified in VAIN and VIN and the only genotype found in SCCs of the vagina and vulva. These findings may suggest, in accordance with other studies, that mRNA assay might be useful in triaging lesions with increased risk of progression to cancer.

1. Introduction

Human papillomavirus (HPV) infection of the female genital tract, is particularly frequent worldwide and its majority is transient, while at the same time, the persistent infections caused by the oncogenic types of HPV are responsible for cancer development. This oncogenic action of HPV is a result of the transformation ability of the high-risk (hr) HPV types' oncoproteins E6 and E7. The oncogenic properties of high-risk HPV reside in the E6 and E7 genes, which if inappropriately expressed in dividing cells deregulate cell division and differentiation [1].

The causal relation of HPV infection with development of cervical cancer has been firmly established. The same does not necessarily apply to vulvar and vaginal squamous cell carcinomas (SCCs), where HPV is responsible for only a smaller percentage, namely, 60–70% of vaginal SCCs [2, 3] and 38–75% of vulvar SCCs [2, 4]. Interestingly, HPV infection is strongly associated with intraepithelial neoplasia of the vagina (VAIN) and vulva (VIN) (93.6% and 84%, resp.) [5]. HPV 16 is by far the commonest HPV type identified in vaginal and vulvar SCCs and precancerous lesions [6]. The above data may vary from population to population, and to our knowledge, no such analysis has been conducted in Greece.

The purpose of the current study was the HPV genotyping, as well as the expression of E6/E7 mRNA from the hrHPV types (16, 18, 31, 33, and 45) in Greek women with VAIN, VIN, and SCCs of the vagina and vulva.

2. Materials and Methods

2.1. Study Population and Collection of Specimens. The sam-ple of this study consisted of 56 paraffin-embedded tissue sections of VAIN, VIN, or vaginal and vulvar SCCs obtained from the Pathology Laboratory of the Regional Anticancer Oncology Hospital of Athens "St. Savvas". The tissues were deparaffinized, and total nucleic acid was extracted using automated extraction (NucliSENS easyMAG, bioMérieux Hellas S.A). Then, genotyping of 24 HPV types was performed with microarray-based assay (PapilloCheck HPV-Screening, Greiner Bio-One GmbH, Germany, cat no. 465 060) as well as the expression of HPV 16, 18, 31, 33, and 45 E6/E7 mRNA, using the commercial real-time NucliSENS EasyQ assay (NucliSENS EasyQ HPV 1.1, bioMérieux Hellas S.A, cat no. 290003).

Although VIN terminology was changed by the International Society for the Study on Vulvovaginal Disease in 2004, we maintained the original terminology which was used during the histological diagnosis [6].

Ethical approval was granted by the ethics committee of the Regional Anticancer Oncology Hospital of Athens "St. Savvas", and all participants provided written informed consent.

2.2. Deparaffinization. Fifteen micrometer sections of paraffin-embedded tissues were deparaffinized by incubation with 100% xylene (Applichem GmbH, Darmstadt, Germany, cat no. 10-20/21-38) at 50°C and washings twice with 100% ethanol (Applichem GmbH, Darmstadt, Germany, cat no. 64-17-5). Then, the pellets were dried at room temperature for 45 minutes. Finally, deparaffinized samples were digested with 100 μL digestion buffer [1 mL TE buffer 1X (Invitrogen corp., Calif, USA, cat no. 12090-015) and 5 μL 50% Tween20 solution (Invitrogen corp., Calif, USA, cat no. 00–3005)] and 5 μL proteinase K solution (Invitrogen corp., Calif, USA, cat no. 25530-049) at 65°C overnight. Proteinase K was deactivated in heat block at 80°C for 15 minutes.

2.3. Extraction of Nucleic Acids. Tissue samples were transferred in lysis buffer (NucliSENS lysis buffer, bioMérieux Hellas S.A, cat no. 200292) for 30 minutes, then total nucleic acid was extracted by the off-board protocol with the NucliSENS easyMAG platform (bioMérieux Hellas S.A), according to the manufacturer's instructions. The nucleic acids were eluted in 55 μL of elution buffer. DNA quality test was carried out using Human Globin, Beta, Primer set kit (Maxim Biotech, Inc., South San Francisco, CA) according to manufacturer's instructions. To assess RNA integrity, 5 μg of RNA per sample was separated on 1% formaldehyde-agarose gel.

2.4. HPV Genotyping (PapilloCheck HPV DNA Microarray). The PapilloCheck HPV-Screening was used. This technology is based on a DNA chip for the type-specific identification of 24 types of HPV (18 high-risk and 6 low-risk types). E1-based PCR was performed according to the manufacturer's guidelines. For each sample, we mixed 19,8 μL PapilloCheck MasterMix, 0,2 μL HotStarTaq plus DNA polymerase (5 U/μL, Qiagen, cat no. 203605), and 5 μL DNA from the tissue sample. Hybridization is followed by mixing 30 μL of the PapilloCheck hybridization buffer in a fresh reaction tube with 5 μL of the PCR product at room temperature and transferring 25 μL of the hybridization mix into each compartment of the chip. We incubated the chip for 15 minutes at room temperature in a humid atmosphere. The chip was washed in 3 washing solutions, centrifuged for 3 minutes at 5000 rpm, and scanned on the CheckScannerTM.

2.5. HPV E6/E7 mRNA Expression (NucliSENS EasyQ HPV Assay). A commercial real-time NucliSENS *EasyQ* assay (NucliSENS EasyQ HPV 1.1, bioMérieux Hellas S.A, cat no. 290003) was performed for the qualitative detection of HPV E6/E7 mRNA of five hrHPV types (16, 18, 31, 33, and 45) according to the manufacturer's instructions. Firstly, three premixes were made by adding reagent sphere diluent (Tris-HCl, 45% DMSO) into reagent spheres (nucleotides, dithiotreitol, and MgCl2). In each premix, we added U1A/HPV 16, HPV 33/45, or HPV 18/31 primer and molecular beacon mixes, KCl stock solution, and NASBA water. Secondly, 10 μL of this premix was distributed to each well in a reaction plate, and the addition of 5 μL RNA followed. The plates were incubated for 4 minutes at 65°C to destabilize secondary structures of RNA, followed by cooling down to 41°C. The reaction was started by addition of enzymes (AMV-RT, RNase H, T7 RNA polymerase, and bovine serum albumin) and measured in real time using the Lambda FL 600 fluorescence reader (Bio-Tek, Winooski, VT) at 41°C for 2 hours and 30 minutes.

2.6. Statistical Analysis. Our data were analyzed using SAS v9.0. Absolute and relative frequencies were used to present the HPV prevalence according to age and histology. Chi-squared tests were performed to assess the statistical significance of any differences in prevalence. *t*-test was used, along with relevant descriptive statistics (mean value, standard deviation, and 95% confidence interval for the mean value) to compare the average age among patients depending on the prevalence of HPV and histology. In all the statistical tests, 5% level of significance was used. Concordance between DNA and mRNA tests was evaluated using the Cohen's kappa statistic.

3. Results

3.1. Vaginal Intraepithelial Neoplasia and Vaginal Squamous Cell Carcinoma. This group consisted of 18 patients with VAIN (mean age 46.2 years) and 4 patients with SCCs (mean age of 61.3 years). 8 VAIN cases were classified as VAIN I

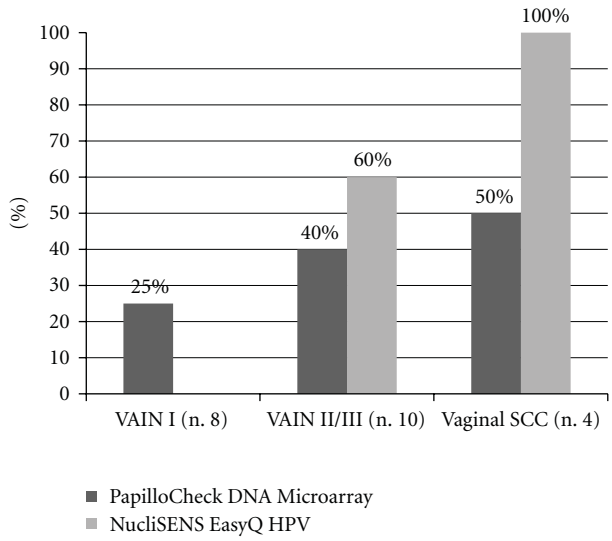

Figure 1: Prevalence of the hr HPV genotypes (16, 18, 31, 33, and 45) detected by both PapilloCheck DNA Microarray and NucliSENS EasyQ HPV assay according to histological status of samples of vagina.

and 10 as VAIN II/III. HPV infection was detected in 56% of VAIN (10/18) and in 50% of vaginal SCCs (2/4). Detectable HPV DNA from at least one of the 24 genotypes was found in 75% (6/8) of cases of VAIN I and 40% (4/10) of VAIN II/III. In VAIN cases, the presence of HPV infection was strongly associated with younger patient age (38.7 versus 55.6 years, $P = 0.006$).

hrHPV types were found in 70% (7/10) and lrHPV types in 30% (3/10) of VAIN cases. Only hrHPVs were detected in vaginal SCCs (2/2). Multiple HPV types were present only in one case (one VAIN II/III sample contained HPV 33 and 53).

The commonest HPV genotype was HPV 42 for VAIN I samples (3/6 cases, 50%), followed by HPV 16 (2/6 cases, 33.3%) and HPV 39 (1/6 cases, 16.7%), while HPV16 and HPV33 were the most common types for VAIN II/III (2/4 cases, 50% each). HPV 16 was the only type associated with HPV-infected vaginal SCCs (2/2 cases, 100%).

Regarding the hrHPV types 16, 18, 31, 33, and 45, 33.3% (6/18) of the VAIN and 50% of vaginal SCCs were HPV DNA positive for at least one of the above types. The detection rates of the five hrHPV types in samples from women with different grades of VAIN and SCCs are provided in Figure 1.

All vaginal SCCs (4/4) and 60% (6/10) of VAIN II/III were positive for HPV E6/E7 mRNA expression. This was statistically significantly higher than VAIN I samples, where no HPV E6/E7 mRNA expression was detected ($P = 0.01$ and $P = 0.002$), respectively (Figure 1).

The expression pattern for hrHPV types 16, 18, 31, 33, and 45, according to VAIN grade and SCCs, is summarized in Table 1.

The concordance between the HPV DNA test (PapilloCheck DNA Microarray) and HPV E6/E7 mRNA test (NucliSENS EasyQ HPV assay) results was poor for patients with VAIN I (75%; kappa = 0.00) and vaginal SCCs (50%; kappa = 0.00), whereas it was good for patients with VAIN II/III (80%; kappa = 0.62) (Table 2).

Table 1: Distribution of different HPV types detected by NucliSENS EasyQ HPV assay.

Histology result	HPV types				
	HPV16	HPV18	HPV31	HPV33	HPV45
VAIN I					
VAIN II/III	4			2	
Vaginal SCCs	4				
Total (*n*.10)	**8**			**2**	
VIN I	4				
VIN II/III	6				2
Vulvar SCCs	3			2	
Total (*n*.17)	**13**			**2**	**2**

Distribution of the five high-risk HPV types determined by NucliSENS EasyQ HPV assay in the vaginal and vulvar tissue samples.

3.2. Vulvar Intraepithelial Neoplasia and Vulvar Squamous Cell Carcinoma. We included 28 patients with VIN, half of which had a low-grade lesion (mean age 35 years) and 6 patients with vulvar SCCs (mean age 62.5 years). VIN cases were not associated with lichen sclerosus, differentiated vulvar intraepithelial neoplasia, or squamous cell hyperplasia. PapilloCheck DNA Microarray detected HPV infection in 71.4% of VIN samples (20/28) and in 50% of vulvar SCCs (3/6). HPV DNA from at least one of the 24 genotypes was detected in 64.3% (9/14) of women with VIN I, and 78.6% of those with VIN II/III (11/14). In VIN cases, the presence of HPV infection was associated with younger patient age (31.7 versus 43.1 years, $P = 0.001$).

PapilloCheck assay detected hrHPV infection in all VIN cases and vulvar SCCs. Multiple infection was detected in 5 cases (3 VIN I cases contained HPV 6/16, HPV 6/16/59 and 11/59, resp., and 2 VIN II/III cases contained HPV 16/53 and HPV 16/51/66, resp.).

HPV16 and HPV59 were the most commonly observed for infected VIN I samples (4/9 cases each and one case with multiple infection HPV 6/16/59, 55.6% each), while HPV16 was the commonest genotype for VIN II/III (9/11 cases, 81.8%) followed by types 18, 51, 52, 53, and 66 (1/11 cases, 9.1% each). In HPV-positive vulvar SCCs, only HPV 16 was present (3/3 cases, 100%).

Regarding the hrHPV types 16, 18, 31, 33, and 45, 53.6% (15/18) of VIN and 50% of vulvar SCCs were HPV DNA positive for at least one of the above HPV types by PapilloCheck DNA Microarray. The detection rates of the five hrHPV types in samples from women with different grades of VIN and SCCs are provided in Figure 2.

42.9% of VIN samples (12/28) and 83.3% of vulvar SCCs (5/6) were positive for HPV E6/E7 mRNA expression. There was a higher prevalence of E6 and E7 mRNA expression in patients with higher-grade lesions as shown in Figure 2. The difference in detection of E6/E7 mRNA expression between VIN I and vulvar SCCs was statistically significant ($P = 0.04$).

Table 2: Concordance between HPV DNA test (PapilloCheck DNA Microarray) and HPV E6/E7 mRNA test (NucliSENS EasyQ HPV assay) by histological status of samples.

	No. of specimens	Number of specimens positive with		Concordance*	%	Kappa value	*P*
		HPV DNA test	E6/E7 mRNA test				
VAIN I	8	2	0	6/8	75.0	0.00	1.00
VAIN II/III	10	4	6	8/10	80.0	0.62	0.08
Vaginal SCCs	4	2	4	2/4	50.0	0.00	1.00
VIN I	14	5	4	13/14	92.9	0.84	0.005
VIN II/III	14	10	8	8/14	57.1	0.09	1.00
Vulvar SCCs	6	3	5	4/6	66.7	0.33	1.00

*The data represent the number of samples for which the results from PapilloCheck DNA Microarray and NucliSENS EasyQ HPV assay were concordant/total number of samples tested.

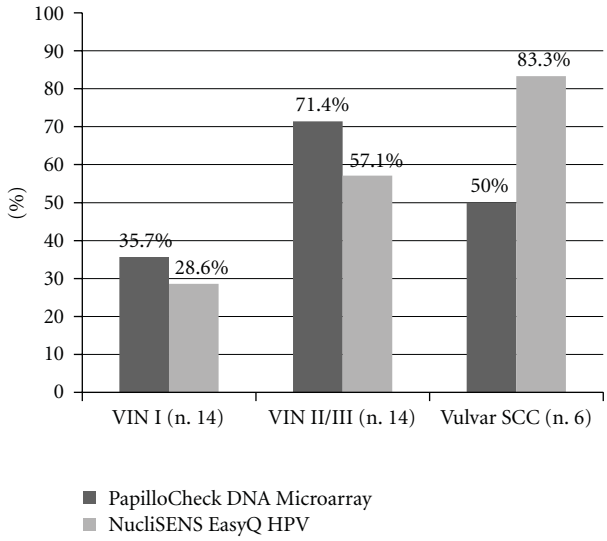

Figure 2: Prevalence of hr HPV genotypes (16, 18, 31, 33 and 45) detected by both PapilloCheck DNA Microarray and NucliSENS EasyQ HPV assay according to histological status of samples of vulva.

HPV16 was the commonest type revealed by NucliSENS EasyQ HPV assay for VIN I, VIN II/III, and vulvar SCCs (4/4 cases: 100%, 6/8 cases: 75%, and 3/5 cases: 60%, resp.) (Table 1).

The concordance between the HPV DNA test (PapilloCheck DNA Microarray) and HPV E6/E7 mRNA test (NucliSENS EasyQ HPV assay) results was very good for samples classified as having VIN I (92.9%; kappa = 0.84) but was poor for patients with VIN II/III (57.1%; kappa = 0.09). In the case of samples classified as having vulvar SCCs, the concordance was fair (66.7%; kappa = 0.33) (Table 2).

