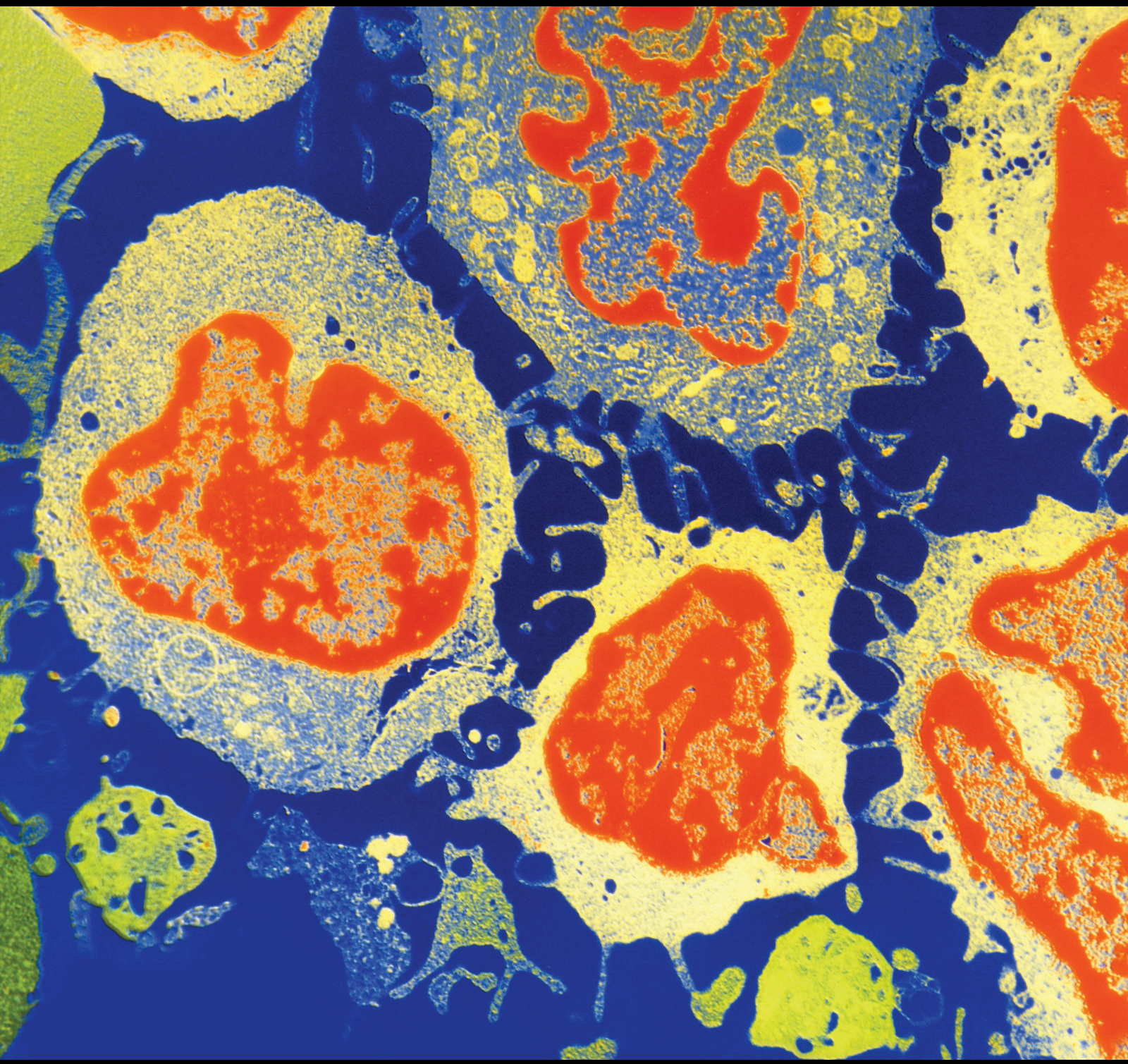
Journal of Oncology

Infection-Associated Cancers

Lead Guest Editor: Hironori Yoshiyama
Guest Editors: Keiji Ueda, Jun Komano, and Hisashi Iizasa



Infection-Associated Cancers

Journal of Oncology

Infection-Associated Cancers

Lead Guest Editor: Hironori Yoshiyama
Guest Editors: Keiji Ueda, Jun Komano, and Hisashi Iizasa

Contents

Hindawi
Journal of Oncology
Volume 2020, Article ID 4979131, 2 pages
https://doi.org/10.1155/2020/4979131

Editorial

Infection-Associated Cancers

Hironori Yoshiyama,[1] **Keiji Ueda**,[2] **Jun Komano**,[3] **and Hisashi Iizasa**[1]

[1]*Department of Microbiology, Shimane University, Izumo, Shimane, Japan*
[2]*Division of Virology, Department of Microbiology and Immunology, Graduate School of Medicine, Osaka University, Osaka, Japan*
[3]*Department of Microbiology, Osaka University of Pharmaceutical Sciences, Takatsuki, Japan*

Correspondence should be addressed to Hironori Yoshiyama; yosiyama@med.shimane-u.ac.jp

Received 18 February 2020; Accepted 18 February 2020; Published 16 March 2020

After the finding by Peyton Rous that filtered extracts from chicken sarcoma generated new sarcoma, numbers of pathogens have been found to be oncogenic. Microorganisms and their metabolites, as well as chronic inflammation, have also been considered to cause cancers. Almost 20% of all cancers worldwide are estimated to be associated with infections. However, we believe cancers caused by infection include many effective preventive measures, which include a potential target for novel cancer diagnostics, therapeutic approaches, and the possibility of prevention by vaccination. In this special issue, we intend to correct papers concerning current knowledge of infection-associated cancers, spanning basic biology, and potential clinical applications. The focus will be on molecular mechanisms to understand infection-attributable cancers, on tumor microenvironment including tumor immune response, on development of novel biomarkers for diagnosis and for predicting prognosis, and on animal models for studying infection-associated cancers. The purpose of this special issue is to present the recent progress in these exciting fields. A brief summary of all accepted papers is provided below.

M. A. Hernández-Luna et al. reviewed the suggested bacterial molecular mechanisms and their possible role in development and progression of gastrointestinal neoplasms, focusing mainly on colon neoplasms, where the bacteria *Fusobacterium nucleatum*, *Escherichia coli*, *Bacteroides fragilis*, and *Salmonella enterica* infect.

The paper by G. Zhang et al. has developed an ELISA assay to detect infection of human beta retrovirus (HBRV). As a result, anti-HBRV antibodies were detected with a significant difference in patients with breast cancer and primary biliary cholangitis compared to controls.

F. I. Bussière et al. showed a novel way for *H. pylori* to promote genome instabilities through the inhibition of TERT levels and telomerase activity in a mice model. The inflammation and ROS-mediated mechanism could play an important role in the early steps of gastric carcinogenesis.

The review by C. K. Chan et al. focused on current situation on human papillomavirus epidemiology in developing countries, where incidence of cervical cancer is high.

The paper by Y. S. Li et al. focused on the multiple functions of deubiquitinating enzymes (DUBs) in RIG-I-like receptors and stimulators of interferon gene-mediated antiviral signaling pathways, oncovirus regulation of NF-κB activation, oncoviral life cycle, and the potential of DUB inhibitors as therapeutic strategies.

A. R. Adams et al. investigated the size of HPV prevalence in female sex workers (FSWs) to provide information for future assessment of the impact of vaccine introduction in Ghana. They found a high HPV prevalence with high risk-HPV genotypes (HPV-16, HPV-35, HPV-33/39/-68, HPV-52/51/59, and HPV-18) among FSWs in the Greater Accra Region and concluded the efficacy of preventable vaccines.

L. S. D. Libera et al. investigated HPV prevalence, genotype distribution, and prognosis aspect in anal cancers in the Midwestern Region of Brazil by a retrospective study. They also reported that gender, histological type, and the presence of distant metastasis were observed as prognostic factors.

F. D. Felice et al. reviewed the evidence-based literature supporting the deintensification strategies in HPV-related oropharyngeal squamous cell carcinoma management, including radiotherapy dose and/or volume reduction, replacement of cisplatin radiosensitising chemotherapy, and the use of transoral surgery. They aimed at raising clinicians in describing the clinical data, the therapeutic implication, and the most promising treatment strategies in HPV-related oropharyngeal cancer scenarios.

Conflicts of Interest

The editors declare that they have no conflicts of interest.

Acknowledgments

We would like to express our gratitude to all authors who made this special issue possible. We hope this collection of articles will be useful to the scientific community.

Hironori Yoshiyama
Keiji Ueda
Jun Komano
Hisashi Iizasa

Hindawi
Journal of Oncology
Volume 2020, Article ID 8958192, 9 pages
https://doi.org/10.1155/2020/8958192

Research Article

Seroprevalence of Human Betaretrovirus Surface Protein Antibodies in Patients with Breast Cancer and Liver Disease

Guangzhi Zhang,[1,2] Kiandokht Bashiri,[1] Mark Kneteman,[1] Kevan Cave,[1] Youngkee Hong,[1] John R. Mackey,[3] Harvey J. Alter,[4] and Andrew L. Mason [1,5]

[1]*Center of Excellence for Gastrointestinal Inflammation and Immunity Research, Division of Gastroenterology, University of Alberta, Edmonton, AB T6G 2E1, Canada*
[2]*National Microbiology Laboratory, Winnipeg, MB R3E 3M4, Canada*
[3]*Department of Medical Oncology, Cross Cancer Institute, University of Alberta, Edmonton, AB, Canada*
[4]*Department of Transfusion Medicine, National Institutes of Health, Bethesda, MD 20892, USA*
[5]*Li Ka Shing Institute of Virology, University of Alberta, Edmonton, AB T6G 2E1, Canada*

Correspondence should be addressed to Andrew L. Mason; andrew.mason@ualberta.ca

Received 30 May 2019; Revised 7 December 2019; Accepted 2 January 2020; Published 27 January 2020

Guest Editor: Hironori Yoshiyama

Mouse mammary tumor virus (MMTV) is a betaretrovirus that plays a causal role in the development of breast cancer and lymphoma in mice. Closely related sequences that share 91–99% nucleotide identity with MMTV have been repeatedly found in humans with neoplastic and inflammatory diseases. Evidence for infection with a betaretrovirus has been found in patients with breast cancer and primary biliary cholangitis and referred to as the human mammary tumor virus and the human betaretrovirus (HBRV), respectively. Using the gold standard technique of demonstrating retroviral infection, HBRV proviral integrations have been detected in cholangiocytes, lymph nodes, and liver of patients with primary biliary cholangitis. However, the scientific biomedical community has not embraced the hypothesis that MMTV like betaretroviruses may infect humans because reports of viral detection have been inconsistent and robust diagnostic assays are lacking. Specifically, prior serological assays using MMTV proteins have produced divergent results in human disease. Accordingly, a partial HBRV surface (Su) construct was transfected into HEK293 to create an ELISA. The secreted HBRV gp52 Su protein was then used to screen for serological responses in patients with breast cancer and liver disease. A greater proportion of breast cancer patients ($n = 98$) were found to have serological reactivity to HBRV Su as compared to age- and sex-matched control subjects (10.2% versus 2.0%, $P = 0.017$, OR = 5.6 [1.25–26.3]). Similarly, the frequency of HBRV Su reactivity was higher in patients with primary biliary cholangitis ($n = 156$) as compared to blood donors (11.5% vs. 3.1%, $P = 0.0024$, OR = 4.09 [1.66–10.1]). While the sensitivity of the HBRV Su ELISA was limited, the assay was highly specific for serologic detection in patients with breast cancer or primary biliary cholangitis, respectively (98.0% [93.1%–99.7%] and 97.0% [93.4%–98.6%]). Additional assays will be required to link immune response to betaretrovirus infection and either breast cancer or primary biliary cholangitis.

1. Introduction

Breast cancer is the most frequent cancer diagnosis among females and a leading cause of cancer deaths worldwide [1, 2]. Several viruses have been linked with the development of human breast cancer, but none have been established as having a causal etiology [3, 4]. One such agent resembles mouse mammary tumor virus (MMTV), a murine betaretrovirus that plays a direct role in the development of breast cancer in mice [5]. Indeed, cloned betaretrovirus nucleotide sequences from humans reportedly share between 91% and 99% identity with various regions of the MMTV genome [6–9]. However, diagnostic assays are lacking to reproducibly detect betaretrovirus infection in humans [10].

MMTV does not encode an oncogene but rather activates growth pathways by insertional mutagenesis to

promote carcinogenesis in mice [11]. The diagnosis of MMTV infection in mice can be challenging. The viral burden is below the limits of detection in blood, and the agent is encoded as an endogenous retrovirus in most mice; therefore, exogenous viral genomic nucleic acid sequences cannot easily be distinguished from the endogenous expression of MMTV [12]. Furthermore, inadequate humoral responses are made by weanling pups infected via ingestion of MMTV in milk due to the tolerizing effects of neonatal infection by the oral route [13]. Accordingly, a diagnosis of MMTV infection is made by assessing the skewing of T-cell receptor V-β subsets to demonstrate the MMTV superantigen effect [14].

Evidence for human infection first surfaced in 1971, when B-type particles resembling MMTV were observed by electron microscopy in the milk of breast cancer patients [15]. Breast cancer patients were also reported to harbor betaretrovirus nucleic acid sequences and/or proteins in various samples, including milk [16], serum [17], salivary glands [18], as well as breast cancer tissue [19], cyst fluid [20], and breast cancer cells in culture [21, 22]. Thereafter, betaretrovirus sequences resembling MMTV were PCR-cloned from breast cancer tissues derived from various countries, and the agent was referred to as the human mammary tumor virus [7, 23–27].

In 2003, a human betaretrovirus (HBRV) was characterized in patients with primary biliary cholangitis (PBC; previously known as primary biliary cirrhosis [28]), an inflammatory autoimmune liver disease. The agent was predominantly detected in perihepatic lymph nodes and was shown to promote the expression of mitochondrial autoantigens in cocultivation studies with cholangiocytes, a well-characterized PBC disease-specific phenotype [9, 29]. Evidence of human betaretrovirus proviral integrations was subsequently demonstrated in PBC patients by ligation-mediated PCR and Illumina sequencing, using a bioinformatics pipeline that ensured the exclusion of all sequences potentially related to murine or HERV sequences. More than 2,200 unique HBRV integrations were identified, and the majority of PBC patients were found to have evidence of proviral integrations linked with HBRV RNA production in cholangiocytes [30]. In clinical trials, PBC patients on combination antiretroviral therapy have shown biochemical and histological improvement with therapy [31–34].

The hypothesis that a betaretrovirus may be linked with human breast cancer has gained little traction over the years because of the inconsistency of findings in different reports, a concern for cross-reactivity with human endogenous retroviruses (HERV) and the low level of viral burden [10, 35, 36]. With regard to the potential for a link with betaretrovirus infection and PBC, investigators have either been unable to detect viral infection [37] or to confirm the specificity of HBRV infection in PBC patients [38]. Furthermore, serological studies using MMTV preparations as substrate have been unable to demonstrate specific antibody reactivity to defined MMTV proteins [37, 39]. While HBRV shares between 93% and 97% amino acid identity with the MMTV envelope protein, consistent differences have been observed between HBRV Env compared to MMTV Env that may alter antigenicity [6]. In the present study, we expressed the HBRV gp52 surface (Su) protein in human cells to create an enzyme-linked immunosorbent assay (ELISA). Herein, we report the seroprevalence of anti-HBRV gp52 Su reactivity in patients with breast cancer, patients with liver disease, and healthy subjects.

2. Materials and Methods

2.1. Ethics. The study protocol was approved by the Human Ethics Review Board from the University of Alberta and institutional review boards/ethics committees at each site. The project was conducted in accordance with the Declaration of Helsinki (1964).

2.2. Patient Samples. A serum panel of breast cancer patients ($n = 98$) and age/sex-matched controls ($n = 102$) was obtained from the Alberta Tomorrow Project, a longitudinal study tracking 55,000 adults in Alberta [40]. Liver disease patient serum was prospectively collected from the hepatology outpatients at the Zeidler Clinic, University of Alberta Hospital from January 2003 to December 2014. Serum from 156 patients with PBC, 46 with primary sclerosing cholangitis (PSC), 16 with autoimmune hepatitis (AIH), 25 with nonalcoholic fatty liver disease (steatosis), 8 with alcoholic liver disease (ALD), 19 with viral hepatitis, 6 with cryptogenic liver disease, and 19 with miscellaneous liver disease. Healthy blood donors' serum samples ($n = 194$) were provided by the Department of Transfusion Medicine, National Institute of Health, Bethesda, MD.

2.3. Recombinant DNA Expression Constructs. The HBRV Su was derived from HBRV sequences obtained from a PBC patients' perihepatic lymph node [6]. The HBRV Su coding sequence was cloned into pcDNA3.1 (Invitrogen) vector along with a TAP tag at the 3′ terminus of the HBRV Su [41] and 4 copies of M-PMV cytoplasmic transport element (CTE) downstream [42]. The expressed HBRV envelope protein sequence corresponds to amino acids 99 to 455 in the surface region that includes the receptor-binding domain, which shares 97% and 98% amino acid identity with MMTV Su [6] (see supplementary material for HBRV Su and MMTV Su alignment; Supplementary Figure 1).

2.4. Cell Culture, Transfection, and Stable Cell Line Generation. HEK293T cells (ATCC) were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco) and 100 μg/ml noromycin. Transfection of HEK293T was performed using PEI as described previously [43]. Briefly, 10^5 cells were seeded in 6-well plates one day before transfection, and 2 μg of each plasmid was used for each well. To generate stable HEK293T cell lines harboring pHBRV Su-TAP-4C FW, the pHBRV Su-TAP-4C FW-puromycin plasmid was linearized with *Pvu*I and transfected into HEK293T cells. Individual clones were selected with puromycin (Invitrogen).

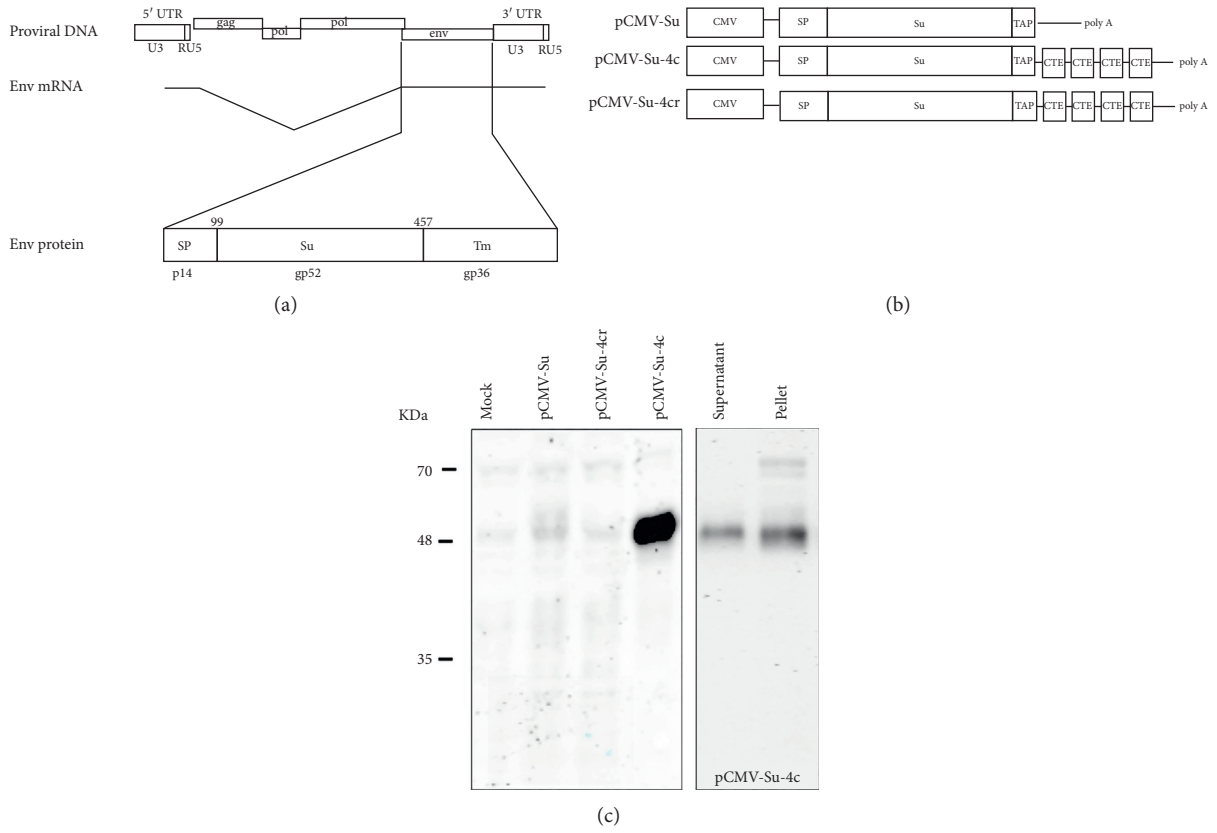


Figure 1: (a) The single spliced mRNA of betaretrovirus Env encodes the signal peptide, surface, and transmembrane proteins. (b) The HBRV Su construct used for mammalian expression contained the cytomegalovirus immediate early promoter, HBRV SP, and Su, a TAP tag; pCMV-Su-4c contained 4 copies of M-PMV CTE inserted in the downstream of Su-TAP in either the sense (pCMV-Su-4c) or the antisense (pCMV-Su-4cr) orientation. (c) Only the pCMV-Su-4c containing the CTE in the correct orientation produced sufficient HBRV Su protein in the cell pellet and supernatant as shown by the western blot analysis.

2.5. Western Blot Analysis. Secreted HBRV Su protein in 400 μl supernatant was precipitated with TCA and dissolved in PBS. Cell lysates were prepared from transfected and stable cells using RIPA buffer with complete proteinase inhibitor (Roche). Approximately 2×10^6 cells were collected and washed twice with ice-cold PBS, incubated with RIPA buffer on ice for 30 min, and centrifuged at 20,000 ×g for 30 minutes. Proteins from cell supernatant and lysate were quantified using the BCA assay (Bio-Rad), and 50 μg and 100 μg of total protein from cell lysate and supernatant, respectively, were resolved by 10% sodium dodecyl sulfate-(SDS-) polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane as previously described (Figure 1) [44].

Western blot analysis was performed using the primary polyclonal goat anti-MMTV envelope gp52 antibody (kindly provided by Dr. Susan Ross, University of Pennsylvania), mouse monoclonal anti-Flag antibody (Sigma-Aldrich), and IRDye goat anti-mouse and rabbit anti-goat secondary antibodies (LI-COR). Reacting membranes were visualized with LI-COR Odyssey infrared imaging system. The anti-MMTV envelope gp52 antibody has demonstrable reactive biliary epithelial cells extracted from a liver transplant recipients with PBC (Supplementary Figure 2), previously shown to have HBRV infection with documented HBRV proviral integrations and HBRV RNA by the QuantiGene assay and *in situ* hybridization [30].

For detection of serological reactivity to HBRV Su, 100 ng of purified protein was resolved on a 10% SDS-PAGE minigel (Bio-Rad) and transferred to nitrocellulose membrane. The membrane was cut into 5 mm wide stripes. Each stripe was incubated with serum from a breast cancer patient or a control (1 : 400 dilution) and IRDye goat anti-human secondary antibody.

2.6. Scale-Up of HBRV Su Production and Purification and Characterization. Stable cells expressing HBRV Su were expanded to 12 × 15 cm cell cultural dishes in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The medium in each plate was replaced with 25 ml Pro293™ CD serum-free medium (Lonza) when cells reached 95% confluence. The medium was collected after 5-6 days of incubation and centrifuged at 3,000 g for 20 min. The clarified medium was adjusted to pH 8.0 and filtered through a 0.22 μm filter before purification.

Purification of HBRV Su was performed on 1 ml Histrap FF crude column and buffers as suggested by the supplier (GE Healthcare) using an ÄKTA explorer 100 (Amersham Pharmacia Biotech). The conditioned medium was loaded to

the equilibrated column at the rate of 1 ml/min, and the column was then washed with 20 ml binding buffer and eluted into 10×0.5 ml fractions using elution buffer. The peak elution fraction was combined and changed to proteins storage buffer by ultrafiltration (Millipore, 30 kDa cutoff limit concentrator, 4000 g for 20 min). The final preparation was aliquoted for storage at −80°C for ELISA. The 10 eluted fractions were assessed by western blot analysis using anti-MMTV Env antibody or anti-FLAG antibody and 10% SDS-PAGE gels stained with Coomassie R-250 blue stain (Bio-Rad). The protein concentration was determined by BCA assay (Pierce) using bovine serum albumin (BSA) as a standard.

2.7. HBRV Su ELISA. ELISA was performed at room temperature with all sera in duplicate using high-binding microplates (Greiner, Monroe, USA). Briefly, wells were coated with 100 μl of 2 ng/μl purified HBRV Su in PBS for 18 hours and blocked with 1% BSA in PBS for 3 hours. Serum was incubated at 100 μl/well at a 1 : 400 dilution in PBS with 1% BSA (Sigma) for 1 hour. A serial dilution of polyclonal anti-MMTV Env was included on each plate as a standard and then incubated with 100 μl/well donkey anti-human and donkey anti-goat secondary antibodies (Jackson Immuno-Research Lab) for 1 hour. The plate was washed 3×5 min after each step using PBS with 0.5% Tween. Plates were developed with 100 μl/well tetramethylbenzidine substrate (TMB, Sigma) for 20 min and then stopped with 50 μl/well 2N H_2SO_4. The absorbance at 450 nm and 540 nm (background) was measured with EMAX Plus Microplate Reader (Molecular Devices, USA) and the cutoff level was established using the reactivity of control samples by adding the mean background level to $3 \times$ S.D. Two-tailed Fisher's exact test was used to assess significant differences in frequency between different groups, followed by calculation of the odds ratio (Baptista–Pike methodology) along with sensitivity, specificity, positive predictive value, negative predictive value, and likelihood ratio (Wilson Brown methodology) using Prism 8 software.

3. Results

3.1. HBRV Su Expression in HEK 293T Cells. A mammalian expression system was employed to express the HBRV Su because prior attempts to express multiple constructs expressing HBRV Env protein in bacteria and baculovirus systems were not sufficiently productive. MMTV Env protein is encoded by a single spliced mRNA in mice, which produces a signal peptide (SP p14), surface (Su gp52), and transmembrane domain (TM gp36) (Figure 1(a)); the Su protein is generated by removal of the signal peptide by signal peptidase and cleavage of the transmembrane domain by cellular Furin. Therefore, a mammalian expression vector pCMV Su-Tap was constructed, using the cytomegalovirus immediate early promoter to drive protein expression and a TAP tag to enable protein purification (Figure 1(b)). Using the pCMV-Su-TAP construct, very little HBRV Su protein was detected in lysates from transfected HEK293T cells (Figure 1(c)). Therefore, an M-PMV cytoplasmic transport element (CTE) was incorporated into the construct to increase protein expression [42]. To this end, two additional Su expression constructs were generated with the 4 copies of M-PMV CTE inserted in the downstream of Su-TAP for expression studies. Following expression in HEK293T, increased production of HBRV Su was observed in cell lysates transfected with the pCMV-Su-Tap-4c but not in cells with the pCMV-Su-Tap-4cr construct that had the CTE arranged in the antisense orientation. Moreover, we were able to detect secreted Su protein in the medium of the cells transfected with the pCMV-Su-Tap-4c plasmid two days after transfection (Figure 1(c)).

3.2. Large-Scale Production and Purification of HBRV Su. Since abundant HBRV Su protein was secreted from 293T cells transfected with the pCMV-Su-Tap-4c plasmids, a strategy was developed to purify the protein directly from a large-scale cell culture medium (Figure 2(a)). Stable 293T cell lines were generated following transfection with the pCMV-Su-Tap-4c plasmid and the cells with the highest Su secretion in the culture medium were expanded to 12×15 cm cell culture dishes using DMEM supplemented with 10% FBS. When cells reached 90–95% confluence, the medium was replaced with serum-free medium and incubated for another 5 days before collection. Approximately 300 ml was obtained for each batch, which was then purified with chromatography to derive 150–200 μg HBRV Su protein. SDS-PAGE revealed that the purified Su protein was homogeneous and devoid of other contaminants. Western blot analysis with polyclonal anti-MMTV Env confirmed that the purified protein was HBRV Su along with select serum from seropositive and negative breast cancer and control samples (Figures 2(b) and 2(c)).

3.3. Detection of Anti-HBRV Su Protein Antibodies by ELISA. The ELISA protocol was established using 200 ng/well of purified HBRV Su. The antibody response was calculated by converting the optical density reading to the equivalent ng/ml reactivity of the positive control, polyclonal anti-MMTV Env antibody. The background reactivity was calibrated using the serum samples from the age/sex-matched healthy controls used as a comparison group for the breast cancer patients. The cutoff level (mean background + $3 \times$ S.D.) was calculated as 61 ng/mL and samples found to be greater than this were considered positive (Figure 3). Accordingly, a greater proportion of breast cancer patients (10.2%) were found to have serological reactivity to HBRV Su versus 2.0% of age- and sex-matched control subjects (Figure 3: $P = 0.017$, OR = 5.6 [1.25–26.3]).

The seroprevalence of HBRV Su reactivity in patients with PBC was comparable to that observed in patients with breast cancer (Figure 3: 11.5% vs. 10.2%). The frequency of HBRV Su reactivity was significantly higher in PBC patients vs. blood donors (11.5% vs. 3.1%, $P = 0.0024$, OR = 4.09 [1.66–10.1]). In prior studies using the gold standard methodology of detecting HBRV integrations in patients' cholangiocytes, subjects with cryptogenic liver disease and

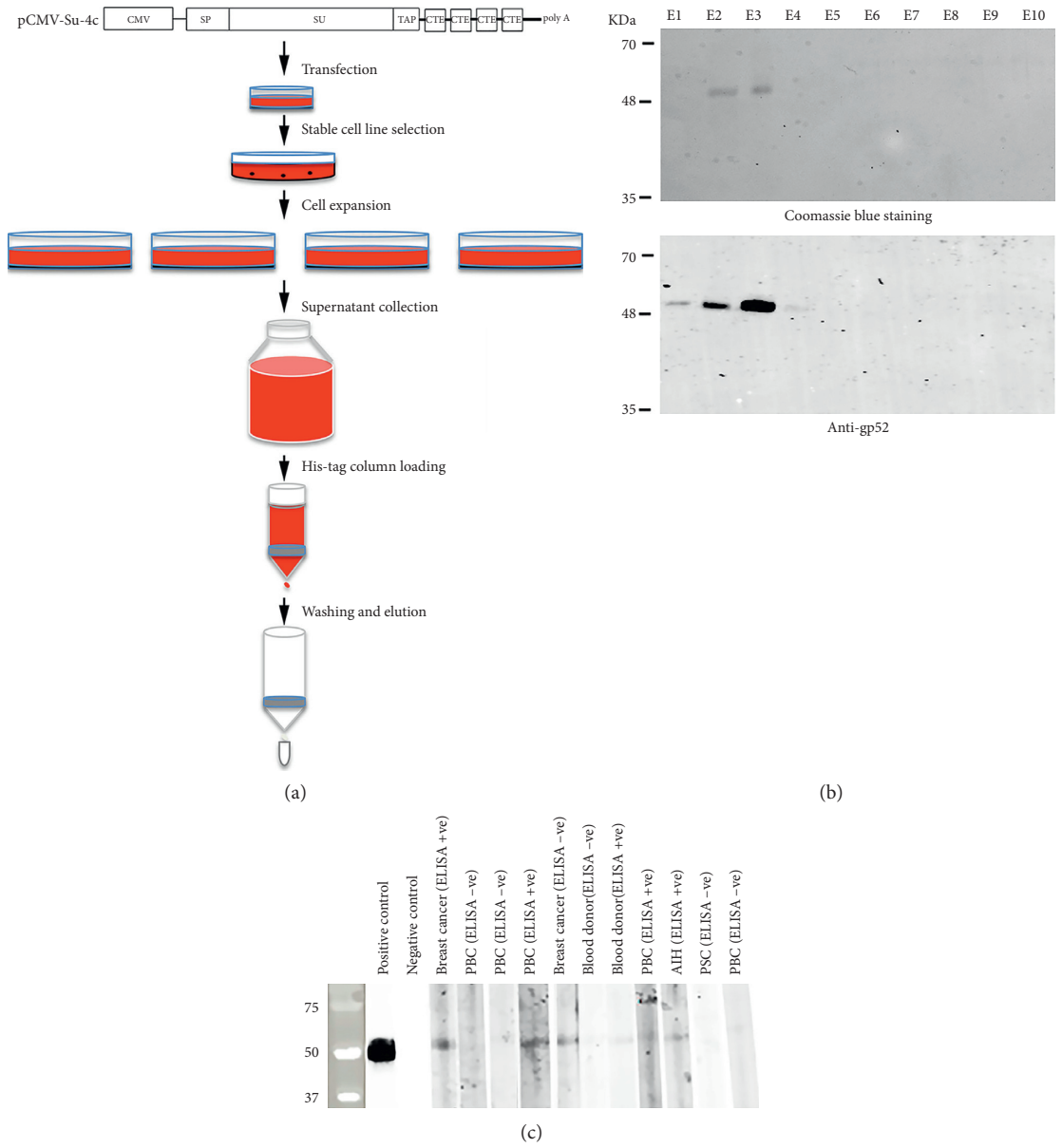


Figure 2: (a) Schematic showing large scale HBRV Su protein purification from the supernatant of HEK293T cells using a His-tag column. (b) Coomassie blue staining and western blot analysis demonstrate the purity of the HBRV gp52 protein using anti-MMTV gp52 Su in sequential elutions. (c) Western blot confirmation of ELISA positive and negative samples demonstrates reactivity using select breast cancer, PBC, and blood donor control samples. The breast cancer serum sample used in lane 7 is positive by western blot and negative by ELISA.