4. Discussion

The goal of this study was to investigate HPV typing and HPV E6/E7 mRNA expression with intraepithelial neoplasia and squamous cell carcinomas of the vagina and vulva. This is the first study to report the association between HPV infection with oncogenic expression and vulvovaginal disease in Greek women.

The role of HPV infection in vulvar intraepithelial neoplasia and squamous cell carcinoma has been confirmed through multiple studies worldwide. Although a similar link exists in vaginal precancerous and cancerous lesions, this has not been firmly established. This in part is due to the fact that VAIN and vaginal carcinomas are less common than their vulvar and cervical counterparts, due to the absence of a susceptible transformation zone and the protective effect of the keratinized vaginal mucosa [7]. Furthermore, it is possible that a proportion of VAIN lesions remain undiagnosed as they are asymptomatic and not easy to visualize during a routine gynecological examination [5]. Nevertheless, we expect an outbreak of VAIN and VIN cases, as well as SCCs of the vulva and vagina, especially in younger women[8, 9]. This is thought to be due to not only the rapid spread of HPV, but also the increased gynecological surveillance and improved diagnostic techniques, aimed at the identification of cervical lesions.

As mentioned above, several studies have previously described the HPV prevalence and genotype distribution in VIN and SCCs of the vulva [3, 4, 10–16]. The results of the current study are generally similar to worldwide VIN and vulvar SCCs data although some differences were observed. In our study, the overall HPV prevalence was 71.4% in VIN and 50% in vulvar carcinoma. A recent international meta-analysis of 14 studies on vaginal and 63 studies on vulvar lesions reported that the overall HPV prevalence in VIN was 84.0% and 40.4% in vulvar SCCs [5]. Similar results were reported by other studies which indicated HPV prevalence of 79.6% in VIN and 40.1% in vulvar SCCs [15, 16] (Table 4).

According to our results, hrHPV infection was the most frequently observed in VIN I cases. This is contrary to data obtained from an older study [3], where low-risk (lr) HPVs were the commonest types. HPV 16 and HPV 59 were the two most frequent genotypes in VIN I, accounting for 35.7%, whereas in the recent international meta-analysis HPV 6 was found to be the commonest genotype [5]. HPV 16 was by far the commonest type in VIN II/III and vulvar SCCs in our study, accounting for 64.3%, similar to other reports [4, 14] (Table 4).

Table 3: Prevalence of HPV in intraepithelial neoplasia and carcinoma of the vagina, by study.

Histologic type	First author	No. of cases	HPV test	HPV prevalence for any and specific genotypes
VAIN I				
	Smith (review)	66	PCR/hybrid capture assays	Any HPV, 98.5% Most common types; 16 (17.9%), 18 (17.9%)
	De Vuyst (meta-analysis)	107	PGMY reverse line blot/SPF 10 line probe assay, blot hybridization	Any HPV, 100% Most common types; 16 (23.4%), 56 (11.0%), 51 (8.8%)
VAIN II/III				
	Smith	166	PCR/hybrid capture assays	Any HPV, 92.6% Most common type; 16 (65.8%)
	De Vuyst	191	PGMY reverse line blot/SPF 10 line probe assay, blot/southern hybridization, restriction fragment analysis, sequencing	Any HPV, 90.1% Most common types; 16 (57.6%), 18 (6.9%), 58 (5.9%)
Vaginal SCC				
	Smith	83	PCR/hybrid capture assays	Any HPV, 65.5% Most common type; 16 (55.4%)
	De Vuyst	136	Reverse line blot assay, INNO-LiPA HPV genotyping, southern hybridization, restriction fragment analysis, sequencing	Any HPV, 69.9% Most common types; 16 (53.7%), 18 (7.6%), 31 (5.6%)

35.7% of VIN I samples harbored hrHPV (16, 18, 31, 33, and 45) and this rose to 71.4% in VIN II/III samples. This is expected as women with hrHPV infection are more likely to progress to high-grade lesions. Interestingly, the percentage of hrHPV dropped to 50% in vulvar SSCs. This suggests that the virus was present only at very low copy numbers and/or that only a specific region of viral DNA was integrated into the host's genome [17].

In our study, HPV prevalence in VAIN samples was 56%. This was significantly lower than those reported by De Vuyst et al. (93,6%) and Smith et al. (95.6%). In vaginal SCCs, HPV prevalence was 50%. This was lower but more comparable to those found in the studies mentioned above (65.5% and 69.9%, resp.) [5, 15] (Table 3).

In a worldwide meta-analysis, the most frequent genotypes were HPV 16, 56, and 51 in patients with VAIN I lesions, HPV 16, 18, and 58 in those with VAIN II/III, and HPV 16, 18, and 31 in those with vaginal SCCs [5] (Table 3). In our study, we detected, in decreasing order, HPV 42, 16, and 39 in VAIN I cases, HPV 16, 33, and 53 in VAIN II/III, cases, and HPV 16 in SCCs. It is interesting to note that HPV 18 was not identified in any of our VAIN or SCC samples, as opposed to what is seen elsewhere, and this may be a geographical variation that needs to be investigated further. Another variation, possibly attributed to our different sample population, was that HPV 42, a lrHPV type, was the most frequently identified type in VAIN I, whereas in the studies by De Vuyst et al. and Smith et al., no lrHPVs were detected in low-grade lesions [5, 15].

Contrary to what was observed in vulvar samples, the proportion of patients with detectable hrHPV (16, 18, 31, 33, and 45) increased progressively as the grade of the vaginal lesion progressed.

Using the NucliSens EasyQ HPV assay, we investigated HPV oncoprotein expression in different grades of dysplasia and carcinoma. The results from E6/E7 mRNA test related well with the grade of lesion. The lowest rates of hrHPV types (16, 18, 31, 33, and 45) E6/E7 mRNA expression were for patients with low-grade vulvar lesions (VIN I), whereas the higher rates were seen in high-grade lesions (VIN II/III), which is in accordance with what is seen in cervical lesions [18–20]. It is possible that VIN I lesions that have detectable E6/E7 mRNA expression are the ones with a potential to progress to higher-grade lesions and malignancy, and therefore, E6/E7 mRNA expression could be used as a screening marker for better surveillance in this subcategory of women. Interestingly, none of the five high-risk types E6/E7 mRNA expression was detected in low-grade vaginal lesions (VAIN I). The above findings probably suggest that VAIN I may not be strictly a precancerous disease, while at the same time, they reflect the transient nature of most HPV infections. On the contrary, VIN II/III, VAIN II/III, and vaginal and vulvar SCCs showed a high prevalence of E6/E7 mRNA expression. For VIN II/III in particular, the rates of E6/E7 mRNA expression that we found were significantly higher than what has been published previously (57.1 versus 38.1%) [21].

In our series, there was a significantly higher detection of HPV 16 by NucliSens EasyQ HPV assay when compared to other hrHPV types for both vaginal and vulvar cases. This indicates that HPV 16 may be related to a different nature of persistent infection and oncoprotein expression in the vagina and vulva in comparison to HPV types 18, 31, 33, and 45.

On the basis of DNA and mRNA assays, DNA from HPV was detected more frequently in vulvar low-grade lesions than E6/E7 mRNA expression. This data possibly reflects

Table 4: Prevalence of HPV in intraepithelial neoplasia and carcinoma of the vulva, by study.

Histologic type	First author	No. of cases	HPV test	HPV prevalence for any and specific genotypes
VIN I				
	Smith (review)	71	PCR/hybrid capture assays	Any HPV, 77.5% Most common types; 6 (23.8%), 16 (14.3%), 56 (1.7%)
	De Vuyst (meta-analysis)	90	PGMY reverse line blot/SPF 10 line probe assay	Any HPV, 67.8% Most common types; 6 (22.4%), 16 (9.8%), 11 (9.0%)
	Garland (original article)	31	Reverse line blot assay	Any HPV, 80.6% Most common types; 6 or 11 (64.5%)
VIN II/III				
	Smith	1340	PCR/hybrid capture assays	Any HPV, 80.4% Most common types; 16 (71.2%), 33 (7.7%)
	De Vuyst	1061	PGMY reverse line blot/SPF 10 line probe assay, sequencing, southern hybridization, restriction fragment-length polymorphism analysis	Any HPV, 85.3% Most common types; 16 (71.9%), 33 (8.0%),18 (5.0%)
	Garland	31	Reverse line blot assay	Any HPV, 87.1% Most common types; 16 (64.5%), 6, or HPV11 (29%)
Vulvar SCC				
	Smith	1379	PCR/hybrid capture assays	Any HPV, 40.1% Most common types; 16 (29.3%), 18 (5.6%)
	De Vuyst	1873	Reverse line blot hybridization, dot blot hybridization, Roche HPV linear array	Any HPV, 40.4% Most common HPV types; 16 (32.2%), 33 (4.5%), 18 (4.4%)

an episomal state or low number of copies of the virus. However, E6/E7 mRNA expression in a number of VIN I indicates that hrHPV may be oncogenically active even before it produces detectable changes in the cell [17].

For vaginal high-grade lesions and carcinomas, as well as vulvar carcinomas, a higher rate of E6/E7 mRNA expression was observed, compared to HPV DNA. This suggests that the presence of E6/E7 oncoproteins is a specific marker for high-grade lesions. Interestingly, 2 cases of VAIN II/III, 1 case of VIN II/III, 2 cases of vaginal SCCs, and 2 cases of vulvar SCCs were positive only for mRNA expression. This may be explained by the fact that total viral DNA has been integrated to the host genome, and therefore, it cannot be detected by the DNA test. It is important to notice that in vulvar high-grade lesions a higher detection rate for HPV DNA was observed compared to E6/E7 mRNA expression. It is possible that these results were due to a very low level of viral transcriptional activity.

The sample in our study was relatively small, primarily due to the low incidence of vulvar and vaginal intraepithelial neoplasias and carcinomas. Thus, we can only provide a rough estimate of the relative importance of each HPV type with regards to vaginal and vulvar cancer and precancer in our population.

Another limitation of our study was that histology types of vulvar SCCs were not available, so a correlation between histology types and HPV infection was not possible. Nevertheless, it has been suggested that differentiated keratinizing SCCS, which occurs more frequently in elderly women, is not associated with HPV infection, whereas nonkeratinizing SCCS, which primarily affects younger women, is caused by hrHPV infection [22]. This was indirectly confirmed in our study, as HPV infection was more likely to be found in younger women.

5. Conclusion

This study described the detection rates and attribution of genital HPV types, as well as the E6/E7 mRNA expression of intraepithelial neoplasias and squamous cell carcinomas of the vagina and vulva in Greek women. In summary, our results showed that a very crucial percentage of HPV was associated with VIN, VAIN, and vaginal/vulvar SCCs, and HPV 16 accounted for most HPV-positive cases. The fact that some cases of vulvar low grade lesions were positive for E6/E7 mRNA expression is also of interest, as it may identify these lesions as more clearly precancerous. A striking increase especially in the incidence of VIN in young women has been

reported in the last decades in some high-resources countries [8, 23, 24].

Further research is required to better assess the role of mRNA testing as a molecular marker for vaginal and vulvar carcinogenesis.

Conflict of Interests

The authors declare that there is no conflict of interests.

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References

[1] M. Stanley, "Pathology and epidemiology of HPV infection in females," *Gynecologic Oncology*, vol. 117, no. 2, pp. S5–S10, 2010.

[2] R. P. Insinga, K. L. Liaw, L. G. Johnson, and M. M. Madeleine, "A systematic review of the prevalence and attribution of human papillomavirus types among cervical, vaginal, and vulvar precancers and cancers in the United States," *Cancer Epidemiology Biomarkers and Prevention*, vol. 17, no. 7, pp. 1611–1622, 2008.

[3] M. Srodon, M. H. Stoler, G. B. Baber, and R. J. Kurman, "The distribution of low and high-risk HPV types in vulvar and vaginal intraepithelial neoplasia (VIN and VaIN)," *The American Journal of Surgical Pathology*, vol. 30, no. 12, pp. 1513–1518, 2006.

[4] B. C. Sutton, R. A. Allen, W. E. Moore, and S. T. Dunn, "Distribution of human papillomavirus genotypes in invasive squamous carcinoma of the vulva," *Modern Pathology*, vol. 21, no. 3, pp. 345–354, 2008.

[5] H. De Vuyst, G. M. Clifford, M. C. Nascimento, M. M. Madeleine, and S. Franceschi, "Prevalence and type distribution of human papillomavirus in carcinoma and intraepithelial neoplasia of the vulva, vagina and anus: a meta-analysis," *International Journal of Cancer*, vol. 124, no. 7, pp. 1626–1636, 2009.

[6] IARC, *Monographs on the Evaluation of Carcinogenic Risks to Humans, Human Papillomaviruses*, vol. 90, IARC Press, Lyon, France, 2007.

[7] M. L. Diaz, "Prevention of cervical, vaginal, and vulval cancers: role of the quadrivalent human papillomavirus (6, 11, 16, 18) recombinant vaccine," *International Journal of Women's Health*, vol. 1, no. 1, pp. 119–129, 2009.

[8] E. A. Joura, A. Losch, M. G. Haider-Angeler, G. Breitenecker, and S. Leodolter, "Trends in vulvar neoplasia: increasing incidence of vulvar intraepitheliai neoplasia and squamous cell carcinoma of the vulva in young women," *Journal of Reproductive Medicine for the Obstetrician and Gynecologist*, vol. 45, no. 8, pp. 613–615, 2000.

[9] H. Li, W. H. Zhang, L. Y. Wu, R. Znang, and P. Bai, "Clinicopathologic study of 24 patients with vulvar intraepithelial neoplasia III," *Zhonghua Zhong Liu Za Zhi* , vol. 27, no. 5, pp. 306–308, 2005.

[10] J. J. Carter, M. M. Madeleine, K. Shera et al., "Human papillomavirus 16 and 18 L1 serology compared across anogenital cancer sites," *Cancer Research*, vol. 61, no. 5, pp. 1934–1940, 2001.

[11] A. P. Pinto, M. C. Lin, G. L. Mutter, D. Sun, L. L. Villa, and C. P. Crum, "Allelic loss in human papillomavirus-positive and -negative vulvar squamous cell carcinomas," *The American Journal of Pathology*, vol. 154, no. 4, pp. 1009–1015, 1999.

[12] Y. T. Kim, N. F. Thomas, T. D. Kessis, E. J. Wilkinson, L. Hedrick, and K. R. Cho, "p53 mutations and clonality in vulvar carcinomas and squamous hyperplasias: evidence suggesting that squamous hyperplasias do not serve as direct precursors of human papillomavirus-negative vulvar carcinomas," *Human Pathology*, vol. 27, no. 4, pp. 389–395, 1996.

[13] B. J. Monk, R. A. Burger, F. Lin, G. Parham, S. A. Vasilev, and S. P. Wilczynski, "Prognostic significance of human papillomavirus DNA in vulvar carcinoma," *Obstetrics & Gynecology*, vol. 85, no. 5, pp. 709–715, 1995.

[14] S. M. Garland, R. P. Insinga, H. L. Sings, R. M. Haupt, and E. A. Joura, "Human papillomavirus infections and vulvar disease development," *Cancer Epidemiology Biomarkers and Prevention*, vol. 18, no. 6, pp. 1777–1784, 2009.

[15] J. S. Smith, D. M. Backes, B. E. Hoots, R. J. Kurman, and J. M. Pimenta, "Human papillomavirus type-distribution in vulvar and vaginal cancers and their associated precursors," *Obstetrics & Gynecology*, vol. 113, no. 4, pp. 917–924, 2009.

[16] D. Bryant, N. Rai, G. Rowlands et al., "Human papillomavirus type distribution in vulvar intraepithelial neoplasia determined using PapilloCheck DNA microarray," *Journal of Medical Virology*, vol. 83, no. 8, pp. 1358–1361, 2011.

[17] P. Cattani, G. F. Zannoni, C. Ricci et al., "Clinical performance of human papillomavirus E6 and E7 mRNA testing for high-grade lesions of the cervix," *Journal of Clinical Microbiology*, vol. 47, no. 12, pp. 3895–3901, 2009.