AIH were found to harbor infection, and in this study, isolated reactivity was observed in subjects with cryptogenic liver disease (16.7%) and AIH (6.3%), whereas other subjects with liver disease were universally negative (Figure 3(b)).

While reactivity in healthy blood donors was incrementally higher than the healthy age/sex-matched comparison group for the breast cancer patients, the difference was not found to be significant (3.1% vs. 2.0%; $P = 0.72$). The sensitivity of the HBRV Su ELISA was limited in detecting reactivity in patients with breast cancer and PBC as compared to their respective control groups (10.2% [5.6%–17.8%] and 11.5% [7.4%–17.5%]), whereas the assay was highly specific for serologic detection in patients with breast cancer and PBC, respectively (98.0% [93.1%–99.7%] and 97.0% [93.4%–98.6%]). Accordingly, the positive predictive values (83.3% [55.2%–97.0%] and 75.0% [55.1%–88.0%]) were diagnostically more useful that the negative predictive values (53.2% [46.1%–60.2%] and 57.7% [52.3%–62.9%]) for patients in the breast cancer and the liver disease study groups.

4. Discussion

This is the first report using an HBRV ELISA for assessing the seroprevalence of infection in patients. Approximately 10% of breast cancer and PBC patients had detectable anti-

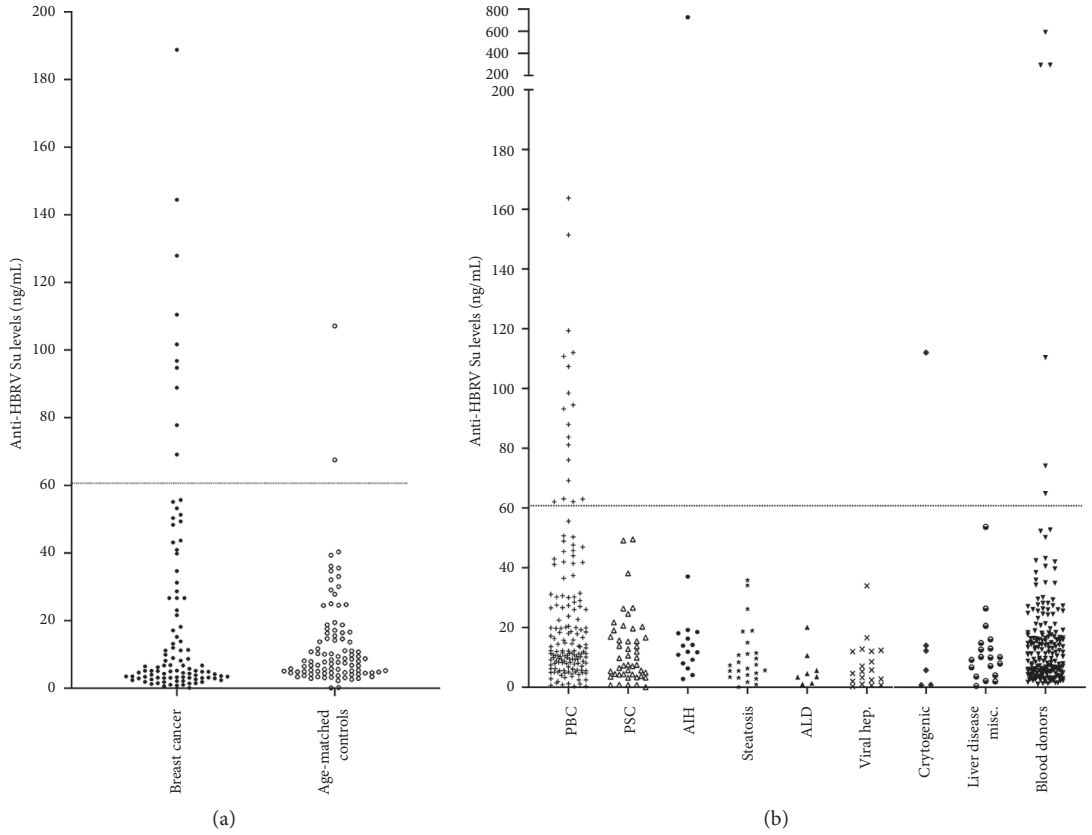


Figure 3: (a) A higher percentage of reactivity to HBRV Su was observed in breast cancer patients' sera versus age/sex-matched healthy controls (10/98 vs. 2/102; $P = 0.017$). (b) Anti-HBRV reactivity was highest in patients with PBC (18/156) and found in AIH (1/16), cryptogenic liver disease (1/6), and healthy blood donors (6/194), whereas reactivity was not observed in patients with PSC, steatosis (NAFLD), ALD, or miscellaneous liver disease (PBC vs. blood donors 11.5% vs. 3.1%, $P = 0.0024$, OR = 4.09 [1.66–10.1]).

HBRV Su, and the test was found to be highly specific for both disorders. The likelihood ratio for having breast cancer with HBRV Su reactivity was 5.2 and for having PBC with HBRV Su reactivity was 3.7; the difference in likelihood ratios probably reflects the chosen control groups for each disorder. Notably, the breast cancer control subjects were mainly middle-aged women and therefore a more suitable control group for the PBC patients, who are also predominantly female; whereas the blood donors were more of an admixture of both sexes. The healthy comparison groups revealed a sizeable population seroprevalence of ~2-3%. These data are in keeping with the hypotheses that HBRV infection may only be disease related in genetically predisposed individuals [10, 45].

Prior seroprevalence studies using MMTV proteins have been widely inconsistent. For example, an ELISA-based study using MMTV proteins demonstrated serological reactivity in 26% of breast cancer patients and 8% of healthy controls [46]. A similar study found no difference between breast cancer patients and their respective controls [47] and a study using 4 strains of MMTV reported only nonspecific reactivity in breast cancer patients, although reactivity consistent with the molecular weights of viral proteins was observed in individual strains of MMTV [39]. In studies of patients with liver disease, MMTV western blot reactivity was attributed to autoreactivity with the antimitochondrial antibody, which is found in up to 95% of patients and used for diagnosing PBC [29], whereas similar MMTV western blot studies employing mitochondrial proteins to remove the autoantibodies from PBC patients' serum demonstrated the presence of signal to the betaretrovirus gp52 surface protein [48]. As the purified antimitochondrial antibody has no reactivity with HBRV Su, we can conclude that humans do make humoral responses to HBRV based on our ELISA.

A second issue to be addressed is that the prevalence of infection detected by the HBRV Su ELISA was somewhat lower than other reports using different techniques to diagnose disease. Indeed, our western blots (Figure 2(c)) show reactivity to one breast cancer sample that was negative by the ELISA, suggesting that our cutoff level may have been too stringent. Using nonserological techniques, a meta-analysis of molecular epidemiological studies reported a prevalence of 40% HBRV infection in Western countries based on PCR detection of betaretrovirus sequences in breast cancer samples [49]. An even higher prevalence of infection has been reported in PBC patients based on the presence of proviral HBRV integrations detected by ligation-mediated PCR and Illumina sequencing, with provirus found in 58% of cholangiocytes from patients with PBC as compared to 7% of nonautoimmune liver disease controls [30]. The

discrepancy of a higher frequency of viral infection in tissue as compared to a lower seroprevalence of anti-HBRV Su reactivity may be partly explained by observations from neonatal mouse infection. Weanling pups have a high risk of developing breast cancer from MMTV infection because they become immunotolerant to viral infection. This occurs because MMTV is taken up in the gut-associated lymphoid tissue along with bacterial lipopolysaccharide, which triggers a cascade of events. The lipopolysaccharide/viral complex engages Toll-like receptor 4 that in turn triggers an IL-4- and IL-6-dependent production of IL-10, which renders the mouse unresponsive to MMTV Su and prevents the formation of neutralizing antibodies [13]. It is currently unknown whether a similar immunological process may occur in humans with HBRV infection. Notably, the cellular immune response to HBRV peptides is more prevalent in patients with liver disease [50].

Our overall goal was to derive a reliable and reproducible diagnostic ELISA to investigate the frequency of HBRV infection. In prior experiments, we used bacterial or baculovirus expressed proteins but failed to generate sufficient amounts of pure viral protein. We also generated serological data using the bacterially expressed Gag proteins, and while a higher seroprevalence was observed in our PBC population as a whole, no significant differences were found between patients and controls with liver disease. Notably, cross reactivity with retroviral Gag (Group Anti-Gen) is a common occurrence in patients with any viral infection due to the positively charged antigenic determinants in capsid and core proteins surrounding the viral genome [51, 52]. For this ELISA, a novel strategy for large-scale production of purified and secreted HBRV Su protein was developed using HEK 293T cells. Three factors contributed to the production of HBRV Su sufficient for multiple ELISAs: these included (i) using multiple copies of CTE downstream of the Su coding region to enhance HBRV Su expression and secretion; (ii) ensuring the stable expression of HBRV Su protein in human cells; and (iii) replacing the FBS containing medium with serum-free medium to remove a source of protein contamination and ensure the high purity of protein after chromatography purification. We can also speculate that the use of HBRV rather than MMTV proteins to assess the betaretrovirus seroprevalence likely improved the accuracy of the assay. Nevertheless, more sensitive assays employing cellular immune responses to viral peptides [50], for example, will be required to improve the sensitivity for the detection of immune response to HBRV.

5. Conclusions

An HBRV ELISA has been constructed by expressing *HBRV env* in HEK293 to produce purified HBRV Su protein. The ELISA detection of HBRV Su antibodies is highly specific for both breast cancer and PBC, but the assay may lack sensitivity as higher prevalence rates for HBRV infection have been recorded using other techniques. Further studies may permit testing whether anti-HBRV is linked with breast cancer, by screening archived predisease serum from patients participating in the Alberta Tomorrow Project who subsequently developed breast cancer. Accordingly, we will be able to study whether anti-HBRV Su predates the development of disease and may act as a biomarker for breast cancer.

Data Availability

The data used to support the findings of this study are included within the supplementary information files and available on request from the corresponding author.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

AM designed and coordinated the study with JRM and HJA. KB, GZ, and AM interpreted data and wrote the article. GZ, MK, KC, and YH performed the majority of the experiments. GZ generated the constructs. All authors read and approved the final manuscript.

Acknowledgments

We thank the Alberta Cancer Foundation, Alberta Innovates Health Solutions, Canadian Institutes for Health Research, and Canadian Liver Foundation for supporting this project as well as Michael Houghton and Michael Sakalian for their assistance.

Supplementary Materials

HBRV Su expression construct and coding sequences; alignment of HBRV Su and MMTV Su proteins as well as anti-MMTV gp52 Su reactivity to biliary epithelial cells cultured from a PBC patient's resected liver following liver transplantation. (*Supplementary Materials*)

References

[1] A. Jemal, F. Bray, M. M. Center, J. Ferlay, E. Ward, and D. Forman, "Global cancer statistics," *CA: A Cancer Journal for Clinicians*, vol. 61, no. 2, pp. 69–90, 2011.

[2] F. Bray, J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, and A. Jemal, "Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries," *CA: A Cancer Journal for Clinicians*, vol. 68, no. 6, pp. 394–424, 2018.

[3] J. S. Lawson, C. Mazzanti, P. Civita et al., "Association of mouse mammary tumor virus with human breast cancer: histology, immunohistochemistry and polymerase chain reaction analyses," *Frontiers in Oncology*, vol. 8, p. 141, 2018.

[4] J. S. Lawson and W. K. Glenn, "Multiple oncogenic viruses are present in human breast tissues before development of virus associated breast cancer," *Infectious Agents and Cancer*, vol. 12, no. 1, p. 55, 2017.

[5] K. Sliva, O. Erlwein, A. Bittner, and B. S. Schnierle, "Murine leukemia virus (MLV) replication monitored with fluorescent proteins," *Virology Journal*, vol. 1, no. 1, Article ID 14, 2004.

[6] L. Xu, M. Sakalian, Z. Shen, G. Loss, J. Neuberger, and A. Mason, "Cloning the human betaretrovirus proviral genome from patients with primary biliary cirrhosis," *Hepatology*, vol. 39, no. 1, pp. 151–156, 2004.

[7] Y Wang, J. F. Holland, I. J. Bleiweiss et al., "Detection of mammary tumor virus env gene-like sequences in human breast cancer," *Cancer Research*, vol. 55, no. 22, pp. 5173–5179, 1995.

[8] Y. Wang, I. Pelisson, S. M. Melana, J. F. Holland, and B. G. Pogo, "Detection of MMTV-like LTR and LTR-env gene sequences in human breast cancer," *International Journal of Oncology*, vol. 18, no. 5, pp. 1041–1044, 2001.

[9] L. Xu, Z. Shen, L. Guo et al., "Does a betaretrovirus infection trigger primary biliary cirrhosis?," *Proceedings of the National Academy of Sciences*, vol. 100, no. 14, pp. 8454–8459, 2003.

[10] A. L. Mason, S. Y. Gilady, and J. R. Mackey, "Mouse mammary tumor virus in human breast cancer," *The American Journal of Pathology*, vol. 179, no. 4, pp. 1588–1590, 2011.

[11] J. C. Cohen, P. R. Shank, V. L. Morris, R. Cardiff, and H. E. Varmus, "Integration of the DNA of mouse mammary tumor virus in virus-infected normal and neoplastic tissue of the mouse," *Cell*, vol. 16, no. 2, pp. 333–345, 1979.

[12] J. P. Dudley, T. V. Golovkina, and S. R. Ross, "Lessons learned from mouse mammary tumor virus in animal models," *ILAR Journal*, vol. 57, no. 1, pp. 12–23, 2016.

[13] M. Kane, L. K. Case, K. Kopaskie et al., "Successful transmission of a retrovirus depends on the commensal microbiota," *Science*, vol. 334, no. 6053, pp. 245–249, 2011.

[14] H. Acha-Orbea and H. R. MacDonald, "Superantigens of mouse mammary tumor virus," *Annual Review of Immunology*, vol. 13, no. 1, pp. 459–486, 1995.

[15] D. H. Moore, J. Charney, B. Kramarsky et al., "Search for a human breast cancer virus," *Nature*, vol. 229, no. 5287, pp. 611–615, 1971.

[16] S. Zotter, C. Kemmer, A. Lossnitzer, H. Grossmann, and B. A. Johannsen, "Mouse mammary tumour virus-related antigens in core-like density fractions from large samples of women's milk," *European Journal of Cancer (1965)*, vol. 16, no. 4, pp. 455–467, 1980.

[17] N. K. Day, S. S. Witkin, N. H. Sarkar et al., "Antibodies reactive with murine mammary tumor virus in sera of patients with breast cancer: geographic and family studies," *Proceedings of the National Academy of Sciences*, vol. 78, no. 4, pp. 2483–2487, 1981.

[18] C. M. Mazzanti, F. Lessi, I. Armogida et al., "Human saliva as route of inter-human infection for mouse mammary tumor virus," *Oncotarget*, vol. 6, no. 21, pp. 18355–18363, 2015.

[19] P. H. Levine, R. Mesa-Tejada, I. Keydar, F. Tabbane, S. Spiefelman, and N. Mourali, "Increased incidence of mouse mammary tumor virus-related antigen in Tunisian patients with breast cancer," *International Journal of Cancer*, vol. 33, no. 3, pp. 305–308, 1984.

[20] S. S. Witkin, N. H. Sarkar, D. W. Kinne, C. N. Breed, R. A. Good, and N. K. Day, "Antigens and antibodies cross-reactive to the murine mammary tumor virus in human breast cyst fluids," *Journal of Clinical Investigation*, vol. 67, no. 1, pp. 216–222, 1981.

[21] S. V. Litvinov and T. V. Golovkina, "Expression of proteins immunologically related to murine mammary tumour virus (MMTV) core proteins in the cells of breast cancer continuous lines MCF-7, T47D, MDA-231 and cells from human milk," *Acta virologica*, vol. 33, no. 2, pp. 137–142, 1989.

[22] I. Keydar, T. Ohno, R. Nayak et al., "Properties of retrovirus-like particles produced by a human breast carcinoma cell line: immunological relationship with mouse mammary tumor virus proteins," *Proceedings of the National Academy of Sciences*, vol. 81, no. 13, pp. 4188–4192, 1984.

[23] F. Zammarchi, M. Pistello, A. Piersigilli et al., "MMTV-like sequences in human breast cancer: a fluorescent PCR/laser microdissection approach," *The Journal of Pathology*, vol. 209, no. 4, pp. 436–444, 2006.

[24] R. Al Dossary, K. R. Alkharsah, and H. Kussaibi, "Prevalence of mouse mammary tumor virus (MMTV)-like sequences in human breast cancer tissues and adjacent normal breast tissues in Saudi Arabia," *BMC Cancer*, vol. 18, no. 1, p. 170, 2018.

[25] M. Hachana, M. Trimeche, S. Ziadi et al., "Prevalence and characteristics of the MMTV-like associated breast carcinomas in Tunisia," *Cancer Letters*, vol. 271, no. 2, pp. 222–230, 2008.

[26] W. Naushad, T. bin Rahat, M. K. Gomez, M. T. Ashiq, M. Younas, and H. Sadia, "Detection and identification of mouse mammary tumor virus-like DNA sequences in blood and breast tissues of breast cancer patients," *Tumor Biology*, vol. 35, no. 8, pp. 8077–8086, 2014.

[27] S. M. Melana, M. A. Picconi, C. Rossi et al., "Detection of murine mammary tumor virus (MMTV) env gene-like sequences in breast cancer from Argentine patients," *Medicina (B Aires)*, vol. 62, no. 4, pp. 323–327, 2002.

[28] A. C. Cheung, A. Montano-Loza, M. Swain et al., "Time to make the change from "primary biliary cirrhosis" to "primary biliary cholangitis"," *Canadian Journal of Gastroenterology and Hepatology*, vol. 29, no. 6, p. 293, 2015.

[29] A. L. Mason, "Is PBC a viral infectious disease?" *Best Practice & Research Clinical Gastroenterology*, vol. 34-35, pp. 27–39, 2018.

[30] W. Wang, S. Indik, S. T. Wasilenko et al., "Frequent proviral integration of the human betaretrovirus in biliary epithelium of patients with autoimmune and idiopathic liver disease," *Alimentary Pharmacology & Therapeutics*, vol. 41, no. 4, pp. 393–405, 2015.

[31] E. Lytvyak, I. Hosamani, A. J. Montano-Loza, L. Saxinger, and A. L. Mason, "Randomized controlled trial: combination antiretroviral therapy with tenofovir-emtricitabine and lopinavir-ritonavir in patients with primary biliary cholangitis," *Canadian Liver Journal*, vol. 2, no. 1, pp. 31–44, 2019.

[32] E. Lytvyak, A. Montano-Loza, L. Saxinger, and A. Mason, "Combination anti-retroviral therapy provides reduction in human betaretrovirus load and durable biochemical responses in patients with primary biliary cirrhosis," *Hepatology*, vol. 62, p. 528A, 2015.

[33] E. Lytvyak, A. J. Montano-Loza, and A. L. Mason, "Combination antiretroviral studies for patients with primary biliary cirrhosis," *World Journal of Gastroenterology*, vol. 22, no. 1, pp. 349–360, 2016.

[34] A. L. Mason, K. D. Lindor, B. R. Bacon, C. Vincent, J. M. Neuberger, and S. T. Wasilenko, "Clinical trial: randomized controlled study of zidovudine and lamivudine for patients with primary biliary cirrhosis stabilized on ursodiol," *Alimentary Pharmacology & Therapeutics*, vol. 28, no. 7, pp. 886–894, 2008.

[35] H. Fukuoka, M. Moriuchi, H. Yano, T. Nagayasu, and H. Moriuchi, "No association of mouse mammary tumor virus-related retrovirus with Japanese cases of breast cancer," *Journal of Medical Virology*, vol. 80, no. 8, pp. 1447–1451, 2008.

[36] M. Motamedifar, M. Saki, and A. Ghaderi, "Lack of association of mouse mammary tumor virus-like sequences in Iranian breast cancer patients," *Medical Principles and Practice*, vol. 21, no. 3, pp. 244–248, 2012.

[37] C. Selmi, S. R. Ross, A. A. Ansari et al., "Lack of immunological or molecular evidence for a role of mouse mammary tumor retrovirus in primary biliary cirrhosis," *Gastroenterology*, vol. 127, no. 2, pp. 493–501, 2004.
[38] H. Johal, G. Marie Scott, R. Jones, C. Camaris, S. Riordan, and W. D. Rawlinson, "Mouse mammary tumour virus-like virus (MMTV-LV) is present within the liver in a wide range of hepatic disorders and unrelated to nuclear p53 expression or hepatocarcinogenesis," *Journal of Hepatology*, vol. 50, no. 3, pp. 548–554, 2009.
[39] J. J. Goedert, C. S. Rabkin, and S. R. Ross, "Prevalence of serologic reactivity against four strains of mouse mammary tumour virus among US women with breast cancer," *British Journal of Cancer*, vol. 94, no. 4, pp. 548–551, 2006.
[40] M. J. Borugian, P. Robson, I. Fortier et al., "The Canadian partnership for tomorrow project: building a pan-Canadian research platform for disease prevention," *Canadian Medical Association Journal*, vol. 182, no. 11, pp. 1197–1201, 2010.
[41] O. Puig, F. Caspary, G. Rigaut et al., "The tandem affinity purification (TAP) method: a general procedure of protein complex purification," *Methods*, vol. 24, no. 3, pp. 218–229, 2001.
[42] H. Wodrich, A. Schambach, and H. G. Krausslich, "Multiple copies of the Mason-Pfizer monkey virus constitutive RNA transport element lead to enhanced HIV-1 gag expression in a context-dependent manner," *Nucleic Acids Research*, vol. 28, no. 4, pp. 901–910, 2000.
[43] G. Zhang, D. Sharon, J. Jovel et al., "Pericentriolar targeting of the mouse mammary tumor virus GAG protein," *PLoS One*, vol. 10, no. 6, Article ID e0131515, 2015.
[44] H. Sanfaçon and G. Zhang, "Analysis of interactions between viral replicase proteins and plant intracellular membranes," *Plant Virology Protocols*, vol. 451, pp. 361–375, 2008.
[45] G. M. Hirschfield, R. W. Chapman, T. H. Karlsen, F. Lammert, K. N. Lazaridis, and A. L. Mason, "The genetics of complex cholestatic disorders," *Gastroenterology*, vol. 144, no. 7, pp. 1357–1374, 2013.
[46] S. S. Witkin, N. H. Sarkar, R. A. Good, and N. K. Day, "An enzyme-linked immunoassay for the detection of antibodies to the mouse mammary tumor virus: application to human breast cancer," *Journal of Immunological Methods*, vol. 32, no. 1, pp. 85–91, 1980.
[47] A. S. Dion, A. J. Girardi, C. C. Williams, A. A. Pomenti, and E. S. Redfield, "Responses of serum from breast cancer patients to murine mammary tumor virus: fact or artifact?" *Journal of the National Cancer Institute*, vol. 79, no. 2, pp. 207–211, 1987.
[48] A. Mason, L. Xu, Z. Shen et al., "Patients with primary biliary cirrhosis make anti-viral and anti-mitochondrial antibodies to mouse mammary tumor virus," *Gastroenterology*, vol. 127, no. 6, pp. 1863-1864, 2004.
[49] F. Wang, J. Hou, Q. Shen et al., "Mouse mammary tumor virus-like virus infection and the risk of human breast cancer: a meta-analysis," *American Journal of Translational Research*, vol. 6, no. 3, pp. 248–266, 2014.
[50] M. Rahbari, "Virological footprint of T-Cell responses in patients with primary biliary cirrhosis," *Canadian Journal of Gastroenterology and Hepatology*, vol. 29, p. 159A, 2015.
[51] A. L. Mason, L. Xu, L. Guo et al., "Detection of retroviral antibodies in primary biliary cirrhosis and other idiopathic biliary disorders," *The Lancet*, vol. 351, no. 9116, pp. 1620–1624, 1998.
[52] S. Karlin and V. Brendel, "Charge configurations in viral proteins," *Proceedings of the National Academy of Sciences*, vol. 85, no. 24, pp. 9396–9400, 1988.

Hindawi
Journal of Oncology
Volume 2019, Article ID 5415761, 13 pages
https://doi.org/10.1155/2019/5415761

Research Article

DNA Hypermethylation Downregulates Telomerase Reverse Transcriptase (TERT) during *H. pylori*-Induced Chronic Inflammation

Françoise I. Bussière,[1] Valérie Michel,[1] Julien Fernandes,[1] Lionel Costa,[1] Vania Camilo,[1] Giulia Nigro,[2] Hilde De Reuse,[1] Laurence Fiette,[3] and Eliette Touati[1]

[1]*Institut Pasteur, Unit of Helicobacter Pathogenesis, CNRS ERL6002, 25-28 Rue du Dr Roux, 75724 Paris cedex 15, France*
[2]*Institut Pasteur, Unit of Molecular Microbial Pathogenesis, INSERM U1202, 25-28 Rue du Dr Roux, 75724 Paris cedex 15, France*
[3]*Institut Pasteur, Unit of Human Histopathology and Animal Model, 25-28 Rue du Dr Roux, 75724 Paris cedex 15, France*

Correspondence should be addressed to Eliette Touati; eliette.touati@pasteur.fr

Received 27 May 2019; Revised 29 August 2019; Accepted 27 September 2019; Published 31 December 2019

Guest Editor: Hisashi Iizasa

Helicobacter pylori infection causes chronic gastritis and is the major risk factor of gastric cancer. *H. pylori* induces a chronic inflammation-producing reactive oxygen species (ROS) which is a source of chromosome instabilities and contributes to the development of malignancy. *H. pylori* also promotes DNA hypermethylation, known to dysregulate essential genes that maintain genetic stability. The maintenance of telomere length by telomerase is essential for chromosome integrity. Telomerase reverse transcriptase (TERT) is the catalytic component of telomerase activity and an important target during host-pathogen interaction. We aimed to investigate the consequences of *H. pylori* on the regulation of *TERT* gene expression and telomerase activity. *In vitro*, *hTERT* mRNA levels and telomerase activity were analysed in *H. pylori*-infected human gastric epithelial cells. In addition, C57BL/6 and INS-GAS mice were used to investigate the influence of *H. pylori*-induced inflammation on TERT levels. Our data demonstrated that, *in vitro*, *H. pylori* inhibits *TERT* gene expression and decreases the telomerase activity. The exposure of cells to lycopene, an antioxidant compound, restores TERT levels in infected cells, indicating that ROS are implicated in this downregulation. *In vivo*, fewer TERT-positive cells are observed in gastric tissues of infected mice compared to uninfected, more predominantly in the vicinity of large aggregates of lymphocytes, suggesting an inflammation-mediated regulation. Furthermore, *H. pylori* appears to downregulate *TERT* gene expression through DNA hypermethylation as shown by the restoration of *TERT* transcript levels in cells treated with 5′-azacytidine, an inhibitor of DNA methylation. This was confirmed in infected mice, by PCR-methylation assay of the *TERT* gene promoter. Our data unraveled a novel way for *H. pylori* to promote genome instabilities through the inhibition of TERT levels and telomerase activity. This mechanism could play an important role in the early steps of gastric carcinogenesis.

1. Introduction

Helicobacter pylori is a gastric pathogen that infects half of the human population worldwide. This bacterium is responsible for chronic inflammation and gastroduodenal diseases, including gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma [1, 2]. *H. pylori* is, to date, the first and only bacterium identified as a type I carcinogenic agent in humans [3]. The complex interplay between bacterial, host, and environmental factors plays a fundamental role in the development of gastric cancer lesions. Prolonged inflammation and long-term persistence of *H. pylori* contribute to gastric carcinogenesis, *via* dysregulation of signaling pathways, cell proliferation, and chromosome instability [4, 5]. *H. pylori* is an efficient inducer of DNA damage such as DNA double-strand breaks

(DSBs) and mutations in the nuclear and mitochondrial DNA [6–9]. The genotoxic activity of *H. pylori* infection is largely associated with chronic inflammation of the gastric mucosa and the resulting oxidative stress, leading to a harmful environment for the host and promotion of carcinogenesis [10]. Oxidative stress is a source of DNA damage and telomere shortening [11]. Recently, a unique *H. pylori*-induced pattern of DNA damage accumulation has been shown preferentially in transcribed regions and in proximal regions of telomeres [12]. *H. pylori* is also a source of aberrant DNA methylation in the host cells [5, 13]. In a previous study, we reported that *H. pylori* inhibits the expression of the transcription factors *USF1* and *USF2* (upstream stimulating factors 1 and 2) genes, by DNA hypermethylation of their promoter region [14]. USF1 and USF2 regulate among others the transcription of *TERT* coding for the telomerase reverse transcriptase (TERT), the major component of telomerase [15, 16].

Telomerase maintains the telomere length essential for chromosome stability and integrity [17]. This ribonucleoprotein is also involved in cell transformation and lymphocyte activation [18]. The telomerase complex includes the reverse transcriptase catalytic subunit (TERT) and a telomerase RNA component (TERC). It elongates telomere ends by adding TTAGGG repeats and prevents telomere shortening during cell division. It is regulated mainly at the TERT transcriptional level [15]. In addition to telomere elongation, hTERT (human TERT) plays a role in diverse cellular processes, such as the transcriptional modulation of Wnt-β-catenin signaling pathway and DNA damage response [19]. Importantly, hTERT is a strategic target for bacterial infection, as previously reported for *Listeria monocytogenes* which promotes hTERT degradation [20].

Telomerase activation is an essential event during the carcinogenesis process, allowing cells to proliferate indefinitely and to avoid apoptosis. In most advanced carcinomas and soft cancer tissues, telomerase expression is upregulated [21]. Increased *hTERT* transcription is observed in more than 85% of tumor cells and is lower in most somatic cells [22]. Slightly elevated levels of *TERT* mRNA and protein were also reported in 45 to 50% of intestinal metaplasia and gastric ulcer cases, and 79% of gastric cancer showed higher TERT levels [23]. In *H. pylori*-positive patients, telomere reduction has been reported in the gastric mucosa [24]. Moreover, reactive oxygen species (ROS) overproduction during *H. pylori*-induced chronic inflammation has been demonstrated as a cause for telomere shortening [25].

In the present study, the consequences of *H. pylori* infection on TERT were investigated *in vitro* in human gastric epithelial cells and in mouse models at an early step of the development of gastric preneoplasia. Our data reveal that *H. pylori* infection downregulates *TERT* gene expression through DNA methylation and thus impairs telomerase activity. Given the role of telomerase in the control of chromosome integrity and epithelial cancer development, these mechanisms could promote the transition between the chronic stage of the infection and the development of neoplasia.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions. *H. pylori* strains B38, isolated from a MALT lymphoma patient [26, 27] (obtained from Pr F. Mégraud, Bordeaux, France), 7.13 and its derivative mutants Δ*cagA* and Δ*cagE* [28] (obtained from Pr RM Peek Jr, Vanderbilt, USA), as well as SS1 [29], a mouse-adapted human strain, were grown on 10% blood agar under microaerobic conditions with the following antibiotics-antifungal cocktail: amphotericin B 2.5 $\mu g \cdot ml^{-1}$, polymyxin B 0.31 $\mu g \cdot ml^{-1}$, trimethoprim 6.25 $\mu g \cdot ml^{-1}$, and vancomycin 12.5 $\mu g \cdot ml^{-1}$. Bacteria lysates were obtained by passage of bacterial suspensions through a French pressure cell as previously described [30]. Protein concentration of supernatants was measured with the DC Protein assay (Biorad, Hercules, CA).

2.2. Cell Culture and Infection. Human adenocarcinoma gastric cell lines AGS (CRl-1739 and ATCC-LGC), MKN45 (CVCL_0434), and KatoIII (CVCL_0371), a gift from Dr C. Figueiredo, Porto, Portugal, were grown in DMEM medium with 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies Corporation, Carlsbad, CA) for AGS and MKN45 cells and with 20% fetal bovine serum for KatoIII cells (Life Technologies Corporation, Carlsbad, CA, USA). Bacteria were added at a multiplicity of infection (MOI) of 20, 50, and 100 bacteria per cell for 12, 24, and 48 h. To inhibit DNA methylation, cells were treated with 5′-azacytidine 1 μM (Sigma Chemical Co., St. Louis, MO) for 3 days, prior to infection for 48 h. To avoid any effect of 5′-azacytidine on bacteria and as similar results were obtained with live bacteria and lysate on *TERT* expression, cells were then treated with *H. pylori* B38 lysate (20 $\mu g \cdot ml^{-1}$), equivalent to 10^8 bacteria for 10^6 epithelial cells. To inhibit ROS production, cells were treated with lycopene 5 μM (Sigma Chemical Co., St. Louis, MO), dissolved in dimethyl sulfoxide (DMSO) 25%, prior to *H. pylori* infection, as previously described [31]. Control cells were incubated with DMSO 2.5% corresponding to the final concentration of DMSO in the vehicle solvent in the culture medium.

For gene expression analysis, total RNA was extracted from cells, as previously described [14]. Proteins were isolated by lysis of cells in NP40 buffer and analysed by Western blot using antibodies against TERT (sc-7212; 1/200; Santa Cruz Biotechnology, CA, USA) and GAPDH (sc-25778; 1/200; Santa Cruz Biotechnology, CA, USA).