[18] I. Kraus, T. Molden, R. Holm et al., "Presence of E6 and E7 mRNA from human papillomavirus types 16, 18, 31, 33, and 45 in the majority of cervical carcinomas," *Journal of Clinical Microbiology*, vol. 44, no. 4, pp. 1310–1317, 2006.

[19] K. Sotlar, H. C. Selinka, M. Menton, R. Kandolf, and B. Bültmann, "Detection of human papillomavirus Type 16 E6/E7 oncogene transcripts in dysplastic and nondysplastic cervical scrapes by nested RT-PCR," *Gynecologic Oncology*, vol. 69, no. 2, pp. 114–121, 1998.

[20] S. Nakagawa, H. Yoshikawa, T. Yasugi et al., "Ubiquitous presence of E6 and E7 transcripts in human papillomavirus-positive cervical carcinomas regardless of its type," *Journal of Medical Virology*, vol. 62, no. 2, pp. 251–255, 2000.

[21] P. Hillemanns and X. Wang, "Integration of HPV-16 and HPV-18 DNA in vulvar intraepithelial neoplasia," *Gynecologic Oncology*, vol. 100, no. 2, pp. 276–282, 2006.

[22] H. P. van de Nieuwenhof, I. A. M. van der Avoort, and J. A. de Hullu, "Review of squamous premalignant vulvar lesions," *Critical Reviews in Oncology/Hematology*, vol. 68, no. 2, pp. 131–156, 2008.

[23] P. L. Judson, E. B. Habermann, N. N. Baxter, S. B. Durham, and B. A. Virnig, "Trends in the incidence of invasive and in situ vulvar carcinoma," *Obstetrics & Gynecology*, vol. 107, no. 5, pp. 1018–1022, 2006.

[24] R. W. Jones, J. Baranyai, and S. Stables, "Trends in squamous cell carcinoma of the vulva: the influence of vulvar intraepithelial neoplasia," *Obstetrics & Gynecology*, vol. 90, no. 3, pp. 448–452, 1997.

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Research Article

Decision-Making about the HPV Vaccine among Ethnically Diverse Parents: Implications for Health Communications

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Objective: To describe parents' knowledge, attitudes, and decision-making with regard to obtaining the HPV vaccine for their daughters. *Methods*: White, Black, and Hispanic parents of daughters who were age eligible to receive the HPV vaccine (9–17 years) were recruited from community settings to participate in focus groups. Parents were asked about knowledge and awareness of HPV, decision-making about HPV vaccine, as well as preferred and actual sources of HPV information. *Results*: Seven focus groups (n = 64 participants) were conducted. Groups were segmented by gender (women = 72%) and race/ethnicity (Black = 59%; White = 23%; Hispanic = 19%). Prevalent themes included: insufficient information to make informed decisions; varied preferences for involvement in decision-making; concerns about vaccine safety; mistrust of medical providers and pharmaceutical companies; and mismatch between actual and preferred sources of information. *Discussion*: Improving communication between providers and caregivers and helping parents to access information necessary for informed decision-making, while alleviating concerns about vaccine safety, may help to improve vaccine acceptance.

1. Introduction

It is well established that recurrent infection with HPV (human papillomavirus) is responsible for most cervical cancers [1]. With the availability of prophylactic HPV vaccines, there is potential to virtually eliminate disparities in cervical cancer, provided that access is assured and uptake is equitable across population groups.

Currently, routine vaccination is recommended for girls aged 11-12, with "catch up" vaccination recommended between ages 13–26 [2]. Vaccine efficacy is highest when administered prior to sexual début [3]. Given the recommended age for administration, parents have decision-making authority for their minor children. Understanding factors that influence parental decision-making about vaccinating their children may improve HPV vaccination uptake.

A number of reviews have summarized sociodemographic, cognitive, and attitudinal factors associated with parental acceptance of HPV vaccination [4, 5]. To date, the majority of studies have been conducted among relatively homogeneous samples in clinical settings. We sought to gain a contextualized understanding of vaccine decisions among a racially/ethnically diverse group of parents. We used focus group methodology to illuminate a wide range of perspectives and yield new insights to inform the design of effective interventions and communication strategies [6].

2. Methods

We developed a focus group guide consisting of open-ended questions (Table 1). Discussion questions were developed from our prior work [7, 8] and existing literature on factors associated with parental acceptance of the HPV vaccine.

During February and May of 2008 we conducted seven focus groups, each with 6–10 parents, Eligible participants

Table 1: Focus group questions.

Construct	Sample question
Sources of health information	Where do you get information about your daughter's health? What is the most credible/believable source of information?
Decision-making about daughter's health	Who makes decisions about your daughter's health? Under what circumstances would you involve your daughter in making a decision about her health care?
Awareness and knowledge of HPV	What do you know about HPV or human papillomavirus? How is it spread? Who is vulnerable to it? What health problems can it cause?
Awareness and knowledge of HPV vaccine	Have you heard about a vaccine for HPV? What have you heard? Who is this vaccine for? What does it do?
Social and subjective norms about HPV vaccination	Are people in your community getting their daughters vaccinated? Why or why not? What would your (spouse/partner; family members; friends) think about your getting your daughter vaccinated for HPV?
Barriers to vaccination	Is it difficult to get this vaccine? Why or why not?
Desired information	What would you need to know before making a decision about vaccinating your daughter(s)?
Preferred sources and settings for HPV information delivery	What would be the best way to deliver information about the HPV vaccine to parents? Where do you prefer to receive information? From what source?

were primary caregivers (female or male, hereafter called "parents") of at least one girl between 9 and 17 years of age. Parents were English or Spanish speaking and self-identified as White, Black, or Hispanic. We recruited participants through health and social service agencies in the Boston metropolitan area through fliers and word of mouth.

Groups were segmented on gender and race/ethnicity, to allow for examination of potential differences across these characteristics. Trained focus group moderators, matched to gender and language of participants, facilitated discussions according to a standardized protocol. Discussions were audio taped, professionally transcribed, and translated. Each discussion lasted 60–90 minutes. Participants received a $50 gift card. The Harvard School of Public Health Institutional Review Board approved this study.

3. Analysis

We analyzed focus group transcripts based on procedures outlined by Corbin and Strauss [9]. Several authors (JDA, MDJ, LT) independently examined transcripts to identify themes and create initial coding categories. We subsequently reviewed categories and supporting quotes to develop a refined coding scheme. Through an iterative process, open codes were collapsed into higher-order categories that reflected emergent themes. Where divergent interpretations of themes occurred, we reevaluated and discussed the original transcripts until consensus was achieved. Data collection was stopped at the point of saturation [10].

4. Results

4.1. Characteristics of Participants. Seven focus groups were conducted with a total of 64 participants. The majority of participants were nonwhite (Black = 59%; Hispanic = 19%; White = 23%) and female (72%). Participants' mean age was 46. Nearly all (98%) reported having health insurance (data not shown).

5. Themes

Themes are presented in the order of discussion, which was driven by the focus group guide. We note where themes were salient or emerged exclusively in particular groups. Refer to Table 2 for sample quotes supporting themes.

5.1. Insufficient Information to Make Informed Decisions. The majority of parents felt that they did not have adequate information about HPV or the vaccine to make an informed decision about vaccinating their children. A number of participants confused HPV with HIV or Hepatitis. Many of the men had never heard of HPV prior to the focus group. In each of the discussions among women, at least one person shared that they had allowed one or more of their daughters get the vaccine, although they felt that they had been inadequately informed or were unprepared for the decision. In some cases, participants reported that they were not given the opportunity to be involved in decision-making.

Table 2: Focus group themes.

Theme	Example quotes
Insufficient information to make informed decisions	"I didn't give it [HPV vaccine] to my daughter when the doctor asked because I wasn't aware of the consequences. I need more research on it before I put her through something like that." *(Black female)* "Men need to get information so that we can get more educated about HPV." *(Black male)* "She got [the vaccine], but I am not well-informed. I don't know what effects it could have on her. I was not given the information . . ." *(Latino female)* "My daughter just got the shot. And I don't even know what she got . . . You know I don't even know how to say it. Papee-glorenoma virus" *(White female)* "They don't explain much to you. They give you a paper with the risks . . ." *(White female)*
Involvement in decision making	"I leave it up to my daughter and her mother to decide what to do about the HPV vaccine." *(Black male)* "I make that decision. Her body is mine until she's 18 years old." *(Black female)* "I would ask some of the women in my family for their opinion . . . my aunt. I would get her opinion and my sister's, too." *(Latino male)* "When I went to the doctor with my daughter, I was asked to leave the office as the doctor wanted to have a private conversation about the HPV vaccine with her. When they were finished, I came back in. I asked the doctor, "What's going on?" The doctor didn't tell me anything . . . I am kind of confused . . . because the doctor didn't say anything to me about it [vaccine]." *(Latino female)* "I think it's better between the daughter and the health care provider" *(White male)* "I would let the decision be up to her, but she should be informed. The more informed she is, the better off she'll be making the decision, and she won't feel forced by you, or anybody else . . ." *(White female)*
Concerns about vaccine safety	"My first thought was, I am sendin' my ten-year old to this clinic to put dead HPV cells in her. What if the HPV that they are shooting in her body . . . what if it comes to life?" *(Black female)* "[They] tried to give me an HPV shot, along with others, while in prison. It was a trial." *(Black male)*
Mistrust-pharmaceutical companies	". . . all those pharmaceuticals care about, let's be honest, is money." *(Black female)* "Are the companies who are putting the HPV vaccine out here . . . are they doing it just for a profit? Or do they actually really care about treatment and prevention?" *(Black female)* "If it's been around and studied for this long, then why is it just now starting to hit the market, and why is it just now starting to get pushed?" *(Black male)*
Mistrust-medical providers	"I was pregnant thirteen times but . . . it's [HPV] never, ever once [taps on table for emphasis] come up with my doctor! Never once have they even mentioned [HPV]!" *(Black female)*
Sources of information	"I don't think the TV commercials explain enough, all they say is "one less, one less, one less." I would like to know how long the vaccine is good for and its effectiveness." *(Black female)* "I know about HPV and the vaccine because they advertise the HPV vaccine a thousand times on Univision and Telemundo." *(Latino female)* "Knowledge is power. We should be getting information about the HPV vaccine from our girls' doctors." *(White female)*
Desired educational materials and strategies	"You can't use big words, you need to make it understandable so that people can understand [the vaccine information] and make decisions." *(Black female)* "For me, the more information channels there is, the better: written, video, discussions . . . Sometimes you don't understand something one way and you understand it in a different format." *(Latino male)* "Sometimes, because you don't know the language, you don't even ask." *(Latino female)*

In other instances, parents reported that they were given a fact sheet, but not the opportunity to ask questions.

5.2. Responsibility for Decision-Making. Parents expressed a wide range of perspectives in terms of the extent of involvement inthe decision-making process they wanted for themselves and their daughters. In general, fathers indicated that they would defer decision-making to female family members (e.g., grandmothers, godmothers) or trusted friends, particularly among the African American and Hispanic groups. The desire for a collaborative decision-making process between the provider, parent, and daughter was commonly expressed by Hispanic mothers. For example, several Hispanic mothers related stories about concern when health care providers discussed the vaccine with their daughter privately and not involving the parent in discussions. This was a cause of concern for these parents, who felt that providers should have involved both the parent and daughter in the decision. The opinion that the primary female caregiver should make decisions (without necessarily involving the daughter) appeared to be most common among African American women. A view more commonly expressed among White women was that the decision could or should be left to the daughter, depending on age and maturity level, and as long as she was adequately informed about the vaccine.

5.3. Concerns about Vaccine Safety. All of the groups raised concerns about vaccine safety. Many shared the opinion that there may be vaccine side effects yet unknown. Concerns about unknown, long-term side effects were most frequently

discussed among African American groups. The most prominent vaccine safety concerns among Hispanic mothers were birth defects and future reproductive health.

5.4. Mistrust of Medical Providers and Pharmaceutical Companies. Mistrust of medical providers was expressed by at least one individual in each of the groups. Females, in particular, expressed skepticism and surprise that they had not previously heard about HPV until the vaccine became available. Some expressed anger at providers for not having previously discussed this "killer virus." Participants also discussed their mistrust of pharmaceutical companies, with some perceiving distribution of vaccine as solely profit driven. Pharmaceutical company mistrust appeared most prevalent among African American groups, where participants drew parallels between being injected with a virus and the Tuskegee experiment.

5.5. Mismatch between Actual Versus Preferred Sources of Information. The majority of parents reported that they preferred to receive vaccine information directly from their daughter's health care provider. At the same time, there was much discussion about feeling rushed at medical appointments, not having sufficient opportunities to ask questions, and as previously mentioned, a sense that health care providers had been withholding information about HPV. While providers were the preferred sources of information, the vast majority of participants stated that their primary source of information about HPV and the vaccine had been television and radio advertisements. Participants in all groups reported seeing advertisements for the vaccine and specifically identified the "One Less" campaign. Many expressed concern that advertisements provided insufficient, misleading, or inaccurate information.

6. Discussion

In this qualitative study of ethnically diverse parents, knowledge and awareness of HPV and the vaccine was low. Parents almost universally reported feeling that they did not have sufficient information to make an informed decision about vaccinating their daughter(s). Concerns about vaccine safety and potential side effects were also prevalent. Often, these concerns were discussed in the context of mistrusting pharmaceutical companies and a concern that the vaccine is being promoted solely for profit. In these respects, our findings are consistent with recent studies of parental knowledge and attitudes toward the HPV vaccine [5, 11].

Our findings shed new light on parental desire for, and experiences with participation in decision-making. Although this study was not specifically designed to test differences in opinion across gender or racial/ethnic groups, we noted variations in themes between groups.

Hispanic mothers more often reported minimal involvement in decision-making despite a desire to take an active role. Most Hispanic and African American men preferred to defer decisions to female caretakers. Whereas Hispanic mothers reported more concern over birth defects and reproductive health. Parents in all groups reported learning about HPV from media sources, yet almost all reported a preference for receiving information from their daughter's health care provider.

Prior to discussion of potential implications, limitations of this study must be noted. This was a sample of convenience, which limits the generalizability of our results. Our goal, however, was not to obtain a representative sample, but rather to hear from parents from a variety of backgrounds and who had diverse perspectives and experiences. It is possible that study volunteers were more interested in HPV and more knowledgeable about the topic compared to the general parent population. If, indeed, participants are assumed to be more knowledgeable than the general population, these findings further emphasize the need for HPV vaccine interventions, as levels of knowledge and awareness were low. Lastly, we were not able to explore inter-ethnic differences across racial groups (e.g., by country of origin or region). We recognize the heterogeneity within each of the groups but were limited in our analysis by sample size. Nevertheless, our data are useful in generating hypotheses that can be tested in future research and for informing health communication intervention development [6].

There are several important implications of this research. Parents need additional information about the vaccine, its benefits, and limitations. Parental reports of inadequate information or involvement in decision-making is cause for concern for many reasons. First, participation of parents in vaccine decisions could increase providers' abilities to assess risks of side effects. For example, providers need to be alerted to a child's allergies or any major illnesses, which could affect vaccine response [2]. Second, parents need to be informed of the potentially serious (but rare) allergic reactions that can occur, as well as the limitations of the vaccine (i.e., it does not protect against other sexually transmitted infections). Third, increased parental knowledge about the vaccine could result in improved rates of vaccine series completion. Currently, most who initiate the vaccine's series fail to receive the required three-dose regimen [12].

Parents reported a strong preference to receive HPV-related information directly from their daughters' health care provider. As such, interventions aimed at improving provider communications with parents about HPV are needed. Provider endorsement has been found to be an important driver of HPV vaccination in previous studies [5]. Providers need information and skills to effectively disseminate accurate information, help manage parental uncertainty, respond to emotional concerns, and facilitate informed decision-making among parents. However, given the mistrust expressed among some groups toward providers, interventions will also need to foster strong provider-patient relationships. While time constraints are a potential barrier to developing relationships with parents, there are ways to address this. For example, provider vaccine recommendations can be followed by reminder mailings and parent education on how to best prepare for a clinical encounter.

Our finding that female caregivers often hold primary responsibility for health decisions, particularly in relation to the sexual health of their daughters, highlights the

importance of targeting HPV health communications. For example, the importance of vaccinating young girls could be discussed with mothers when they themselves present for cervical cancer screening. In addition, the finding that many parents rely on female family members and friends for input on health-related decisions suggests that targeting key members within an individual's social network could be potentially effective for the dissemination of HPV vaccine information and adherence to provider recommendations.

Finally, to address parental mistrust of pharmaceutical companies, it will be important to deliver interventions that are not associated with or financially tied to the pharmaceutical industry. This may help to improve program credibility and mitigate mistrust. Given some of the public's mistrust of vaccines in general, providing information through trusted and credible sources will be critical.

In conclusion, given that there is variation among ethnically diverse parents in the degree of desired involvement in decision-making, it would be prudent for providers to inquire about and respect preferences for involvement. Understanding the diverse models of parental decision-making about HPV vaccination would enable the development and evaluation of much needed targeted health communication interventions to promote vaccine uptake and completion.

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References

[1] A. E. Grulich, F. Jin, E. L. Conway, A. N. Stein, and J. Hocking, "Cancers attributable to human papillomavirus infection," *Sexual Health*, vol. 7, no. 3, pp. 244–252, 2010.

[2] Centers for Disease Control and Prevention, "HPV (Human Papilloma Virus) Vaccine What You Need to Know," http://www.cdc.gov/vaccines/pubs/vis/downloads/vis-hpv-gardasil.pdf. Accessed May 30, 2011.

[3] A. Koulova, J. Tsui, K. Irwin, P. Van Damme, R. Biellik, and M. T. Aguado, "Country recommendations on the inclusion of HPV vaccines in national immunization programmes among high-income countries, June 2006-January 2008," *Vaccine*, vol. 26, no. 51, pp. 6529–6541, 2008.

[4] N. T. Brewer, G. B. Chapman, F. X. Gibbons, M. Gerrard, K. D. McCaul, and N. D. Weinstein, "Meta-analysis of the relationship between risk perception and health behavior: the example of vaccination," *Health Psychology*, vol. 26, no. 2, pp. 136–145, 2007.

[5] M. E. Fernández, J. D. Allen, R. Mistry, and J. A. Kahn, "Integrating clinical, community, and policy perspectives on human papillomavirus vaccination," *Annual Review of Public Health*, vol. 31, pp. 235–252, 2010.

[6] R. A. Krueger and M. A. Casey, *Focus Groups: A Practical Guide for Applied Research*, Sage, Thousand Oaks, Calif, USA, 3rd edition, 2000.

[7] J. D. Allen, H. C. Fantasia, H. Fontenot, S. Flaherty, and J. Santana, "College men's knowledge, attitudes, and beliefs about the human papillomavirus infection and vaccine," *Journal of Adolescent Health*, vol. 45, no. 5, pp. 535–537, 2009.

[8] M. de Jesus, L. Parast, R. C. Shelton et al., "Actual vs preferred sources of human papillomavirus information among black, white, and Hispanic parents," *Archives of Pediatrics and Adolescent Medicine*, vol. 163, no. 11, pp. 1066–1067, 2009.

[9] J. Corbin and A. Strauss, *Basics of Qualitative Research: Techniques and Procedures for Developing Grounded Theory*, Sage, Thousand Oaks, Calif, USA, 2007.

[10] H. Bernard, *Research Methods in Anthropology: Qualitative and Quantitative Approaches*, AltaMira, Lanhan, Md, USA , 4th edition, 2006.

[11] J. D. Allen, G. D. Coronado, R. S. Williams et al., "A systematic review of measures used in studies of human papillomavirus (HPV) vaccine acceptability," *Vaccine*, vol. 28, no. 24, pp. 4027–4037, 2010.

[12] L. D. Taylor, S. Hariri, M. Sternberg, F. Dunne, and L. E. Markowitz, "Human papillomavirus vaccine converage in the United States, National Health and Nutrition Examination Survey, 2007-2008," *Preventive Medicine*, vol. 52, no. 2, pp. 398–400, 2011.

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Clinical Study

Performance of a New HPV Cervi-Collect Collection and Transportation Kit

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Background. Liquid-based Pap (L-Pap) media are used for Pap and human papillomavirus (HPV) testing. *Objectives*. To compare RealTi*m*e High Risk (HR) HPV testing of a new collection kit (Cervi-Collect) and PreservCyt L-Pap specimens. To determine ease of use and safety of Cervi-Collect. *Methods*. L-Pap samples ($n = 203$) were tested with HC2 and RealTi*m*e HR HPV and Cervi-Collect with RealTi*m*e HR HPV. Discordant samples were genotyped. *Results*. L-Pap and Cervi-Collect specimens tested by RealTi*m*e HR HPV showed 93.1% agreement (Kappa 0.86). RealTi*m*e HR HPV and HC2 on L-Pap had 90.3% agreement (Kappa 0.80). RealTi*m*e HR HPV on Cervi-Collect and HC2 on L-Pap showed 88.2% agreement (Kappa 0.76). Sixteen of 21 samples which were HC2 negative and RealTi*m*e HR HPV positive on L-Pap or Cervi-Collect contained HR HPV genotypes. Eleven healthcare collectors were in strong agreement on a usability and safety questionnaire. *Conclusion*. Cervi-Collect samples were easy to collect and showed strong agreement with L-Pap samples tested with RealTi*m*e HR HPV or HC2.

1. Introduction

High-risk human papillomaviruses (HR HPV) are a major cause of cervical cancer [1]. HR HPV testing either adjunctively with cytology or as the primary screening test has shown increased sensitivity for detecting CIN2+ precancerous lesions when compared with Pap testing alone [2]. ThinPrep PreservCyt Solution and SurePath Preservative Fluid are transportation and storage media enabling Pap and HPV testing. PreservCyt liquid-based (L-Pap) medium has been validated with the Abbott RealTi*m*e HR HPV assay. In cases where Pap testing is performed using non-L-Pap samples or HPV testing is performed as the primary screening method, a cervical specimen is collected for HPV testing. A collection brush and transportation medium kit (Cervi-Collect) was designed by Abbott Molecular for testing with the Abbott RealTi*m*e HR HPV assay. The principles and analytical performance of this assay have been described [3], and there are several reports comparing it to HC2 in archived samples [4–6] and to various DNA and RNA detection methods in L-Pap samples [7–9].

The aims were as follows: (a) to compare the performance of the RealTi*m*e HR HPV assay by testing Cervi-Collect and PreservCyt L-Pap specimens, (b) to compare the RealTi*m*e HR HPV and HC2 assays on L-Pap specimens, (c) to test discordant samples in a linear array (LA) assay, and (d) to analyze the strength of agreement of healthcare workers on ease of use and safety of the collection device and its package insert using a questionnaire.

2. Material and Methods

A total of 203 women attending a women's health clinic undergoing a routine gynecological exam or a follow-up exam due to an abnormal Pap or positive HR HPV test signed consent to have 2 cervical specimens collected: the first was collected with a Cervex-Brush (Rovers Medical devices, Oss, The Netherlands) and placed into an L-Pap PreservCyt

collection medium tube (Hologic Inc, Marlborough, Mass, USA) and the second was collected with the Cervi-Collect brush and placed into a Cervi-Collect transportation tube. Specimen collection was performed according to the respective manufacturers' instructions. The PreservCyt sample was processed for cytology in the Pathology Laboratory at the Juravinski Hospital, Hamilton, ON, Canada, and the remainder of the sample was sent to the Infection Research Laboratory (IRL) at the St. Joseph's Healthcare Hamilton, Hamilton, ON, Canada. Both samples were received within 24 hours in the IRL.

2.1. HC2 Testing. The L-Pap sample was tested for HR HPV with the HC2 test (Qiagen, Gaithersburg, Md, USA) at the IRL according to the package insert. Previous positive and negative clinical samples were included with each run as controls. Samples were scored negative if relative light units/cutoff (RLU/CO) ratios were <1.0, indeterminate when ≥1.0 and <2.5, and positive when ≥2.5. Indeterminate samples were repeated in duplicates: a sample with an RLU/CO ratio ≥1.0 in either replicate was considered positive.

2.2. RealTime HR HPV Testing. The Cervi-Collect sample and one milliliter of the L-Pap sample were packaged and shipped to Abbott Diagnostics in Wiesbaden, Germany, where they were tested in a blinded fashion with the RealTi*m*e HR HPV assay on the Abbott *m*2000 instrument. The automated test procedure consisted of sample preparation, reaction assembly, real-time PCR, and result reporting [3]. During sample preparation using the Abbott *m*2000*sp*, 0.4 mL of sample was processed using the Abbott *m*Sample Preparation System$_{\text{DNA}}$ where it was lysed with chaotropic reagents, allowing the DNA to be captured on magnetic microparticles. The bound purified DNA was washed and then eluted. An amplification master mix was created with AmpliTaq Gold enzyme (Roche Molecular Systems Inc., Branchburg, NJ, USA), magnesium chloride, and an oligonucleotide reagent containing primers, probes, and dNTPs. The PCR reaction was then assembled in a 96-well optical reaction plate by combining aliquots of the master mix and the extracted DNA eluate. Thermocycling and fluorescence detection of the amplified products were carried out in the Abbott *m*2000 real-time PCR instrument, and results were automatically reported. The assay detects 14 HR HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) with type specific detection for types 16 and 18 and detection of the other 12 non-HPV 16/18 types as a group. A separate detection category of β-globin is included as an internal control to validate sample adequacy, DNA recovery, and PCR efficiency. Results for each sample were reported based on all three HPV signals, corresponding to HPV16, HPV 18, and non-HPV 16/18 HR types, as well as the internal control signal.

2.3. LA Testing. Samples which showed discordant results after testing by HC2 and RealTi*m*e HR HPV assays were tested using the LA HPV Genotyping Test (Roche Molecular Systems Inc., Branchburg, NJ, USA) following the manufacturer's protocol. PCR was performed in a final reaction volume of 100 μL containing 50 μL of kit master mix. The genotyping strips were visually interpreted using the HPV reference guide provided in the kit package insert. The same high-risk genotypes represented in the Abbott assay were considered high risk.

Table 1: Agreement between Cervi-Collect and PreservCyt L-Pap specimens tested by the Abbott RealTi*m*e HR HPV assay.

		Abbott RealTi*m*e HR HPV with Cervi-Collect	
		+	−
Abbott RealTi*m*e HR HPV with PreservCyt L-Pap	+	84	8
	−	6	105

Positive agreement—85.7% (84/98); Negative agreement—88.2% (105/119); Overall agreement—93.1% (189/203) (kappa = 0.86).

2.4. Questionnaires. Sample collectors (physicians and nurses) were asked to complete a questionnaire rating whether the product labeling information was adequate and easy to understand in the following areas: the intended use statement, the instructions for safe use, collection, storage, and transport, and limitation of use statement in the package insert. They also evaluated the usability aspects (such as whether the kit package was easy to open, whether the tube cap was easy to take off and replace, and whether any leakage was present) as well as the safety aspects for the collection kit and instructions. In total, eleven questions were answered by each of the eleven collectors. Each question was answered on a scale of 1 to 5, with 5 indicating strong agreement with a statement and 1 if there was strong disagreement. The overall rating across all collectors for each question was calculated as the combined score as a percentage of a maximal score of 55 (i.e., 11 times 5).

2.5. Statistical Analysis. Agreement between tests was assessed by kappa statistic (κ).

3. Results

There was strong agreement between the L-Pap and Cervi-Collect specimens tested by RealTi*m*e HR HPV (Table 1). The positive agreement was 85.7% (84/98), negative agreement was 88.2% (105/119), and overall agreement was 93.1% (189/203) (Kappa 0.86). There were 8 L-Pap samples with insufficient volume for HC2 testing (4 were from patients who were negative in Cervi-Collect and L-Pap samples and 4 were positive in both by RealTi*m*e HR HPV). Table 2 shows agreement between RealTi*m*e HR HPV and HC2 performed on 195 L-Pap specimens. The assays agreed on 73 positives and 103 negatives. There were 15 samples which were positive by the RealTi*m*e HR HPV test and negative by HC2, and 4 other samples which were positive by HC2 but negative by the RealTi*m*e HR HPV test. The positive agreement was 79.3% (73/92), negative agreement was 84.4% (103/122), and the overall agreement was 90.3%

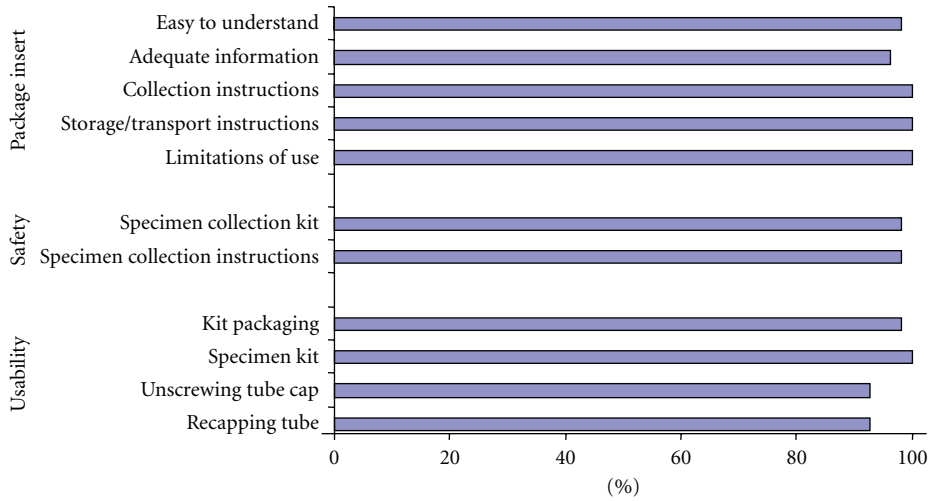

Figure 1: Evaluation of the Cervi-Collect package insert, safety, and usability rated by 11 healthcare collectors. (Each of the eleven questionnaire categories were graded by the collectors, with 5 being the most favorable because of total agreement with the statement. The final rating was based on the combined score from all the collectors as a percentage of the maximal score of 55. E.g., 100% is representative of 11 healthcare collectors giving a combined score of 55).

Table 2: Correlations between Abbott RealTi*me* HR HPV and Hybrid Capture 2 with PreservCyt L-Pap Samples.

		Abbott RealTi*me* HR HPV with PreservCyt L-Pap	
		+	−
Hybrid Capture 2 with PreservCyt L-Pap	+	73	4
	−	15	103

Positive agreement—79.3% (73/92); Negative agreement—84.4% (103/122); Overall agreement—90.3% (176/195) (kappa = 0.80).

Table 3: Correlation between Abbott RealTi*me* HR HPV with Cervi-Collect and Hybrid Capture 2 with PreservCyt L-Pap Samples.

		Abbott RealTi*me* HR HPV with Cervi-Collect	
		+	−
Hybrid Capture 2 with PreservCyt L-Pap	+	70	7
	−	16	102

Positive Agreement—75.3% (70/93); Negative agreement—81.6% (102/125); Overall agreement—88.2% (172/195) (kappa = 0.76).

(176/195) (Kappa 0.80). When the RealTi*me* HR HPV test was performed on Cervi-Collect specimens and HC2 was performed on L-Pap (Table 3), positive agreement was 75.3% (70/93), negative agreement was 81.6% (102/125), and overall agreement was 88.2% (172/195) (Kappa 0.76).

Table 4 summarizes the results of LA testing of 28 discordant samples from the 3 testing strategies (HC2 on L-Pap, RealTi*me* HR HPV on L-Pap, and RealTi*me* HR HPV on Cervi-Collect). Samples from 16 of 21 patients with a negative HC2 result and a positive RealTi*me* HR PCR result obtained either from L-Pap or Cervi-Collect samples contained HR HPV genotypes. Samples from 4 patients (026, 040, C121, and 190) which were positive by HC2 and negative by the RealTi*me* HR HPV assay in the L-Pap and Cervi-Collect samples contained low-risk HPV genotypes. Three patients (099, C169, and C193), which were HC2 and RealTi*me* positive in L-Pap but were negative in the Cervi-Collect sample, contained HR genotypes.