2.3. Measurement of Intracellular ROS. The production of ROS was assessed using the ROS-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCF-DA) (Sigma Aldrich) as previously described [32]. In brief, the H_2-DCF-DA probe freely enters the cells where it is cleaved to nonfluorescent and impermeant product, which is later oxidized by ROS to DCF, a fluorescent compound. For these assays, 4×10^4 MKN45 cells were plated in 96-well plates, in quintuplicate. The following day, these cells were treated with 10 μM H_2-DCF-DA for 30 minutes at 37°C and washed 3 times with PBS. Afterwards, cells were exposed for 24 h to

either different concentrations of bacterial extracts ($20\,\mu g{\cdot}ml^{-1}$, $50\,\mu g{\cdot}ml^{-1}$, or $100\,\mu g{\cdot}ml^{-1}$) obtained from the 7.13 *H. pylori* strain or the vehicle control. H_2O_2 (5 mM) was used as a positive control, and WT MKN45 cells were used as a reference for ROS production at basal levels. DCF fluorescence was measured using an excitation/emission wavelength of 488/530 nm with an Infinite M200PRO microplate reader (Tecan).

2.4. Animal Infection

2.4.1. Ethical Statement. Mouse experiments were carried out in strict accordance with the recommendations in the Specific Guide for the Care and Use of Laboratory Animals of the Institut Pasteur, according to the European Directives (2010/63/UE). The project was approved by the Comité d'Éthique en Expérimentation Animale (CETEA), Institut Pasteur and the Ministère de l'Enseignement Supérieur et de la Recherche, France (Ref 00317.02).

Two different mouse models were used in this study. The first model consists of six-week-old specific pathogen-free (SPF) C57BL/6 male mice (Charles Rivers, France), which were orogastrically infected with *H. pylori* SS1 (10^7 cfu/$100\,\mu l$) for 12 and 18 months ($n = 6$/group). Control mice received peptone trypsin broth alone. The second model corresponds to INS-GAS mice, which are transgenic for the human gastrin, leading to an exacerbated development of gastric neoplasia in the presence of *H. pylori,* as early as 7–9 months after infection [33]. Three couples of SPF INS-GAS/FVB mice were kindly provided by Pr. T.C. Wang (Columbia University, NY, USA) and bred at the animal facility of the Institut Pasteur. Six-week-old INS-GAS/FVB male mice ($n = 6$/group) were infected with *H. pylori* SS1 as described above, for 8 months. At each time point, mice were sacrificed and stomachs were collected and used for the quantification of gastric colonization, histological analysis, RNA extraction, and genomic DNA isolation as previously described [6, 14].

2.5. PCR and Real-Time qPCR Analysis. RNA extraction and cDNA synthesis were performed as previously described [14]. Gene expression in human gastric epithelial cells was measured by real-time quantitative PCR (qPCR) analysis using TaqMan® Gene Expression Assays (Applied Biosystems, Thermo Fischer Scientific, France). TaqMan gene expression primers were *hTERT* (Hs99999022_m1) and *18S* (Hs99999901_s1) (Applied Biosystems, Thermo Fischer Scientific, France) as the endogenous control. For mouse analysis, primers were *mTERT* (Mn01352136-m1) and *GAPDH* (Mn99999915-g1) as an endogenous control (Applied Biosystems, Thermo Fischer Scientific, France). Quantitative PCR was performed in triplicate. The expression of hTERT and mTERT was normalized to Ct values obtained for *18S* and GAPDH, respectively, using the ΔCt formula: Ct gene–Ct housekeeping gene. For each experiment, fold changes for *TERT* RNA levels were determined from this calculation for infected samples to the uninfected control $2^{-(\Delta\Delta Ct)}$, for at least two independent biological and three technical replicates.

2.6. Detection of Protein Levels by Western Blot. After co-culture with *H. pylori*, cells were lysed in NP-40 buffer containing protease inhibitors; $20\,\mu g$ per lane were separated on a 12% Mini-PROTEAN® TGX Stain-Free™ Precast Gel (BioRad) and transferred onto Trans-Blot® Turbo™ Midi PVDF Transfer Packs using a Trans-Blot® Turbo™ Transfer System (BioRad). TERT (H-231) antibodies (Ref sc-7212, Santa Cruz Biotechnology, CA, USA; dilution 1/500) and GAPDH ((FL-335) sc-25778, Santa Cruz Biotechnology, CA, USA; 1/100) were used, followed by a goat anti-rabbit IgG-HRP (sc-2054, Santa Cruz Biotechnology, CA, USA; 1/10000). Detection was performed using the Clarity™ Western ECL Substrate (BioRad) and revealed using a ChemiDoc XRS (Bio-Rad). Western blot data were quantified by densitometry using Image Lab software (Bio-Rad).

2.7. Telomerase Repeat Amplification Protocol Assay (TRAP Assay). Telomerase activity was analysed by TRAP assay [34] using a TRAPeze® telomerase detection kit (Chemicon-Millipore, Billerica, MA), according to the manufacturer's instructions. In brief, the telomerase activity in cell extracts (150 ng) was evaluated by its ability to extend the 3′ end of an oligonucleotide substrate with telomeric repeats (GGTTAG). The primary telomerase products were then amplified by PCR, generating a ladder of products with 6 base increments starting at 50 nucleotides length. Reaction products were detected by electrophoresis on 12.5% non-denaturing polyacrylamide gel (PAGE) stained with SYBR® Green followed by UV detection (Gel Doc System, Bio-Rad).

2.8. Histology and Immunohistochemistry. For both mouse models, C57BL/6 and INS-GAS, stomachs from uninfected or *H. pylori* SS1-infected mice were fixed in 4% formalin and then embedded in low-melting point paraffin (Poly Ethylene Glycol Distearate; Sigma, USA). Four μm thick paraffin sections were deparaffinised in absolute ethanol, air-dried, and then stained with hematoxylin-eosin (H&E) or used for immunolabeling. Immunostaining of B and T lymphocytes was performed using anti-CD45R (RM2600, 1/40, Invitrogen, Carlsbad, CA, USA) and anti-CD3 (A0452; 1/75, DAKO, Carpinteria, CA, USA), respectively. *In situ* expression of TERT was visualized by immunodetection with a rabbit polyclonal antibody against telomerase catalytic subunit (Ref 600-401-252; 1 : 200; Rockland Immunochemicals Inc., Gilbertsville, PA, USA). The staining was revealed using peroxidase detection as previously described [6, 14].

2.9. Determination of DNA Methylation Status in the Mouse TERT-Promoter Region. Two distinct regions of the *mTERT*-promoter region were selected between nucleotides −7 to −326 (segment I) and −791 to −1028 (segment II) (see Figure 1(b)). Segment I corresponds to a CpG island region including GC boxes and a noncanonical E-box (−197 to

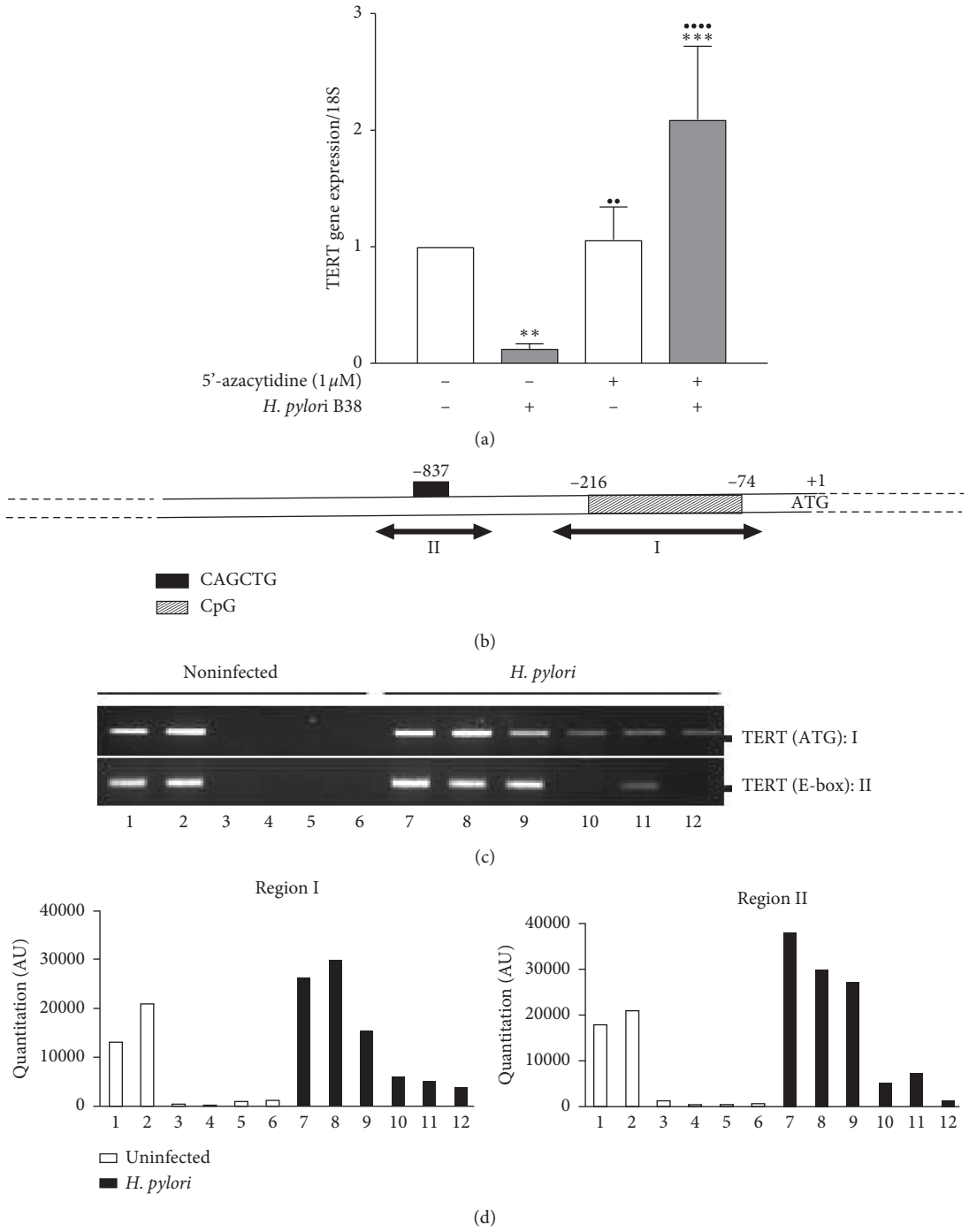


FIGURE 1: DNA methylation downregulates *hTERT* gene expression during *H. pylori* infection. (a) AGS gastric epithelial cells were treated with 5′-azacytidine (1 μM) for 3 days before incubation with *H. pylori* B38 lysate (20 μg·ml^{-1}) for 48 h. Quantification of *hTERT* gene expression was performed by real-time qPCR. 5′-azacytidine treatment restores *hTERT* gene expression in cells stimulated with *H. pylori* B38 lysate. Results are expressed as mean ± SD of at least 2 independent experiments in duplicate. $p < 0.0001$; one-way ANOVA Kruskal–Wallis test followed by Dunn's multiple comparison (infected *versus* uninfected $^{**}p < 0.01$; $^{***}p < 0.001$; infected *versus* azacytidine treated ± infection $^{••}p < 0.01$; $^{••••}p < 0.0001$) (b) Structure of the *mTERT* gene-promoter region in mice, containing a CpG island (hatched box, I and E-box element (black box, II)). (c) DNA methylation status of *mTERT*-promoter regions analysed by promoter methylation PCR assay, on genomic DNA isolated from the gastric mucosa of *H. pylori* SS1-infected (18 months) and uninfected mice, as described in the *Experimental procedures*. A representative gel of amplified methylated DNA is reported (upper panel) with each well corresponding to one mouse. (d) Quantification for each amplified methylated DNA fragment using BIO-PROFIL Bio-1D++ (Biosystems) software (lower panel), showing *H. pylori*-induced DNA hypermethylation in both *mTERT*-promoter regions I and II.

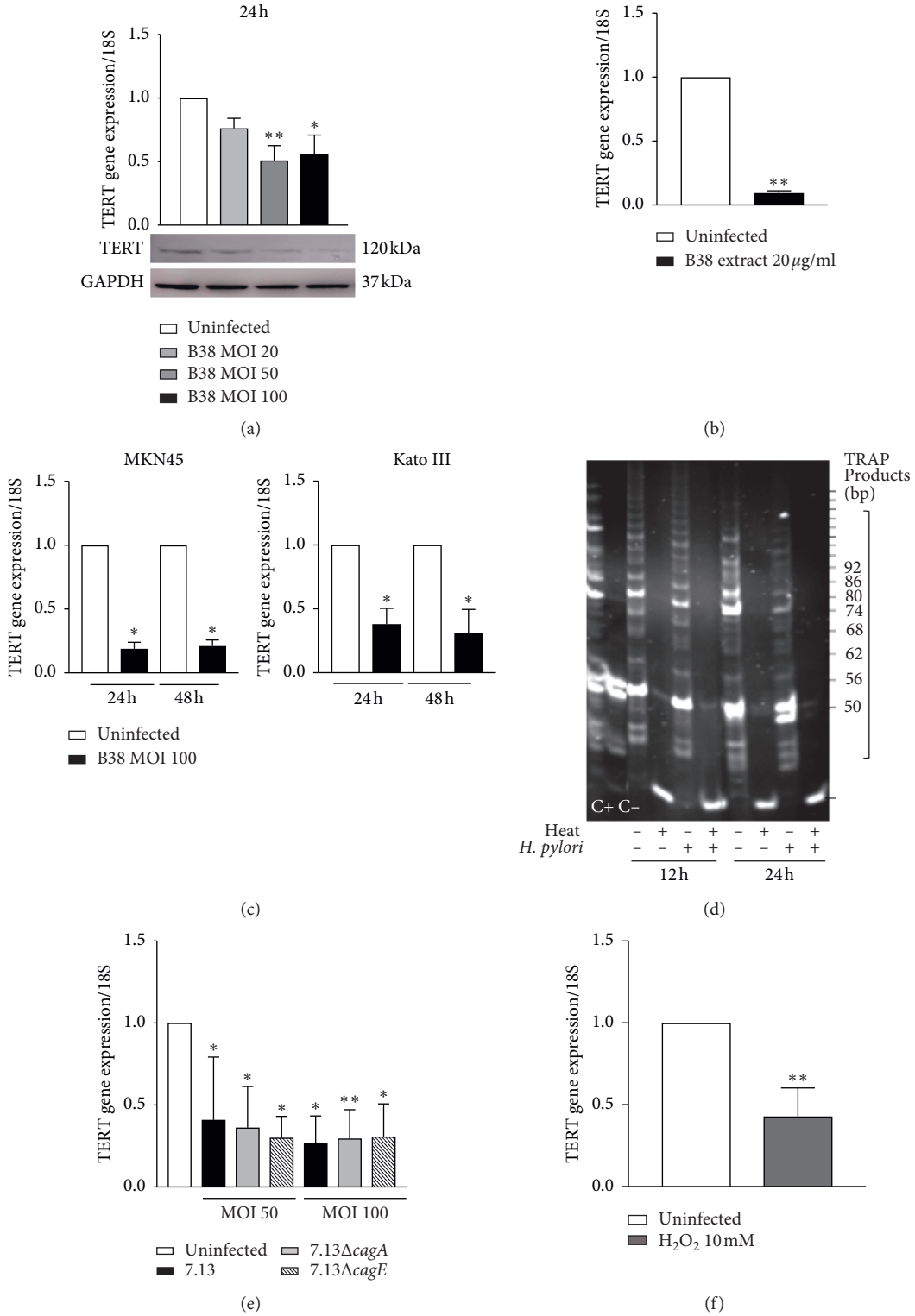
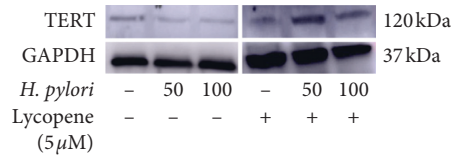


Figure 2: Continued.



(g)

Figure 2: *H. pylori* inhibits *hTERT* gene expression and telomerase activity in gastric epithelial cells. (a) *hTERT* gene expression (upper panel) was measured by real-time qPCR, and protein levels were analysed by western blot (lower panel) in human gastric epithelial cell lines AGS infected with *H. pylori* B38 at MOI 20, 50, and 100 for 24 h. (b) *hTERT* gene expression measured in AGS cells treated with *H. pylori* B38 bacterial extracts ($20\,\mu g{\cdot}ml^{-1}$) for 24 h. (c) MKN45 and KatoIII gastric epithelial cell lines infected with *H. pylori* B38 for 24 h and 48 h at MOI 100. (d) Telomerase activity analysed by TRAPeze® assay in AGS cell extracts prepared from cells infected with *H. pylori* B38 for 12 h and 24 h (MOI 100). C+, positive control using commercial telomerase-positive cell extracts; C−, negative control. For each analysed condition, heat-inactivated cell extracts obtained after incubation at 85°C for 10 min were also analysed. The displayed gel is representative of 2 independent experiments performed in duplicate. (e) *hTERT* gene expression is also inhibited in AGS cells infected by the *H. pylori* strain 7.13 (MOI 50 and 100) in a CagA- and CagE-independent manner. $^{\S}p < 0.001$, one-way ANOVA analysis followed by Dunn's multiple comparison (infected versus uninfected $^{*}p < 0.05$; $^{**}p < 0.01$). (f) Oxidative stress generated by exposure of cells to H_2O_2 10 mM for 24 h inhibits the *hTERT* gene expression. (g) Representative western blot of AGS cells infected 24 h with *H. pylori* 7.13 as in (d) and treated with lycopene 5 μM. Lycopene abolished the *H. pylori*-mediated inhibition of TERT. Results are expressed as mean ± SD of three independent experiments (infected versus uninfected $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). $p < 0.001$, one-way ANOVA Kruskal–Wallis followed by Dunn's multiple comparison (infected *versus* uninfected $^{*}p < 0.05$; $^{**}p < 0.01$) (a and e).

−202) [35]. Segment II presents a canonical E-box sequence at position −837. DNA methylation status was analysed using the Promoter Methylation PCR assay (Panomics, Redwood City, CA). Genomic DNA was extracted from 18-month-infected and uninfected mouse stomachs as previously described [6] and digested with *Ban*II restriction enzyme (New England Biolabs, Ipswich, MA). The methylated DNA was isolated according to the manufacturer's instructions; segments I and II were amplified by PCR using the following primers: 5′-GCCCGAGAAGCATTCTGTAG-3′ and 5′-CACTGAGAGTCCACGACGAA-3′ for the segment I, and 5′-GAAAGCTGAAGGCACCAAAG-3′ and 5′-GATGGCAGCTCTGCTAGGTT-3′ for the segment II (GenBank NG_055506.1). The PCR products were detected by agarose gel electrophoresis (Gel Doc System, Bio-Rad), and the band intensities were quantified by using Quantity One software (Bio-Rad).

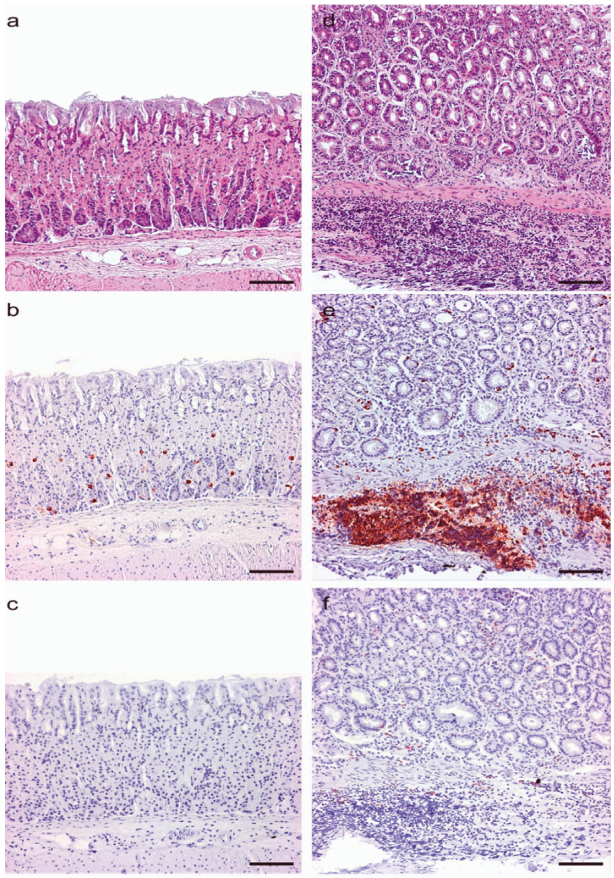
2.10. Statistical Analysis. Statistical analysis was performed using the Student's *t* test or Mann–Whitney test, after being assessed for normality of samples distribution, for comparison between two groups. The one-way ANOVA Kruskal–Wallis test was used for comparison of more than 2 groups, followed by Dunn's multiple comparison to compare the mean rank of each column to the mean rank of the control column. Results were expressed as mean ± SD of separate experiments. A p value ≤0.05 was considered significant using GraphPad Prism® 8 (GraphPad Software Inc., La Jolla, CA, USA).

3. Results

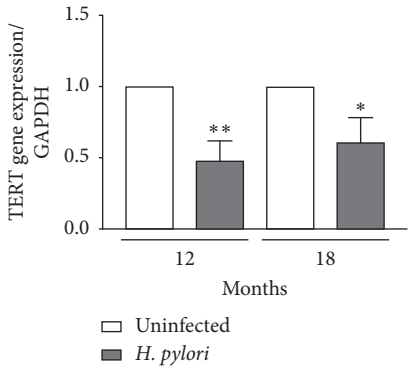
3.1. hTERT Expression and Telomerase Activity Are Downregulated in H. pylori-Infected Gastric Epithelial Cells. Human *TERT* (*hTERT*) gene expression was measured by RT-qPCR in the gastric epithelial cell line AGS, infected for 24°h with *H. pylori* strain B38, a clinical isolate from a MALT lymphoma patient [26, 27]. As compared to controls, *hTERT* mRNA levels were decreased in infected cells after 24°h (Figure 2(a), upper panel). A similar inhibition was observed at the TERT protein level (Figure 2(a), lower panel). The same inhibitory effect on *hTERT* gene expression was seen in cells treated with *H. pylori* B38 bacterial extract ($20\,\mu g{\cdot}ml^{-1}$) for 24°h (Figure 2(b)), suggesting that this downregulation does not require a direct bacterium-epithelial cell interaction and involves one (or more) soluble bacterial factors. The inhibition of *hTERT* gene expression by *H. pylori* was also confirmed in several gastric epithelial cell lines, MKN45 and KatoIII (Figure 2(c)).

Under our experimental conditions of infection, we verified that the decrease of TERT levels was not due to apoptosis. After 24 h and 48°h infection with *H. pylori* strain B38, 77.3% and 70% of the cells were negative for annexin V and 7-aminoactinomycin D staining (live cells), respectively, as compared to 79% and 75.5% in the controls.

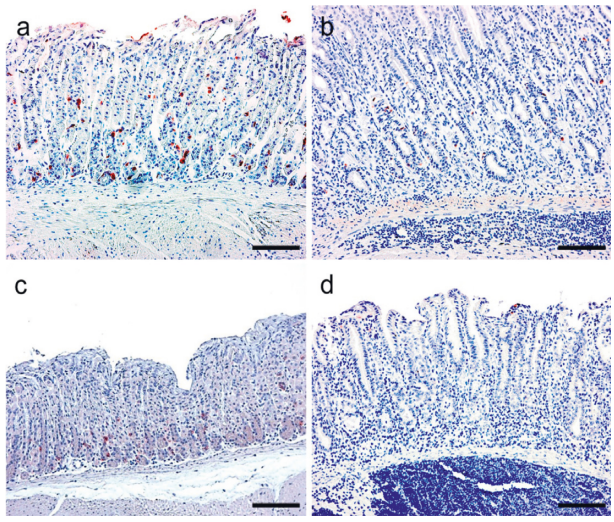
We next examined the consequences of *H. pylori* infection on telomerase activity using the Telomeric Repeat Amplification Protocol (TRAP) assay [34], which allows the ability of the telomerase to add telomeric repeats at the 3′end of an oligonucleotide substrate to be determined. As reported in Figure 2(d), a lower telomerase activity was observed when testing the protein extracts of *H. pylori* B38-infected cells, as indicated by the lower intensity and smaller size of the DNA fragments synthesised by these samples, compared to protein extracts from uninfected cells. This effect is particularly observed at 24°h after infection, compared to the pattern of DNA fragments obtained with heat-inactivated samples and samples from the uninfected condition at the same time point (Figure 2(d)). Thus, *H. pylori* infection inhibits *hTERT* gene expression and telomerase activity. As reported in Figure 2(e), *hTERT* gene expression is also downregulated in AGS cells infected with the oncogenic *H. pylori* strain 7.13 [28] and with the isogenic



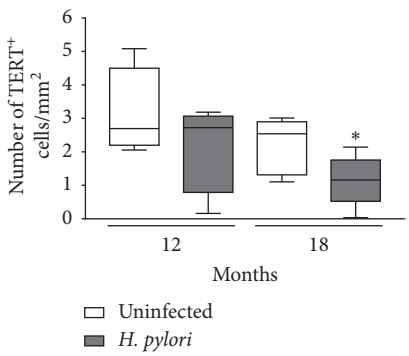
(a)



(b)



(c)



(d)

Figure 3: Continued.

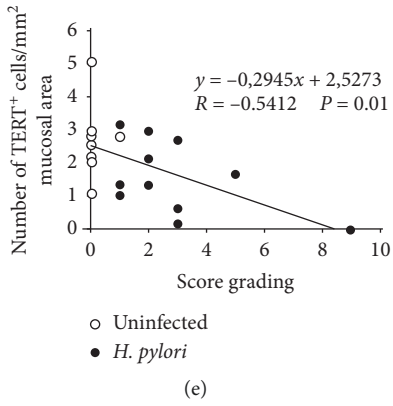


Figure 3: *H. pylori* infection decreases *mTERT* expression in the gastric mucosa of C57BL/6 mice, in the presence of large B lymphocyte aggregates. (a, d) H&E staining and immunostaining of B (b, e) and T (c, f) lymphocytes in gastric sections in infected mice, 12 months after *H. pylori* SS1 infection (d, e, f) and in control mice (a, b, c). Inflammatory infiltrates were observed in the stomach of infected mice, in the lamina propria and submucosa (c). High number of B lymphocytes (e) and a low number of T lymphocytes (f) were present in the inflammatory cell infiltrates in the infected gastric submucosa (e) compared to uninfected (b and c, respectively). Sections of the stomach from the uninfected mice were negative for both B (b) and T (c) lymphocyte staining. Original magnification ×4, bar: 250 μm (a, b, c), and ×10, bar: 100 μm (d, e, f). (b) *mTERT* gene expression in gastric tissues of *H. pylori* SS1-infected mice at 12 and 18 months after infection quantified by real-time qPCR (Taqman). Results are expressed as means ± SD of three independent experiments (infected versus uninfected $^{*}p < 0.05$; $^{**}p < 0.01$). (c) TERT immunolabeling in gastric tissue sections from uninfected mice (a, c) and *H. pylori* SS1-infected (b, d) after 12 (a, b) and 18 (c, d) months. Lower TERT staining is observed in the gastric mucosa in the area of the inflammatory B lymphocyte infiltrates in infected samples. Original magnification: ×10, bar: 100 μm (a, b), and ×4, bar: 250 μm (c, d). (d) Number of TERT-positive cells/mm^2 mucosal area in gastric tissue sections of uninfected and infected samples at 12 and 18 months. Results are expressed as mean ± SD (infected versus uninfected $^{*}p < 0.05$) according to Mann–Whitney analysis. (e) Inverse correlation between the number of TERT-positive cells/mm^2 mucosal area and the total score grading inflammatory lesions in uninfected (white symbols) and infected mice (black symbol), indicating that TERT level decreases with the exacerbation of gastric inflammation. Each symbol represents one mouse.

mutants 7.13 Δ*cagA* and 7.13 Δ*cagE* deficient for the oncogenic protein CagA [36] and the virulence factor CagE required for a functional type IV secretion system, respectively [37]. These results indicate that *H. pylori* downregulates *hTERT* gene expression through a CagA- and CagE-independent mechanism.

The production of ROS has been reported during *H. pylori* infection [10, 38, 39] and is confirmed under our experimental conditions in *H. pylori*-infected gastric epithelial cells (Supplementary materials; Figure S1). Similar to *H. pylori* infection, the exposure of cells to hydrogen peroxide (H_2O_2) led to a significant decrease of *hTERT* gene expression (Figure 2(f)). In addition, the treatment of cells with lycopene, an efficient singlet oxygen quencher [40], previously shown to prevent ROS production in *H. pylori*-infected cells [31], abolished the inhibitory effect of the infection on the TERT protein levels (Figure 2(g)). These data support that the inhibition of hTERT level during the infection could be regulated by an ROS-mediated mechanism.

3.2. Downregulation of TERT Gene Expression in H. Pylori SS1-Infected Mice Is Associated with Chronic Inflammation. We then took advantage of the ability of the *H. pylori* strain SS1 to chronically colonize (i.e., for several months) the gastric mucosa of mice [29], to investigate *mTERT* gene expression in C57BL/6 mice infected for 12 and 18 months. The measure of *H. pylori* gastric colonization loads confirmed that mice were successfully infected (Supplementary materials; Figure S2(a)). Histological analysis of infected stomachs showed active gastritis as indicated by the semiquantitative evaluation of histological score grading of the inflammatory lesions (Supplementary materials; Figure S2(b)), as previously reported under the same conditions of infection [6, 14]. Hyperplastic gastric lesions and more severe metaplasia were observed in mice infected for 18 months (Supplementary materials; Figure S2(b)). Large inflammatory cell aggregates mostly constituted of B lymphocytes, as shown by the antigen B220-positive staining, were observed in the gastric mucosa of mice after 12 and 18 months of infection (Figures 3(a) and 3(e)). In contrast, no T lymphocytes were found in the inflammatory infiltrates (Figures 3(a) and 3(f)).

mTERT gene expression was quantified by RT-qPCR in the gastric tissues of mice. *H. pylori* inhibited *mTERT* gene expression after 12 and 18 months of infection ($p = 0.0028$ and $p = 0.017$, respectively) (Figure 3(b)). Under these conditions, immunohistochemistry analysis of TERT on gastric tissue sections (Figure 3(c)) showed a significantly lower number of TERT-positive gastric cells at 18 months (2.8-fold), compared to uninfected mice (Figure 3(d)). It is important to note the absence of TERT staining in the vicinity of the large aggregates of lymphocytes in the gastric mucosa and submucosa, at both 12 and 18 months of infection (Figure 3(c)). In accordance with this, we found that the number of TERT-positive cells is inversely correlated with the inflammatory score grading (Figure 3(e)), suggesting that lower TERT levels correlate with an exacerbation of inflammatory lesions. The *mTERT* gene expression

was also investigated in INS-GAS transgenic mice. These mice, which develop gastric neoplasia in the presence of *H. pylori* infection [33], are a powerful tool to study the early events of gastric carcinogenesis associated with the infection. As expected, *H. pylori* SS1-infected INS-GAS mice showed more severe lesions than infected C57BL/6 mice, with atypical gastric hyperplasia and high-grade dysplasia after 8 months (Supplementary materials; Figures S3(a) and S3(c)). At 8 months after infection, *mTERT* gene expression was lower in infected INS-GAS mice as compared to uninfected. These data support that the increase in the severity of gastric lesions is inversely correlated with TERT levels.

3.3. Downregulation of mTERT Gene Expression during H. Pylori Infection Is Mediated by DNA Hypermethylation. A potential link between *H. pylori*-related promoter CpG islands methylation and telomere shortening has been suggested in the gastric mucosa of infected patients [41]. In addition, multiple levels of regulation of *hTERT* gene expression have been previously reported by methylation of CpG islands at its promoter region [42]. In order to determine if DNA hypermethylation could be involved in the *H. pylori*-mediated inhibition of the *TERT* gene expression, we first tested the effect of a pretreatment of AGS cells with 5′-azacytidine, an inhibitor of DNA methylation, before incubation with *H. pylori* B38 extracts. Under this condition, *hTERT* gene expression was restored to control levels in cells exposed to bacterial extracts (Figure 1(a)). These data suggest that *H. pylori*-induced DNA hypermethylation is responsible for the downregulation of *hTERT* gene expression during the infection.

Aberrant DNA methylation is frequently associated with chronic inflammation, as observed in gastritis patients [43]. We investigated, in the mouse model, the DNA methylation status at the promoter region of the *mTERT* gene (Figure 1(b)), on genomic DNA samples extracted from the gastric mucosa of uninfected C57BL/6 mice and mice infected for 18 months with *H. pylori*. Using a promoter PCR methylation assay, two regions of the *mTERT* promoter were analysed including the CpG island (I) in the core-promoter region and an upstream segment containing a canonical E-box (II) (Figure 1(b)). In both cases (I and II), higher amounts of DNA-methylated fragments were observed by PCR amplification in infected mice (3-fold), compared to uninfected mice (Figures 1(c) and 1(d)). These data suggest that, in the presence of gastric chronic inflammation and preneoplastic lesions in mice, *H. pylori* induces DNA hypermethylation at the promoter region of the *mTERT* gene, leading to the downregulation of its expression.

4. Discussion

Impaired telomerase activity and shortened telomere length are associated with genetic instability and an increased risk of gastric cancer [44]. Telomerase could constitute an important target during the interaction of *H. pylori* with gastric epithelial cells. In the present study, we demonstrated that *H. pylori* infection leads to inhibition of *TERT* gene expression through DNA hypermethylation and impairment of telomerase activity, in human gastric epithelial cells. The decrease in TERT levels is confirmed in *H. pylori*-infected mice after 12 and 18 months, together with the induction of inflammation and exacerbation of the severity of gastric lesions. These results were also validated in the INS-GAS mouse model that presents *H. pylori*-induced gastric preneoplasia at 8 months after infection. In accordance with our data, previous studies reported that *H. pylori* infection causes telomere shortening [24, 25]. Moreover, *H. pylori* eradication in gastritis patients was shown to result in increased telomere length and telomerase activity [45]. Importantly, a preferential and massive accumulation of DNA damage close to the telomeric regions, associated with the impairment of DNA repair systems, has been reported in *H. pylori*-infected cells and could trigger loss of telomeres [12]. Our data indicate that telomerase dysfunction, resulting in shortened telomere length, can be considered as a key event at the early steps of gastric carcinogenesis during *H. pylori* infection.

Telomerase deficiency and telomere dysfunction have been reported during chronic inflammatory diseases and contribute to inflammation-associated pathogenesis [46, 47] *H. pylori* infection is characterized by an infiltration of polymorphonuclear cells within the gastric mucosa, as observed in infected mice [6]. In previous studies, the pathogenicity of *H. pylori* infection has been shown to be related to chronic inflammation-associated oxidative stress and DNA damage [48]. Both *H. pylori* and inflammatory cells constitute a source of ROS [10]. In the present study, the *TERT* gene expression was found to be decreased in H_2O_2-treated gastric epithelial cells *in vitro*, indicating an ROS-mediated downregulation. In accordance with these results, the treatment of *H. pylori*-infected cells with the antioxidant lycopene led to the restoration of TERT mRNA and protein levels. Thus, our findings suggest that the decrease of TERT-positive cells in the gastric mucosa of infected mice might be an ROS-mediated regulation due to the oxidative stress generated during inflammation. In line with this, it was reported that low-grade chronic inflammation in mice can directly promote ROS-mediated telomeric DNA damage, which is repaired less efficiently than elsewhere in the chromosome [49, 50].

DNA methylation plays a key role during the early steps of carcinogenesis [51]. In *H. pylori*-infected individuals, high levels of CpG methylation have been associated with a higher risk of gastric cancer [43, 52, 53]. A potential link between telomere length shortening and promoter CpG island methylation has been described in the gastric mucosa of *H. pylori*-positive patients [41]. Importantly, ROS-induced oxidative stress during chronic inflammation is associated with aberrant DNA hypermethylation of tumor suppressor gene-promoter region [54]. Furthermore, HOCl and HOBr produced by polymorphonuclear cells during inflammation are also able to interact with DNA and promote DNA methylation [55]. Our data show that *H. pylori* induces DNA hypermethylation at the E-box and CpG island present in the core-promoter region of the *mTERT* gene, leading to the downregulation of its expression. However, the shutting down of TERT expression could, in addition, result from

indirect mechanisms through DNA methylation of genes coding for transcriptional regulators as tumor suppressors. The transcription factors USF1 and USF2 activate the transcription of *hTERT* through E-box interaction [15, 16]. Indeed, we previously showed that *H. pylori* induces DNA hypermethylation in the promoter region of *USF1* and *USF2* genes, inhibiting their expression, and consequently resulting in diminished USF1/USF2-E-box binding at the *hTERT* promoter [14].

In *H. pylori*-infected mice, we showed a decrease in *mTERT* gene expression as early as the stage of gastritis and the initial development of preneoplastic lesions. Decrease in *TERT* gene expression has direct consequences on telomerase activity, as we observed in *H. pylori*-infected gastric epithelial cells, *in vitro*. Both models of *H. pylori* infection showed the accumulation of DNA damage [6–8], predominantly observed at the ends of chromosomal arms [12]. Importantly, *H. pylori* DNA damage activity is associated with the impairment of DNA repair systems and p53 deficiency [5], and it plays an important role at the origin of genomic translocations and chromosome end fusion observed in gastric tumors [12]. Dysregulation of the DNA repair system and telomerase activity play a pivotal, decisive role in the decision at the cross-road between the preneoplastic stage and cancer development. At premalignant stages, telomerase deficiency is associated with shortened telomeres, leading to chromosomal instabilities, cell cycle arrest, and replicative senescence. This step needs the activation of the p53-mediated DNA damage response. However, during *H. pylori* infection, the p53-mediated DNA damage response is deficient, thus increasing chromosome instabilities and consequently the promotion of tumorigenesis [56]. This mechanism needs the reactivation of telomerase and maintenance of telomere length. Between the premalignant stage and cancer, *TERT* expression is thus reactivated resulting in unlimited cellular proliferation and tumorigenesis [57], as described during hepatocarcinogenesis (HCC) to enable malignant transformation and HCC development [58]. *TERT* expression has been reported to be reactivated in 85% of all cancers [59]. Importantly, reactivation of *TERT* expression is also associated with *TERT*-promoter mutations, currently found in many types of cancers. As an example in melanoma, T > G at −57 base pairs from the transcription start site (TSS) generates an E-twenty-six (ETS) transcription factor-binding site that leads to the upregulation of *TERT* transcription [60]. Therefore, we propose that telomerase deficiency, together with *H. pylori*-induced chronic inflammation, promotes accumulation of chromosome instabilities, driving cell transformation at the earliest stage toward preneoplastic phase. It is during later stages of carcinogenesis that the activation of *TERT* expression and telomerase activity may occur, resulting in an uncontrolled proliferation pattern and tumorigenesis, previously reported in gastric cancer [61, 62].

5. Conclusions

In conclusion, our study demonstrates that *H. pylori* infection inhibits *TERT* gene expression through DNA hypermethylation at its promoter region. This downregulation occurs during chronic gastritis and the development of preneoplastic lesions. This regulation is mediated through ROS production induced by the infection and chronic inflammation. The decrease in TERT levels is associated with a progressive shortening of telomeres with direct consequences on cell differentiation and proliferation, thus contributing to the early steps of the gastric carcinogenesis process.

Data Availability

The data used to support the findings of this study are included within the manuscript.

Disclosure

Françoise I. Bussière is presently at Infectiologie et Santé Publique, (ISP), INRA, Université de Tours, UMR 1282, 37380, Nouzilly, France. Julien Fernandes is presently at Institut Pasteur, UtechS-C2RT, 25–28 Rue du Dr Roux, 75724 Paris cedex 15, France. Lionel Costa is presently at Institut Cochin, INSERM U1016, CNRS UMR 8104, Université Paris Descartes, 75014, Paris, France. Vania Camilo is presently at Portuguese Oncology Institute of Porto (IPO Porto), Cancer Biology & Epigenetics Group, Rua Dr. Antonio Bernardino de Almeida, Porto, Portugal. Laurence Fiette is presently at IMMR, Institut Mutualiste Montsouris, 42 Bd Jourdan, 75014, Paris, France.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

The authors thank Pr. F. Mégraud (University of Bordeaux V. Ségalen, Bordeaux, France) for the *H. pylori* strain B38, Pr. RM Peek Jr (University of Vanderbilt, USA) and Dr. C. Varon (University of Bordeaux, France) for the strains 7.13 and related mutants, Dr. C. Figueiredo (Institute of Molecular Pathology and Immunology Porto, Portugal) for providing us the KatoIII cell line, and Pr. Timothy C Wang (Columbia University, NY, USA) for sending us the INS-GAS mice. The authors also thank Dr. A. Danckaert (Institut Pasteur, Paris, France) for her precious advices on the statistical analysis and Dr. R. Wheeler (Institut Pasteur, Paris, France) for reading the manuscript. This work was supported by the Odyssey Reinsurance Company which also supported J. Fernandes and the INCA Consortium/European FP6 Program (LSHC-CT-2005-018704) which provided a fellowship to F.I. Bussière.

Supplementary Materials

Figure S1: *H. pylori* induces ROS production in gastric epithelial cells. MKN45 cells treated with H2DCFDA were exposed to increasing concentrations of bacterial extracts (BEs) of *H. pylori* strain 7.13 or to a vehicle control. WT MKN45 (mock) cells were used to assess basal levels of ROS,

and H_2O_2 (5 mM) was used as a positive control. Intracellular DCF fluorescence (readout of ROS production) was measured using an excitation/emission wavelength of 488/530. Results are expressed as means ± SEM of a representative experiment ($n = 3$). Treated cells compared to vehicle control: $^{***}p < 0.001$; $^{****}p < 0.0001$. Figure S2: gastric inflammatory and preneoplastic lesions are induced in *H. pylori* chronically infected mice. (a) Quantification of stomach colonization by *H. pylori* SS1 after 12 and 18 months of infection. Each symbol corresponds to a single mouse. (b) Semiquantitative evaluation of histologic lesions induced by *H. pylori* in the gastric mucosa of mice. The microscopic changes (inflammation, hyperplasia, and metaplasia) were semiquantitatively scored on H&E-stained paraffin sections from 0 to 5 according to Eaton and Coll [4]. The scores of inflammation were similar at 12 and 18 months after infection. However, hyperplasia was only observed in mice infected for 18 months; at this stage, histologic lesions are associated with a higher severity of metaplasia as compared to the lesions observed in 12-month infected mice. Infected mice compared to noninfected $^{*}p < 0.05$. Figure S3: *H. pylori* inhibits mTERT gene expression in the gastric mucosa of INS-GAS transgenic mice. INS-GAS transgenic mice were chronically infected with *H. pylori* SS1 for 8 months, and gastric lesions were compared to noninfected mice as described in Materials and Methods. (a) Representative histological changes in gastric mucosa of *H. pylori* infected (b) and noninfected (a) mice. (b) Quantification of *H. pylori* gastric colonization at 8 months after infection. Each symbol corresponds to a single mouse. (c) Semiquantitative evaluation of the histologic lesions induced by *H. pylori* in the gastric mucosa of mice. The microscopic changes (inflammation, hyperplasia, and metaplasia) were scored from 0 to 5 on H&E-stained paraffin sections, according to [65]; Original magnification: ×4, bar: 250 μm (a); ×10, bar: 100 μm (b). The gastric mucosa of noninfected mice was thickened due to the presence of hyperplasia, dilatation of gastric glands, and metaplasia that occur spontaneously in the INS-GAS mice. In the infected mice, the severity of these lesions is higher than in noninfected mice. (d) mTERT gene expression quantified by real-time RT-PCR from RNA isolated from the gastric mucosa of infected and noninfected mice. Values represent the mean ± SEM of three independent measurements for each group of mice. Infected mice compared to noninfected; $^{*}p < 0.05$; $^{**}p < 0.01$. (*Supplementary Materials*)

References

[1] R. M. Peek and J. E. Crabtree, "Helicobacter infection and gastric neoplasia," *The Journal of Pathology*, vol. 208, no. 2, pp. 233–248, 2006.

[2] P. Correa, "*Helicobacter pylori* and gastric cancer: state of the art," *Cancer Epidemiology Biomarkers and Prevention*, vol. 5, no. 6, pp. 477–481, 1996.

[3] IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, "Infection with *Helicobacter pylori*," in *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans Schistosomes, liver flukes and Helicobacter pylori*, vol. 61, pp. 177–240, International Agency for Research on Cancer, Lyon, France, 1994.

[4] J. C. Atherton, "The pathogenesis ofhelicobacter pylori-induced gastro-duodenal diseases," *Annual Review of Pathology: Mechanisms of Disease*, vol. 1, no. 1, pp. 63–96, 2006.

[5] D. M. Hardbower, R. M. Peek Jr., and K. T. Wilson, "At the bench: *Helicobacter pylori*, dysregulated host responses, DNA damage, and gastric cancer," *Journal of Leukocyte Biology*, vol. 96, no. 2, pp. 201–212, 2014.

[6] E. Touati, V. Michel, J.-M. Thiberge, N. Wuscher, M. Huerre, and A. Labigne, "Chronic Helicobacter pylori infections induce gastric mutations in mice," *Gastroenterology*, vol. 124, no. 5, pp. 1408–1419, 2003.

[7] A. M. D. Machado, C. Figueiredo, E. Touati et al., "Helicobacter pylori infection induces genetic instability of nuclear and mitochondrial DNA in gastric cells," *Clinical Cancer Research*, vol. 15, no. 9, pp. 2995–3002, 2009.

[8] I. M. Toller, K. J. Neelsen, M. Steger et al., "Carcinogenic bacterial pathogen *Helicobacter pylori* triggers DNA double-strand breaks and a DNA damage response in its host cells," *Proceedings of the National Academy of Sciences*, vol. 108, no. 36, pp. 14944–14949, 2011.

[9] E. Touati, "When bacteria become mutagenic and carcinogenic: lessons from *H. pylori*," *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, vol. 703, no. 1, pp. 66–70, 2010.

[10] L. D. Butcher, G. den Hartog, P. B. Ernst, and S. E. Crowe, "Oxidative stress resulting from *Helicobacter pylori* infection contributes to gastric carcinogenesis," *Cellular and Molecular Gastroenterology and Hepatology*, vol. 3, no. 3, pp. 316–322, 2017.

[11] T. von Zglinicki, G. Saretzki, W. Döcke, and C. Lotze, "Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence?," *Experimental Cell Research*, vol. 220, no. 1, pp. 186–193, 1995.

[12] M. Koeppel, F. Garcia-Alcalde, F. Glowinski, P. Schlaermann, and T. F. Meyer, "*Helicobacter pylori* infection causes characteristic DNA damage patterns in human cells," *Cell Reports*, vol. 11, no. 11, pp. 1703–1713, 2015.

[13] T. Niwa, T. Tsukamoto, T. Toyoda et al., "Inflammatory processes triggered by *Helicobacter pylori* infection cause aberrant DNA methylation in gastric epithelial cells," *Cancer Research*, vol. 70, no. 4, pp. 1430–1440, 2010.

[14] F. I. Bussière, V. Michel, S. Mémet et al., "*H. pylori*-induced promoter hypermethylation downregulates USF1 and USF2 transcription factor gene expression," *Cellular Microbiology*, vol. 12, no. 8, pp. 1124–1133, 2010.

[15] M. Yago, R. Ohki, S. Hatakeyama, T. Fujita, and F. Ishikawa, "Variant forms of upstream stimulatory factors (USFs) control the promoter activity ofhTERT, the human gene encoding the catalytic subunit of telomerase," *FEBS Letters*, vol. 520, no. 1–3, pp. 40–46, 2002.

[16] M. K. Ramlee, J. Wang, W. X. Toh, and S. Li, "Transcription regulation of the human telomerase reverse transcriptase (hTERT) gene," *Genes*, vol. 7, no. 8, p. 50, 2016.

[17] C. Autexier and N. F. Lue, "The structure and function of telomerase reverse transcriptase," *Annual Review of Biochemistry*, vol. 75, no. 1, pp. 493–517, 2006.

[18] Y. Qian, L. Yang, and S. Cao, "Telomeres and telomerase in T cells of tumor immunity," *Cellular Immunology*, vol. 289, no. 1-2, pp. 63–69, 2014.

[19] P. Martínez and M. A. Blasco, "Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins," *Nature Reviews Cancer*, vol. 11, no. 3, pp. 161–176, 2011.

[20] A. Samba-Louaka, F. Stavru, and P. Cossart, "Role for telomerase in *Listeria monocytogenes* infection," *Infection and Immunity*, vol. 80, no. 12, pp. 4257–4263, 2012.

[21] N. R. Forsyth, W. E. Wright, and J. W. Shay, "Telomerase and differentiation in multicellular organisms: turn it off, turn it on, and turn it off again," *Differentiation*, vol. 69, no. 4-5, pp. 188–197, 2002.

[22] E. Hiyama and K. Hiyama, "Clinical utility of telomerase in cancer," *Oncogene*, vol. 21, no. 4, pp. 643–649, 2002.

[23] M. C. Duarte, E. Babeto, K. R. M. Leite et al., "Expression of TERT in precancerous gastric lesions compared to gastric cancer," *Brazilian Journal of Medical and Biological Research*, vol. 44, no. 2, pp. 100–104, 2011.

[24] H. Kuniyasu, Y. Kitadai, H. Mieno, and W. Yasui, "*Helicobactor pylori* infection is closely associated with telomere reduction in gastric mucosa," *Oncology*, vol. 65, no. 3, pp. 275–282, 2003.

[25] W.-P. Lee, M.-C. Hou, K.-H. Lan et al., "*Helicobacter pylori*-induced chronic inflammation causes telomere shortening of gastric mucosa by promoting PARP-1-mediated non-homologous end joining of DNA," *Archives of Biochemistry and Biophysics*, vol. 606, pp. 90–98, 2016.

[26] P. Lehours, A. Menard, S. Dupouy et al., "Evaluation of the association of nine *Helicobacter pylori* virulence factors with strains involved in low-grade gastric mucosa-associated lymphoid tissue lymphoma," *Infection and Immunity*, vol. 72, no. 2, pp. 880–888, 2004.

[27] J.-M. Thiberge, C. Boursaux-Eude, P. Lehours et al., "From array-based hybridization of *Helicobacter pylori* isolates to the complete genome sequence of an isolate associated with MALT lymphoma," *BMC Genomics*, vol. 11, no. 1, p. 368, 2010.

[28] A. T. Franco, D. A. Israel, M. K. Washington et al., "Activation of -catenin by carcinogenic Helicobacter pylori," *Proceedings of the National Academy of Sciences*, vol. 102, no. 30, pp. 10646–10651, 2005.

[29] A. Lee, J. O'Rourke, M. De Ungria, B. Robertson, G. Daskalopoulos, and M. Dixon, "A standardized mouse model of Helicobacter pylori infection: introducing the Sydney strain," *Gastroenterology*, vol. 112, no. 4, pp. 1386–1397, 1997.

[30] K. T. Wilson, K. S. Ramanujam, H. L. Mobley, R. F. Musselman, S. P. James, and S. J. Meltzer, "*Helicobacter pylori* stimulates inducible nitric oxide synthase expression and activity in a murine macrophage cell line," *Gastroenterology*, vol. 111, no. 6, pp. 1524–1533, 1996.

[31] S. H. Jang, J. W. Lim, T. Morio, and H. Kim, "Lycopene inhibits Helicobacter pylori-induced ATM/ATR-dependent DNA damage response in gastric epithelial AGS cells," *Free Radical Biology and Medicine*, vol. 52, no. 3, pp. 607–615, 2012.

[32] S.-Z. Ding, Y. Minohara, X. J. Fan et al., "*Helicobacter pylori* infection induces oxidative stress and programmed cell death in human gastric epithelial cells," *Infection and Immunity*, vol. 75, no. 8, pp. 4030–4039, 2007.

[33] T. C. Wang, C. A. Dangler, D. Chen et al., "Synergistic interaction between hypergastrinemia and Helicobacter infection in a mouse model of gastric cancer," *Gastroenterology*, vol. 118, no. 1, pp. 36–47, 2000.

[34] A. M. Burger, "Standard TRAP assay," *Methods in Molecular Biology*, vol. 191, pp. 109–124, 2002.

[35] I. Horikawa, Y. J. Chiang, T. Patterson et al., "Differential cis-regulation of human versus mouse TERT gene expression in vivo: identification of a human-specific repressive element," *Proceedings of the National Academy of Sciences*, vol. 102, no. 51, pp. 18437–18442, 2005.

[36] S. Odenbreit, J. Puls, B. Sedlmaier, E. Gerland, W. Fischer, and R. Haas, "Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion," *Science*, vol. 287, no. 5457, pp. 1497–1500, 2000.

[37] S. Kutter, R. Buhrdorf, J. Haas, W. Schneider-Brachert, R. Haas, and W. Fischer, "Protein subassemblies of the *Helicobacter pylori* Cag type IV secretion system revealed by localization and interaction studies," *Journal of Bacteriology*, vol. 190, no. 6, pp. 2161–2171, 2008.

[38] K. Nagata, H. Yu, M. Nishikawa et al., "Helicobacter pyloriGenerates superoxide radicals and modulates nitric oxide metabolism," *Journal of Biological Chemistry*, vol. 273, no. 23, pp. 14071–14073, 1998.

[39] O. Handa, Y. Naito, and T. Yoshikawa, "*Helicobacter pylori*: a ROS-inducing bacterial species in the stomach," *Inflammation Research*, vol. 59, no. 12, pp. 997–1003, 2010.

[40] P. Di Mascio, S. Kaiser, and H. Sies, "Lycopene as the most efficient biological carotenoid singlet oxygen quencher," *Archives of Biochemistry and Biophysics*, vol. 274, no. 2, pp. 532–538, 1989.

[41] T. Tahara, T. Shibata, M. Okubo et al., "Demonstration of potential link between *Helicobacter pylori* related promoter CpG island methylation and telomere shortening in human gastric mucosa," *Oncotarget*, vol. 7, pp. 43989–43996, 2016.

[42] D. D. Lee, R. Leão, M. Komosa et al., "DNA hypermethylation within TERT promoter upregulates TERT expression in cancer," *Journal of Clinical Investigation*, vol. 129, no. 4, p. 1801, 2019.

[43] M. Kaise, T. Yamasaki, J. Yonezawa, J. Miwa, Y. Ohta, and H. Tajiri, "CpG island hypermethylation of tumor-suppressor genes in *H. pylori*-infected non-neoplastic gastric mucosa is linked with gastric cancer risk," *Helicobacter*, vol. 13, no. 1, pp. 35–41, 2008.

[44] L. Hou, S. A. Savage, M. J. Blaser et al., "Telomere length in peripheral leukocyte DNA and gastric cancer risk," *Cancer Epidemiology Biomarkers & Prevention*, vol. 18, no. 11, pp. 3103–3109, 2009.

[45] R. Aslan, A. Bektas, A. Bedir et al., "*Helicobacter pylori* eradication increases telomere length in gastric mucosa," *Hepatogastroenterology*, vol. 60, no. 123, pp. 601–604, 2013.

[46] B. Usselmann, M. Newbold, A. G. Morris, and C. U. Nwokolo, "Deficiency of colonic telomerase in ulcerative colitis," *The American Journal of Gastroenterology*, vol. 96, no. 4, pp. 1106–1112, 2001.

[47] A. Zhang, J. Wang, B. Zheng et al., "Telomere attrition predominantly occurs in precursor lesions during in vivo carcinogenic process of the uterine cervix," *Oncogene*, vol. 23, no. 44, pp. 7441–7447, 2004.

[48] D. M. Hardbower, T. de Sablet, R. Chaturvedi, and K. T. Wilson, "Chronic inflammation and oxidative stress," *Gut Microbes*, vol. 4, no. 6, pp. 475–481, 2013.

[49] V. Kordinas, A. Ioannidis, and S. Chatzipanagiotou, "The telomere/telomerase system in chronic inflammatory diseases. Cause or effect?," *Genes*, vol. 7, no. 9, p. 60, 2016.

[50] T. von Zglinicki, "Oxidative stress shortens telomeres," *Trends in Biochemical Sciences*, vol. 27, no. 7, pp. 339–344, 2002.

[51] G. H. Kang, Y. H. Shim, H. Y. Jung, W. H. Kim, J. Y. Ro, and M. G. Rhyu, "CpG island methylation in premalignant stages of gastric carcinoma," *Cancer Research*, vol. 61, no. 7, pp. 2847–2851, 2001.

[52] T. Nakajima, S. Yamashita, T. Maekita, T. Niwa, K. Nakazawa, and T. Ushijima, "The presence of a methylation fingerprint of

Helicobacter pylori infection in human gastric mucosae," *International Journal of Cancer*, vol. 124, no. 4, pp. 905–910, 2009.

[53] T. Maekita, K. Nakazawa, M. Mihara et al., "High levels of aberrant DNA methylation in *Helicobacter pylori*-infected gastric mucosae and its possible association with gastric cancer risk," *Clinical Cancer Research*, vol. 12, no. 3, pp. 989–995, 2006.

[54] Q. Wu and X. Ni, "ROS-mediated DNA methylation pattern alterations in carcinogenesis," *Current Drug Targets*, vol. 16, no. 1, pp. 13–19, 2015.

[55] V. Valinluck and L. C. Sowers, "Inflammation-mediated cytosine damage: a mechanistic link between inflammation and the epigenetic alterations in human cancers," *Cancer Research*, vol. 67, no. 12, pp. 5583–5586, 2007.

[56] M. T. Hayashi, A. J. Cesare, T. Rivera, and J. Karlseder, "Cell death during crisis is mediated by mitotic telomere deprotection," *Nature*, vol. 522, no. 7557, pp. 492–496, 2015.

[57] D. Hanahan, "Benefits of bad telomeres," *Nature*, vol. 406, no. 6796, pp. 573-574, 2000.

[58] J.-C. Nault, M. Ningarhari, S. Rebouissou, and J. Zucman-Rossi, "The role of telomeres and telomerase in cirrhosis and liver cancer," *Nature Reviews Gastroenterology & Hepatology*, vol. 16, no. 9, pp. 544–558, 2019.

[59] S. C. Akincilar, B. Unal, and V. Tergaonkar, "Reactivation of telomerase in cancer," *Cellular and Molecular Life Sciences*, vol. 73, no. 8, pp. 1659–1670, 2016.

[60] S. Horn, A. Figl, P. S. Rachakonda et al., "TERT promoter mutations in familial and sporadic melanoma," *Science*, vol. 339, no. 6122, pp. 959–961, 2013.

[61] I. K. Chung, K. Y. Hwang, I. H. Kim et al., "*Helicobacter pylori* and telomerase activity in intestinal metaplasia of the stomach," *The Korean Journal of Internal Medicine*, vol. 17, no. 4, pp. 227–233, 2002.

[62] H. Kameshima, A. Yagihashi, T. Yajima et al., "*Helicobacter pylori* infection: augmentation of telomerase activity in cancer and noncancerous tissues," *World Journal of Surgery*, vol. 24, no. 10, pp. 1243–1249, 2000.

Hindawi
Journal of Oncology
Volume 2019, Article ID 3257939, 11 pages
https://doi.org/10.1155/2019/3257939

Review Article

Human Papillomavirus Infection and Cervical Cancer: Epidemiology, Screening, and Vaccination—Review of Current Perspectives

Chee Kai Chan,[1] Gulzhanat Aimagambetova,[1] Talshyn Ukybassova,[2] Kuralay Kongrtay,[1] and Azliyati Azizan[1]

[1]*Department of Biomedical Sciences, Nazarbayev University School of Medicine, 5/1, Zhanybek and Kerey Khans St., Astana, Kazakhstan*
[2]*University Medical Center, National Research Center for Mother and Child Health, Turan Ave., 32, Astana, Kazakhstan*

Correspondence should be addressed to Gulzhanat Aimagambetova; gulzhanat.aimagambetova@nu.edu.kz

Received 27 May 2019; Revised 4 September 2019; Accepted 9 September 2019; Published 10 October 2019

Guest Editor: Hironori Yoshiyama

Viral infections contribute as a cause of 15–20% of all human cancers. Infection by oncogenic viruses can promote different stages of carcinogenesis. Among many types of HPV, around 15 are linked to cancer. In spite of effective screening methods, cervical cancer continues to be a major public health problem. There are wide differences in cervical cancer incidence and mortality by geographic region. In addition, the age-specific HPV prevalence varies widely across different populations and showed two peaks of HPV positivity in younger and older women. There have been many studies worldwide on the epidemiology of HPV infection and oncogenic properties due to different HPV genotypes. However, there are still many countries where the population-based prevalence has not yet been identified. Moreover, cervical cancer screening strategies are different between countries. Organized cervical screening programs are potentially more effective than opportunistic screening programs. Nevertheless, screening programs have consistently been associated with a reduction in cervical cancer incidence and mortality. Developed countries have achieved such reduced incidence and mortality from cervical cancer over the past 40 years. This is largely due to the implementation of organized cytological screening and vaccination programs. HPV vaccines are very effective at preventing infection and diseases related to the vaccine-specific genotypes in women with no evidence of past or current HPV infection. In spite of the successful implementation of the HPV vaccination program in many countries all over the world, problems related to HPV prevention and treatment of the related diseases will continue to persist in developing and underdeveloped countries.

1. Introduction

According to the World Health Organization's (WHO) statistics, common cancers are one of the most prevalent causes of mortality worldwide with 8.2 million deaths in 2012, and this trend has not changed in recent years. Viral infections contribute to 15–20% of all human cancers, whereby several viruses play considerable roles in the multistage development of malignant cancers. Over the past two decades, it has become obvious that several viruses play an important role in the development of human cancers. Around 15% to 20% of cancer cases are associated with viral infections. Oncogenic viruses can facilitate various stages of carcinogenesis [1]. One of the viruses contributing to the statistics of cancerous diseases is human papillomavirus (HPV). HPV is a virus that can be sexually transmitted, and high-risk HPV DNA is found to be present in 99.7% of cervical cancer specimens [2]. Within 12 to 24 months of exposure to the virus, 90% of HPV infections are cleared or become inactive. However, infections by the high-risk HPV types persist which then increase the risk of progression to cervical cancer [3].

HPV is a double-stranded DNA virus belonging to the Papovaviridae family. Almost 200 HPV types have been identified with more than 40 types colonizing the genital tract. All HPV infection types are divided into two groups based on their carcinogenic properties; these are high risk

and low risk. High-risk types include 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 68, and 59. Others are classified as potential high-risk (which are 53, 66, 70, 73, and 82). Currently, it is well known and proven that HPV16 and 18 are the most virulent high-risk genotypes, causing about 70% of all invasive cervical cancer in the world [4].

At the present time, we have a relatively clear picture of HPV infection's natural history, oncogenic properties, screening, and prevention algorithms. However, HPV infection rates continue to persist, especially in developing countries, where cervical cancer incidence and prevalence are still high. This is due to different reasons, which include low socioeconomic status, lack of population awareness, and inadequately implemented screening and vaccination programs. Thus, it is necessary to continue this discussion and to refocus attention of specialists and population worldwide to HPV infection and related diseases. The aim of this review article is to summarize updated information regarding the aforementioned aspects of HPV infection and related cancers, including also discussions about the HPV genome and molecular events leading to cancer development following an HPV infection. Enhanced knowledge of HPV status and cancer progression events contributes to the improvement of the future management of patients with cervical lesions; this in turn can help mitigate cervical cancer progression among HPV-infected women.

2. The HPV Genome

Papillomavirus genome is comprised of a small double-stranded and highly conserved DNA with an approximate size of 8000 base pairs and consists of three regions. The molecular biology of this small DNA molecule is complex. There are six early proteins, three regulatory proteins (E1, E2, and E4) and three oncoproteins (E5, E6, and E7) encoded in 4000 base pairs (bp) that participate in viral replication and transformation of cell. Another 3000 bp region of DNA molecule encodes two structural proteins L1 and L2 that compose the capsid of virus. The viral DNA replication and transcriptional regulatory elements are controlled by a long control region (LCR) that is encoded in a 1000 bp region [5].

Upon the viral evolution, accumulation of numerous lineage-defining genetic variations in these regions can lead to speciation into separate HPV types. Sequence variations such as single-nucleotide polymorphisms or genetic mutations within L1, LCR, E6, and E7 regions of HPV can determine families, relatedness, and phylogeny of the HPV types. HPV type can be defined as an entity based on the more than 10% difference in the DNA sequence of the L1 gene between two genomes. However, the difference between 2% and 10% determines the HPV subtypes. In addition, the variants are entities that define less than 2% of dissimilarities between HPV genomes. According to recent studies, there are 60 out of 160 HPV types associated with mucosal epithelia and categorized as Alphapapillomavirus genus (alpha-PV) [6]. Furthermore, alpha-PV can be classified into nine groups: alpha-5 (HPV23, 51, 69, and 82), alpha-6 (HPV30, 53, 56, and 66), alpha-7 (HPV18, 39, 45, 59, 68, 70, 85, and 97), and alpha-9 (HPV16, 31, 33, 35, 52, 58, and 67), which include mostly the oncogenic high-risk types [7]. However, there are also Betapapillomavirus and Gammapapillomavirus genus that have not been investigated in detail yet [8].

According to Papillomavirus Nomenclature Committee, each HPV type can be differentiated into phylogenetic lineages in terms of geographic distribution, pathogenicity, regulation of transcription, and immunological response [9]. The alpha-9 HPV16 type has been further classified into four phylogenetic lineages: A, B, C, and D. Phylogeny A is divided further into four sublineages A1, A2, A3, and A4. Sublineages A1, A2, and A3 include European HPV DNA sequences while A4 includes Asian sequences isolated worldwide. Lineage B is classified as B1 and B2 sublineages, which comprise the African HPV sequences. Lineage C is also referred to as African sequences. Lineage D consists of 3 sublineages: D1, D2, and D3 that include Asian-American and North American sequences. HPV intratypic molecular variants can be distinguished based on oncogenic potentials in spite of their phylogenetic relatedness. Several research studies associate the HPV16 lineage D as being more tumorigenic in comparison with the other lineages [10].

3. Association between HPV Infection and Cervical Lesions

The vast majority of HPV infections are transitory and become undetectable in 12–24 months [4, 11–14]. However, in some women whose infections continue to persist, the risk of developing precancerous conditions is significant. Many studies confirmed that persistent infection with an oncogenic HPV type is the main risk factor for detecting a cervical intraepithelial neoplasia (CIN) that may range from CIN1 to CIN3 and cancer [12, 13, 15]. In the VIVIANE study, the researchers found that HPV33 and HPV16 were associated with the highest risk of CIN development, followed by HPV18, HPV31, and HPV45 [13].