Figure 1 summarizes the outcomes from the questionnaires. Four of 11 categories received a full score (100%) out of a maximal score of 55 (5 from all 11 collectors), and the other 7 categories were graded at the maximum by most collectors (8 or greater) with an overall rating between 93% and 98%. The lower scores (93%) were recorded in categories for unscrewing and recapping the tube.

4. Discussion

The new Cervi-Collect kit compared well to PreservCyt when tested by the RealTi*me* HR HPV assay (Table 1), showing strong agreement of 93.1% (Kappa = 0.86). Analysis of the 98 samples which were positive in either sample type from Table 1 showed that 27 were positive in the type 16 signal (with or without the non-HPV 16/18 HR HPV signal), 12 were positive in the type 18 signal, 3 were positive in both the HPV 16 and 18 signals, and the rest were positive only in the non-HPV 16/18 HR HPV signal. The higher agreement between the two RealTi*me* HR HPV results for different transport media compared to that between RealTi*me* HR HPV and HC2 was mainly due to more positives in agreement ($n = 84$ in Table 1 versus 70 or 73 in Tables 2 and 3, resp.).

Comparing assays in Tables 2 and 3 showed more cases of HC2 negative/RealTi*me* HR HPV positive than HC2 positive/RealTi*me* HR HPV negative samples. These differences are consistent with findings in other studies [7–9] which showed that the RealTi*me* HR HPV assay detected the same number or more cases of HPV infection than the HC2 test.

Table 4: Comparison of discordant samples tested by linear array (LA).

Patient number	RealTi*m*e HR HPV on Cervi-Collect	RealTi*m*e HR HPV on L-Pap	HC2 on L-Pap	HPV genotypes[1]
025	HR HPV	HR HPV	NEG	**59**, **66**, **68**, 81
029	HR HPV	HR HPV	NEG	**45**
058	HR HPV	HR HPV	NEG	**51**
084	HR HPV	HR HPV	NEG	**16**, **18**, **39**, **51**, 54, **66**, CP6108
085	HR HPV	HR HPV	NEG	**51**, **66**
C104	HR HPV	HR HPV	NEG	**31**, 62
C129	HR HPV	HR HPV	NEG	**39**, **66**
C158	HR HPV	HR HPV	NEG	**52**
C156	HPV 18	HPV 18	NEG	**18**, 84
177	HPV 16	HPV 16	NEG	**16**
C112	HR HPV	Not Detected	NEG	NEG
C131	HR HPV	Not Detected	NEG	NEG
C173	HR HPV	Not Detected	NEG	**16**, **59**, 62, 70
C182	HPV 16	Not Detected	NEG	**16**, 40, 53, 55
186	HPV 16	Not Detected	NEG	81, CP6108
C167	HPV 18	Not Detected	NEG	**18**, 42, **73**
081	Not Detected	HR HPV	NEG	**35**, **52**, **59**
C128	Not Detected	HR HPV	NEG	**18**
099	Not Detected	HR HPV	POS	**51**, 54, **56**, 62
C169	Not Detected	HR HPV	POS	**56**, 84
060	Not Detected	HR HPV	NEG	NEG
070	Not Detected	HPV 16	NEG	**16**
095	Not Detected	HPV 16	NEG	NEG
C193	Not Detected	HPV 16	POS	**16**
026	Not Detected	Not Detected	POS	IS39
040	Not Detected	Not Detected	POS	40, 53, CP6108
C121	Not Detected	Not Detected	POS	53
190	Not Detected	Not Detected	POS	81, 84

[1] High-risk HPV genotypes are bolded.

The HR HPV positive samples that were not detected by the HC2 test contained HR genotypes by the LA test (Table 4). There were 16 patients positive by RealTi*m*e HR HPV in the Cervi-Collect sample and negative by HC2 in the L-Pap sample, 13 of which contained HR HPV by LA testing. Ten of the 13 were also positive by RealTi*m*e HR HPV in the L-Pap samples. Of the total 28 discordant samples, 24 were positive for HPV and 19 showed the presence of HR HPV genotypes by LA testing. Of these 19 samples, 13 Cervi-Collect samples were identified as HR HPV positive by the RealTi*m*e HR HPV assay, 16 L-Pap samples positive by RealTi*m*e HR HPV, and 3 L-Pap samples positive by HC2 (Table 4). The study was not designed to follow patients to colposcopy and biopsy, so one can only speculate what the significance of these additional positive infections would be in predicting precancerous lesions. Examination of the 7 samples positive by HC2 and negative by RealTi*m*e HR HPV on Cervi-Collect revealed 3 samples that were confirmed positive by LA and RealTi*m*e HR HPV on the L-Pap sample. All 3 samples contained a low level of HPV targets as indicated by results from both assays. Because the new collection device was experimental, the L-Pap sample was required to be collected first and the Cervi-Collect brush was used to collect the second sample. Low levels of target, collection order, and analytical sensitivity differences for HC2, RealTi*m*e HR HPV, and LA may contribute to variability of assay comparison. The other 4 samples, only positive by HC2 testing, were shown to contain no HR HPV but a variety of low-risk (LR) genotypes by LA (Table 4, patients 26, 40, C121, and 190). Cross-reactivity of the HC2 test with low-risk HPV genotypes has been reported previously. Sandri et al. [10] showed that low risk genotypes such as HPV types 6, 42, 62, 71, 73, and 81 were found to be reactive in the HR HC2 test. Castle et al. [11] showed that genotypes not targeted in the HR HC2 panel most often testing positive were HPV 82 (80%), HPV 70 (59.1%), and HPV 67 (56.3%).

Analysis of the questionnaire scores (Figure 1) showed that 4 of the eleven categories received the maximum score of 5 by all respondents (55 = 100%). Unscrewing and recapping the tube received scores of 3 or 4 by 3 of the eleven collectors suggesting that these maneuvers may be difficult for some collectors due to a certain degree of dexterity required.

5. Conclusion

Because cervical samples may be collected specifically for HPV testing, a system suitable for the collection, transportation, and storage of specimens for the detection of HR HPV DNA by the Abbott RealTi*m*e HR HPV was developed and evaluated. Cervi-Collect was designed to achieve efficient cervical collection, optimal sample stability, and compatibility with the automated sample preparation instrument (Abbott *m*2000*sp*) as the primary input tube. This study demonstrated excellent performance of the Cervi-Collect samples for the detection of HR HPV when tested with RealTi*m*e HR HPV compared with the PreservCyt L-Pap samples tested with RealTi*m*e HR HPV or HC2. The Cervi-Collect samples were not evaluated as a source for cytological examination. Healthcare collectors showed strong agreement with the usability and safety design features of Cervi-Collect and its package insert. Further studies need to be conducted to determine the versatility of this new collection kit for other anatomical sites such as the vagina [12], anus [13], and oropharynx [14] as well as other sexually transmitted infections [15, 16].

References

[1] F. X. Bosch, A. Lorincz, N. Muñoz et al., "The causal relation between human papillomavirus and cervical cancer," *Journal of Clinical Pathology*, vol. 55, no. 4, pp. 244–265, 2002.

[2] M. H. Mayrand, E. Duarte-Franco, I. Rodrigues et al., "Human papillomavirus DNA versus Papanicolaou screening tests for cervical cancer," *The New England Journal of Medicine*, vol. 357, no. 16, pp. 1579–1588, 2007.

[3] S. Huang, N. Tang, W. B. Mak et al., "Principles and analytical performance of Abbott RealTi*m*e high risk HPV test," *Journal of Clinical Virology*, vol. 45, no. 1, pp. S13–S17, 2009.

[4] N. Tang, S. Huang, B. Erickson et al., "High-risk HPV detection and concurrent HPV 16 and 18 typing with Abbott RealTi*m*e high risk HPV test," *Journal of Clinical Virology*, vol. 45, no. 1, pp. S25–S28, 2009.

[5] M. Poljak, A. Kovanda, B. J. Kocjan et al., "The Abbott RealTi*m*e high risk HPV test: comparative evaluation of analytical specificity and clinical sensitivity for cervical carcinoma and CIN 3 lesions with the Hybrid Capture 2 HPV DNA test," *Acta Dermatovenerologica Alpina, Pannonica et Adriatica*, vol. 18, no. 3, pp. 94–103, 2009.

[6] V. Kaliterna, S. Ž. Lepej, and A. Vince, "Comparison between the Abbott RealTi*m*e high risk HPV assay and the hybrid capture 2 assay for detecting high-risk human papillomavirus DNA in cervical specimens," *Journal of Medical Microbiology*, vol. 58, no. 12, pp. 1662–1663, 2009.

[7] S. Huang, B. Erickson, N. Tang et al., "Clinical performance of Abbott RealTi*m*e high risk HPV test for detection of high-grade cervical intraepithelial neoplasia in women with abnormal cytology," *Journal of Clinical Virology*, vol. 45, no. 1, pp. S19–S23, 2009.

[8] P. Halfon, D. Benmoura, A. Agostini et al., "Evaluation of the clinical performance of the Abbott RealTi*m*e high-risk HPV for carcinogenic HPV detection," *Journal of Clinical Virology*, vol. 48, no. 4, pp. 246–250, 2010.

[9] J. Cuzick, L. Ambroisine, L. Cadman et al., "Performance of the Abbott RealTi*m*e high-risk HPV test in women with abnormal cervical cytology smears," *Journal of Medical Virology*, vol. 82, no. 7, pp. 1186–1191, 2010.

[10] M. T. Sandri, P. Lentati, E. Benini et al., "Comparison of the digene HC2 assay and the roche AMPLICOR human papillomavirus (HPV) test for detection of high-risk HPV genotypes in cervical samples," *Journal of Clinical Microbiology*, vol. 44, no. 6, pp. 2141–2146, 2006.

[11] P. E. Castle, D. Solomon, C. M. Wheeler et al., "Human papillomavirus genotype specificity of hybrid capture 2," *Journal of Clinical Microbiology*, vol. 46, no. 8, pp. 2595–2604, 2008.

[12] R. L. Winer, Q. Feng, J. P. Hughes et al., "Concordance of self-collected and clinician-collected swab samples for detecting human papillomavirus DNA in women 18 to 32 years of age," *Sexually Transmitted Diseases*, vol. 34, no. 6, pp. 371–377, 2007.

[13] J. M. Palefsky, "Anal squamous intraepithelial lesions in human immunodeficiency virus-positive men and women," *Seminars in Oncology*, vol. 27, no. 4, pp. 471–479, 2000.

[14] T. Ramqvist and T. Dalianis, "Oropharyngeal cancer epidemic and human papillomavirus," *Emerging Infectious Diseases*, vol. 16, no. 11, pp. 1671–1677, 2010.

[15] M. A. Chernesky, E. W. Hook, D. H. Martin et al., "Women find it easy and prefer to collect their own vaginal swabs to diagnose Chlamydia trachomatis or Neisseria gonorrhoeae infections," *Sexually Transmitted Diseases*, vol. 32, no. 12, pp. 729–733, 2005.

[16] J. R. Schwebke, S. C. Morgan, and G. B. Pinson, "Validity of self-obtained vaginal specimens for diagnosis of trichomoniasis," *Journal of Clinical Microbiology*, vol. 35, no. 6, pp. 1618–1619, 1997.

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Review Article

Biomarkers of Cervical Dysplasia and Carcinoma

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Although cervical cytology screening has decreased the incidence of cervical cancer in industrialized countries, HPV-related cervical disease, including premalignant and malignant lesions, continues to represent a major burden on the health care system. Some of the problems include the potential for either under- or overtreatment of women due to decreased specificity of screening tests as well as significant interobserver variability in the diagnosis of cervical dysplastic lesions. Although not completely elucidated, the HPV-driven molecular mechanisms underlying the development of cervical lesions have provided a number of potential biomarkers for both diagnostic and prognostic use in the clinical management of these women.

1. Introduction

Cervical cancer remains a leading cause of morbidity and mortality worldwide, with an estimated incidence of 470,000 [1]. Approximately 230,000 women die each year from cervical cancer; over 190,000 of these women are from developing countries in South America, sub-Saharan Africa, and the Far East [2]. In the United States, the incidence of invasive cervical cancer is much lower; the American Cancer Society estimated that in 2010, there were approximately 12,200 new cases, with the number of estimated deaths at 4,210 [3]. The differences in incidence are attributed mainly to the utilization of cytological screening in numerous industrialized countries during the latter half of the 20th century [4]. In the US, the main burden of cervical disease manifests as a much higher number of premalignant lesions, including low grade cervical intraepithelial neoplasia (CIN1) (over 1.4 million new cases) and high grade lesions (CIN2/3) (330,000 new cases) [5]. Overall, the clinical management of patients with cervical premalignant and malignant lesions represents a significant burden on the health care system. Although improved methods are needed to improve the accuracy of cervical cancer screening, it is also important to consider that the vast majority of cervical cancer deaths worldwide occur in women that have never been screened.

The association between cervical premalignant and malignant epithelial lesions and human papillomaviruses (HPV) has been well established [6, 7]. There are over 100 defined HPV types, and these have been subdivided into high-risk (HR-HPV) and low-risk (LR-HPV) categories, based on their association with cervical cancer [8, 9]. Although the majority of women with HR-HPV infections have only transient infections that do not lead to malignant transformation of the cervical mucosa, HR-HPV is the etiologic agent of virtually all cases of cervical cancer. Dunne et al. found that while the overall prevalence of HPV infection (including both low-risk and high-risk types) in US women between the ages of 14 and 59 years of age was 26.8% ($n = 1921$), the prevalence of high risk HPVs was 15.2%. Furthermore, there was a marked peak in HR-HPV infection in women between the ages of 20 and 24, with a prevalence of 29% [10]. The vast majority of HPV infections (up to 90%) regress spontaneously, without treatment, after a few months [11, 12]. If the viral infection persists, however, the risk of developing a precancerous lesion increases as well as the risk of developing an invasive carcinoma [12, 13]. This underscores the importance of accurate diagnosis as well as identification of those lesions at highest risk for progression.

Histological examination of colposcopy-guided biopsies is still considered the "gold standard" in the assessment of

cervical lesions; however, the histologic assessment of these lesions is limited to the interpretation of the morphology, with little to no information regarding the risk of persistence, progression, or regression. In addition, histologic assessment of cervical lesions is complicated by interobserver variability [14]. The main interpretive categories include distinguishing normal from dysplasia (CIN) of any grade and low-grade (CIN1) lesions from high-grade (CIN2/3) lesions. Errors in histologic diagnosis lead to either overtreatment of patients who will not benefit from intervention or, conversely, undertreatment of patients with clinically significant high-grade lesions that received false negative diagnoses. The HPV life cycle and molecular events leading to cellular transformation, while not completely elucidated, have provided insight into potential biomarkers that can be used as adjunctive tests to improve diagnostic accuracy of cervical lesions as well as, identify those patients at risk for progression to cancer. This paper focuses on those biomarkers that appear to be most relevant in the clinical management of patients with HPV-related cervical disease.

2. HPV Review

Human papillomaviruses (HPVs) are a diverse group of viruses (numbering more than 100) that can infect numerous epithelial sites and cause a variety of epithelial lesions, including common warts, verrucas, laryngeal papillomas, and genital condylomata, depending on the type of HPV [15]. The different types that infect the female genital tract have been divided into high-risk types (HR-HPV, including types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68) and low-risk types (LR-HPV, including types 6, 11, 40, 42, 54, and 57). The LR-HPVs are associated with benign exophytic genital warts (condylomata acuminata) and are rarely associated with high-grade squamous intraepithelial lesions (HSILs) or invasive squamous cancers. Conversely, HR-HPVs, especially HPV-16, the most prevalent virus infecting the cervix, are associated with the entire spectrum of CIN lesions as well as, invasive squamous carcinomas. Recent studies have demonstrated that HR-HPV types account for almost 90% of all cervical infections [16].

The HPV genome consists of a double-stranded circular genome that includes early and late open reading frames (ORFs). The early ORFs E1 through E7 encode proteins that are involved in the regulation of DNA replication and cell proliferation, while the late ORFs L1 and L2 encode the two viral capsid proteins [15]. CIN1 lesions reflect high levels of HPV episomal replication, the so-called "productive infections." In these cases, the E1/E2 open reading frames serve as negative regulators of E6 and E7. In CIN 2 or more severe lesions (CIN2+), however, arrested squamous maturation no longer supports effective HPV DNA replication, and the copy number of HPV DNA is generally low. In these lesions, a transforming event, often associated with disruption of E1/E2 by the integration of the HPV genome into the host genome, results in the unregulated expression of E6 and E7. Overexpression of E6 promotes cell-cycle progression by promoting degradation of p53, allowing cell-cycle progression even in the face of genomic damage, while E7 promotes the degradation of Rb, resulting in the release of transcription factor E2F and cell-cycle progression. The degradation of Rb also results in the hypomethylation of the $p16^{INK4a}$ promoter, enabling high-level overexpression of $p16^{INK4a}$ [17]. The identification of these major components in HPV-mediated oncogenesis provides potential targets for clinically relevant biomarkers.

3. HPV DNA

The most widely used and extensively investigated biomarker in the management of cervical disease is HPV DNA testing. There are a wide range of HPV detection techniques, including in situ hybridization, and genotyping assays, including molecular amplification assays with or without genotyping [17]. The *Digene* HPV test, which uses Hybrid Capture 2 (HC2) technology, and the Cervista HPV HR assay are the only methods that currently have FDA approval for diagnostic testing in the United States.

The *Digene* HPV Test (Qiagen, Valencia, Calif, USA) was the first HPV test that was licensed by the FDA (United States Food and Drug Administration). This test is a solution-phase hybridization assay that uses RNA probes complementary to HPV DNA, resulting in signal amplification. This test detects the presence of 13 HR-HPV types (16/18/31/33/35/39/45/51/52/56/58/59/68) or 5 low-risk types (6, 11, 42, 43, and 44). The assay is usually performed using only the HR-HPV probe set, since LR-HPV is not clinically significant. In the ASCUS-LSIL Triage Study (ALTS), HC2 was shown to provide more effective triage of ASCUS cytology than a repeat cytology examination [5]. Other randomized large studies have reported that 50% to 70% more precancerous lesions may be diagnosed when HPV testing is incorporated in primary screening [18–20]. Another advantage is that because a negative result excludes the risk of HPV-related disease in subsequent years, screening intervals may safely be increased to 3 to 5 years in those patients with a negative result. Other advantages include good interlaboratory reproducibility [21] and ease of use. One of the disadvantages is that this assay does not produce information on individual HPV types; instead, the presence of at least one of the high-risk or low-risk types is reported. This is a significant limitation, since persistent infection with HR-HPV is a risk factor for progression to cervical cancer and with the advent of HPV vaccines, it is increasingly relevant to perform HPV genotyping to identify oncogenic HPV vaccine types [22]. HPV genotyping is of clinical interest, since the risk of developing a precancerous lesion is between 10%, and 15% with HPV types 16 and 18, and below 3% for all other high-risk types combined. Genotyping information could provide more information regarding risk-stratification as well as persistence of infection [23].

The Cervista HPV HR test (Hologic, Bedford, Mass, USA) detects the presence of 14 HPV types designated as high risk by the International Agency for Research on Cancer (IARC), consisting of 16/18/31/33/35/39/45/51/52/

56/58/59/66/68. This assay utilizes Invader chemistry, a signal amplification method for the detection of specific nucleic acid sequences. This method comprises two isothermal reactions: a primary reaction on the targeted DNA sequence and a secondary reaction that produces a fluorescent signal. In a comparison between the Digene and Cervista assays, the Cervista assay demonstrated 100% sensitivity in the detection of CIN 3 or worse and 98% sensitivity for the detection of CIN 2 or worse [24]. After adjustments to compensate for potential bias related to availability of biopsy histologic diagnoses, the expected clinical performance of the HPV HR test is 95.49% sensitivity, specificity 63.3%, PPV 10.1%, and NPV 99.7%. In this study, the authors reported that the Cervista assay had a lower false-positive rate compared to the Digene assay (attributed to cross-reactivity with low-risk HPV types). Some other strengths of the Cervista assay include an internal positive control to determine the presence of sufficient DNA and the presence of potentially interfering substances and requiring a smaller sample volume compared to other assays. However, IARC determined that there is limited evidence to conclude HPV 66 is carcinogenic; although the prevalence of HPV 66 in women is low, this reclassification may have a marginal impact on the false-positivity rate [25]. Also, similar to the Digene assay, information regarding individual HPV types is not provided. To address this, a DNA-based genotyping assay was developed as well.

The Cervista HPV 16/18 test (Hologic, Bedford, Mass, USA) has been approved by the FDA for use in conjunction with the Cervista HPV HR test. This test utilizes the same Invader chemistry used by the HPV HR test in the analysis of cervical cytology specimens. Clinical validation and analytical performance studies report that the Cervista HPV 16/18 genotyping test demonstrated a high degree of analytical sensitivity, and specificity, and performed as expected in women with ASC-US cytology who were positive for HR HPV [25, 26]. These studies support the utilization of the genotyping test in the proper clinical context.

The polymerase chain reaction (PCR) method of detecting HPV enables the sensitive amplification of even small amounts of HPV DNA, enabling the evaluation of extracts of formalin-fixed histologic sections which generally yield fragmented DNA [17]. Briefly, the two major types of available PCR assays are type-specific and consensus sequence assays. The type-specific assays amplify a single HPV genotype, necessitating multiple separate PCR assays and increasing the cost for genotyping each sample. The consensus assays detect a wide range of HPV types, most commonly using primers that target the L1 region. Once the sample is amplified, there are a number of methods that may be used to determine the specific type of HPV, including nucleic acid hybridization, restriction fragment length polymorphism, and sequencing. There are, however, cost and other considerations currently that limit clinical application.

Overall, HPV DNA testing has a sensitivity above 90% for the detection of underlying CIN2+ lesions but has generally poor specificity for underlying clinically significant lesions, because most positive cases represent only transient infections rather than providing evidence of cervical mucosal transformation [27]. As a result, HPV testing is useful for the triage of women with ASCUS cytology but is generally not used for the triage of women under the age of 30 with other cytologic diagnostic test results. HPV testing in women over the age of 30, in combination with liquid-based cervical cytology, can, however, be used to increase the screening interval in women over age 30 due to the high negative predictive value of HPV testing. Although HPV genotyping provides more information regarding a patient's risk for progression, individual typing assays are not commonly used for routine cervical cancer screening.

4. HPV Viral Load

Viral load may be a useful marker in predicting the risk of progression. High viral load is often considered to be indicative of persistent infection and progression, while low viral load has been interpreted to reflect HPV viral clearance. A fundamental pitfall of this concept, however, is that CIN1 lesions reflect productive infections and may have thousands of viral copies/cell in upper layers of the cervical mucosa, but CIN2/3 and SCC lesions may have as low as a single copy of viral DNA/cell (commonly integrated into the host genome but not supporting viral replication) [28]. Thus, there is at least a theoretically increased risk of false negative HPV test results in high-grade lesions compared to low grade lesions, unless the high grade lesional cell sample also includes cells that are derived from a coexisting low-grade productive infection. HPV viral copy number may be determined using PCR assays that target type-specific HPV DNA and normalize to the total human DNA present [29]. In a study by Carcopino et al., HPV 16 and 18 viral loads were related to the severity of the cervical lesion [30] although as suggested above, these results may have been impacted by the presence of cells that were actually derived from low-grade lesions.

5. HPV mRNA

Assays for the detection of E6/E7 mRNA have been developed based on the concept that E6/E7 expression results in a transforming event with unregulated cell-cycle progression due to degradation of p53 and Rb [31]. The PreTect HPV-Proofer assay (NorChip AS, Klokkarstua Norway) is a commercially available assay (in Europe only) to detect E6/E7 mRNA from five HR-HPV types (16, 18, 31, 33, and 45) [32]. A positive HPV-Proofer result is indicative of E6/E7 integration and identifies a high risk of persistent infection. In a study by Molden et al., a comparison of HPV DNA and mRNA was performed on women with an initial diagnosis of ASCUS or LSIL on cervical cytology, with a 2-year follow-up period [33]. In this study, women with a positive HPV-Proofer assay were approximately 70 times more likely to be diagnosed with CIN2 or greater than women who tested negative. Consensus PCR testing for HPV was also performed; women who tested positive were 6 times more likely to be diagnosed with CIN2 or greater than women who tested negative. These results suggest that the HPV-Proofer assay is as sensitive but is more specific than HPV PCR for the detection of underlying high-grade lesions.

The APTIMA HPV assay is another commercially available test for mRNA detection (Gen-Probe, San Diego, Calif, USA). The APTIMA HPV assay detects mRNA from 14 HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) using liquid-based cervical cytology specimens. The assay involves target capture, target amplification, and the detection of the amplification products. This assay is currently in clinical trials, with a focus on the identification of women that are at high risk for persistent infection.

6. HPV L1 Capsid Protein

The L1 capsid protein represents approximately 90% of the total protein on the virus surface and is generally detectable during the reproductive phase of HPV infection. The L1 protein is abundant in productive infections [34, 35]; conversely, it is found only in rare cases of CIN3, and it is not produced in carcinomas [34]. In general, CIN2/3 lesions are unlikely to support productive HPV infection, because viral maturation depends on squamous maturation that, by definition, is arrested in CIN2/3. There have been a few studies evaluating the prognostic significance of L1 status; although the studies are relatively small, it has been suggested that L1 status may have utility in the prediction of disease progression [35–37]. A recent study by Galgano et al., however, found that L1 was neither sensitive nor specific for the detection of CIN2/3 lesions [38].

7. $p16^{INK4a}$ and Ki-67

$p16^{INK4a}$ is a tumor-suppressor protein and cyclin-dependent kinase (cdk) inhibitor that blocks cdk4- and cdk6-mediated pRb phosphorylation to inhibit E2F-dependent transcription and cell-cycle progression [39]. In most cervical carcinomas, the functional inactivation of pRb by HPV E7 results in the overexpression of $p16^{INK4a}$ and the accumulation of the protein in cells. $p16^{INK4a}$ is thus a surrogate marker of HPV E7-mediated pRb catabolism, providing evidence of transformation of the cervical mucosa [40, 41]. $p16^{INK4a}$ has been successfully deployed for the classification of HPV-related disease for several reasons [42]: (1) the expression of $p16^{INK4a}$ is directly linked to the HPV oncogenic action, since continuous expression of E7 is necessary to maintain the malignant phenotype, (2) the expression of $p16^{INK4a}$ is independent of the HPV type, and therefore, genotyping does not need to be performed, and (3) the expression of $p16^{INK4a}$ by cycling cells is a specific marker of HPV-E7 overexpression or other events that inactivate Rb [43]. Immunohistochemical analysis has demonstrated that diffuse staining for $p16^{INK4a}$ is present in almost all cases of CIN2, CIN3, and squamous cell carcinoma (as well as in endocervical glandular neoplasia); however, it is rarely detected in benign squamous mucosa or CIN1 lesions associated with LR-HPV [40, 41]. One limitation of the analysis of $p16^{INK4a}$ as a marker of cervical neoplasia is that focal and occasionally diffuse expression can also be observed in benign endocervical intercalated columnar cells, in tuboendometrial metaplasia, and in cervical endometriosis [44]. The expression of $p16^{INK4a}$ in these cells, however, denotes no premalignant potential. Focal staining can also be detected in the lower third of some CIN1 lesions and in the upper third of the epithelium in a few cases of squamous metaplasia. The diffuse pattern of $p16^{INK4a}$ expression within the lower third of the squamous mucosa, however, is highly specific for CIN1+ lesions, and diffuse expression in glandular epithelial cells usually reflects endocervical glandular neoplasia (lesions with some but not all features of AIS), AIS, or invasive adenocarcinoma.

For cervical tissue punch and cone biopsies, immunohistochemistry for $p16^{INK4a}$ has been reported to reduce interobserver disagreement when compared with diagnosis of H&E stained sections [45–47]. In one study, 496 cervical histology H&E-stained slides (each representing an independent case, either punch or cone biopsy) were evaluated by 6 pathologists. Interobserver agreement for punch biopsies was moderate (mean $\kappa = 0.49$) and substantial for cone biopsies (mean $\kappa = 0.63$) [45]. The addition of $p16^{INK4a}$-immunostained, consecutive slides read together with the H&E-stained slides significantly improved the interobserver agreement for the interpretation of both punch and cone biopsies. For the punch biopsies, the κ value increased from 0.49 (moderate agreement) to 0.64 (substantial agreement), and the κ value for the cone biopsies increased from 0.64 to 0.70. A subsequent study by Bergeron et al. [48] addressed the utility of $p16^{INK4a}$ testing for both increasing interobserver agreement as well as increasing diagnostic accuracy. In this study, H&E-stained slides from 500 cases (comprising cervical punch and cone biopsies) were interpreted by twelve community pathologists. These interpretations were compared to the "gold standard" diagnoses established by three expert gynecologic pathologists. After a "washout" period of at least four weeks, the same H&E-stained slides were reassessed by the twelve pathologists, but this time in conjunction with $p16^{INK4a}$-immunostained matched slides. The pathologists were blinded to their original diagnoses as well as the gold standard diagnoses. Overall, diagnostic accuracy for high-grade CIN was significantly improved with the addition of $p16^{INK4a}$-immunostained slides. The mean sensitivity increased from 0.77 to 0.87 (an increase in sensitivity of 13%). The number of missed high-grade CIN cases was reduced by 45%. The number of cases with a gold standard diagnosis of CIN 3 that were missed by the community-based pathologists was decreased by 60%. Importantly, this gain in sensitivity was not associated with a relevant loss in specificity. Also, the interobserver agreement of the community-based pathologists for categorizing lesions as high-grade CIN versus CIN 1 or negative for dysplasia significantly improved with the addition of the $p16^{INK4a}$-immunostained slides, with a κ coefficient of 0.749 (for H&E stained slides only, the κ coefficient was 0.566). Also demonstrated in this study was the relative ease with which accurate, reproducible interpretation of $p16^{INK4a}$-immunostained slides can be implemented into clinical practice [48]. In addition to improving diagnostic accuracy and reproducibility, the use of $p16^{INK4a}$ immunohistochemistry may help in identifying CIN1 lesions that are associated with HR-HPV types; these lesions are at an increased risk for progression to high-grade dysplasia or carcinoma [49].

$p16^{INK4a}$ has also recently emerged as a sensitive and specific diagnostic adjunct for underlying CIN2+ lesions in cervical cytology specimens [50, 51]. Most studies that have evaluated the use of $p16^{INK4a}$ as an immunocytochemical diagnostic adjunct have relied on the use of scoring criteria that depend on both the morphologic interpretation of $p16^{INK4a}$ positive cells and on the use of quantitative thresholds to establish positive test results [50]. Samarawardana et al. established rigorous criteria for scoring $p16^{INK4a}$ test results that was associated with decreased sensitivity and negative predictive value but improved specificity and positive predictive value compared with most of the previous reports of $p16^{INK4a}$ test performance. Denton et al. used a different scoring system in the evaluation of $p16^{INK4a}$ test results and also demonstrated that the use of $p16^{INK4a}$ immunostaining on cytology provides significantly better specificity than HR-HPV for the triage of ASC-US and LSIL cytology cases [51]. It is important to emphasize that the primary value of $p16^{INK4a}$ and other cervical cancer biomarkers is to improve test specificity rather than sensitivity relative to HPV testing.