Natural history of CIN lesions is different depending on its grade. CIN1 is a low-grade squamous intraepithelial lesion (LSIL). According to statistical data, over 70–80% of CIN1 lesions spontaneously regress without treatment or become undetectable [11, 16]. Thus, CIN1 reflects a state of infection rather than a stage in disease development. Detection of CIN1 following HPV infection does not therefore automatically represent disease progression. Furthermore, obvious clearance may be attributed to an inability to detect the infection [13]. Therefore, clearance rates should be interpreted with caution.

CIN2 and CIN3 are considered high-grade dysplasia or high-grade squamous intraepithelial lesion (HSIL); however, they are different whereby CIN2 less commonly progresses to cancer. CIN2 develops in two different ways; the annual regression rate of CIN2 in adult women is estimated to range from 15 to 23%, with up to 55% regressing by 4–6 years [16, 17], whereas approximately 2% of CIN2 lesions develop to CIN3 within the same period. CIN3 is considered a true precancer with the potential to progress to invasive cancer at a rate of 0.2% to 4% within 12 months [16, 18]. Untreated CIN3 has a 30% probability of becoming invasive cancer

over a 30-year period, although only about 1% of properly treated CIN3 will become invasive [12, 16, 18, 19]. Adenocarcinoma of the cervix is distinct from squamous cell carcinoma as it arises from the glandular epithelium of the endocervical canal and its immediate precursor is adenocarcinoma in situ. The time from HPV infection to cervical cancer development is typically 20 years; therefore, rapid progression of cervical cancers rarely occurs [20].

The link between high-risk HPV types and cervical cancer development contributed to the introduction of novel screening programs. For example, testing for the presence of high-risk HPV is recommended as a screening tool by the WHO and the European Guidelines for Quality Assurance for Cervical Cancer Screening [21, 22]. HPV testing has been found to be effective in detection of precancerous cervical lesions particularly in population-based cervical screening programs [23]. The establishment of the causal link between HPV and cervical cancer, along with an understanding of the epidemiology and natural history of HPV infection, has led to a new model for cervical carcinogenesis: HPV acquisition, HPV persistence, progression to precancer, and invasion [24], which helps guide age-appropriate interventions to prevent cervical cancer.

4. Pathogenesis of Cervical Cancer Development following HPV Infection

Cervical cancerogenesis can be defined as the complex mechanism of uncontrolled cellular division that can involve HPV gene integration together with other cellular changes and epigenetic factors. As the HPV infection occurs, the DNA can undergo mutations under the cellular and other environmental conditions leading to viral DNA integration and operation with the host DNA synthesis machinery. As a result, virus can escape cellular and immune defense mechanisms while promoting cell proliferation and inhibiting cellular apoptotic mechanisms.

Oncogenic potential of HPV16 depends on the regulation of viral transcriptional factors. At the initiation of viral infection, the HPV16 genome can be presented as unintegrated small DNA molecule also called episome and results in benign and precancerous lesions of the cervix. However, HPV16 can integrate its genome into the host genome, which in turn can lead to the development of cervical carcinoma and cervical intraepithelial neoplasia grade III [10]. Viral genome integration in combination with dysregulation of the E2 protein, which is a repressor of the oncoprotein, contributes towards the carcinogenic process. These events cause overexpression of E6 and E7 proteins that eventually contribute to viral carcinogenesis by altering cellular apoptotic mechanism [5, 10]. Overexpression of E6 and E7 alone is insufficient to contribute to the cancerogenesis as other genetic and epigenetic factors also need to be established.

There are many types of HPV, which are found to be associated with cancerous diseases—16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 types [4]. The most carcinogenic HPV type is HPV16, and 50% of all cervical cancers are associated with HPV16 [15]. In HPV16-positive cells, it is found that E6 and E7 viral genes are retained integrated into the host genome and are expressed, although in some HPV16-infected cells E6/E7 overexpression can be absent. Moreover, E6/E7 overexpression is also found in cells infected by other HPV types [25, 26]. E6 and E7 are small proteins of 150 and 100 amino acids without any known enzymatic activity, but they can influence the host cell activity by binding with cellular proteins. E6, for example, binds with E6-associated binding protein (E6AP), a ubiquitin ligase leading to a structural change in E6 allowing it to bind with p53, the cell cycle control tumor suppressor protein to form a trimeric complex E6/E6AP/p53 (Figure 1).

This binding leads to the degradation of p53 and thus leads to cell proliferation. E7, on the other hand, binds pRb causing its inactivation and degradation. Both the low-risk and high-risk E7 protein has been shown to target the pRB family members including p107 (RBL1) and p130 (RBL2) for degradation [27]. pRb downregulates E2F a transcription factor. As pRb is deactivated by E7, E2F is upregulated and cell proliferation genes are activated. Furthermore, E6 and E7 have been shown to form complexes with hundreds of other proteins in the host cell [28–30] and it will be interesting to understand the functions and consequence of what these complexes do. It is important to note that E6 and E7 transforming and oncogenic properties involve other cancer pathways not involving p53 or pRB. For example, E7 stimulates telomerase activity [31] and E6/E7 has been shown to deregulate miRNA linked to carcinogenesis [32]. E7 has also been shown to interact with histone deacetylase-(HDAC1-3-) enhancing E2F activation that is associated with differentiation and viral replication [33].

miRNA plays an important role in the posttranscriptional control of the expression of host genes. Recent studies proposed that HPV E6, E7, and E5 oncoproteins regulate the host miRNA profile. In HPV-associated cervical cancer cells, a number of miRNAs such as miR-21, miR-143, and miR-9 are overexpressed, thus targeting CCL20 (chemokine (C-C) motif ligand) and promoting migration of HPV16-positive cancerous cells. However, overexpression of some miRNAs such as miR-203 inhibits HPV amplification. Thus, in the HPV-infected cancer cells, miR-203 is suppressed by HPV E7 gene overexpression, leading to the induction of viral replication. Deregulation of miRNA expression can occur mostly due to epigenetic methylation of miRNA promoters [34].

E6 belonging to tumorigenic HPV types harbors a PDZ binding motif (PBM) at the C terminus which facilitates the binding of E6 to a number of proteins containing the PDZ site. The binding of E6 to these proteins leads to inactivation and degradation. Examples of such proteins include potential tumor suppressors such as Dlg [35], MAGI-1 [36], and Scribble [37, 38].

The epigenetic control of viral and host gene expression plays an important role in carcinogenesis by involving changes in DNA methylation, modifications of histones, and noncoding RNA profile. Cervical carcinogenesis is strongly associated with persistent HPV infection that can further affect both the host genome and the viral genome methylation process [34].

E6 and E7 have been shown to bind DNA methyltransferases (DNMT) which impairs their activity leading to hypermethylation of CpG islands which can eventually lead to possible silencing of host tumor suppressors [30, 39]. Some studies showed decreased methylation of the upstream

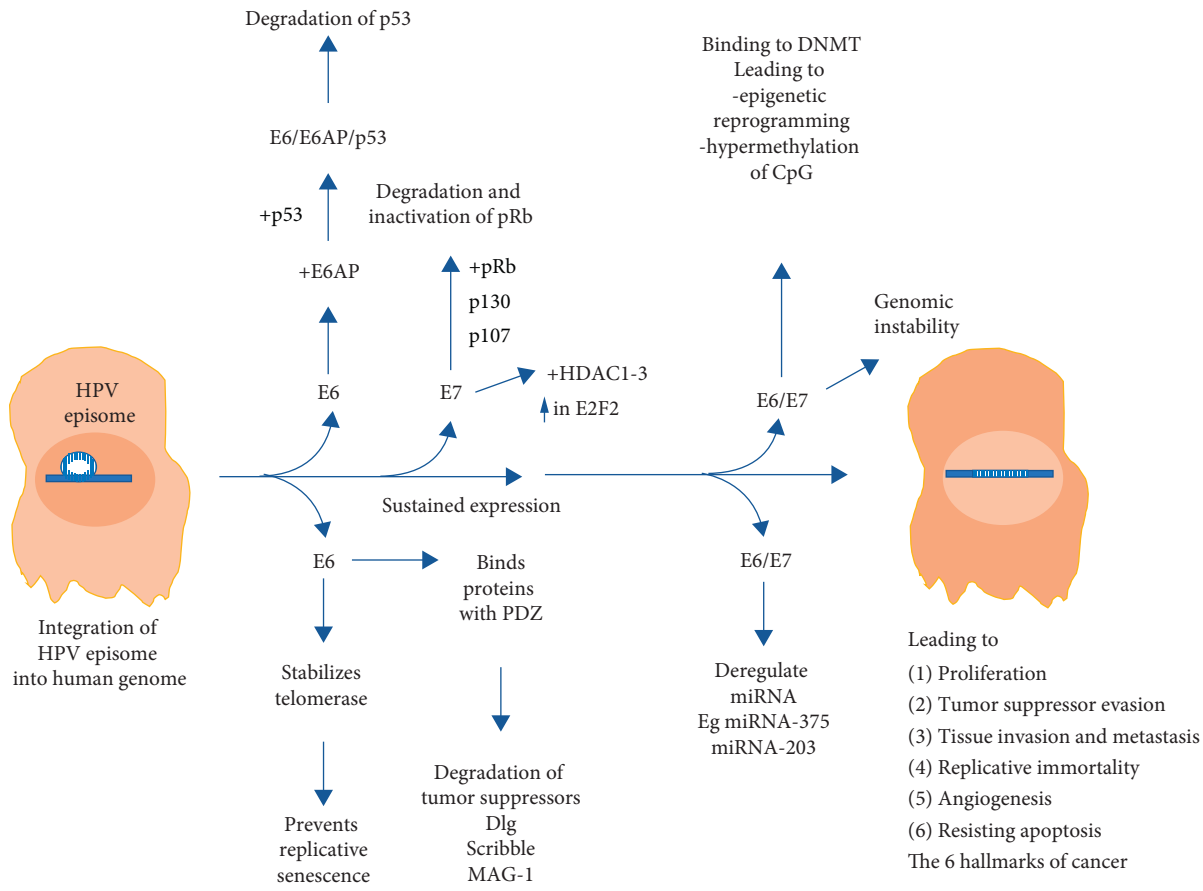


Figure 1: Progression of cervical cancerogenesis which involves HPV gene integration, leading to sustained expression of E6 and E7, impacting and dysregulating the various pathways including the inactivation and degradation of p53 and pRB that lead to uncontrolled cellular division, proliferation, tumor suppressor evasion, and other features of tumorigenicity.

regulatory region (URR) in the cervical cancer cells in comparison with normal cells, whereas other studies described increased methylation of the viral genome [40]. These studies' discrepancies can be explained by the viral life cycle stages, type of HPV genome integrations, cervical cancer stages, and other factors. However, methylation of viral DNA can be defined as the host cellular defense mechanism. So, it is still poorly investigated if HPV DNA methylation is beneficial for viral cancerogenesis [34].

It has been suggested that increased methylation of CpG dinucleotides within E2 binding site (E2BS) on the host genome can modify interaction of different factors and result in abnormal cell differentiation with further disease progression [34]. As a result, this hypermethylation event reduces the binding affinity of the viral regulatory protein E2 to E2BS, thus leading to E6 and E7 overexpression and further epigenetic inhibition of tumor suppressor genes [10]. Some studies suggest that CpG region methylation can be used as a biomarker of cervical cancer detection.

5. Epidemiology of Cervical Cancer

Cervical cancer is the leading genital cancer among women worldwide, with almost half a million new cases per year (GLOBOCAN, 2012) [41]. In 2015, 526,000 women developed cervical cancer worldwide and caused 239,000 deaths [42]. The majority of cervical cancer cases are squamous cell carcinoma [41]. In spite of effective screening methods, cervical cancer continues to be a major public health problem [4].

The mortality from cervical cancer varies in different geographic regions. The age-standardized incidence rate for cervical cancer is much lower in developed countries at 5.0 per 100,000 compared to developing countries at 8.0 per 100,000 [43]. Similarly, the age-standardized mortality rate for cervical cancer is lower in developed nations at 2.2 per 100,000 compared with developing nations at 4.3 per 100,000. For example, in sub-Saharan Africa, there were 34.8 new cases and 22.5 deaths per 100,000 women, while in Western Asia there were only 4.4 new cases and 1.9 deaths per 100,000 women in 2012 [44]. In comparison, Northern America is found to be the region with the third-lowest cervical cancer rate in the world [43].

Limited statistical data are available on cervical cancer in Central Asia [43]. From the existing sources, it is found that the incidence rates of cervical cancer in many countries of Central Asia are quite high (ranging from 9.9 per 100,000 women in Tajikistan to 29.4 per 100,000 in Kazakhstan) compared to Europe (ranging from 4.0 per 100.000 in Finland and 7.0 per 100.000 in Germany) [43–45]. Approximately 25,700 women are diagnosed with cervical cancer and 12,700 die from this disease annually in the

Central Asian countries [46]. The mortality rates range from 4.9 per 100,000 women in Tajikistan to 11.2 per 100,000 in Kyrgyzstan [41, 46]. The indicators are higher than in Western European countries (incidence rates ranging from 2.1 per 100,000 women in Malta to 12.2 per 100,000 in Portugal; mortality rates ranging from 0.8 per 100,000 women in Iceland to 3.6 per 100,000 in Portugal) [46].

Cervical cancer has a bimodal age distribution with the majority of cases occurring among women in their 30s and 40s, the age at which women are often raising families and ensuring the financial viability of their families and communities. In addition to the risk of death, cervical cancer is associated with increased morbidity, including bleeding, pain, and kidney failure, which are difficult to treat, especially in communities with poor access to healthcare [47].

6. Prevalence of HPV in the General Population and in Cervical Cancer Patients

HPV infections are widespread all over the world; however, prevalence and type distribution are heterogeneous [48]. The age-specific HPV prevalence varies in young and advanced age women populations [49]. A comprehensive meta-analysis assessing the global prevalence of cervical HPV infection among women without cervical lesions revealed that almost 12% of females worldwide are positive for HPV DNA [50].

There have been many studies worldwide on the epidemiology of HPV infection and oncogenic properties due to different HPV genotypes [4]. One of the international studies found that 10.4% of patients with normal cytology have been detected with either high- or low-risk HPV types. Women in less developed countries and those who are younger than 25 years old have a higher prevalence, ranging from 15 to 45% [50]. The highest HPV prevalence was observed in sub-Saharan Africa (24%), Eastern Europe (21.4%), and Latin America (16.1%) and the lowest in Northern America (4.7%) and Western Asia (1.7%). The HPV type 16 was the most common virus worldwide with prevalence rates accounting for 32.3% of all infections in Southern Asia, 28.9% in Southern Europe, 24.4% in Western Europe, 24.3% in Northern America, and 12% in Africa [51].

According to the Extended Middle East and North Africa (EMENA) study, in the Middle East, the incidence of HPV shows lower rates compared to the rest of the world [52]. For instance, in Qatar HPV prevalence among the general population of women with normal or abnormal cytology recently estimated 6.1% [52]. The authors detected the presence of various HPV genotypes with a high prevalence of low-risk HPV types, particularly type 81.

Very limited data are available on HPV prevalence, incidence, and genotype-specific dissemination in Central Asia and Eastern Europe. For example, according to the report of HPV Information Centre (2017), no data on the epidemiology of HPV infection are available in Kazakhstan (which is a Central Asian country), and only a few articles on the epidemiology of HPV infection in Kazakhstan were published in international peer-reviewed journals and several articles in local medical journals [53]. The authors' findings demonstrated that 43.6% of the patients attending gynecologic clinic were HPV positive. The most prevalent types detected were HPV16 (18.4%) and HPV18 (9.22%), followed by HPV types 33, 51, and 52 (nearly 5% each) [53].

The prevalence of HPV infection among Africans is higher than in the European population with 26.3% in Nigeria, 47.9% in Guinea, 41% in South Africa, and 38.8–42.3% in Kenya [54, 55]. Possibly high prevalence of HPV among women in sub-Saharan African countries is more prominent due to high exposure of human immunodeficiency virus (HIV) in the country, and cervical cancer may become epidemic if cervical cancer knowledge is not increased and the barriers for early screening services will still exist [56].

Other studies highlighted that some special populations have a higher risk of acquiring HPV infection. A study investigated the prevalence of HPV infection among the adolescent population in Uganda has shown significantly high distribution of high-risk HPV types (16, 18, 31, 52, and 58), which is 51.4% [57]. The reasons for such high prevalence were explained by sexual behavior, which includes early age of sexual debut and multiple sexual partners. Those factors put young women at higher risk of HPV infection [50].

With the development of highly sensitive HPV DNA testing, studies have confirmed that most cervical cancer specimens have detectable HPV DNA, and greater than 90% contain DNA for HPV16, 18, 31, 33, 39, 45, 52, or 58 [7]. It should be noted that women who develop cervical cancer have often had the same type of high-risk HPV detected in cervical specimens 3 to 5 years prior to their cancer incidence. Unfortunately, HPV genotyping can only detect current infection; therefore, we are not able to understand when in the lifetime HPV has had a carcinogenic effect [58].

Some investigators have identified regional differences in the prevalence of squamous cell carcinoma linked to HPV infection. In a meta-analysis of 85 studies, which included 10,058 women with cervical cancer, HPV16 prevalence predominated in squamous cell carcinoma, ranging from 46% in Asia to 63% in North America. The second most prevalent type was HPV18, found in 10–14% of squamous cell carcinoma specimens. The frequency of adenocarcinoma among all invasive cervical cancers also remains significant. It ranges from 4% in Africa to 32% in North America. As expected, high-risk HPV type 18 was found to be dominant in adenocarcinoma cases with a prevalence that ranges from 37% to 41%. The next most common HPV types are type 16 and type 45, which were found in 26–36% and 5–7% of samples, respectively [50]. According to the meta-analysis that included 133 studies and 14,595 women, combination of HPV16 and 18 contributes to 74–77% of squamous cell carcinoma in Europe and North America, and 65–70% of squamous cell carcinoma in Africa, Asia, and South/Central America [50]. While data from meta-analyses are limited by their reliance on the HPV DNA testing methods of each individual study, multiple studies collecting samples from large cohorts have confirmed the presence of the same HPV types in invasive cervical cancer specimens.

Several international studies investigated the prevalence of HPV types in invasive cervical cancer specimens. One of those studies explored the most prevalent types in 1918

women with cervical cancer. For that purpose, cervical cancer cells were directly tested for HPV types and the researchers found the following HPV types to be the most prevalent: HPV16, 18, 45, 31, 33, 52, 58, and 35 [59]. Similarly, an international study conducted in 38 countries tested invasive cervical cancer paraffin block samples from 10,575 women for the presence of certain HPV types. The researchers found HPV DNA in 8977 of the samples, which comprise 85% of all specimens. HPV16 or 18 was detected in 71%, and types 31, 33, 35, 45, 52, and 58 were detected in an additional 20% of the HPV-positive samples.

Having a high incidence and mortality from cervical cancer makes the screening program very important. Enhancing public awareness of underlying causal factors is a high priority for developing an appropriate cancer control and prevention program.

7. Cervical Cancer Screening

It is well known that cervical cancer screening can reduce cervical cancer incidence and mortality [60]. Cervical cancer screening strategies are different between countries. Some countries have population-based programs, whereby women in the target population are individually identified and invited to attend the screening. In opportunistic screening, invitations depend on the individual's decision or on encounters with healthcare providers. Organized cervical screening programs may achieve high participation at regular intervals with equal access, and high-quality standards for diagnosis, thus potentially more effective than opportunistic screening [61, 62]. Examples of organized programs for cervical cancer screenings exist in high-income countries such as the United Kingdom, Australia, Canada, Finland, the Netherlands, and Singapore. On the other hand, Eastern European countries have an opportunistic screening with lower-screening coverage and lower-immunization coverage and show high cervical cancer incidence and mortality rates [42]. In most of the Central Asian countries, the Caucasus region, the Russian Federation, and the Western countries of the former Soviet Union, cervical cancer screening is mainly opportunistic and characterized by cytology testing, using Romanowsky staining and generally low or unreported coverage [63]. Nevertheless, cervical cancer screening contributes to a decrease in cervical cancer incidence and mortality [64].

HPV vaccine was introduced later. Developed countries have accomplished reduction of cervical cancer incidence and mortality during the last 40 years due to the introduction of cytological smear screening [65]. For instance, since the introduction of the Pap smear cytology testing in the 1950s and 1960s, cervical cancer incidence and mortality have declined in the United States with organized cervical cancer screening programs and screening rates of 83% [66]. In the Northern European countries, an organized screening program was established in the 1960s and their effects on cervical cancer incidence and mortality have been accurately investigated [62]. However, in the greater part of Europe, evaluation systems are insufficient and nonstandard.

At the same time, cervical cancer prevalence remains at a high level in developing countries of Central and South-East Asia, Africa, and Eastern Europe, where cervical cancer screening programs are not properly implemented due to a variety of reasons (socioeconomic, geographic, etc.). Population coverage by screening program in developing countries ranges between 6 and 8% [67]. In recent years, international recommendations for screening have been developed to include HPV testing, where available [68]. Despite marked advances in knowledge about cervical cancer and effective screening, cervical cancer screening programs have variable efficacy depending on availability of resources, implementation strategies, quality of laboratory and pathology testing, and community awareness [69]. Effective cytological screening of cervical specimens and HPV genotyping require materials and specialists that are complicated and expensive for many low-income countries [70]. Even in developed countries with advanced healthcare systems and long-standing cervical cancer screening modalities, population coverage is not perfect [71].

There is also discrepancy in the frequency of the screening tests among countries and age groups [72]. In developed countries like England and the USA, screening is scheduled every 3 years for women aged 21–29; starting from 30 years old until 65 years old, the screening tests are recommended for every 5 years [73–75]. Results of the population-based survey of adults aged 50–70 in England suggest that although awareness of the purpose of early detection screening is high, awareness that screening can prevent cancer is low across all demographic groups [74].

In most of the developing countries of Africa, Central Asia, South-East Asia, Eastern Europe, screening is scheduled every 5 years or even rarer [72, 76]. However, there are several exclusions. For instance, in Kyrgyzstan, republic of Central Asia, there is no cervical cancer screening program at all [63]. In South Africa, a national cervical screening policy was formulated in 2000 and allowed for three free cervical smear tests, conducted at 10-year intervals from the age of 30 years [72]. This policy has been implemented in some areas; however, there is currently no population-wide screening program in South Africa.

Although the recommended screening modalities for cervical cancer have contributed to a reduction in cervical cancer incidence and mortality due to cervical cancer, the benefits of cervical cancer screening are yet to be fully realized in countries with poorly organized screening programs for women at risk. The updated WHO recommendations for cervical cancer screening and prevention are summarized in Table 1 [21].

It is also noteworthy that even in countries with organized screening services, these benefits are not maximized in underserved, uninsured, and underrepresented populations due to factors such as cost, access problems, anxiety, discomfort with the screening procedure, and fear of cancer or poor health literacy, all of which contribute to poor outcomes for cervical cancer [77].

Incorporation of HPV testing into cervical cancer screening strategies has the potential to allow both increased disease detection and increased length of screening intervals (decreasing harms such as psychosocial impact of screening positive, additional clinical visits and procedures, and treatment of lesions destined to resolve).

Table 1: WHO recommendations on cervical cancer screening and prevention in the low- and middle-income countries.

	Primary prevention: vaccination		Secondary prevention: screening
Methods	Inclusion of HPV vaccine in the national immunization schedule: (i) Bivalent (ii) Tetravalent		(i) Cervical cytology (conventional Pap smear and liquid-based) (ii) Visual inspection with acetic acid (VIA)/visual inspection with Lugol's iodine (VILI) (iii) HPV testing for high-risk HPV types (i.e., types 16, 18,31, 33, 45, and 58)
Target age group (years) and gender	Girls 9–14 years old	Girls over 15 years old	Women 30–49 years old
Frequency and intervals	2 doses 6-month interval	3 doses Bivalent: 0, 1, 6 months; tetravalent: 0, 2, 6 months	(i) Once in life time (ii) Once in 10 years (iii) Once in 5 years (a) VIA/cytology every 3- to 5-year interval; (b) HPV testing minimum every 5-year interval
Programmatic consideration	School-based delivery strategy		(i) Organized program (ii) Unorganized/opportunistic/sporadic initiatives (a) Screen-and-treat approach (b) Screen-diagnose-treat approach

8. HPV Vaccination

Statistical data from the recent years show that utilization of HPV vaccines is very effective for preventing infection and disease related to the specific HPV genotypes [78]. Vaccination programs have been very successfully implemented in many countries all over the world [78, 79].

There are three commercially prophylactic vaccines available; these are Cervarix (a bivalent vaccine against HPV16 and HPV18), Gardasil (a tetravalent against HPV6, 11, 16, and 18), and Gardasil 9 (9-valent vaccine against HPV6, 11, 16, 18, 31, 33, 45, 52, and 58). They are noninfectious subunit vaccines containing viral-like particles (VLP) derived from the assembly of the recombinant expression of L1 major capsid protein of the HPV in yeast (Gardasil) and in insect cells (Cervarix). Administration of the vaccine is carried out by intramuscular injection with three doses of prime/boost series over a 6-month period. Early analysis shows that even a single dose can reduce infection and is effective in preventing the persistent incidence of infection and premalignant neoplasia [80]. The exceptionally strong and lasting antibody response has been well documented; for example, the 100% seroconversion rate in young healthy women, preadolescent boys, and girls with antibody response remains stable for over a decade [81]. The exact molecular mechanism however is still elusive in humans as HPV hosting organism, and presently, there is no human model to study the mechanism except on transgenic mouse models and xenograft models [38]. Screening remains to be the only form of prevention for 2 to 3 generations of women beyond the adolescent target age for vaccination [82].

At the present time, we have an abundance of evidence from multiple countries, with a different level of HPV vaccination coverage and implementation strategies that show the vaccines are effective [78]. In developing countries with long-standing screening programs, catch-up vaccination cohorts and established registration have demonstrated reductions in the diagnosis of CIN in screening women due to vaccination [78]. For example, the researchers from Scotland show a reduction of low- and high-grade CIN associated with high uptake of the HPV bivalent vaccine at the population level [83]. Results from one of the recent studies from Japan demonstrated that women aged 20–24 years who received HPV vaccination had significantly lower rates of abnormal cervical cytology results when compared to those who did not receive the vaccine [79]. An Australian study found that vaccination employing tetravalent HPV vaccine helps to reduce cases of HSIL and LSIL in females [84]. Research findings from Canada suggest that the HPV vaccination was moderately effective in preventing HSIL among adolescents but far less effective in the older age groups, especially among those with a history of abnormal cytology [85].

Taking into consideration the present efforts to increase HPV vaccinations for primary cervical cancer prevention, early detection of precancerous cervical lesions through screening remains to be very important in order to timely diagnose and reduce cervical cancer incidence and mortality. This is especially true in low-income regions where HPV vaccination has not yet implemented and supported at the governmental level [86]. Developed countries, with well-established cervical cancer screening programs, have achieved an impressive reduction in cervical cancer incidence and mortality, while developing countries with lack of HPV vaccination and/or worse modalities of screening programs still have a high level of adverse outcomes [87]. These discrepancies in HPV vaccination envelopment could explain the differences in incidence, prevalence, and mortality linked to cervical cancer in different countries in the world.

HPV vaccination for the prevention of high-risk HPV types is expected to reduce cervical cancer burden [88]. Supporting HPV vaccines' effectiveness against cervical

cancer is difficult due to the long period between initial infection and cancer development. Surrogate markers therefore have been proposed to determine vaccines' effectiveness on a shorter term, such as population-based continuous monitoring of high-grade precursor lesions such as CIN3 [89]. Statistics received from studies that covered large cohorts of women after implementation of Cervarix or Gardasil have shown that both vaccines are effective in order to reduce the frequency of precancerous lesions associated with the vaccine genotypes [90]. On the other hand, even the nonavalent Gardasil vaccine cannot prevent all cervical cancer cases due to type specificity and time of implementation.

There are also apparent limitations and the public health challenges in attempting to implement HPV vaccination programs. These limitations and challenges include the vaccine's type specificity, required to be given prior to exposure, the three-dose schedule, ethical issues in targeting age group of early adolescence, and potential communication challenges around HPV being a sexually transmitted infection [78]. Therefore, large groups of women in advanced age who have not received vaccination are still under the risk of cervical cancer development. Furthermore, HPV screening and vaccination being complementary preventive options are often implemented as separate and noncoordinated public health programs. Therefore, to address this inaccuracy, the recently created "HPV FASTER" protocols aim at combining both strategies with the purpose of accelerating the reduction of cervical cancer incidence and mortality, making the programs both cost-effective and sustainable [91]. The proposal of "HPV FASTER" protocol is to offer HPV vaccination to women in a broad age range of 9 to 45 years irrespective of HPV status.

In developing countries, reduction of cervical cancer incidence and mortality could be achieved only with governmental guidance by the implementation of sustainable and effective screening and vaccination programs.

9. Conclusion

Cervical cancer is associated with considerable morbidity and mortality all over the world. It is well known that one of the main causative agents for cervical cancer is high-risk HPV strains, and this type of malignancy is preventable. High incidence of cervical cancer with considerable mortality is an evidence of HPV infection abundance with the absence of the HPV screening and low public awareness of the problem. Substantial incidence and mortality from cervical cancer make the screening program very important. Enhancing public awareness of underlying causal factors is a priority that should be emphasized for prevention programs. Incorporation of HPV testing into screening strategies has a high potential to decrease morbidity and mortality from cervical cancer. The knowledge of HPV prevalence and type distribution could contribute to the successful vaccination program implementation. The educational health promotion projects for the population should be provided to reinforce the knowledge and conversance of this public health problem. From the review given here, it is clear that the HPV screening along with the vaccination program should be implemented and supported at a governmental level in developing countries with high incidence and mortality of cervical cancer.

Abbreviations

WHO: World Health Organization
HPV: Human papillomavirus
DNA: Deoxyribonucleic acid
CIN: Cervical intraepithelial neoplasia
LSIL: Low-grade squamous intraepithelial lesion
HSIL: High-grade squamous intraepithelial lesion
DNMT: DNA methyltransferases
EMENA: Extended Middle East and North Africa
Pap test: Papanicolaou test
VIA: Visual inspection with acetic acid
VILI: Visual inspection with Lugol's iodine.

Conflicts of Interest

The authors declare that they have no conflicts of interests with respect to this paper.

Authors' Contributions

CC and GA compiled, analyzed, and reviewed data and prepared the manuscript. TU and KK contributed information related to HPV genotypes, epidemiology, and cervical cancer pathogenesis. AA provided intellectual input to contribute towards manuscript preparation and edited the manuscript. All authors reviewed and approved the final manuscript.

Acknowledgments

The authors would like to acknowledge the Nazarbayev University School of Medicine for the support that enabled completion of this review article.

References

[1] M. E. McLaughlin-Drubin and K. Munger, "Viruses associated with human cancer," *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, vol. 1782, no. 3, pp. 127–150, 2008.

[2] J. M. M. Walboomers, M. V. Jacobs, M. M. Manos et al., "Human papillomavirus is a necessary cause of invasive cervical cancer worldwide," *The Journal of Pathology*, vol. 189, no. 1, pp. 12–19, 1999.

[3] A. Asiaf, S. T. Ahmad, S. O. Mohammad, and M. A. Zargar, "Review of the current knowledge on the epidemiology, pathogenesis, and prevention of human papillomavirus infection," *European Journal of Cancer Prevention*, vol. 23, no. 3, pp. 206–224, 2014.

[4] R. Reid, C. R. Stanhope, B. R. Herschman, E. Booth, G. D. Phibbs, and J. P. Smith, "Genital warts and cervical cancer. I. Evidence of an association between subclinical papillomavirus infection and cervical malignancy," *Cancer*, vol. 50, no. 2, pp. 377–387, 1982.

[5] Y. Jing, T. Wang, Z. Chen et al., "Phylogeny and polymorphism in the long control regions E6, E7, and L1 of HPV

Type 56 in women from southwest China," *Molecular Medicine Reports*, vol. 17, no. 5, pp. 7131–7141, 2018.

[6] H.-U. Bernard, R. D. Burk, Z. Chen, K. van Doorslaer, H. Z. Hausen, and E.-M. de Villiers, "Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments," *Virology*, vol. 401, no. 1, pp. 70–79, 2010.

[7] R. D. Burk, A. Harari, and Z. Chen, "Human papillomavirus genome variants," *Virology*, vol. 445, no. 1-2, pp. 232–243, 2013.

[8] S. Pande, N. Jain, B. K. Prusty et al., "Human papillomavirus type 16 variant analysis of E6, E7, and L1 genes and long control region in biopsy samples from cervical cancer patients in North India," *Journal of Clinical Microbiology*, vol. 46, no. 3, pp. 1060–1066, 2008.

[9] V. Ramas, S. Mirazo, S. Bonilla, D. Ruchansky, and J. Arbiza, "Analysis of human papillomavirus 16 E6, E7 genes and Long Control Region in cervical samples from Uruguayan women," *Gene*, vol. 654, pp. 103–109, 2018.

[10] M. Lehoux, C. M. D'Abramo, and J. Archambault, "Molecular mechanisms of human papillomavirus-induced carcinogenesis," *Public Health Genomics*, vol. 12, no. 5-6, pp. 268–280, 2009.

[11] R. P. Insinga, E. J. Dasbach, and E. H. Elbasha, "Epidemiologic natural history and clinical management of Human Papillomavirus (HPV) Disease: a critical and systematic review of the literature in the development of an HPV dynamic transmission model," *BMC Infectious Diseases*, vol. 9, no. 1, p. 119, 2009.

[12] M. Stanley, "Pathology and epidemiology of HPV infection in females," *Gynecologic Oncology*, vol. 117, no. 2, pp. S5–S10, 2010.