Ki-67 is a proliferation marker that is confined to the parabasal cell layer of normal stratified squamous mucosa but shows expression in the stratified squamous epithelium in CIN lesions in correlation with the extent of disordered maturation. Although Ki-67 has been used as a diagnostic adjunct for the classification of cervical tissue specimens [52, 53], the expression of Ki-67 alone does not discriminate HPV-mediated dysplasia versus benign proliferating cells in benign reactive processes, which limits its use in cytologic specimens as a specific marker of underlying CIN or glandular neoplasia. Recent large-scale studies from Europe and pilot studies from the US, however, show that a dual stain approach for $p16^{INK4a}$ and Mib-1, (using the CINtec Plus kit from MTM Laboratories, Westborough, Mass, USA) can be used to score cases positive on the basis of a single dual stained epithelial cell, independent of cell morphology, resulting in sensitivity that rivals HPV testing but with specificity that is greater than that provided by HR-HPV testing [54–60].

8. DNA Aneuploidy

HPV infection may lead to DNA hypermethylation (discussed below), disruption of the normal cell cycle, and chromosomal aberrations, all of which may lead to changes in DNA content. Studies using DNA-cytometry of Feulgen-stained cytology material to assess ploidy have demonstrated significant differences in aneuploidy between HSILs and LSILs: 79% aneuploid versus 4%, respectively [61]. Subsequent studies have reported a strong association between highly aneuploid squamous cells and HR-HPV [62] as well as a positive predictive value of 81.8% for CIN 2 for 9c cells [63]. A prospective study by Grote et al. demonstrated a significant increase in DNA aneuploidy in cervical cytology material from patients with CIN 1 (54%) and CIN 2 (64.3%) to CIN 3 or greater (83.3%) on subsequent biopsies [64]. In a preliminary retrospective study assessing the utility of DNA ploidy in the management of ASC cytology specimens, Lorenzato et al. suggest that the combined use of HR-HPV testing and DNA ploidy measurement on ASCUS cytology specimens may improve the triage of women who have to undergo colposcopy as well as identify patients with a diagnosis of ASC-H at higher risk for CIN 2 or greater lesions [65]. DNA image cytometry has become increasingly standardized and represents an objective and highly reproducible diagnostic procedure [64].

9. ProExC Test

The ProExC test (BD TriPath Imaging, Burlington, NC, USA) is a recently developed immunocytochemical assay for the detection of minichromosome maintenance proteins (MCMs) and in previous formulations, Topoisomerase 2α, in cervical cytology slides, as a marker of aberrant S-phase induction and underlying high-grade dysplasia. MCMs are members of the DNA licensing factor family that are required for the origination of DNA replication and are overexpressed in cervical high-grade dysplasia and carcinoma [66–68]. Preliminary studies demonstrated that the ProExC test is consistently positive in HSILs and negative in normal cytologic specimens [69, 70] but to date, the ProExC assay has not come into widespread utilization as a diagnostic adjunct for cervical cytology due at least in part to expression of MCMs in some benign cycling squamous and glandular cells [71, 72]. Further confirmation of the performance of this test as a diagnostic adjunct for cervical cytology will depend on the results of large-scale trials including biopsy correlation and clinical outcome correlation.

10. Methylation Markers

As part of the search for novel and relevant biomarkers in cervical disease, attention has been focused on methylated genes. Of particular interest is that $p16^{INK4a}$ has been found to be inactivated in numerous cancers due to mutations and epigenetic alterations (patterns of altered gene expression mediated by mechanisms that do not affect the primary DNA sequence) [73]. Methylation of a CpG island within the $p16^{INK4a}$ exon1α has been associated with a variety of malignant tumors, such as nonsmall cell lung cancer, colorectal cancer or pancreatic cancer [74–76]. Several groups have analyzed cervical cancers for p16 exon1α methylation, with frequencies ranging from 19% to 61% [77–84]; however, most of these studies reported methylation data without confirmation of the expression level. Nehls et al. performed a detailed analysis of $p16^{INK4a}$ exon1α methylation, with comparison to $p16^{INK4a}$ expression, using both cell lines and clinical samples [85]. They found composite or complete methylation of $p16^{INK4a}$ exon1α without any influence on $p16^{INK4a}$ expression and concluded that methylation in this region does not suppress $p16^{INK4a}$ expression. Wentzensen et al. recently published a systematic literature review of studies analyzing the utility of methylation markers in cervical cancer [86]. They identified 51 studies analyzing 68 different genes for methylation across all stages of cervical carcinogenesis. This group found that the published data was highly

heterogeneous; for 7 genes, there was a reported range of methylation frequencies in cervical cancers of greater than 60% between studies. They did identify 3 markers, DAPK1, CADM1, and RARB, which showed elevated methylation in cervical cancers consistently across studies. Thus, based on these findings, no methylation markers can yet be utilized in cervical cancer screening or triage settings. Similar to other diagnostic molecular approaches, large, well-powered epidemiologic studies are still needed to identify and validate candidate methylation markers of cervical neoplasia.

11. FISH

Fluorescent in situ hybridization (FISH) technology has increasingly been recognized as a valuable tool to evaluate cervical dysplasia [87, 88]. Studies have demonstrated that one of the most consistent chromosomal abnormalities identified in cervical carcinoma is gain of chromosome arm 3q, which is detected in approximately 70% of cervical carcinomas [89, 90]. These extra copies result in a gain of the human telomerase RNA gene (TERC) located in the 3q26 region. The gene product, telomerase, is involved in chromosome maintenance by providing telomere stability and regulating telomere length. In a study by Caraway et al., FISH analysis for gain of chromosome 3q was performed on cervicovaginal liquid-based preparations, and results were compared with cytologic diagnosis and concurrent/subsequent biopsies [91]. Patients with HSIL or squamous cell carcinoma cytologic diagnoses had significantly higher percentages of cells with 3q26 gain than patients with negative or ASC-US diagnoses. Seppo et al. demonstrated that a fully automated FISH scoring system can detect gain of 3q in liquid cytology samples [92]. Another study evaluated HPV DNA and telomerase using a different assay (telomeric repeat amplification protocol, TRAP) as diagnostic adjuncts in cervical cytology specimens [93]. Interestingly, telomerase showed a low sensitivity (29.9%) for biopsy-confirmed CIN 2/3; the study concluded that the TRAP assay for telomerase is unlikely to be used as a diagnostic adjunct. FISH analysis for 3q appears to hold more promise as a useful biomarker.

12. Conclusion

Despite the tremendous progress that has been achieved in the screening and management of women with HPV-related cervical disease, there is still a need for clinically robust biomarkers to further refine the screening, triage, and management of women. In this paper, we focused on those biomarkers that have the greatest utility in the clinical setting, such as those that will increase screening and diagnostic accuracy of cervical specimens and tissue biopsies, and we provide information regarding the risk for progression to a more severe lesion. Examples of these include HPV DNA testing for the effective triage of women with abnormal cervical cytology, and the use of $p16^{INK4a}$ immunohistochemistry to increase diagnostic accuracy of dysplastic lesions. Merely identifying the presence of HPV infection is not sufficient, as multiple studies have demonstrated. We expect that in the future, in addition to cervical cytology, more advanced techniques, including HPV genotyping, will be used to identify and triage those women most likely to harbor a clinically significant cervical lesion. Assays for HPV viral load and mRNA detection may be useful in both the triage of abnormal cervical cytology, and detecting persistent infection, which is associated with an increased risk for disease progression. We also reviewed new applications of technologies such as FISH and the detection of DNA methylation; although their clinical utility is still under investigation, they have the potential to provide valuable information in the identification of disease, risk of disease progression, and clinical management of patients. Although cervical cancer biomarkers will provide increasingly detailed and important information in countries that have organized screening programs, their utility will depend on the resolution of social and economic factors that have precluded the utilization of cervical cancer screening programs in developing nations.

References

[1] World Health Organization, *Comprehensive Cervical Cancer Control: A Guide to Essential Practice*, WHO, Geneva, Switzerland, 2006.

[2] "Stat bite: worldwide cervical and uterine cancer incidence and mortality, 2002," *Journal of the National Cancer Institute*, vol. 98, no. 15, p. 1031, 2006.

[3] A. Jemal, R. Siegel, J. Xu, and E. Ward, "Cancer statistics, 2010," *CA Cancer Journal for Clinicians*, vol. 60, no. 5, pp. 277–300, 2010.

[4] L. Gustafsson, J. Pontén, M. Zack, and H. O. Adami, "International incidence rates of invasive cervical cancer after introduction of cytological screening," *Cancer Causes and Control*, vol. 8, no. 5, pp. 755–763, 1997.

[5] M. Schiffman and D. Solomon, "Findings to date from the ASCUS-LSIL Triage Study (ALTS)," *Archives of Pathology and Laboratory Medicine*, vol. 127, no. 8, pp. 946–949, 2003.

[6] M. H. Schiffman, H. M. Bauer, R. N. Hoover et al., "Epidemiologic evidence showing that human papillomavirus infection causes most cervical intraepithelial neoplasia," *Journal of the National Cancer Institute*, vol. 85, no. 12, pp. 958–964, 1993.

[7] J. M. Walboomers, M. V. Jacobs, M. M. Manos et al., "Human papillomavirus is a necessary cause of invasive cervical cancer worldwide," *Journal of Pathology*, vol. 189, no. 1, pp. 12–19, 1999.

[8] J. K. Wolf and P. T. Ramirez, "The molecular biology of cervical cancer," *Cancer Investigation*, vol. 19, no. 6, pp. 621–629, 2001.

[9] H. Zur Hausen, "Papillomaviruses in human cancers," *Proceedings of the Association of American Physicians*, vol. 111, no. 6, pp. 581–587, 1999.

[10] E. F. Dunne, E. R. Unger, M. Sternberg et al., "Prevalence of HPV infection among females in the United States," *Journal of the American Medical Association*, vol. 297, no. 8, pp. 813–819, 2007.

[11] M. Schiffman, P. E. Castle, J. Jeronimo, A. C. Rodriguez, and S. Wacholder, "Human papillomavirus and cervical cancer," *Lancet*, vol. 370, no. 9590, pp. 890–907, 2007.

[12] M. Schiffman, "Integration of human papillomavirus vaccination, cytology, and human papillomavirus testing," *Cancer*, vol. 111, no. 3, pp. 145–153, 2007.

[13] N. F. Schlecht, S. Kulaga, J. Robitaille et al., "Persistent human papillomavirus infection as a predictor of cervical intraepithelial neoplasia," *Journal of the American Medical Association*, vol. 286, no. 24, pp. 3106–3114, 2001.

[14] M. H. Stoler and M. Schiffman, "Interobserver reproducibility of cervical cytologic and histologic interpretations: realistic estimates from the ASCUS-LSIL Triage Study," *Journal of the American Medical Association*, vol. 285, no. 11, pp. 1500–1505, 2001.

[15] J. Doorbar, "Papillomavirus life cycle organization and biomarker selection," *Disease Markers*, vol. 23, no. 4, pp. 297–313, 2007.

[16] M. H. Stoler, "Human papillomavirus biology and cervical neoplasia: implications for diagnostic criteria and testing," *Archives of Pathology and Laboratory Medicine*, vol. 127, no. 8, pp. 935–939, 2003.

[17] D. Dehn, K. C. Torkko, and K. R. Shroyer, "Human papillomavirus testing and molecular markers of cervical dysplasia and carcinoma," *Cancer*, vol. 111, no. 1, pp. 1–14, 2007.

[18] N. W. J. Bulkmans, L. Rozendaal, P. J. Snijders et al., "POBASCAM, a population-based randomized controlled trial for implementation of high-risk HPV testing in cervical screening: design, methods and baseline data of 4402 women," *International Journal of Cancer*, vol. 110, no. 1, pp. 94–101, 2004.

[19] M. H. Mayrand, E. Duarte-Franco, I. Rodrigues et al., "Human papillomavirus DNA versus Papanicolaou screening tests for cervical cancer," *New England Journal of Medicine*, vol. 357, no. 16, pp. 1579–1588, 2007.

[20] P. Naucler, W. Ryd, S. Törnberg et al., "Human papillomavirus and Papanicolaou tests to screen for cervical cancer," *New England Journal of Medicine*, vol. 357, no. 16, pp. 1589–1597, 2007.

[21] P. Davies, J. Kornegay, and T. Iftner, "Current methods of testing for human papillomavirus," *Best Practice and Research: Clinical Obstetrics and Gynaecology*, vol. 15, no. 5, pp. 677–700, 2001.

[22] T. C. Wright and M. Schiffman, "Adding a test for human papillomavirus DNA to cervical-cancer screening," *New England Journal of Medicine*, vol. 348, no. 6, pp. 489–490, 2003.

[23] M. J. Khan, P. E. Castle, A. T. Lorincz et al., "The elevated 10-Year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice," *Journal of the National Cancer Institute*, vol. 97, no. 14, pp. 1072–1079, 2005.

[24] L. R. Johnson, C. R. Starkey, J. Palmer et al., "A comparison of two methods to determine the presence of high-risk HPV cervical infections," *American Journal of Clinical Pathology*, vol. 130, no. 3, pp. 401–408, 2008.

[25] M. H. Einstein, M. G. Martens, F. A. Garcia et al., "Clinical validation of the Cervista HPV HR and 16/18 genotyping tests for use in women with ASC-US cytology," *Gynecologic Oncology*, vol. 118, no. 2, pp. 116–122, 2010.

[26] D. A. Bartholomew, R. D. Luff, N. B. Quigley, M. Curtis, and M. C. Olson, "Analytical performance of Cervista® HPV 16/18 genotyping test for cervical cytology samples," *Journal of Clinical Virology*, vol. 51, no. 1, pp. 38–43, 2011.

[27] C. L. Peyton, P. E. Gravitt, W. C. Hunt et al., "Determinants of genital human papillomavirus detection in a US population," *Journal of Infectious Diseases*, vol. 183, no. 11, pp. 1554–1564, 2001.

[28] P. J. Snijders, A. J. van den Brule, and C. J. Meijer, "The clinical relevance of human papillomavirus testing: relationship between analytical and clinical sensitivity," *Journal of Pathology*, vol. 201, no. 1, pp. 1–6, 2003.

[29] P. E. Gravitt, C. Peyton, C. Wheeler, R. Apple, R. Higuchi, and K. V. Shah, "Reproducibility of HPV 16 and HPV 18 viral load quantitation using TaqMan real-time PCR assays," *Journal of Virological Methods*, vol. 112, no. 1-2, pp. 23–33, 2003.

[30] X. Carcopino, M. Henry, D. Benmoura et al., "Determination of HPV type 16 and 18 viral load in cervical smears of women referred to colposcopy," *Journal of Medical Virology*, vol. 78, no. 8, pp. 1131–1140, 2006.

[31] J. B. Hudson, M. A. Bedell, D. J. McCance, and L. A. Laimins, "Immortalization and altered differentiation of human keratinocytes in vitro by the E6 and E7 open reading frames of human papillomavirus type 18," *Journal of Virology*, vol. 64, no. 2, pp. 519–526, 1990.

[32] T. Molden, I. Kraus, F. Karlsen, H. Skomedal, and B. Hagmar, "Human papillomavirus E6/E7 mRNA expression in women younger than 30 years of age," *Gynecologic Oncology*, vol. 100, no. 1, pp. 95–100, 2006.

[33] T. Molden, J. F. Nygård, I. Kraus et al., "Predicting CIN2$^+$ when detecting HPV mRNA and DNA by PreTect HPV-Proofer and consensus PCR: a 2-year follow-up of women with ASCUS or LSIL Pap smear," *International Journal of Cancer*, vol. 114, no. 6, pp. 973–976, 2005.

[34] P. Birner, B. Bachtiary, B. Dreier et al., "Signal-amplified colorimetric in situ hybridization for assessment of human papillomavirus infection in cervical lesions," *Modern Pathology*, vol. 14, no. 7, pp. 702–709, 2001.

[35] P. Melsheimer, S. Kaul, S. Dobeck, and G. Bastert, "Immunocytochemical detection of HPV high-risk type L1 capsid proteins in LSIL and HSIL as compared with detection of HPV L1 DNA," *Acta Cytologica*, vol. 47, no. 2, pp. 124–128, 2003.

[36] H. Griesser, H. Sander, R. Hilfrich, B. Moser, and U. Schenck, "Correlation of immunochemical detection of HPV L1 capsid protein in pap smears with regression of high-risk HPV positive mild/moderate dysplasia," *Analytical and Quantitative Cytology and Histology*, vol. 26, no. 5, pp. 241–245, 2004.