[13] S. R. Skinner, C. M. Wheeler, B. Romanowski et al., "Progression of HPV infection to detectable cervical lesions or clearance in adult women: analysis of the control arm of the VIVIANE study," *International Journal of Cancer*, vol. 138, no. 10, pp. 2428–2438, 2016.

[14] P. Brianti, E. De Flammineis, and S. R. Mercuri, "Review of HPV-related diseases and cancers," *New Microbiologica*, vol. 40, no. 2, pp. 80–85, 2017.

[15] L. Mirabello, M. A. Clarke, C. W. Nelson et al., "The intersection of HPV epidemiology, genomics and mechanistic studies of HPV-mediated carcinogenesis," *Viruses*, vol. 10, no. 2, 2018.

[16] A.-B. Moscicki, Y. Ma, C. Wibbelsman et al., "Rate of and risks for regression of cervical intraepithelial neoplasia 2 in adolescents and young women," *Obstetrics and Gynecology*, vol. 116, no. 6, pp. 1373–1380, 2010.

[17] A. F. Rositch, A. E. Burke, R. P. Viscidi, M. I. Silver, K. Chang, and P. E. Gravitt, "Contributions of recent and past sexual partnerships on incident human papillomavirus detection: acquisition and reactivation in older women," *Cancer Research*, vol. 72, no. 23, pp. 6183–6190, 2012.

[18] P. E. Gravitt, A. F. Rositch, M. I. Silver et al., "A cohort effect of the sexual revolution may be masking an increase in human papillomavirus detection at menopause in the United States," *The Journal of Infectious Diseases*, vol. 207, no. 2, pp. 272–280, 2013.

[19] M. R. McCredie, K. J. Sharples, C. Paul et al., "Natural history of cervical neoplasia and risk of invasive cancer in women with cervical intraepithelial neoplasia 3: a retrospective cohort study," *The Lancet Oncology*, vol. 9, no. 5, pp. 425–434, 2008.

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

[21] WHO, *Comprehensive Cervical Cancer Control: A Guide to Essential Practice*, WHO, Geneva, Switzerland, 2nd edition, 2014.

[22] M. Arbyn, A. Anttila, J. Jordan et al., "European guidelines for quality assurance in cervical cancer screening. Second edition--summary document," *Annals of Oncology*, vol. 21, no. 3, pp. 448–458, 2010.

[23] G. Ronco, J. Dillner, K. M. Elfström et al., "Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of four European randomised controlled trials," *The Lancet*, vol. 383, no. 9916, pp. 524–532, 2014.

[24] S. K. Kjær, K. Frederiksen, C. Munk, and T. Iftner, "Long-term absolute risk of cervical intraepithelial neoplasia grade 3 or worse following human papillomavirus infection: role of persistence," *Journal of the National Cancer Institute*, vol. 102, no. 19, pp. 1478–1488, 2010.

[25] E. Argyri, E. Tsimplaki, D. Daskalopoulou et al., "E6/E7 mRNA expression of high-risk HPV types in 849 Greek women," *Anticancer Research*, vol. 33, no. 9, pp. 4007–4011, 2013.

[26] 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 Dec.

[27] B. Zhang, W. Chen, and A. Roman, "The E7 proteins of low- and high-risk human papillomaviruses share the ability to target the pRB family member p130 for degradation," *Proceedings of the National Academy of Sciences*, vol. 103, no. 2, pp. 437–442, 2006.

[28] E. A. White, R. E. Kramer, M. J. A. Tan, S. D. Hayes, J. W. Harper, and P. M. Howley, "Comprehensive analysis of host cellular interactions with human papillomavirus E6 proteins identifies new E6 binding partners and reflects viral diversity," *Journal of Virology*, vol. 86, no. 24, pp. 13174–13186, 2012.

[29] G. Neveu, P. Cassonnet, P.-O. Vidalain et al., "Comparative analysis of virus-host interactomes with a mammalian high-throughput protein complementation assay based on Gaussia princeps luciferase," *Methods*, vol. 58, no. 4, pp. 349–359, 2012.

[30] M. Schiffman, J. Doorbar, N. Wentzensen et al., "Carcinogenic human papillomavirus infection," *Nature Reviews Disease Primers*, vol. 2, no. 1, p. 16086, 2016.

[31] R. Katzenellenbogen, "Telomerase induction in HPV infection and oncogenesis," *Viruses*, vol. 9, no. 7, p. E180, 2017.

[32] H. Jung, B. L. Phillips, and E. K. Chan, "miR-375 activates p21 and suppresses telomerase activity by coordinately regulating HPV E6/E7, E6AP, CIP2A, and 14-3-3ζ," *Molecular Cancer*, vol. 13, no. 1, p. 80, 2014.

[33] C. Moody, "Mechanisms by which HPV induces a replication competent environment in differentiating keratinocytes," *Viruses*, vol. 9, no. 9, 2017.

[34] I. J. Groves and N. Coleman, "Pathogenesis of human papillomavirus-associated mucosal disease," *The Journal of Pathology*, vol. 235, no. 4, pp. 527–538, 2015.

[35] S. S. Lee, R. S. Weiss, and R. T. Javier, "Binding of human virus oncoproteins to hDlg/SAP97, a mammalian homolog of the Drosophila discs large tumor suppressor protein," *Proceedings of the National Academy of Sciences*, vol. 94, no. 13, pp. 6670–6675, 1997.

[36] B. A. Glaunsinger, S. S. Lee, M. Thomas, L. Banks, and R. Javier, "Interactions of the PDZ-protein MAGI-1 with

adenovirus E4-ORF1 and high-risk papillomavirus E6 oncoproteins," *Oncogene*, vol. 19, no. 46, pp. 5270–5280, 2000.

[37] S. Nakagawa and J. M. Huibregtse, "Human Scribble (vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase," *Molecular and Cellular Biology*, vol. 20, no. 21, pp. 8244–8253, 2000.

[38] K. Hoppe-Seyler, F. Bossler, J. A. Braun, A. L. Herrmann, and F. Hoppe-Seyler, "The HPV E6/E7 oncogenes: key factors for viral carcinogenesis and therapeutic targets," *Trends in Microbiology*, vol. 26, no. 2, pp. 158–168, 2018.

[39] W. A. Burgers, L. Blanchon, S. Pradhan, Y. d. Launoit, T. Kouzarides, and F. Fuks, "Viral oncoproteins target the DNA methyltransferases," *Oncogene*, vol. 26, no. 11, pp. 1650–1655, 2007.

[40] D. Hong, F. Ye, W. Lu et al., "Methylation status of the long control region of HPV 16 in clinical cervical specimens," *Molecular Medicine Reports*, vol. 1, pp. 555–560, 2008.

[41] J. Ferlay, I. Soerjomataram, R. Dikshit et al., "Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012," *International Journal of Cancer*, vol. 136, no. 5, pp. E359–E386, 2015.

[42] E. Altobelli, L. Rapacchietta, V. F. Profeta, and R. Fagnano, "HPV-vaccination and cancer cervical screening in 53 WHO European Countries: an update on prevention programs according to income level," *Cancer Medicine*, vol. 8, no. 5, pp. 2524–2534, 2019.

[43] L. Bruni, L. Barrionuevo-Rosas, G. Albero et al., "Human papillomavirus and related diseases in Kazakhstan," Summary Report, ICO Information Centre on HPV and Cancer (HPV Information Centre), Barcelona, Spain, 2014.

[44] International Agency for Research on Cancer, *European Guidelines for Quality Assurance in Cervical Cancer Screening*, International Agency for Research on Cancer, Lyon, France, 2007.

[45] P. A. Cohen, A. Jhingran, A. Oaknin, and L. Denny, "Cervical cancer," *The Lancet*, vol. 393, no. 10167, pp. 169–182, 2019 Jan 12.

[46] F. Bray, J. Lortet-Tieulent, A. Znaor, M. Brotons, M. Poljak, and M. Arbyn, "Patterns and trends in human papillomavirus-related diseases in central and Eastern Europe and central Asia," *Vaccine*, vol. 31, pp. H32–H45, 2013.

[47] J. Ferlay, H.-R. Shin, F. Bray, D. Forman, C. Mathers, and D. M. Parkin, "Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008," *International Journal of Cancer*, vol. 127, no. 12, pp. 2893–2917, 2010.

[48] O. Adegoke, S. Kulasingam, and B. Virnig, "Cervical cancer trends in the United States: a 35-year population-based analysis," *Journal of Women's Health*, vol. 21, no. 10, pp. 1031–1037, 2012.

[49] G. 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.

[50] L. Bruni, M. Diaz, X. Castellsagué, E. Ferrer, F. X. Bosch, and S. de Sanjosé, "Cervical human papillomavirus prevalence in 5 continents: meta-analysis of 1 million women with normal cytological findings," *The Journal of Infectious Diseases*, vol. 202, no. 12, pp. 1789–1799, 2010.

[51] S. de Sanjosé, M. Diaz, X. Castellsagué et al., "Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis," *The Lancet Infectious Diseases*, vol. 7, no. 7, pp. 453–459, 2007.

[52] D. Bansal, A. A. Elmi, S. Skariah et al., "Molecular epidemiology and genotype distribution of Human Papillomavirus (HPV) among Arab women in the State of Qatar," *Journal of Translational Medicine*, vol. 12, no. 1, p. 300, 2014.

[53] L. Niyazmetova, G. Aimagambetova, N. Stambekova et al., "Application of molecular genotyping to determine prevalence of HPV strains in Pap smears of Kazakhstan women," *International Journal of Infectious Diseases*, vol. 54, pp. 85–88, 2017.

[54] M. L. Tornesello, R. Cassese, N. De Rosa et al., "High prevalence of human papillomavirus infection in Eastern European and West African women immigrants in South Italy," *APMIS*, vol. 119, no. 10, pp. 701–709, 2011.

[55] H. De Vuyst, M. R. Parisi, A. Karani et al., "The prevalence of human papillomavirus infection in Mombasa, Kenya," *Cancer Causes and Control*, vol. 21, no. 12, pp. 2309–2313, 2010.

[56] J. Kangmennaang, E. O. Onyango, I. Luginaah, and S. J. Elliott, "The next Sub Saharan African epidemic? A case study of the determinants of cervical cancer knowledge and screening in Kenya," *Social Science and Medicine*, vol. 197, pp. 203–212, 2018.

[57] M. K. Grabowski, P. E. Gravitt, R. H. Gray et al., "Trends and determinants of human papillomavirus concordance among human immunodeficiency virus-positive and -negative heterosexual couples in rakai, Uganda," *Journal of Infectious Diseases*, vol. 215, no. 5, pp. 772–780, 2017.

[58] C. M. Wheeler, "Natural history of human papillomavirus infections, cytologic and histologic abnormalities, and cancer," *Obstetrics and Gynecology Clinics of North America*, vol. 35, no. 4, pp. 519–536, 2008.

[59] 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.

[60] International Agency for Research on Cancer, "Cervix cancer screening. International agency for research on cancer," in *IARC Handbooks of Cancer Prevention*, vol. 10, pp. 1–302, IARC Press, Lyon, France, 2005.

[61] A. Zucchetto, G. Ronco, P. Giorgi Rossi et al., "Screening patterns within organized programs and survival of Italian women with invasive cervical cancer," *Preventive Medicine*, vol. 57, no. 3, pp. 220–226, 2013.

[62] L. Bucchi, F. Baldacchini, S. Mancini et al., "Estimating the impact of an organised screening programme on cervical cancer incidence: a 26-year study from northern Italy," *International Journal of Cancer*, vol. 144, no. 5, 2018.

[63] S. I. Rogovskaya, I. P. Shabalova, I. V. Mikheeva et al., "Human papillomavirus prevalence and type-distribution, cervical cancer screening practices and current status of vaccination implementation in Russian Federation, the Western countries of the former Soviet Union, Caucasus region and Central Asia," *Vaccine*, vol. 31, no. 7, pp. H46–H58, 2013.

[64] L. Peirson, D. Fitzpatrick-Lewis, D. Ciliska, and R. Warren, "Screening for cervical cancer: a systematic review and meta-analysis," *Systematic Reviews*, vol. 2, no. 1, p. 35, 2013.

[65] G. Engholm, J. Ferlay, and N. Christensen, *NORDCAN: Cancer Incidence, Mortality, Prevalence and Survival in the Nordic Countries, Version 6.1*, Cancer Registries, Danish Cancer Society, Copenhagen, Denmark, 2014.

[66] A. Virginia, "Screening for cervical cancer: U.S. Preventive services task force recommendation statement," *Annals of Internal Medicine*, vol. 156, pp. 880–890, 2012.

[67] S. L. Sudenga, A. F. Rositch, W. A. Otieno, and J. S. Smith, "Knowledge, attitudes, practices, and perceived risk of cervical cancer among Kenyan women: brief report," *International Journal of Gynecologic Cancer*, vol. 23, no. 5, pp. 895–899, 2013.

[68] World Health Organization, *WHO Guidance Note: Comprehensive Cervical Cancer Prevention and Control: A Healthier Future for Girls and Women*, World Health Organization, Geneva, Switzerland, 2013.

[69] S. B. Glick, A. R. Clarke, A. Blanchard, and A. K. Whitaker, "Cervical cancer screening, diagnosis and treatment interventions for racial and ethnic minorities: a Systematic Review," *Journal of General Internal Medicine*, vol. 27, no. 8, pp. 1016–1032, 2012.

[70] R. Sankaranarayanan, R. Anorlu, G. Sangwa-Lugoma, and L. A. Denny, "Infrastructure requirements for human papillomavirus vaccination and cervical cancer screening in Sub-Saharan Africa," *Vaccine*, vol. 31, pp. F47–F52, 2013.

[71] A. White, T. D. Thompson, M. C. White et al., "Cancer screening test use—United States, 2015," *MMWR. Morbidity and Mortality Weekly Report*, vol. 66, no. 8, pp. 201–206, 2017.

[72] B. Olson, B. Gribble, J. Dias et al., "Cervical cancer screening programs and guidelines in low- and middle-income countries," *International Journal of Gynecology and Obstetrics*, vol. 134, no. 3, pp. 239–246, 2016.

[73] R. A. Smith, K. S. Andrews, D. Brooks et al., "Cancer screening in the United States, 2017: a review of current American Cancer Society guidelines and current issues in cancer screening," *CA: A Cancer Journal for Clinicians*, vol. 67, no. 2, pp. 100–121, 2017.

[74] K. Canfell, F. Sitas, and V. Beral, "Cervical cancer in Australia and the United Kingdom: comparison of screening policy and uptake, and cancer incidence and mortality," *Medical Journal of Australia*, vol. 185, no. 9, pp. 482–486, 2006.

[75] Centers for Disease Control and Prevention (CDC), "Cervical cancer screening among women aged 18-30 years - United States, 2000-2010," *Morbidity and Mortality Weekly Report*, vol. 61, no. 51-52, pp. 1038–1042, 2013.

[76] R. Gupta, S. Gupta, R. Mehrotra, and P. Sodhani, "Cervical cancer screening in resource-constrained countries: current status and future directions," *Asian Pacific Journal of Cancer Prevention*, vol. 18, no. 6, pp. 1461–1467, 2017.

[77] C. C. Gunderson, E. K. Nugent, D. S. McMeekin, and K. N. Moore, "Distance traveled for treatment of cervical cancer," *International Journal of Gynecological Cancer*, vol. 23, no. 6, pp. 1099–1103, 2013.

[78] J. M. L. Brotherton, "Impact of HPV vaccination: achievements and future challenges," *Papillomavirus Research*, vol. 7, pp. 138–140, 2019.

[79] H. Tanaka, H. Shirasawa, D. Shimizu et al., "Preventive effect of human papillomavirus vaccination on the development of uterine cervical lesions in young Japanese women," *Journal of Obstetrics and Gynaecology Research*, vol. 43, no. 10, pp. 1597–1601, 2017.

[80] J. Schiller and D. Lowy, "Explanations for the high potency of HPV prophylactic vaccines," *Vaccine*, vol. 36, no. 32, pp. 4768–4773, 2018.

[81] M. Nygård, A. Saah, C. Munk et al., "Evaluation of the long-term anti-human papillomavirus 6 (HPV6), 11, 16, and 18 immune responses generated by the quadrivalent HPV vaccine," *Clinical and Vaccine Immunology*, vol. 22, no. 8, pp. 943–948, 2015.

[82] United States Food and Drug Administration, "FDA approves gardasil 9 for prevention of certain cancers caused by five additional types of HPV," August 2018, https://www.esmo.org/Oncology-News/FDA-Approves-Gardasil-9-for-Prevention-of-Certain-Cancers-Caused-by-Five-Additional-Types-of-HPV.

[83] T. Palmer, L. Wallace, K. G. Pollock et al., "Prevalence of cervical disease at age 20 after immunisation with bivalent HPV vaccine at age 12-13 in Scotland: retrospective population study," *BMJ*, vol. 365, p. l1161, 2019.

[84] D. M. Gertig, J. M. Brotherton, A. C. Budd, K. Drennan, G. Chappell, and A. M. Saville, "Impact of a population-based HPV vaccination program on cervical abnormalities: a data linkage study," *BMC Medicine*, vol. 11, no. 1, p. 227, 2013.

[85] S. M. Mahmud, E. V. Kliewer, P. Lambert, S. Bozat-Emre, and A. A. Demers, "Effectiveness of the quadrivalent human papillomavirus vaccine against cervical dysplasia in manitoba, Canada," *Journal of Clinical Oncology*, vol. 32, no. 5, pp. 438–443, 2014.

[86] N. G. Campos, V. Tsu, J. Jeronimo, M. Mvundura, and J. J. Kim, "Evidence-based policy choices for efficient and equitable cervical cancer screening programs in low-resource settings," *Cancer Medicine*, vol. 6, no. 8, pp. 2008–2014, 2017.

[87] M. C. White and F. L. Wong, "Preventing premature deaths from breast and cervical cancer among underserved women in the United States: insights gained from a national cancer screening program," *Cancer Causes and Control*, vol. 26, no. 5, pp. 805–809, 2015.

[88] M. Drolet, É. Bénard, M.-C. Boily et al., "Population-level impact and herd effects following human papillomavirus vaccination programmes: a systematic review and meta-analysis," *The Lancet Infectious Diseases*, vol. 15, no. 5, pp. 565–580, 2015.

[89] C. A. Wong, M. Saraiya, S. Hariri et al., "Approaches to monitoring biological outcomes for HPV vaccination: challenges of early adopter countries," *Vaccine*, vol. 29, no. 5, pp. 878–885, 2011.

[90] S. M. Garland, S. K. Kjaer, N. Muñoz et al., "Impact and effectiveness of the Quadrivalent human papillomavirus vaccine: a systematic review of 10 years of real-world experience," *Clinical Infectious Diseases*, vol. 63, no. 4, pp. 519–527, 2016.

[91] F. X. Bosch, C. Robles, M. Díaz et al., "HPV-FASTER: broadening the scope for prevention of HPV-related cancer," *Nature Reviews Clinical Oncology*, vol. 13, no. 2, pp. 119–132, 2015.

Hindawi
Journal of Oncology
Volume 2019, Article ID 5636272, 12 pages
https://doi.org/10.1155/2019/5636272

Review Article

The Four Horsemen in Colon Cancer

Marco Antonio Hernández-Luna,[1] Sergio López-Briones,[1] and Rosendo Luria-Pérez[2]

[1]*Department of Medicine and Nutrition, University of Guanajuato, Campus Leon, Leon, Guanajuato, Mexico*
[2]*Unit of Investigative Research on Oncological Diseases, Children's Hospital of Mexico Federico Gomez, Mexico City, Mexico*

Correspondence should be addressed to Rosendo Luria-Pérez; rluria@protonmail.com

Received 8 June 2019; Accepted 26 August 2019; Published 29 September 2019

Guest Editor: Hironori Yoshiyama

Worldwide, neoplasms of the gastrointestinal tract have a very high incidence and mortality. Among these, colorectal cancer, which includes colon and rectum malignancies, representing both highest incidence and mortality. While gallbladder cancer, another neoplasm associated to gastrointestinal tract occurs less frequently. Genetic factors, inflammation and nutrition are important risk factors associated with colorectal cancer development. Likewise, pathogenic microorganisms inducing intestinal dysbiosis have become an important scope to determine the role of bacterial infection on tumorigenesis. Interestingly, in human biopsies of different types of gastrointestinal tract cancer, the presence of different bacterial strains, such as *Fusobacterium nucleatum*, *Escherichia coli*, *Bacteroides fragilis* and *Salmonella enterica* have been detected, and it has been considered as a high-risk factor to cancer development. Therefore, pathogens infection could contribute to neoplastic development through different mechanisms; including intestinal dysbiosis, inflammation, evasion of tumoral immune response and activation of pro-tumoral signaling pathways, such as β catenin. Here, we have reviewed the suggested bacterial molecular mechanisms and their possible role on development and progression of gastrointestinal neoplasms, focusing mainly on colon neoplasms, where the bacteria *Fusobacterium nucleatum*, *Escherichia coli*, *Bacteroides fragilis* and *Salmonella enterica* infect.

1. Introduction

Worldwide, neoplasms affecting gastrointestinal tract are among the most frequent in incidence and mortality [1]. Gastrointestinal tract neoplasms are including: colon, rectum, stomach, pancreas, biliary tract and esophagus [2]. The main factors associated with development of gastrointestinal tract malignancies are alcohol consumption and smoking [3–5], high fat diets [6–9]; as well as, ageing, gender and race [10–13]. In addition, pathogenic microorganisms such as viruses and bacteria infecting the gastrointestinal tract, are being studied as possible triggers for development of neoplasms. In this regard, the role of *Helicobacter pylori* in the development of gastric cancer has been extensively studied [14]. However, other bacteria have also been associated with development of gastrointestinal neoplasms, especially in colon, rectum and gallbladder. This review describes the possible roles of *Fusobacterium nucleatum*, *Escherichia coli*, *Bacteroides fragilis* and *Salmonella enterica* on cancer development. These bacteria have been considered as emerging pathogenic bacteria associated with development of colorectal cancer, which includes colon and rectum neoplasms, [15]. Here, we have focused on colon cancer, a neoplasia with a very high incidence on worldwide population, registering in 2018; 850,000 new cases and a mortality rate of 550,000 individuals [1].

2. *Fusobacterium nucleatum*

Fusobacterium nucleatum (*F. nucleatum*) is an adherent and invasive Gram-negative anaerobic bacterium. *F. nucleatum* resides mainly in oral cavity and is usually associated with periodontal disease [16]. Nevertheless, in last years, this bacterium has been detected in primary lesions [17], biopsies [18, 19], and stools [20] of patients with colon cancer, so bacterium has also been linked to development and progression of this neoplasia. In addition, different regions of human colon are colonized by *F. nucleatum* [21]. However,

in patients with colon cancer, *F. nucleatum* has been located mainly on cecum and rectum [22, 23], where it is preferentially localized into tumor tissue [24, 25]. An important factor associated with *F. nucleatum* recruitment into tumor is over-expression of Gal-GalNAc molecules by tumor cells, which promote bacterial adhesion via Fap2 protein [26]. Likewise, high levels of anti-*Fusobacterium* IgA and IgG antibodies have been detected in sera of colon cancer patients [27], which could be used as biomarkers in early diagnosis of this neoplasia. Additionally, infection by *F. nucleatum* has been associated with a low survival of colon cancer patients [28], as well as increased resistance to chemotherapy treatment [29].

Previous studies have reported the association of *F. nucleatum* and colon cancer, although the presence of this bacterium in infected people is highly variable and inconsistent. In this regard, infection with *F. nucleatum* has been detected in 15% of North American population with colon cancer, while more than 60% of infected patients have been found in Chinese population [25, 28, 30, 31]. Interestingly, common characteristics found in all colon cancer patients with *F. nucleatum* infection were microsatellite instability (MSI), methylation phenotype of CpG island (CIMP), as well as *BRAF* and *KRAS* genes mutations [23, 25, 32].

On the other hand, infection with *F. nucleatum* in C57BL/6 $APC^{Min/+}$ mice induced tumorigenesis regardless of colitis development [20], unlike the infection by enterotoxigenic *Bacteroides fragilis*, which initially produces colitis and subsequently tumors [33]. Therefore, several mechanisms inducing tumor by *F. nucleatum* have been proposed, including β catenin signaling pathway activation, which is upregulated in colon cancer [34]. In this pathway, β catenin is phosphorylated by PAK-1 through *F. nucleatum*-TLR4 interaction [35]. Likewise, binding of *F. nucleatum* FadA adhesin to E-cadherin expressed on host cells activates the Wnt/β catenin pathway promoting cell proliferation [36]. Additionally, a significant decrease on expression of TOX family proteins (thymocyte selection-associated high-mobility group box) after *F. nucleatum* infection has been shown [37]. These proteins regulate important cellular functions such as growth, apoptosis, DNA repair and metastatic processes [38]. Interestingly, an important decrease on TOX family proteins expression has been associated with advanced tumors.

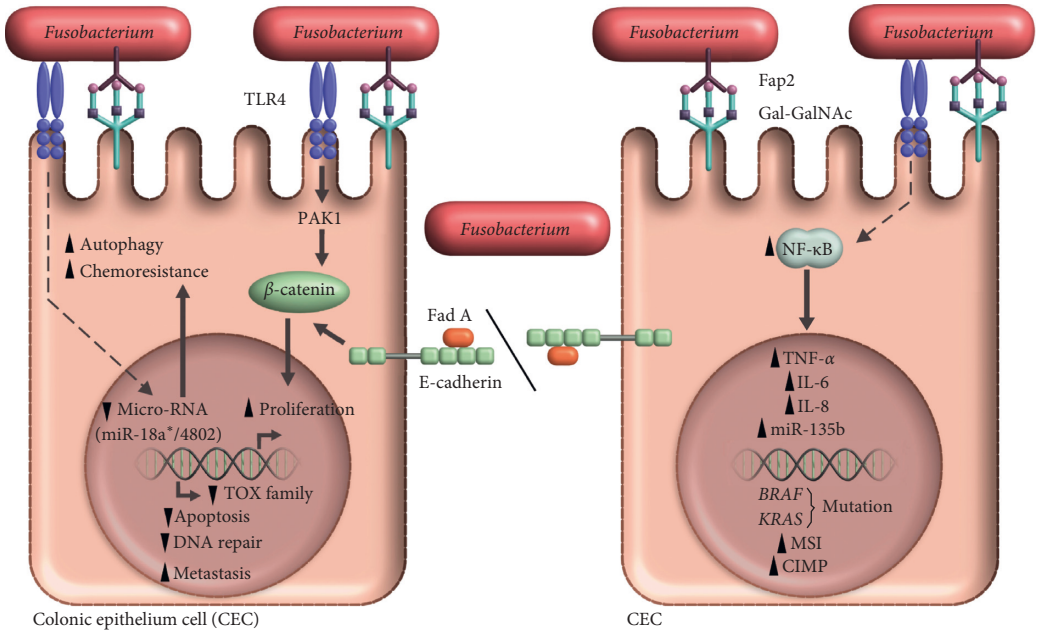
Another mechanism associated with development and progression of colon cancer induced by *F. nucleatum* have been linked to inflammation. Thus, in colon cancer patients infected with *F. nucleatum*, an important increase on TNF-α and IL-10 expression levels have been shown in adenomas, a precursor lesion of colon cancer [17]; while into tumor, IL-6 and IL-8 increased levels were induced by *F. nucleatum*. Both IL-6 and IL-8 are proinflammatory cytokines regulated by NF-κB transcription factor, a link between inflammation and cancer; and NF-κB activation has been also shown in colon cancer [18, 36]. Additionally, *F. nucleatum* infection increased the chemokine CCL20 expression [39], a chemokine related with both colon cancer progression [40], and Th17 + lymphocytes mediated inflammatory response [41]. Likewise, *F. nucleatum* induced inflammation could be regulated by microRNAs, such as miR-135b; because a correlation between *F. nucleatum* and miR-135b overexpression in colon cancer patients has been found [42]. So it has suggested that miR-135b could also be used as a biomarker in early detection of colon cancer [43]. However, the role of *F. nucleatum* in development and progression of colon cancer remains to be understood.

Finally, microsatellite instability (MSI) in colon cancer has been linked to capability to evade immune response by *F. nucleatum* infected tumor cells [31]. In this fact, CD3+ [32], and T CD4+ lymphocytes subsets were decreased into the tumor after *F. nucleatum* infection [37], but proportions of T CD8+, CD45RO+, or FOXP3+ lymphocytes subsets were not modified [32]. In addition, the binding of *F. nucleatum* Fap2 protein with TIGIT [44], a receptor with tyrosine-based inhibitory motif (ITIM) expressed on NK cells [45], leads to an important decreased on lymphocytes infiltration into tumor. This way, tumor is protected from an effective immune cells attack [44]. The proposed mechanisms are summarized in Figure 1(a).

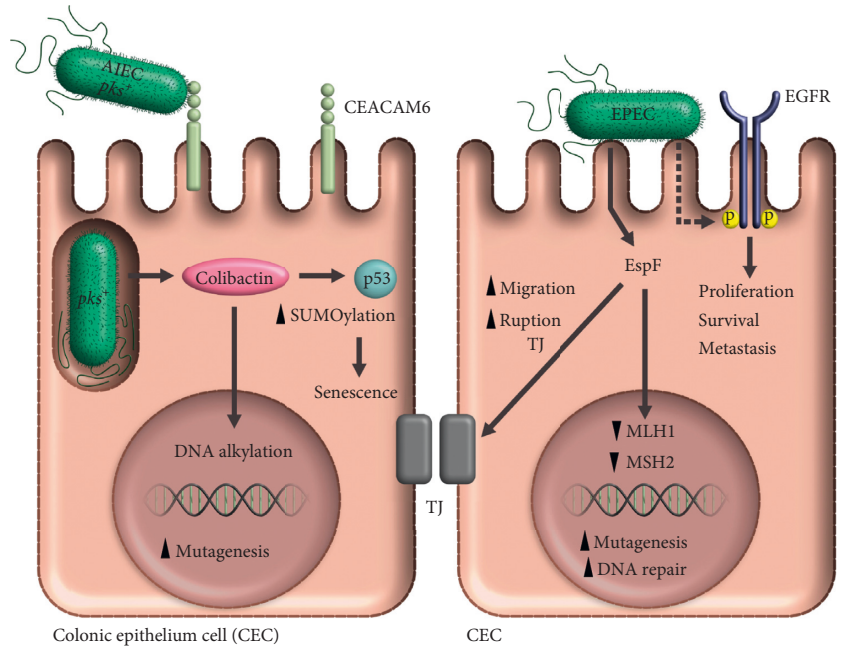
3. *Escherichia coli*

Escherichia coli (*E. coli*) is a Gram-negative bacterium widely distributed in nature, including human intestinal microbiome. The *E. coli* strains are classified into 5 phylogenetic groups: A, B1, B2, D, and E [46]. The main *E. coli* strains associated with human disease belong to B2 group and are also related to colon cancer [47, 48]. To date, the role of pathogenic *E. coli* strains in carcinogenesis is not completely known; however, chronic inflammation in gastrointestinal tract that they promote has been suggested as the trigger mechanism [49]. Because, this chronic inflammation induces pathologies such as Crohn's disease [50], an important risk factor to develop colon cancer [51]. Alternatively, molecular mechanisms induced directly by bacteria have been described. *In vitro* studies have shown that pathogenic strains such as Adherent-Invasive *Escherichia coli* (AIEC) and Enteropathogenic *Escherichia coli* (EPEC), secrete cyclomodulin colibactin [52] and effector protein EspF [53], respectively, which are involved in development and progression of colon cancer. Although the specific mechanisms associated to colon cancer induced by pathogenic *E. coli* have started to become elucidated recently. The molecular mechanisms associated to colon cancer and pathogenic *E. coli* are described in Figure 1(b).

3.1. Adherent-Invasive Escherichia coli. The main pathogenic *E. coli* strain found in tumor tissue from colon cancer patients is Adherent-Invasive *Escherichia coli* or AIEC [54]. On infection, AIEC binds to CEACAM6 (cellular adhesion receptor associated to carcinoembryonic antigen) [55], which is overexpressed on intestinal epithelial cells of both Crohn's disease and colon cancer patients [56]. To date, it is still unknown what induces overexpression of CEACAM6 on the intestinal epithelium in these patients, although it has been shown that IL-6 is related to induction of CEACAM6



(a)



(b)

Figure 1: Oncogenic activity of *Fusobacterium nucleatum* and *Escherichia coli.* (a) Gal-GalNAc overexpression in colon cells promotes the recruitment of *Fusobacterium nucleatum* via the Fap2 protein. After interacting with TLR4, the bacterium activates the protein PAK 1 and in turn, β catenin; the latter can also be activated through the effect of FadA on E-Cadherin. Activation of these signaling pathways promotes cellular proliferation and decreases proteins of the TOX family, which are associated with decreased apoptosis, failures in DNA repair and increased metastases. Likewise, bacterial interaction with TLR4 and its signaling via MYD88, modulates specific microRNAs that activate the autophagy associated with chemotherapy resistance. Also, *Fusobacterium nucleatum* increases the inflammatory process characterized by the presence of cytokines such as TNF-α, IL-6 and IL-8, that are regulated by the transcription factor NF-κB, whose increased activation has also been documented in colon cancer. *Fusobacterium nucleatum* has also been shown to be associated with the development of mutations in the genes *BRAF* and *KRAS*, microsatellite instability (MSI) and the methylation phenotype in CpG islands (CIMP). (b). The Adherent Invasive *Escherichia coli* strain (AIEC) colonizes the intestinal epithelium and uses CEACAM6 to invade the cells of the colonic epithelium; once internalized, it produces colibactin, a cyclomodulin encoded by the *pks* island, that damages DNA by alkylation and promotes the development of mutations. Colibactin also fosters cellular senescence by favoring SUMOylation of p53. Infection with the Enteropathogenic *Escherichia coli* (EPEC) strain, promotes the autophosphorylation of EGFR, a protein associated with an increase in proliferation, survival and metastases; it also decreases the expression of the DNA repair proteins, MLH1 and MSH2, and favors the rupture of tight junctions, a process involved in the development of metastases. All these EPEC-dependent mechanisms have been associated with the EspF protein.

expression [57]. Additionally, it well is known that infection with AIEC stimulates IL-6 production [58]. Taking all these finding together, it is suggested that AIEC could regulate its own infective capacity on intestinal epithelium by both increasing IL-6 production and CEACAM6 expression, and when bacterium has penetrated and invaded the intestinal epithelium, carcinogenesis could be induced through secretion of colibactin, although the true mechanism is not completely known.