[37] R. Hilfrich and J. Hariri, "Prognostic relevance of human papillomavirus L1 capsid protein detection within mild and moderate dysplastic lesions of the cervix uteri in combination with p16 biomarker," *Analytical and Quantitative Cytology and Histology*, vol. 30, no. 2, pp. 78–82, 2008.

[38] M. T. Galgano, P. E. Castle, K. A. Atkins, W. K. Brix, S. R. Nassau, and M. H. Stoler, "Using Biomarkers as objective standards in the diagnosis of cervical biopsies," *American Journal of Surgical Pathology*, vol. 34, no. 8, pp. 1077–1087, 2010.

[39] H. S. Zhang, A. A. Postigo, and D. C. Dean, "Active transcriptional repression by the Rb-E2F complex mediates G1 arrest triggered by p16INK4a, TGFβ, and contact inhibition," *Cell*, vol. 97, no. 1, pp. 53–61, 1999.

[40] J. T. Keating, A. Cviko, S. Riethdorf et al., "Ki-67, cyclin E, and p16INK4 are complimentary surrogate biomarkers for human papilloma virus-related cervical neoplasia," *American Journal of Surgical Pathology*, vol. 25, no. 7, pp. 884–891, 2001.

[41] R. Klaes, T. Friedrich, D. Spitkovsky et al., "Overexpression of p16ink4a as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri," *International Journal of Cancer*, vol. 92, no. 2, pp. 276–284, 2001.

[42] K. Cuschieri and N. Wentzensen, "Human papillomavirus mRNA and p16 detection as biomarkers for the improved diagnosis of cervical neoplasia," *Cancer Epidemiology Biomarkers and Prevention*, vol. 17, no. 10, pp. 2536–2545, 2008.

[43] C. M. Beauséjour, A. Krtolica, F. Galimi et al., "Reversal of human cellular senescence: roles of the p53 and p16 pathways," *EMBO Journal*, vol. 22, no. 16, pp. 4212–4222, 2003.

[44] B. Tringler, C. J. Gup, M. Singh et al., "Evaluation of p16INK4a and pRb expression in cervical squamous and glandular neoplasia," *Human Pathology*, vol. 35, no. 6, pp. 689–696, 2004.

[45] L. C. Horn, A. Reichert, A. Oster et al., "Immunostaining for p16INK4a used as a conjunctive tool improves interobserver agreement of the histologic diagnosis of cervical intraepithelial neoplasia," *American Journal of Surgical Pathology*, vol. 32, no. 4, pp. 502–512, 2008.

[46] R. Klaes, A. Benner, T. Friedrich et al., "p16INK4a immunohistochemistry improves interobserver agreement in the diagnosis of cervical intraepithelial neoplasia," *American Journal of Surgical Pathology*, vol. 26, no. 11, pp. 1389–1399, 2002.

[47] Q. Zhang, L. Kuhn, L. A. Denny, M. De Souza, S. Taylor, and T. C. Wright, "Impact of utilizing p16INK4A immunohistochemistry on estimated performance of three cervical cancer screening tests," *International Journal of Cancer*, vol. 120, no. 2, pp. 351–356, 2007.

[48] C. Bergeron, J. Ordi, D. Schmidt, M. J. Trunk, T. Keller, and R. Ridder, "Conjunctive p16INK4a testing significantly increases accuracy in diagnosing high-grade cervical intraepithelial neoplasia," *American Journal of Clinical Pathology*, vol. 133, no. 3, pp. 395–406, 2010.

[49] N. Murphy, M. Ring, A. G. Killalea et al., "p16INK4A as a marker for cervical dyskaryosis: CIN and cGIN in cervical biopsies and ThinPrep smears," *Journal of Clinical Pathology*, vol. 56, no. 1, pp. 56–63, 2003.

[50] P. Samarawardana, D. L. Dehn, M. Singh et al., "p16^{INK4a} is superior to high-risk human papillomavirus testing in cervical cytology for the prediction of underlying high-grade dysplasia," *Cancer Cytopathology*, vol. 118, no. 3, pp. 146–156, 2010.

[51] K. J. Denton, C. Bergeron, P. Klement, M. J. Trunk, T. Keller, and R. Ridder, "The sensitivity and specificity of p16INK4a cytology vs HPV testing for detecting high-grade cervical disease in the triage of ASC-US and LSIL Pap cytology results," *American Journal of Clinical Pathology*, vol. 134, no. 1, pp. 12–21, 2010.

[52] A. J. Kruse, J. P. Baak, P. C. de Bruin et al., "Ki-67 immunoquantitation in cervical intraepithelial neoplasia (CIN): a sensitive marker for grading," *Journal of Pathology*, vol. 193, no. 1, pp. 48–54, 2001.

[53] A. J. Kruse, J. P. Baak, P. C. de Bruin, F. R. van de Goot, and N. Kurten, "Relationship between the presence of oncogenic HPV DNA assessed by polymerase chain reaction and Ki-67 immunoquantitative features in cervical intraepithelial neoplasia," *Journal of Pathology*, vol. 195, no. 5, pp. 557–562, 2001.

[54] M. Singh, D. Mockler, A. Akalin et al., "Dual localization of p16INK4a and Ki-67 detects high grade cervical intraepithelial neoplasia and cancer," *Cancer Cytopathology*. In press.

[55] K. U. Petry, D. Schmidt, S. Scherbring et al., "Triaging Pap cytology negative, HPV positive cervical cancer screening results with p16/Ki-67 Dual-stained cytology," *Gynecologic Oncology*, vol. 121, no. 3, pp. 505–509, 2011.

[56] D. Schmidt, C. Bergeron, K. J. Denton, and R. Ridder, "p16/ki-67 dual-stain cytology in the triage of ASCUS and LSIL papanicolaou cytology: results from the European equivocal or mildly abnormal Papanicolaou cytology study," *Cancer Cytopathology*, vol. 119, no. 3, pp. 158–166, 2011.

[57] H. Liu, J. Shi, M. Wilkerson et al., "Immunohistochemical detection of p16INK4a in liquid-based cytology specimens on cell block sections," *Cancer*, vol. 111, no. 2, pp. 74–82, 2007.

[58] J. Eleutério Jr., P. C. Giraldo, A. K. Gonçalves et al., "Prognostic markers of high-grade squamous intraepithelial lesions: the role of p16INK4a and high-risk human papillomavirus," *Acta Obstetricia et Gynecologica Scandinavica*, vol. 86, no. 1, pp. 94–98, 2007.

[59] J. L. Meyer, D. W. Hanlon, B. T. Andersen, O. F. Rasmussen, and K. Bisgaard, "Evaluation of p16INK4a expression in ThinPrep cervical specimens with the CINtec p16INK4a assay: correlation with biopsy follow-up results," *Cancer*, vol. 111, no. 2, pp. 83–92, 2007.

[60] N. Wentzensen, C. Bergeron, F. Cas, S. Vinokurova, and M. von Knebel Doeberitz, "Triage of women with ASCUS and LSIL cytology: use of qualitative assessment of p16INK4a positive cells to identify patients with high-grade cervical intraepithelial neoplasia," *Cancer*, vol. 111, no. 1, pp. 58–66, 2007.

[61] R. Bollmann, M. Bollmann, D. E. Henson, and M. Bodo, "DNA cytometry confirms the utility of the Bethesda System for the classification of Papanicolaou smears," *Cancer*, vol. 93, no. 3, pp. 222–228, 2001.

[62] R. Bollmann, G. Méhes, R. Torka, N. Speich, C. Schmitt, and M. Bollmann, "Human papillomavirus typing and DNA ploidy determination of squamous intraepithelial lesions in liquid-based cytologic samples," *Cancer*, vol. 99, no. 1, pp. 57–62, 2003.

[63] R. Bollmann, G. Méhes, N. Speich, C. Schmitt, and M. Bollmann, "Aberrant, highly hyperdiploid cells in human papillomavirus-positive, abnormal cytologic samples are associated with progressive lesions of the uterine cervix," *Cancer*, vol. 105, no. 2, pp. 96–100, 2005.

[64] H. J. Grote, H. V. Nguyen, A. G. Leick, and A. Böcking, "Identification of progressive cervical epithelial cell abnormalities using DNA image cytometry," *Cancer*, vol. 102, no. 6, pp. 373–379, 2004.

[65] M. Lorenzato, S. Caudroy, J. M. Nou et al., "Contribution of DNA ploidy image cytometry to the management of ASC cervical lesions," *Cancer*, vol. 114, no. 4, pp. 263–269, 2008.

[66] A. D. Santin, F. Zhan, E. Bignotti et al., "Gene expression profiles of primary HPV16- and HPV18-infected early stage cervical cancers and normal cervical epithelium: identification of novel candidate molecular markers for cervical cancer diagnosis and therapy," *Virology*, vol. 331, no. 2, pp. 269–291, 2005.

[67] N. Murphy, M. Ring, C. C. Heffron et al., "p16^{INK4}A, CDC6, and MCM5: predictive biomarkers in cervical preinvasive neoplasia and cervical cancer," *Journal of Clinical Pathology*, vol. 58, no. 5, pp. 525–534, 2005.

[68] D. P. Malinowski, "Molecular diagnostic assays for cervical neoplasia: emerging markers for the detection of high-grade cervical disease," *BioTechniques*, pp. 17–23, 2005.

[69] M. T. Siddiqui, K. Hornaman, C. Cohen, and A. Nassar, "ProEx C immunocytochemistry and high-risk human papillomavirus DNA testing in papanicolaou tests with atypical squamous cell (ASC-US) cytology: correlation study with histologic biopsy," *Archives of Pathology and Laboratory Medicine*, vol. 132, no. 10, pp. 1648–1652, 2008.

[70] K. R. Shroyer, P. Homer, D. Heinz, and M. Singh, "Validation of a novel immunocytochemical assay for topoisomerase II-α and minichromosome maintenance protein 2 expression in cervical cytology," *Cancer*, vol. 108, no. 5, pp. 324–330, 2006.

[71] T. N. Oberg, B. R. Kipp, J. A. Vrana et al., "Comparison of p16^{INK4}a and ProEx C immunostaining on cervical ThinPrep® cytology and biopsy specimens," *Diagnostic Cytopathology*, vol. 38, no. 8, pp. 564–572, 2010.

[72] P. Conesa-Zamora, A. Doménech-Peris, F. J. Orantes-Casado et al., "Effect of human papillomavirus on cell cycle-related proteins p16, Ki-67, cyclin D1, p53, and ProEx C in precursor lesions of cervical carcinoma: a tissue microarray study," *American Journal of Clinical Pathology*, vol. 132, no. 3, pp. 378–390, 2009.

[73] S. B. Baylin and J. E. Ohm, "Epigenetic gene silencing in cancer—a mechanism for early oncogenic pathway addiction?" *Nature Reviews Cancer*, vol. 6, no. 2, pp. 107–116, 2006.

[74] S. A. Belinsky, K. J. Nikula, W. A. Palmisano et al., "Aberrant methylation of p16INK4a is an early event in lung cancer and a potential biomarker for early diagnosis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 20, pp. 11891–11896, 1998.

[75] E. Miranda, A. Destro, A. Malesci et al., "Genetic and epigenetic changes in primary metastatic and nonmetastatic colorectal cancer," *British Journal of Cancer*, vol. 95, no. 8, pp. 1101–1107, 2006.

[76] M. Schutte, R. H. Hruban, J. Geradts et al., "Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas," *Cancer Research*, vol. 57, no. 15, pp. 3126–3130, 1997.

[77] H. J. Yang, V. W. Liu, Y. Wang et al., "Detection of hypermethylated genes in tumor and plasma of cervical cancer patients," *Gynecologic Oncology*, vol. 93, no. 2, pp. 435–440, 2004.

[78] S. M. Dong, H. S. Kim, S. H. Rha, and D. Sidransky, "Promoter hypermethylation of multiple genes in carcinoma of the uterine cervix," *Clinical Cancer Research*, vol. 7, no. 7, pp. 1982–1986, 2001.

[79] D. H. Jeong, M. Y. Youm, Y. N. Kim et al., "Promoter methylation of p16, DAPK, CDH1, and TIMP-3 genes in cervical cancer: correlation with clinicopathologic characteristics," *International Journal of Gynecological Cancer*, vol. 16, no. 3, pp. 1234–1240, 2006.

[80] Z. Lin, M. Gao, X. Zhang et al., "The hypermethylation and protein expression of p16INK4A and DNA repair gene O6-methylguanine-DNA methyltransferase in various uterine cervical lesions," *Journal of Cancer Research and Clinical Oncology*, vol. 131, no. 6, pp. 364–370, 2005.

[81] J. S. Lea, R. Coleman, A. Kurien et al., "Aberrant p16 methylation is a biomarker for tobacco exposure in cervical squamous cell carcinogenesis," *American Journal of Obstetrics and Gynecology*, vol. 190, no. 3, pp. 674–679, 2004.

[82] S. Kang, J. W. Kim, G. H. Kang et al., "Comparison of DNA hypermethylation patterns in different types of uterine cancer: cervical squamous cell carcinoma, cervical adenocarcinoma and endometrial adenocarcinoma," *International Journal of Cancer*, vol. 118, no. 9, pp. 2168–2171, 2006.

[83] A. K. Virmani, C. Muller, A. Rathi, S. Zoechbauer-Mueller, M. Mathis, and A. F. Gazdar, "Aberrant methylation during cervical carcinogenesis," *Clinical Cancer Research*, vol. 7, no. 3, pp. 584–589, 2001.

[84] Y. F. Wong, T. K. Chung, T. H. Cheung et al., "Methylation of p16(INK4A) in primary gynecologic malignancy," *Cancer Letters*, vol. 136, no. 2, pp. 231–235, 1999.

[85] K. Nehls, S. Vinokurova, D. Schmidt et al., "p16 methylation does not affect protein expression in cervical carcinogenesis," *European Journal of Cancer*, vol. 44, no. 16, pp. 2496–2505, 2008.

[86] N. Wentzensen, M. E. Sherman, M. Schiffman, and S. S. Wang, "Utility of methylation markers in cervical cancer early detection: appraisal of the state-of-the-science," *Gynecologic Oncology*, vol. 112, no. 2, pp. 293–299, 2009.

[87] X. Wang, B. Zheng, S. Li et al., "Automated detection and analysis of fluorescent in situ hybridization spots depicted in digital microscopic images of Pap-smear specimens," *Journal of Biomedical Optics*, vol. 14, no. 2, Article ID 021002, 2009.

[88] K. Heselmeyer-Haddad, K. Sommerfeld, N. M. White et al., "Genomic amplification of the human telomerase gene (TERC) in Pap smears predicts the development of cervical cancer," *American Journal of Pathology*, vol. 166, no. 4, pp. 1229–1238, 2005.

[89] K. Heselmeyer, E. Schröck, S. du Manoir et al., "Gain of chromosome 3q defines the transition from severe dysplasia to invasive carcinoma of the uterine cervix," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 1, pp. 479–484, 1996.

[90] D. G. Allen, D. J. White, A. M. Hutchins et al., "Progressive genetic aberrations detected by comparative genomic hybridization in squamous cell cervical cancer," *British Journal of Cancer*, vol. 83, no. 12, pp. 1659–1663, 2000.

[91] N. P. Caraway, A. Khanna, M. Dawlett et al., "Gain of the 3q26 region in cervicovaginal liquid-based pap preparations is associated with squamous intraepithelial lesions and squamous cell carcinoma," *Gynecologic Oncology*, vol. 110, no. 1, pp. 37–42, 2008.

[92] A. Seppo, G. R. Jalali, R. Babkowski et al., "Gain of 3q26: a genetic marker in low-grade squamous intraepithelial lesions (LSIL) of the uterine cervix," *Gynecologic Oncology*, vol. 114, no. 1, pp. 80–83, 2009.

[93] E. A. Jarboe, L. C. Thompson, D. Heinz, J. A. McGregor, and K. R. Shroyer, "Telomerase and Human Papillomavirus as Diagnostic Adjuncts for Cervical Dysplasia and Carcinoma," *Human Pathology*, vol. 35, no. 4, pp. 396–402, 2004.