3.2. Colibactin and the pks Island. Colibactin is a cyclomodulin encoded in the genotoxic *pks* island (polyketide island). The *pks* island has been found in different *E. coli* strains [59, 60]. Colibactin is a secondary metabolite produced by non-ribosomal peptide synthetase (NRPS)–polyketide synthase (PKS) (NRPS-PKS). Although the synthesis of colibactin is not completely known, it has been shown that a multi-enzymatic complex is required in which several genes of *pks* island participate [61, 62]. The main role of colibactin in carcinogenesis has been associated with DNA damage [63], by acting as an alkylating agent [64, 65], inducing DNA mutations and promoting tumor development.

On the other hand, because of the synthesis of colibactin has not yet been achieved, which has prevented the understanding of the molecular mechanism of this cyclomodulin, most studies designed to evaluate the role of colibactin in carcinogenesis have been limited to study the *pks* island function. *In vitro* infection of cell lines with *E. coli pks* + strains induced a cell cycle arrest, aneuploidy and tetraploidy [66, 67]; as well as, cell senescence via miR-20a-5P, which inhibits the expression of SUMO-specific protease 1 (SENP-1) [52]. SENP-1 is a protein that induce deSUMOylation of p53 [68], an important transcription factor involved in regulation of cellular senescence and development of colon cancer [69]. On the other hand, the role of the *pks* island has been evaluated in experimental murine models. The inflammatory environment in mice intestinal epithelium induced upon infection, both spreading of *E. coli pks*+ and increased risk of colon cancer were produced [49, 70]. In a xenotransplant murine model, infection with *E. coli pks* + strains lead to a significant increase in tumor size, while infection with *E. coli pks*–strains do not [52].

3.3. Enteropathogenic Escherichia coli. Enteropathogenic *Escherichia coli* or EPEC, is the second pathogenic strain of *E. coli* associated to colon cancer [71, 72], and it has been suggested that EPEC infection might be involved in some molecular pathways involved in colorectal tumorigenesis [72]. *In vitro* studies have shown that infection with EPEC stimulates macrophage-inhibitory cytokine-1 (MIC-1) production, a cytokine related to metastasis by inducing both, increasing survival and spreading of tumor cells through a GTPase Rho A-dependent pathway [73]. Likewise, autophosphorylation of EGFR receptor, was induced upon EPEC infection [74]; this is a upstream activator of both prosurvival phosphoinositide 3-kinase/Akt and proinflammatory mitogen-activated protein (MAP) kinase pathways. These molecular mechanisms have been associated with colon cancer [75], and poor prognosis in patients [76].

However, it has been shown that EPEC can degrade EGFR receptor via EspF protein [77]; this effector protein is internalized to epithelial cells through the *E. coli* type III secretion system [78]. Interestingly, this process can be inhibited by EspZ, another protein that is also internalized into epithelial cell through the same secretion system [77]. On the other hand, EspF has also been associated with other mechanism inducing cancer, such as decreasing levels of DNA repair proteins MLH1 and MSH2 (mismatch repair MMR) [53, 71], which are widely related to colon cancer [79]. Further, EspF could also contribute to colon cancer metastasis by promoting detachment and dissemination of tumor cells through rupturing tight junction proteins such as Occludin and Claudin on intestinal epithelium [80].

Finally, other proteins produced by pathogenic *E. coli* strains and related to carcinogenesis have been studied. These proteins include: (1) *Cytolethal distending toxin* (CDT), which blocks cell cycle [81], and induces malignant transformation of epithelial cells [82], (2) *Cycle inhibiting factor* (Cif), which induces nuclear DNA elongation on cells and stimulates DNA synthesis even when infected cells are not actively dividing [83] and (3) *Cytotoxic Necrotizing Factor 1* (CNF1), which induces gene transcription and cellular proliferation by GTPases activation [84].

4. *Bacteroides fragilis*

The *bacteroides* is a normal inhabitant of human intestine and represent about 30% of intestinal microbiota [85]. These bacteria have a very important role on mucosal immune system development [86], and intestinal homeostasis [87]. *Bacteroides fragilis* (*B. fragilis*) is classified within *bacteroides* species and is an anaerobic Gram-negative bacterium colonizing about 0.5% to 2% of whole human intestine [86, 88, 89]. Two *Bacteroides fragilis* strain has been described: (a) non-toxigenic *B. fragilis* or NTBF and (b) toxigenic *B. fragilis* or ETBF, which is characterized by a 6 kb pathogenicity island encoding to a metalloproteinase, also known as *B. fragilis* toxin (BFT) or fragilysin [90], of which 3 isoforms have been identified [91].

It has been shown that while NTBF has a protective effect against the development of colitis and colon cancer [92], ETBF has been associated with a wide variety of clinical manifestations ranging from a simple diarrhea to inflammatory bowel disease and colitis [93], both considered as high-risk factors to develop colon cancer. ETBF has already been associated to colon cancer [88], because bacteria has been detected in stool and biopsies obtained from colon cancer patients [94], particularly in early cancer stages [95]. However, a very low proportion of ETBF has been detected in stools from healthy individuals [96].

Although role of enterotoxigenic *B. fragilis* in development of colon cancer has not been completely described; different studies have shown that carcinogenesis induced by ETBF is through BFT toxin, which is present in ETBF but not in NTBF bacteria strains. BTF toxin is a multifunctional protein; thus, it could induce to tumorigenesis through

several mechanisms including activation of c-Myc [97], and consequently an increase on spermine oxidase (SMO) expression [98], an enzyme increasing reactive oxygen species (ROS), which favors cellular injury and carcinogenesis.

Another possible mechanism of ETBF toxin-mediated carcinogenesis, could be through host immune system dysregulation, inducing the recruitment and accumulation of Treg lymphocytes in intestinal lamina in response to bacteria [99], which subsequently suppress the mucosal Th1 response and polarizing to Th17 lymphocytes response [100] by increasing IL-17 secretion [33]. Interestingly, increased levels of IL-17 have been detected on early weeks post-infection, after that; its expression was decreased. However, in $APC^{Min/+}$ mice, the early and temporary increased on IL-17 was enough to trigger tumorigenesis [101]. On this regards, it has been suggested that activation of Stat3 [102] and NF-κB [103] pathways by immune responding cells and colonic epithelial cells (CECs) may be involved [104]. Furthermore, ETBF also polarizes IL-17-secreting TCR$\gamma\delta$+ T lymphocytes [105], promoting the differentiation and recruitment of myeloid-derived suppressor cells (MDSC) into the tumor [106, 107], which has been associated with a poor prognosis of colon cancer patients [108]. Because IL-17 up regulates CXCL1, CXCL2 and CXCL5 chemokines expression, also has been involved on MDSC recruitment [104]. Additionally, T lymphocyte proliferation is inhibited by high levels of Nitric Oxide (NO), and arginase 1 (Arg1) a potent metabolic enzyme induced and produced by an increase on MDSC population [107], this way several mechanisms of evasion of anti-tumor immune response by tumor cells are generated.

Finally, ETBF could trigger carcinogenesis through β catenin pathway activation, by disrupting the adherent E-cadherin gap junctions, similar than *F. nucleatum*, [109, 110]. The molecular carcinogenic mechanisms of ETBF are summarized in Figure 2(a).

5. *Salmonella enterica*

Salmonella enterica represents a broad range of bacteria, including serotypes such as *Salmonella* Typhi (*S.* Typhi), *Salmonella* Paratyphi (*S.* Paratyphi), *Salmonella* Enteritidis (*S.* Enteritidis) and *Salmonella* Typhimurium (*S.* Typhimurium) [111]. In recent years, development of colon cancer [112], gallbladder cancer [113], and other gastrointestinal tract neoplasms have been associated with *Salmonella enterica* infection. Also, It has been found that bacteria may modulate host immune response [114], promoting carcinogenesis by both DNA damage and increasing proliferation, as well as cell migration through induction of chronic inflammation [115]. At least, two proteins of *Salmonella enterica* have been associated with an increased risk of developing colon cancer. The former is typhoid toxin; a cyclomodulin sharing features with the *E. coli* CDT [116], increasing cellular survival and promoting intestinal dysbiosis [117]. Both mechanisms are involved with development of inflammatory bowel disease and colon cancer [118]. The second protein is AvrA, an effector protein secreted by bacteria through type III Secretion System [119], and it has been detected in stool samples from colon cancer patients [120].

Thus, the main protein of *Salmonella enterica* associated with carcinogenesis is AvrA. It has been suggested that most important role of AvrA in colon cancer may be related to inflammatory and immune response dysregulation, through several mechanisms such as: inhibition of NF-$_K$B signaling pathway [121], inhibition of IL-12, INF-γ and TNF-α secretion [122], inhibition of IL-6 transcription and increasing on IL-10 transcription [123]. On the other hand, AvrA has been associated to tumors on intestinal epithelium through activation of Wnt/β catenin, inducing cellular proliferation [124], by both β catenin phosphorylation (activation) and deubiquitination (decreased degradation) [125]. These mechanism are important in signaling pathway associated with colon cancer development [126]. Likewise, JAK/STAT signaling pathway is activated by AvrA [127], which regulates several mechanisms such as: apoptosis, cellular proliferation and differentiation, as well as inflammatory response, all these important events involved in carcinogenesis [128]. Additionally, the function of p53 transcription factor is affected by AvrA acetyl transferase activity [129], leading to cell cycle arrest and inhibition of apoptosis by decreasing pro-apoptotic proteins (such as Bax), dependent of p53 acetylation [130]. The carcinogenic mechanisms associated to *Salmonella enterica* are summarized in Figure 2(b).

5.1. Salmonella enterica and Gallbladder Cancer. Gallbladder cancer is the main type of neoplasia affecting the biliary tract. Worldwide, the incidence of this neoplasia is low. Interestingly, it has been shown that gallbladder cancer occurs more frequently in geographic regions with a high incidence of *Salmonella* infection [113, 131–134]. Therefore, a greater interest has been generated in searching for a possible association between *Salmonella* infection and development of gallbladder cancer. On this respect, Typhoidal *Salmonella* serotypes as *S.* Typhi and *S.* Paratyphi have been detected in most of the biopsies from patients with gallbladder cancer [113, 135–137], however, DNA traces of Non-typhoidal *Salmonella* serotypes as *S.* Typhimurium and *S.* Choleraesuis have also been found in gallbladder cancer biopsies [135]. These findings have suggested that *Salmonella* (which may be undetected for years, because it can produce biofilm on cholesterol biliary stones [138]), could represent an important risk factor in development of gallbladder cancer [132], because inflammation and epithelial injury associated to cholelithiasis is induced by *Salmonella* [139] and cholelithiasis is a common clinical manifestation in most patients with gallbladder cancer [137]. However, the mechanism triggering carcinogenesis by *Salmonella enterica* in gallbladder is not completely known, but it has been suggested that a chronic inflammation of gallbladder is induced [140], after bacteria arrival to gallbladder from either blood circulation or bile [141].

Additionally, recruitment of some immune cells, including activated macrophages expressing COX-2 is increased upon *Salmonella enterica* infection [142]. COX-2 is an important enzyme that promotes the development of gastrointestinal tract tumors [143, 144]. Also, bacteria induced inflammation leads to mutations of *TP53* gene,

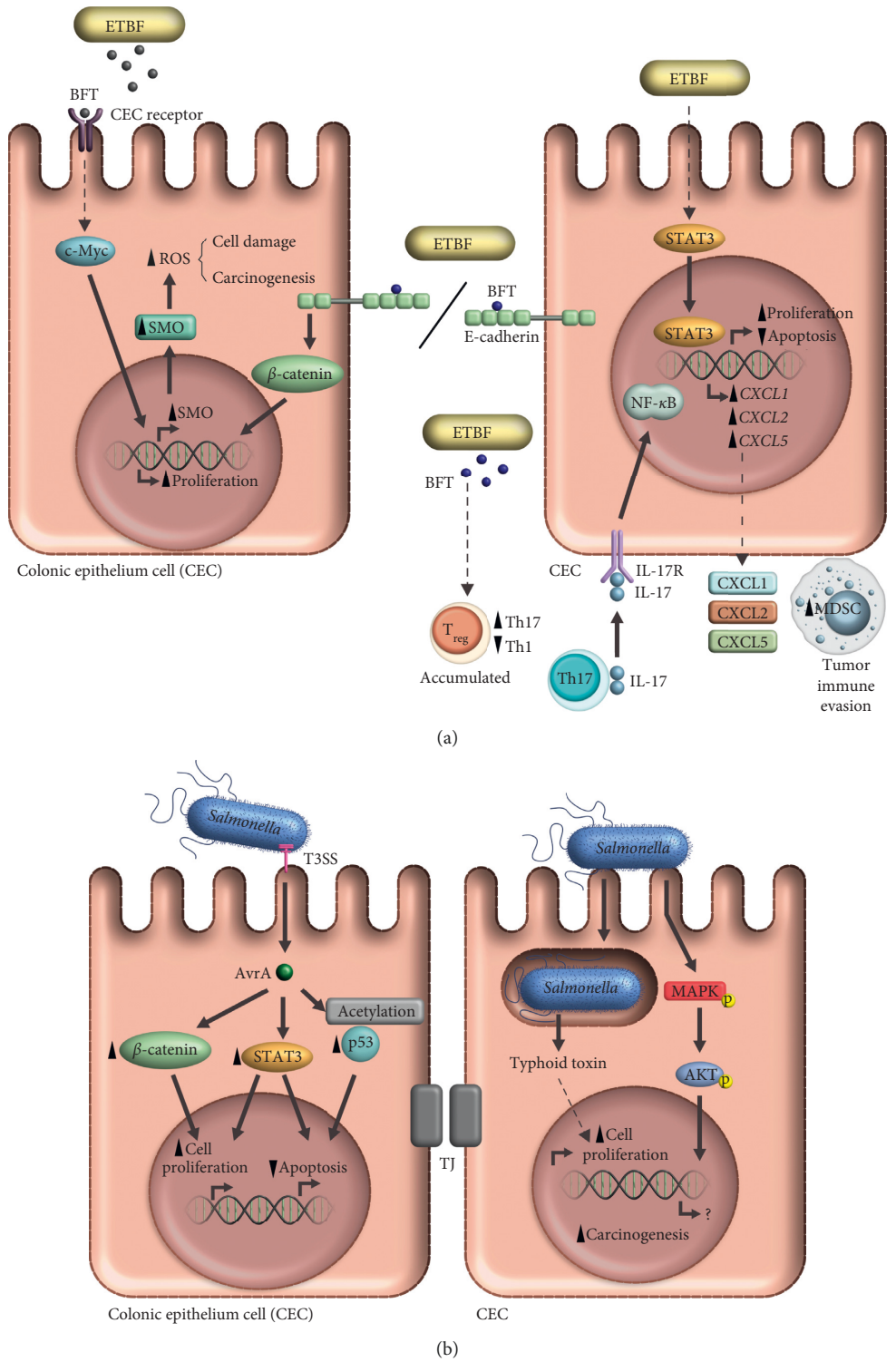


Figure 2: Oncogenic activity of *Bacteroides fragilis* and *Salmonella enterica*. (a) Enterotoxigenic *Bacteroides fragilis* (ETBF) stimulates carcinogenesis in colonic epithelium through the BFT toxin. This toxin leads to an increase in reactive oxygen species (ROS) by inducing spermine oxidase expression via c-Myc. Likewise, BFT cuts E-cadherin, thus activating β catenin which stimulates cellular proliferation. BFT also modulates the host's immune response by promoting Treg lymphocytes to polarize the response to Th17 lymphocytes, thus increasing IL-17 secretion which in turn, activates NF-κB in the colonic epithelium; this results in the secretion of the chemokines CXCL1, CXCL2 and CXCL5 that recruit MDSC, thus favoring evasion from the immune response. The presence of ETBF has also been associated with STAT3 activation. (b). *Salmonella enterica* releases two proteins that promote carcinogenesis: the typhoid toxin that induces cellular proliferation, and the AvrA protein that is internalized via the Type 3 Secretion System(T3SS). AvrA activates the β catenin and STAT3 pathways, and also causes the acetylation of p53. Additionally, *Salmonella enterica* leads to the activation of the MAPK/AKT pathway. The activation of these pathways promotes an increase in proliferation and cellular differentiation, and decreases apoptosis.

increasing the risk of developing gallbladder cancer [145]. Finally, *in vitro* infection of cell lines and gallbladder organoids with *S.* Typhimurium, led to malignant transformation though MAPK and AKT signaling pathways activation. Similarly, *in vivo* activation of these signaling pathways resulted in tumor development in mice [134].

6. Conclusions

Recently, the number of publications referring an association between pathogenic bacteria and development of gastrointestinal tumors, has increased exponentially. The best example and widely reported is *Helicobacter pylori* and gastric cancer. However, emerging bacteria such as *Fusobacterium nucleatum*, *Escherichia coli*, *Bacteroides fragilis* and *Salmonella enterica* have also been involved in development of cancer, particularly colon cancer.

In this review, it is suggested that infection by pathogenic bacteria may be a high-risk factor associated with the development of neoplasms in gastrointestinal tract. Mechanisms such as, inflammation, modulation and evasion of immune response and activation of signaling pathways, such as the β-catenin pathway; all are potential triggers of carcinogenesis.

The inducing tumor mechanisms can be evaluated in murine models, such as $APC^{Min/+}$, a specific mice model to study intestinal tumorigenesis [146]. In this experimental model, developing colon cancer mechanisms by *Fusobacterium nucleatum*, *Escherichia coli*, *Bacteroides fragilis* and *Salmonella enterica* have been identified. However, effects of coinfection with these bacteria and tumor development remains to be analyzed, because ETBF and *E. coli pks* + strains have been found simultaneously in patients with adenomatous polyps, a precursor lesion of colon cancer [147]. Nevertheless, ETBF is a very common bacterium in colon cancer patients but also in healthy individuals [96], so it remains to be elucidated whether ETBF has a role on induction of carcinogenesis. Another possible mechanism through bacteria may trigger cancer is by biofilm. This structure produced by a community of bacteria, more common in ascending colon [148], could increases carcinogenic metabolites concentration, such as polyamines [149], which are related to an important increase on reactive oxygen species. In addition, biofilm has been associated with decreased expression of E-cadherin on colonic epithelial cell, an over activation of IL-6 and Stat3 in epithelial cell [148], all these mechanisms are involved in colon cancer. The mechanisms above described, are used by *Fusobacterium nucleatum*, *Escherichia coli*, *Bacteroides fragilis* and *Salmonella enterica*. Therefore, further studies are required to understand the specific roles of these four bacteria in development of neoplasms on gastrointestinal tract, specifically in colon cancer.

7. Future Perspectives

Worldwide, colon cancer has very high incidence and mortality. Here we have described that infection with either bacteria such as *F. nucleatum*, *E. coli*, *B. fragilis* or *S. enterica* represent an important risk factor that promote cell transformation (carcinogenesis). In this regards, detection of promoting carcinogenesis bacterial proteins, such as cyclomodulin, colibactin, BFT, AvrA or EspF may be used as a biomarker for early detection of colon cancer, as it has been proposed for Fap2 [150]. Because early detection of tumor can increase both healing and survival. Moreover, it would generate new and appropriate strategies to block bacterial proteins activity, thus complementing the traditional treatment to neoplasms of gastrointestinal tract.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Acknowledgments

R. L. P. Acknowledges support from CONACYT (CB-2013-01-222446, INFR-2015-01-255341, PN-2015-01-1537) and Federal Funds (HIM-2016-114 SSA. 1333, HIM-2017-041 SSA. 1325).

References

[1] F. Bray, J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, and A. Jemal, "Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries," *CA: A Cancer Journal for Clinicians*, vol. 68, no. 6, pp. 394–424, 2018.

[2] M. F. Bijlsma, A. Sadanandam, P. Tan, and L. Vermeulen, "Molecular subtypes in cancers of the gastrointestinal tract," *Nature Reviews Gastroenterology & Hepatology*, vol. 14, no. 6, pp. 333–342, 2017.

[3] S.-Y. Park, L. R. Wilkens, V. W. Setiawan, K. R. Monroe, C. A. Haiman, and L. Le Marchand, "Alcohol intake and colorectal cancer risk in the multiethnic cohort study," *American Journal of Epidemiology*, vol. 188, no. 1, pp. 67–76, 2019.

[4] G. Vanella, L. Archibugi, S. Stigliano, and G. Capurso, "Alcohol and gastrointestinal cancers," *Current Opinion in Gastroenterology*, vol. 35, no. 2, pp. 107–113, 2019.

[5] I. O. Fagunwa, M. B. Loughrey, and H. G. Coleman, "Alcohol, smoking and the risk of premalignant and malignant colorectal neoplasms," *Best Practice & Research Clinical Gastroenterology*, vol. 31, no. 5, pp. 561–568, 2017.

[6] K. Triff, M. W. McLean, E. Callaway, J. Goldsby, I. Ivanov, and R. S. Chapkin, "Dietary fat and fiber interact to uniquely modify global histone post-translational epigenetic programming in a rat colon cancer progression model," *International Journal of Cancer*, vol. 143, no. 6, pp. 1402–1415, 2018.

[7] S. Nadella, J. Burks, A. Al-Sabban et al., "Dietary fat stimulates pancreatic cancer growth and promotes fibrosis of the tumor microenvironment through the cholecystokinin receptor," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 315, no. 5, pp. G699–G712, 2018.

[8] S. Arita, Y. Kinoshita, K. Ushida, A. Enomoto, and K. Inagaki-Ohara, "High-fat diet feeding promotes stemness and precancerous changes in murine gastric mucosa mediated by leptin receptor signaling pathway," *Archives of Biochemistry and Biophysics*, vol. 610, pp. 16–24, 2016.

[9] A. J. Fowler, A. L. Richer, R. M. Bremner, and L. J. Inge, "A high-fat diet is associated with altered adipokine production and a more aggressive esophageal adenocarcinoma

phenotype in vivo," *The Journal of Thoracic and Cardiovascular Surgery*, vol. 149, no. 4, pp. 1185–1191, 2015.

[10] A. C. Tramontano, R. Nipp, N. D. Mercaldo, C. Y. Kong, D. Schrag, and C. Hur, "Survival disparities by race and ethnicity in early esophageal cancer," *Digestive Diseases and Sciences*, vol. 63, no. 11, pp. 2880–2888, 2018.

[11] H. Ashktorab, S. S. Kupfer, H. Brim, and J. M. Carethers, "Racial disparity in gastrointestinal cancer risk," *Gastroenterology*, vol. 153, no. 4, pp. 910–923, 2017.

[12] G. Luo, Y. Zhang, P. Guo, L. Wang, Y. Huang, and K. Li, "Global patterns and trends in stomach cancer incidence: age, period and birth cohort analysis," *International Journal of Cancer*, vol. 141, no. 7, pp. 1333–1344, 2017.

[13] M. Øines, L. M. Helsingen, M. Bretthauer, and L. Emilsson, "Epidemiology and risk factors of colorectal polyps," *Best Practice & Research Clinical Gastroenterology*, vol. 31, no. 4, pp. 419–424, 2017.

[14] H. Maleki Kakelar, A. Barzegari, J. Dehghani et al., "Pathogenicity of Helicobacter pylori in cancer development and impacts of vaccination," *Gastric Cancer*, vol. 22, no. 1, pp. 23–36, 2019.

[15] K. Tamas, A. M. E. Walenkamp, E. G. E. de Vries et al., "Rectal and colon cancer: not just a different anatomic site," *Cancer Treatment Reviews*, vol. 41, no. 8, pp. 671–679, 2015.

[16] Y. W. Han, "*Fusobacterium nucleatum*: a commensal-turned pathogen," *Current Opinion in Microbiology*, vol. 23, pp. 141–147, 2015.

[17] A. N. McCoy, F. Araujo-Perez, A. Azcarate-Peril, J. J. Yeh, R. S. Sandler, and T. O. Keku, "Fusobacterium is associated with colorectal adenomas," *PLoS One*, vol. 8, no. 1, Article ID e53653, 2013.

[18] M. Castellarin, R. L. Warren, J. D. Freeman et al., "*Fusobacterium nucleatum* infection is prevalent in human colorectal carcinoma," *Genome Research*, vol. 22, no. 2, pp. 299–306, 2012.

[19] A. D. Holt, D. Gevers, C. S. Pedamallu et al., "Genomic analysis identifies association of Fusobacterium with colorectal carcinoma," *Genome Research*, vol. 22, no. 2, pp. 292–298, 2012.

[20] A. D. Baselga, E. Chun, L. Robertson et al., "*Fusobacterium nucleatum* potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment," *Cell Host & Microbe*, vol. 14, no. 2, pp. 207–215, 2013.

[21] J. El-Omar, A. White, C. Ambrose, J. McDonald, and E. Allen-Vercoe, "Phenotypic and genotypic analyses of clinical *Fusobacterium nucleatum* and *Fusobacterium periodonticum* isolates from the human gut," *Anaerobe*, vol. 14, no. 6, pp. 301–309, 2008.

[22] K. Mima, Y. Cao, A. T. Chan et al., "*Fusobacterium nucleatum* in colorectal carcinoma tissue according to tumor location," *Clinical and Translational Gastroenterology*, vol. 7, no. 11, Article ID e200, 2016.

[23] M. Ito, S. Kanno, K. Nosho et al., "Association of *Fusobacterium nucleatum* with clinical and molecular features in colorectal serrated pathway," *International Journal of Cancer*, vol. 137, no. 6, pp. 1258–1268, 2015.

[24] H. J. Yoshii, J. H. Kim, J. M. Bae, H. J. Kim, N.-Y. Cho, and G. H. Kang, "Prognostic impact of *Fusobacterium nucleatum* depends on combined tumor location and microsatellite instability status in stage II/III colorectal cancers treated with adjuvant chemotherapy," *Journal of Pathology and Translational Medicine*, vol. 53, no. 1, pp. 40–49, 2019.

[25] T. Tahara, E. Yamamoto, H. Suzuki et al., "*Fusobacterium* in colonic flora and molecular features of colorectal carcinoma," *Cancer Research*, vol. 74, no. 5, pp. 1311–1318, 2014.

[26] J. Shureiqi, J. E. M. Emgård, G. Zamir et al., "Fap2 mediates *Fusobacterium nucleatum* colorectal adenocarcinoma enrichment by binding to tumor-expressed Gal-GalNAc," *Cell Host & Microbe*, vol. 20, no. 2, pp. 215–225, 2016.

[27] H. F. Mellul, L. F. Li, S. H Guo et al., "Evaluation of antibody level against *Fusobacterium nucleatum* in the serological diagnosis of colorectal cancer," *Scientific Reports*, vol. 6, Article ID 33440, 2016.

[28] K. Mima, R. Nishihara, Z. R. Qian et al., "*Fusobacterium nucleatum* in colorectal carcinoma tissue and patient prognosis," *Gut*, vol. 65, no. 12, pp. 1973–1980, 2016.

[29] T. Kostic, F. Guo, Y. Yu et al., "*Fusobacterium nucleatum* promotes chemoresistance to colorectal cancer by modulating autophagy," *Cell*, vol. 170, no. 3, pp. 548–563, 2017.

[30] Y.-Y. Chen, Q.-X. Ge, J. Cao et al., "Association of *Fusobacterium nucleatum* infection with colorectal cancer in Chinese patients," *World Journal of Gastroenterology*, vol. 22, no. 11, pp. 3227–3233, 2016.

[31] T. Hamada, X. Zhang, K. Mima et al., "*Fusobacterium nucleatum* in colorectal cancer relates to immune response differentially by tumor microsatellite instability status," *Cancer Immunology Research*, vol. 6, no. 11, pp. 1327–1336, 2018.

[32] K. Song, Y. Sukawa, R. Nishihara et al., "*Fusobacterium nucleatum* and T Cells in colorectal carcinoma," *JAMA Oncology*, vol. 1, no. 5, pp. 653–661, 2015.

[33] S. Kostic, K.-J. Rhee, E. Albesiano et al., "A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses," *Nature Medicine*, vol. 15, no. 9, pp. 1016–1022, 2009.

[34] A. Housseau, F. Amerizadeh, S. ShahidSales et al., "Therapeutic potential of targeting Wnt/β-catenin pathway in treatment of colorectal cancer: rational and progress," *Journal of Cellular Biochemistry*, vol. 118, no. 8, pp. 1979–1983, 2017.

[35] Y. Chen, Y. Peng, J. Yu et al., "Invasive *Fusobacterium nucleatum* activates beta-catenin signaling in colorectal cancer via a TLR4/P-PAK1 cascade," *Oncotarget*, vol. 8, no. 19, pp. 31802–31814, 2017.

[36] M. R. Rubinstein, X. Wang, W. Liu, Y. Hao, G. Cai, and Y. W. Han, "*Fusobacterium nucleatum* promotes colorectal carcinogenesis by modulating E-cadherin/β-catenin signaling via its FadA adhesin," *Cell Host & Microbe*, vol. 14, no. 2, pp. 195–206, 2013.

[37] T. Chen, Q. Li, X. Zhang et al., "TOX expression decreases with progression of colorectal cancers and is associated with CD4 T-cell density and *Fusobacterium nucleatum* infection," *Human Pathology*, vol. 79, pp. 93–101, 2018.

[38] X. Yu and Z. Li, "TOX gene: a novel target for human cancer gene therapy," *American Journal of Cancer Research*, vol. 5, no. 12, pp. 3516–3524, 2015.

[39] X. Ye, R. Wang, R. Bhattacharya, D. R. Boulbes et al., "*Fusobacterium nucleatum* subspecies *animalis* influences proinflammatory cytokine expression and monocyte activation in human colorectal tumors," *Cancer Prevention Research*, vol. 10, no. 7, pp. 398–409, 2017.

[40] V. O. Petrosino, C. Rubie, K. Kölsch et al., "CCR6/CCL20 chemokine expression profile in distinct colorectal malignancies," *Scandinavian Journal of Immunology*, vol. 78, no. 3, pp. 298–305, 2013.

[41] C.-C. Chin, C.-N. Chen, H.-C. Kuo et al., "Interleukin-17 induces CC chemokine receptor 6 expression and cell migration in colorectal cancer cells," *Journal of Cellular Physiology*, vol. 230, no. 7, pp. 1430–1437, 2015.

[42] M. A. Proença, J. M. Biselli, M. Succi et al., "Relationship between *Fusobacterium nucleatum*, inflammatory mediators

and microRNAs in colorectal carcinogenesis," *World Journal of Gastroenterology*, vol. 24, no. 47, pp. 5351–5365, 2018.

[43] K. Lee and L. R. Ferguson, "MicroRNA biomarkers predicting risk, initiation and progression of colorectal cancer," *World Journal of Gastroenterology*, vol. 22, no. 33, pp. 7389–7401, 2016.

[44] C. Gur, Y. Ibrahim, B. Isaacson et al., "Binding of the Fap2 protein of *Fusobacterium nucleatum* to human inhibitory receptor TIGIT protects tumors from immune cell attack," *Immunity*, vol. 42, no. 2, pp. 344–355, 2015.

[45] Y. Shussman, H. Peng, R. Sun et al., "Contribution of inhibitory receptor TIGIT to NK cell education," *Journal of Autoimmunity*, vol. 81, pp. 1–12, 2017.

[46] R. R. Chaudhuri and I. R. Henderson, "The evolution of the *Escherichia coli* phylogeny," *Infection, Genetics and Evolution*, vol. 12, no. 2, pp. 214–226, 2012.

[47] N. P. Deshpande, M. R. Wilkins, H. M. Mitchell, and N. O. Kaakoush, "Novel genetic markers define a subgroup of pathogenic *Escherichia coli* strains belonging to the B2 phylogenetic group," *FEMS Microbiology Letters*, vol. 362, no. 22, 2015.

[48] A. Swidsinski, M. Khilkin, D. Kerjaschki et al., "Association between intraepithelial *Escherichia coli* and colorectal cancer," *Gastroenterology*, vol. 115, no. 2, pp. 281–286, 1998.

[49] J. C. Arthur, E. Perez-Chanona, M. Mühlbauer et al., "Intestinal inflammation targets cancer-inducing activity of the microbiota," *Science*, vol. 338, no. 6103, pp. 120–123, 2012.

[50] A. Rhodes, J. Boudeau, P. Bulois et al., "High prevalence of adherent-invasive *Escherichia coli* associated with ileal mucosa in Crohn's disease," *Gastroenterology*, vol. 127, no. 2, pp. 412–421, 2004.

[51] M. C. Mattar, D. Lough, M. J. Pishvaian, and A. Charabaty, "Current management of inflammatory bowel disease and colorectal cancer," *Gastrointestinal Cancer Research*, vol. 4, no. 2, pp. 53–61, 2011.

[52] A. Cougnoux, G. Dalmasso, R. Martinez et al., "Bacterial genotoxin colibactin promotes colon tumour growth by inducing a senescence-associated secretory phenotype," *Gut*, vol. 63, no. 12, pp. 1932–1942, 2014.

[53] O. D. Pezet, K. M. Scanlon, and M. S. Donnenberg, "An *Escherichia coli* effector protein promotes host mutation via depletion of DNA mismatch repair proteins," *MBio*, vol. 4, no. 3, Article ID e00152, 2013.

[54] E. Buc, D. Dubois, P. Sauvanet et al., "High prevalence of mucosa-associated E. coli producing cyclomodulin and genotoxin in colon cancer," *PLoS One*, vol. 8, no. 2, Article ID e56964, 2013.

[55] N. Barnich, F. A. Carvalho, A.-L. Glasser et al., "CEACAM6 acts as a receptor for adherent-invasive *E. coli*, supporting ileal mucosa colonization in Crohn disease," *Journal of Clinical Investigation*, vol. 117, no. 6, pp. 1566–1574, 2007.

[56] K. S. Darfeuille-Michaud, J.-T. Kim, S.-J. Lee et al., "Overexpression and clinical significance of carcinoembryonic antigen-related cell adhesion molecule 6 in colorectal cancer," *Clinica Chimica Acta*, vol. 415, pp. 12–19, 2013.

[57] R. Kim, G. H. Watzig, S. Tiwari, S. Rose-John, and H. Kalthoff, "Interleukin-6 trans-signaling increases the expression of carcinoembryonic antigen-related cell adhesion molecules 5 and 6 in colorectal cancer cells," *BMC Cancer*, vol. 15, p. 975, 2015.

[58] P. Lapaquette, M.-A. Bringer, and A. Darfeuille-Michaud, "Defects in autophagy favour adherent-invasive *Escherichia coli* persistence within macrophages leading to increased pro-inflammatory response," *Cellular Microbiology*, vol. 14, no. 6, pp. 791–807, 2012.

[59] E. Lee and Y. Lee, "Prevalence of *Escherichia coli* carrying *pks* islands in bacteremia patients," *Annals of Laboratory Medicine*, vol. 38, no. 3, pp. 271–273, 2018.

[60] J. R. Johnson, B. Johnston, M. A. Kuskowski, J.-P. Nougayrede, and E. Oswald, "Molecular epidemiology and phylogenetic distribution of the *Escherichia coli pks* genomic island," *Journal of Clinical Microbiology*, vol. 46, no. 12, pp. 3906–3911, 2008.

[61] S. G. Van Lanen, "SAM cycles up for colibactin," *Nature Chemical Biology*, vol. 13, no. 10, pp. 1059–1061, 2017.

[62] L. Zha, Y. Jiang, M. T. Henke et al., "Colibactin assembly line enzymes use S-adenosylmethionine to build a cyclopropane ring," *Nature Chemical Biology*, vol. 13, no. 10, pp. 1063–1065, 2017.

[63] M. I. Vizcaino and J. M. Crawford, "The colibactin warhead crosslinks DNA," *Nature Chemistry*, vol. 7, no. 5, pp. 411–417, 2015.

[64] E. P. Balskus, "Colibactin: understanding an elusive gut bacterial genotoxin," *Natural Product Reports*, vol. 32, no. 11, pp. 1534–1540, 2015.

[65] M. R. Wilson, Y. Jiang, P. W. Villalta et al., "The human gut bacterial genotoxin colibactin alkylates DNA," *Science*, vol. 363, no. 6428, 2019.

[66] G. Cuevas-Ramos, C. R. Petit, I. Marcq, M. Boury, E. Oswald, and J.-P. Nougayrede, "*Escherichia coli* induces DNA damage in vivo and triggers genomic instability in mammalian cells," *Proceedings of the National Academy of Sciences*, vol. 107, no. 25, pp. 11537–11542, 2010.

[67] J.-P. Nougayrede, S. Homburg, F. Taieb et al., "*Escherichia coli* induces DNA double-strand breaks in eukaryotic cells," *Science*, vol. 313, no. 5788, pp. 848–851, 2006.

[68] K. E. Yates, G. A. Korbel, M. Shtutman, I. B. Roninson, and D. DiMaio, "Repression of the SUMO-specific protease Senp1 induces p53-dependent premature senescence in normal human fibroblasts," *Aging Cell*, vol. 7, no. 5, pp. 609–621, 2008.

[69] A. K. Pandurangan, T. Divya, K. Kumar, V. Dineshbabu, B. Velavan, and G. Sudhandiran, "Colorectal carcinogenesis: insights into the cell death and signal transduction pathways: a review," *World Journal of Gastrointestinal Oncology*, vol. 10, no. 9, pp. 244–259, 2018.

[70] J. C. Arthur, R. Z. Gharaibeh, M. Muhlbauer et al., "Microbial genomic analysis reveals the essential role of inflammation in bacteria-induced colorectal cancer," *Nature Communications*, vol. 5, p. 4724, 2014.

[71] O. D. Maddocks, A. J. Short, M. S. Donnenberg, S. Bader, and D. J. Harrison, "Attaching and effacing *Escherichia coli* downregulate DNA mismatch repair protein in vitro and are associated with colorectal adenocarcinomas in humans," *PLoS One*, vol. 4, no. 5, Article ID e5517, 2009.

[72] A. Magdy, M. Elhadidy, M. E. Abd Ellatif et al., "Enteropathogenic *Escherichia coli* (EPEC): does it have a role in colorectal tumourigenesis? a prospective cohort study," *International Journal of Surgery*, vol. 18, pp. 169–173, 2015.

[73] H. J. Choi, J. Kim, K. H. Do, S.-H. Park, and Y. Moon, "Enteropathogenic *Escherichia coli*-induced macrophage inhibitory cytokine 1 mediates cancer cell survival: an in vitro implication of infection-linked tumor dissemination," *Oncogene*, vol. 32, no. 41, pp. 4960–4969, 2013.

[74] J. L. Roxas, A. Koutsouris, and V. K. Viswanathan, "Enteropathogenic *Escherichia coli*-induced epidermal growth factor receptor activation contributes to physiological

alterations in intestinal epithelial cells," *Infection and Immunity*, vol. 75, no. 5, pp. 2316–2324, 2007.

[75] Z. Chen, S. Gao, D. Wang, D. Song, and Y. Feng, "Colorectal cancer cells are resistant to anti-EGFR monoclonal antibody through adapted autophagy," *American Journal of Translational Research*, vol. 8, no. 2, pp. 1190–1196, 2016.

[76] M. De Robertis, L. Loiacono, C. Fusilli et al., "Dysregulation of EGFR pathway in EphA2 cell subpopulation significantly associates with poor prognosis in colorectal cancer," *Clinical Cancer Research*, vol. 23, no. 1, pp. 159–170, 2017.

[77] J. L. Garcia-Foncillas, K. Ryan, G. Vedantam, and V. K. Viswanathan, "Enteropathogenic *Escherichia coli* dynamically regulates EGFR signaling in intestinal epithelial cells," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 307, no. 3, pp. G374–G380, 2014.

[78] S. J. Elliott, V. Sperandio, J. A. Giron et al., "The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE- and non-LEE-encoded virulence factors in enteropathogenic and enterohemorrhagic *Escherichia coli*," *Infection and Immunity*, vol. 68, no. 11, pp. 6115–6126, 2000.

[79] M. S. Pino and D. C. Chung, "Microsatellite instability in the management of colorectal cancer," *Expert Review of Gastroenterology & Hepatology*, vol. 5, no. 3, pp. 385–399, 2011.

[80] J. Peralta-Ramirez, J. M. Hernandez, R. Manning-Cela et al., "EspF Interacts with nucleation-promoting factors to recruit junctional proteins into pedestals for pedestal maturation and disruption of paracellular permeability," *Infection and Immunity*, vol. 76, no. 9, pp. 3854–3868, 2008.

[81] T. Fais, J. Delmas, A. Serres, R. Bonnet, and G. Dalmasso, "Impact of CDT toxin on human diseases," *Toxins*, vol. 8, no. 7, 2016.

[82] V. Graillot, I. Dormoy, J. Dupuy et al., "Genotoxicity of cytolethal distending toxin (CDT) on isogenic human colorectal cell lines: potential promoting effects for colorectal carcinogenesis," *Frontiers in Cellular and Infection Microbiology*, vol. 6, p. 34, 2016.

[83] F. Taieb, J.-P. Nougayrède, C. Watrin, A. Samba-Louaka, and E. Oswald, "*Escherichia coli* cyclomodulin Cif induces G2arrest of the host cell cycle without activation of the DNA-damage checkpoint-signalling pathway," *Cellular Microbiology*, vol. 8, no. 12, pp. 1910–1921, 2006.

[84] A. Fabbri, S. Travaglione, and C. Fiorentini, "*Escherichia coli* cytotoxic necrotizing factor 1 (CNF1): toxin biology, in vivo applications and therapeutic potential," *Toxins*, vol. 2, no. 2, pp. 283–296, 2010.

[85] M. Arumugam, J. Raes, E. Pelletier et al., "Enterotypes of the human gut microbiome," *Nature*, vol. 473, no. 7346, pp. 174–180, 2011.

[86] H. M. Wexler, "Bacteroides: the good, the bad, and the nitty-gritty," *Clinical Microbiology Reviews*, vol. 20, no. 4, pp. 593–621, 2007.

[87] J. Xu and J. I. Gordon, "Honor thy symbionts," *Proceedings of the National Academy of Sciences*, vol. 100, no. 18, pp. 10452–10459, 2003.

[88] C. L. Sears, A. L. Geis, and F. Housseau, "*Bacteroides fragilis* subverts mucosal biology: from symbiont to colon carcinogenesis," *Journal of Clinical Investigation*, vol. 124, no. 10, pp. 4166–4172, 2014.

[89] J. Holton, "Enterotoxigenic *Bacteroides fragilis*," *Current Infectious Disease Reports*, vol. 10, no. 2, pp. 99–104, 2008.

[90] J. V. Pierce and H. D. Bernstein, "Genomic diversity of enterotoxigenic strains of *Bacteroides fragilis*," *PLoS One*, vol. 11, no. 6, Article ID e0158171, 2016.

[91] C. L. Sears, "The toxins of *Bacteroides fragilis*," *Toxicon*, vol. 39, no. 11, pp. 1737–1746, 2001.

[92] Y. K. Lee, P. Mehrabian, S. Boyajian et al., "The protective role of *Bacteroides fragilis* in a murine model of colitis-associated colorectal cancer," *mSphere*, vol. 3, no. 6, 2018.

[93] K.-J. Rhee, S. Wu, X. Wu et al., "Induction of persistent colitis by a human commensal, enterotoxigenic *Bacteroides fragilis*, in wild-type C57BL/6 mice," *Infection and Immunity*, vol. 77, no. 4, pp. 1708–1718, 2009.

[94] J. I. Sartor, A. Aitchison, R. V. Purcell, R. Greenlees, J. F. Pearson, and F. A. Frizelle, "Screening for enterotoxigenic *Bacteroides fragilis* in stool samples," *Anaerobe*, vol. 40, pp. 50–53, 2016.

[95] R. V. Purcell, J. Pearson, A. Aitchison, L. Dixon, F. A. Frizelle, and J. I. Keenan, "Colonization with enterotoxigenic *Bacteroides fragilis* is associated with early-stage colorectal neoplasia," *PLoS One*, vol. 12, no. 2, Article ID e0171602, 2017.

[96] A. Boleij, E. M. Hechenbleikner, A. C. Goodwin et al., "The *Bacteroides fragilis* toxin gene is prevalent in the colon mucosa of colorectal cancer patients," *Clinical Infectious Diseases*, vol. 60, no. 2, pp. 208–215, 2015.

[97] A. V. Platz, G. S. Krasnov, A. V. Lipatova et al., "The dysregulation of polyamine metabolism in colorectal cancer is associated with overexpression of c-Myc and C/EBPbeta rather than enterotoxigenic *Bacteroides fragilis* infection," *Oxidative Medicine and Cellular Longevity*, vol. 2016, Article ID 2353560, 11 pages, 2016.

[98] A. C. Goodwin, C. E. D. Shields, S. Wu et al., "Polyamine catabolism contributes to enterotoxigenic *Bacteroides fragilis*-induced colon tumorigenesis," *Proceedings of the National Academy of Sciences*, vol. 108, no. 37, pp. 15354–15359, 2011.

[99] A. L. Casero and F. Housseau, "Procarcinogenic regulatory T cells in microbial-induced colon cancer," *OncoImmunology*, vol. 5, no. 4, Article ID e1118601, 2016.

[100] A. L. Geis, H. Fan, X. Wu et al., "Regulatory T-cell response to enterotoxigenic *Bacteroides fragilis* colonization triggers IL17-dependent colon carcinogenesis," *Cancer Discovery*, vol. 5, no. 10, pp. 1098–1109, 2015.

[101] C. E. DeStefano Shields, S. W. Van Meerbeke, F. Housseau et al., "Reduction of murine colon tumorigenesis driven by enterotoxigenic *Bacteroides fragilis* using cefoxitin treatment," *Journal of Infectious Diseases*, vol. 214, no. 1, pp. 122–129, 2016.

[102] E. C. Wick, S. Rabizadeh, E. Albesiano et al., "Stat3 activation in murine colitis induced by enterotoxigenic *Bacteroides fragilis*," *Inflammatory Bowel Diseases*, vol. 20, no. 5, pp. 821–834, 2014.

[103] S. Housseau, J. Powell, N. Mathioudakis, S. Kane, E. Fernandez, and C. L. Sears, "*Bacteroides fragilis* enterotoxin induces intestinal epithelial cell secretion of interleukin-8 through mitogen-activated protein kinases and a tyrosine kinase-regulated nuclear factor- B pathway," *Infection and Immunity*, vol. 72, no. 10, pp. 5832–5839, 2004.

[104] L. Chung, E. Thiele Orberg, A. L. Geis et al., "*Bacteroides fragilis* toxin coordinates a pro-carcinogenic inflammatory cascade via targeting of colonic epithelial cells," *Cell Host & Microbe*, vol. 23, no. 2, pp. 203–214, 2018.

[105] F. Tam, S. Wu, E. C. Wick et al., "Redundant innate and adaptive sources of IL17 production drive colon tumorigenesis," *Cancer Research*, vol. 76, no. 8, pp. 2115–2124, 2016.

[106] P. Iyadorai, D. Wu, C. Ni et al., "$\gamma\delta$T17 cells promote the accumulation and expansion of myeloid-derived suppressor

cells in human colorectal cancer," *Immunity*, vol. 40, no. 5, pp. 785–800, 2014.

[107] E. Chen, H. Fan, A. J. Tam et al., "The myeloid immune signature of enterotoxigenic *Bacteroides fragilis*-induced murine colon tumorigenesis," *Mucosal Immunology*, vol. 10, no. 2, pp. 421–433, 2017.

[108] E. Ganguly, R. Euvrard, M. Thibaudin et al., "Accumulation of MDSC and Th17 cells in patients with metastatic colorectal cancer predicts the efficacy of a FOLFOX-bevacizumab drug treatment regimen," *Cancer Research*, vol. 76, no. 18, pp. 5241–5252, 2016.

[109] A. G. Bengrine-Lefevre, S. A. Shiryaev, and A. Y. Strongin, "Distinct interactions with cellular E-cadherin of the two virulent metalloproteinases encoded by a *Bacteroides fragilis* pathogenicity island," *PLoS One*, vol. 9, no. 11, Article ID e113896, 2014.

[110] S. Wu, K. J. Rhee, M. Zhang, A. Franco, and C. L. Sears, "*Bacteroides fragilis* toxin stimulates intestinal epithelial cell shedding and gamma-secretase-dependent E-cadherin cleavage," *Journal of Cell Science*, vol. 120, no. 11, pp. 1944–1952, 2007.

[111] S. Spanò, "Mechanisms of *Salmonella* Typhi host restriction," *Biophysics of Infection*, vol. 915, pp. 283–294, 2016.

[112] L. Mughini-Gras, M. Schaapveld, J. Kramers et al., "Increased colon cancer risk after severe *Salmonella* infection," *PLoS One*, vol. 13, no. 1, Article ID e0189721, 2018.

[113] J. Koshiol, A. Wozniak, P. Cook et al., "*Salmonella enterica* serovar Typhi and gallbladder cancer: a case-control study and meta-analysis," *Cancer Medicine*, vol. 5, no. 11, pp. 3310–3235, 2016.

[114] D. L. Levine, A. Chaudhary, and S. I. Miller, "Salmonellae interactions with host processes," *Nature Reviews Microbiology*, vol. 13, no. 4, pp. 191–205, 2015.

[115] H. Kuper, H.-O. Adami, and D. Trichopoulos, "Infections as a major preventable cause of human cancer," *Journal of Internal Medicine*, vol. 248, no. 3, pp. 171–183, 2000.

[116] F. Grasso and T. Frisan, "Bacterial genotoxins: merging the DNA damage response into infection biology," *Biomolecules*, vol. 5, no. 3, pp. 1762–1782, 2015.

[117] L. Del Bel Belluz, R. Guidi, I. S. Pateras et al., "The typhoid toxin promotes host survival and the establishment of a persistent asymptomatic infection," *PLOS Pathogens*, vol. 12, no. 4, Article ID e1005528, 2016.

[118] M. Kang and A. Martin, "Microbiome and colorectal cancer: unraveling host-microbiota interactions in colitis-associated colorectal cancer development," *Seminars in Immunology*, vol. 32, pp. 3–13, 2017.

[119] Z. Ye, E. O. Petrof, D. Boone, E. C. Claud, and J. Sun, "*Salmonella* effector AvrA regulation of colonic epithelial cell inflammation by deubiquitination," *The American Journal of Pathology*, vol. 171, no. 3, pp. 882–892, 2007.

[120] R. Lu, M. Bosland, Y. Xia, Y. G. Zhang, I. Kato, and J. Sun, "Presence of *Salmonella* AvrA in colorectal tumor and its precursor lesions in mouse intestine and human specimens," *Oncotarget*, vol. 8, no. 33, pp. 55104–55115, 2017.

[121] X. Liu, R. Lu, Y. Xia, S. Wu, and J. Sun, "Eukaryotic signaling pathways targeted by *Salmonella* effector protein AvrA in intestinal infection in vivo," *BMC Microbiology*, vol. 10, no. 1, p. 326, 2010.

[122] R. Lu, S. Wu, X. Liu, Y. Xia, Y. G. Zhang, and J. Sun, "Chronic effects of a *Salmonella* type III secretion effector protein AvrA in vivo," *PLoS One*, vol. 5, no. 5, Article ID e10505, 2010.

[123] R. Lu, X. Liu, S. Wu et al., "Consistent activation of the β-catenin pathway by *Salmonella* type-three secretion effector protein AvrA in chronically infected intestine," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 303, no. 10, pp. G1113–G1125, 2012.

[124] X. Liu, R. Lu, S. Wu, and J. Sun, "*Salmonella* regulation of intestinal stem cells through the Wnt/β-catenin pathway," *FEBS Letters*, vol. 584, no. 5, pp. 911–916, 2010.

[125] R. Lu, S. Wu, Y.-g. Zhang et al., "Enteric bacterial protein AvrA promotes colonic tumorigenesis and activates colonic beta-catenin signaling pathway," *Oncogenesis*, vol. 3, no. 6, p. e105, 2014.

[126] Z. Li, B. Vogelstein, and K. W. Kinzler, "Phosphorylation of beta-catenin at S33, S37, or T41 can occur in the absence of phosphorylation at T45 in colon cancer cells," *Cancer Research*, vol. 63, no. 17, pp. 5234-5235, 2003.

[127] R. Lu, S. Wu, Y. G. Zhang et al., "*Salmonella* protein AvrA activates the STAT3 signaling pathway in colon cancer," *Neoplasia*, vol. 18, no. 5, pp. 307–316, 2016.

[128] R. Lu, Y. G. Zhang, and J. Sun, "STAT3 activation in infection and infection-associated cancer," *Molecular and Cellular Endocrinology*, vol. 451, pp. 80–87, 2017.

[129] S. Wu, Z. Ye, X. Liu et al., "*Salmonella* typhimurium infection increases p53 acetylation in intestinal epithelial cells," *American Journal of Physiology-Gastrointestinal and Liver Physiology*, vol. 298, no. 5, pp. G784–G794, 2010.

[130] H. Yamaguchi, N. T. Woods, L. G. Piluso et al., "p53 acetylation is crucial for its transcription-independent proapoptotic functions," *Journal of Biological Chemistry*, vol. 284, no. 17, pp. 11171–11183, 2009.

[131] E. C. Lazcano-Ponce, J. F. Miquel, N. Munoz et al., "Epidemiology and molecular pathology of gallbladder cancer," *CA: A Cancer Journal for Clinicians*, vol. 51, no. 6, pp. 349–364, 2001.

[132] V. Nagaraja and G. D. Eslick, "Systematic review with meta-analysis: the relationship between chronic *Salmonella* typhicarrier status and gall-bladder cancer," *Alimentary Pharmacology & Therapeutics*, vol. 39, no. 8, pp. 745–750, 2014.

[133] G. Randi, S. Franceschi, and C. La Vecchia, "Gallbladder cancer worldwide: geographical distribution and risk factors," *International Journal of Cancer*, vol. 118, no. 7, pp. 1591–1602, 2006.

[134] T. Scanu, R. M. Spaapen, J. M. Bakker et al., "*Salmonella* manipulation of host signaling pathways provokes cellular transformation associated with gallbladder carcinoma," *Cell Host & Microbe*, vol. 17, no. 6, pp. 763–774, 2015.

[135] P. Song, S. G. Barreto, B. Sahoo et al., "Non-typhoidal *Salmonella* DNA traces in gallbladder cancer," *Infectious Agents and Cancer*, vol. 11, p. 12, 2016.

[136] S. Dongol, C. N. Thompson, S. Clare et al., "The microbiological and clinical characteristics of invasive *Salmonella* in gallbladders from cholecystectomy patients in Kathmandu, Nepal," *PLoS One*, vol. 7, no. 10, Article ID e47342, 2012.

[137] Y. D. Walawalkar, R. Gaind, and V. Nayak, "Study on *Salmonella* Typhi occurrence in gallbladder of patients suffering from chronic cholelithiasis-a predisposing factor for carcinoma of gallbladder," *Diagnostic Microbiology and Infectious Disease*, vol. 77, no. 1, pp. 69–73, 2013.

[138] R. W. Crawford, R. Rosales-Reyes, M. d. l. L. Ramirez-Aguilar, O. Chapa-Azuela, C. Alpuche-Aranda, and J. S. Gunn, "Gallstones play a significant role in *Salmonella* spp. gallbladder colonization and carriage," *Proceedings of*

the National Academy of Sciences, vol. 107, no. 9, pp. 4353–4358, 2010.

[139] C. H. C. Pilgrim, R. T. Groeschl, K. K. Christians, and T. C. Gamblin, "Modern perspectives on factors predisposing to the development of gallbladder cancer," *HPB*, vol. 15, no. 11, pp. 839–844, 2013.

[140] A. Menendez, E. T. Arena, J. A. Guttman et al., "*Salmonella* infection of gallbladder epithelial cells drives local inflammation and injury in a model of acute typhoid fever," *The Journal of Infectious Diseases*, vol. 200, no. 11, pp. 1703–1713, 2009.

[141] C. M. Parry, T. T. Hien, G. Dougan, N. J. White, and J. J. Farrar, "Typhoid fever," *New England Journal of Medicine*, vol. 347, no. 22, pp. 1770–1782, 2002.

[142] S. Carotti, M. P. L. Guarino, M. Cicala et al., "Effect of ursodeoxycholic acid on inflammatory infiltrate in gallbladder muscle of cholesterol gallstone patients," *Neurogastroenterology & Motility*, vol. 22, no. 8, pp. 866–e232, 2010.

[143] Y. Ye, M. Liu, H. Yuan et al., "COX-2 regulates Snail expression in gastric cancer via the Notch1 signaling pathway," *International Journal of Molecular Medicine*, vol. 40, no. 2, pp. 512–522, 2017.

[144] L. Sorski, R. Melamed, P. Matzner et al., "Reducing liver metastases of colon cancer in the context of extensive and minor surgeries through β-adrenoceptors blockade and COX2 inhibition," *Brain, Behavior, and Immunity*, vol. 58, pp. 91–98, 2016.

[145] J. A. Espinoza, C. Bizama, P. García et al., "The inflammatory inception of gallbladder cancer," *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, vol. 1865, no. 2, pp. 245–254, 2016.

[146] A. R. Moser, C. Luongo, K. A. Gould, M. K. McNeley, A. R. Shoemaker, and W. F. Dove, "ApcMin: a mouse model for intestinal and mammary tumorigenesis," *European Journal of Cancer*, vol. 31, no. 7-8, pp. 1061–1064, 1995.

[147] C. M. Dejea, P. Fathi, J. M. Craig et al., "Patients with familial adenomatous polyposis harbor colonic biofilms containing tumorigenic bacteria," *Science*, vol. 359, no. 6375, pp. 592–597, 2018.

[148] C. M. Anders, E. C. Wick, E. M. Hechenbleikner et al., "Microbiota organization is a distinct feature of proximal colorectal cancers," *Proceedings of the National Academy of Sciences*, vol. 111, no. 51, pp. 18321–18326, 2014.

[149] C. H. Stein, C. M. Dejea, D. Edler et al., "Metabolism links bacterial biofilms and colon carcinogenesis," *Cell Metabolism*, vol. 21, no. 6, pp. 891–897, 2015.

[150] L. A. Uritboonthai Jr., A. C. F. Afable, P. J. O. Belza et al., "Immunogenicity of a Fap2 peptide mimotope of *Fusobacterium nucleatum* and its potential use in the diagnosis of colorectal cancer," *Infectious Agents and Cancer*, vol. 13, p. 11, 2018.

Hindawi
Journal of Oncology
Volume 2019, Article ID 6018269, 10 pages
https://doi.org/10.1155/2019/6018269

Research Article

Human Papillomavirus and Anal Cancer: Prevalence, Genotype Distribution, and Prognosis Aspects from Midwestern Region of Brazil

Larisse Silva Dalla Libera,[1] **Keila Patrícia Almeida de Carvalho**,[2] **Jéssica Enocencio Porto Ramos**,[3] **Lázara Alyne Oliveira Cabral**,[3] **Rita de Cassia Goncalves de Alencar**,[4] **Luísa Lina Villa**,[5] **Rosane Ribeiro Figueiro Alves**,[1] **Silvia Helena Rabelo Santos**,[2] **Megmar Aparecida dos Santos Carneiro**,[2] **and Vera Aparecida Saddi**[1,3]

[1]*Universidade Federal de Goiás, Postgraduate Program in Health Sciences, Faculty of Medicine, Goiânia, Goiás, CEP 74605-020, Brazil*
[2]*Universidade Federal de Goiás, Institute of Tropical Pathology and Public Health, Goiânia, Goiás, CEP 74605-050, Brazil*
[3]*Pontifical Catholic University of Goiás, Postgraduate Program in Environmental Sciences and Health, Goiânia, Goiás, CEP 74605-010, Brazil*
[4]*Department of Pathology, Hospital Araújo Jorge, Goiânia, Goiás, CEP 74605-050, Brazil*
[5]*Cancer Institute of the State of São Paulo, São Paulo, SP, CEP 01246-000, Brazil*

Correspondence should be addressed to Larisse Silva Dalla Libera; larisse.dalla@gmail.com

Received 5 March 2019; Revised 16 July 2019; Accepted 3 September 2019; Published 18 September 2019

Guest Editor: Hironori Yoshiyama

Background. Approximately 90% of all anal cancers are associated with human papillomavirus (HPV), especially high-risk genotypes such as HPVs 16 and 18. *Objective*. To investigate the clinical and prognostic aspects of anal cancers associated with the presence, as well as the genotypic distribution of human papillomavirus (HPV). *Methods*. A retrospective study carried out over a 10-year period, using clinical and molecular data, with PCR analysis and reverse hybridization (INNO-LIPA kit), in anal cancers. The data analysis was done using descriptive univariate statistics, and the survival curves were made using the Kaplan–Meier and log-rank methods. *Results*. Of the 81 formalin-fixed and paraffin-embedded specimens, HPV prevalence was 69% and was significantly higher in squamous cell carcinomas (SCC) than in other anal tumors ($p = 0.0001$). Female patients had a higher prevalence of HPV ($p = 0.01$). Multiple infections were detected in 14.3% of cases. The most prevalent genotypes were HPVs 16, 33, and 18. The overall survival at 60 months was 44.3%, and the prognostic factors included gender ($p = 0.008$) with greater survival for men (52.9%) in comparison to women (29.6%), histological type ($p = 0.01$), SCC (54.4%), adenocarcinomas (37.5%), other carcinomas (14.2%), and the presence of distant metastasis ($p = 0.01$). Survival was not influenced by the presence of HPV ($p = 0.54$). *Conclusions*. The association of HPV to anal cancer was found in this study, especially in SCC. However, the presence of HPV did not influence the prognosis of patients with anal cancer.

1. Introduction

Approximately 5% of all cancers worldwide are associated with human papillomavirus (HPV), and the proportion of anal cancer attributed to HPV is 90%, with genotypes 16 and 18 found in more than 70% of these cancers [1–5]. Anal cancer is a rare tumor that corresponds to approximately 2% of cancers that affect the gastrointestinal system [6]. About

48,000 new cases of anal cancer are diagnosed every year worldwide, with a peak incidence in the age group between 58 and 64 years [7–9].

In Brazil, data on anal cancer are scarce, but it is estimated to correspond to 1% to 2% of all colorectal cancers [10]. In 2010, 274 deaths from anal cancer were recorded in the country, with 98 cases in men and 176 cases in women [11]. In 2013, 348 deaths were recorded, with 106 in men and 242 in women. In 2014, 1,100 new cases of anal cancer were estimated in Brazil [10]. According to the Population Based Cancer Registry of Goiânia, between 1989 and 2008, a total of 117 cases of anal cancer were diagnosed in the city, but more recent data were not available. A study carried out in Goiânia, a city in the Midwest region of Brazil, described 42 cases of anal cancer, of which 38 were tested for HPV DNA and 76% were positive for the presence of HPV DNA [12].

The most common histological type of anal cancer is squamous cell carcinoma (SCC), followed by adenocarcinoma [2, 6, 13]. HPV can be considered an essential factor for the development of SCC, as well as its precursor lesions such as anal intraepithelial neoplasia (AIN) [14, 15]. Several factors contribute to HPV-induced anal carcinogenicity, such as early onset of sexual activity, sexual practices involving anal intercourse, prior exposure to high-risk HPV genotypes, history of anogenital injury, and other cancers associated with HPV. The major risk groups include men who have sex with men, transsexual women, and individuals carrying human immunodeficiency virus (HIV) [14–18].

The role of HPV in the prognosis of anal carcinomas is poorly understood, as well as its influence on the clinical aspects of this tumor [14–16]. Although patients diagnosed with primary cancers associated with HPV respond well to treatment, there is a risk for a second exposure or relapse of HPV infection [14, 18]. Classical aspects of staging, such as tumor size, metastatic lymph node involvement, and distant metastasis, remain the main factors that influence the prognosis of anal carcinomas [6, 19, 20].

The goal of this study was to evaluate the prognostic, sociodemographic, and clinical aspects of individuals with anal cancer associated with the detection and genotypic distribution of HPV, as well as the influence of HPV infection on the prognosis of this tumor.

2. Methodology

2.1. Type of Study and Sample Selection. This is a retrospective study that investigated HPV prevalence and genotype distribution in a group of anal cancer patients assisted in a cancer reference center from Goiânia, a middle-sized city in the Midwest region of Brazil, during a period of 10 years.

A sample size calculation was not performed since we aimed to include in the study all the cases that were diagnosed as anal cancer in the Pathology Department of the center. Our reference sample came from the registry of the Pathology Laboratory, so initially, a list of 140 patients diagnosed with anal cancers from 2000 to 2010 was consulted. After pathological/clinical review, 85 cases of anal cancers were considered eligible. A description of the inclusion and exclusion criteria of the cases was presented in Figure 1.

The selected cases were those that presented histopathological diagnoses of anal cancer confirmed by two pathologists, those with clinical data available in the medical records, and those with paraffin blocks available and sufficient for molecular analyses. Cases that were not confirmed as primary anal cancer were excluded. Since we aimed to evaluate five-year overall survival, the retrospective study that was initiated in 2016 considered patients that were diagnosed until 2010.

This study was approved by the Research Ethics Committee of the Association to Combat Cancer in Goiás (CEP/ACCG) under CEP: 272,288.

2.2. Preparation of Samples. After selection of the paraffin blocks containing the tumor specimens, each block was serially sectioned with the use of a microtome and the sections packed in properly identified 2 ml sterile microtubes. The microtome knives were changed between samples, and the equipment was cleaned with ethanol for each new block. From each block, slides containing tumor fragments were prepared and stained using hematoxylin and eosin and reviewed by a pathologist. The diagnosis of anal carcinoma was confirmed for each case, based on the classification criteria for tumors from the World Health Organization [21].

2.3. DNA Extraction. Viral DNA extraction was performed using the phenol-chloroform-isoamyl alcohol method; the paraffin removal was done with the organic solvent xylol and cell digestion performed with proteinase-K. The final DNA precipitation was done with isopropanol and DNA purification with 70% ethanol. As dewaxing of the sample can lead to tissue loss and consequent degradation of the DNA present in the sample [22], the amount of DNA extracted and its purity were evaluated by spectrophotometry (Thermo Scientific NanoDrop Products). The samples were submitted to polymerase chain reaction (PCR) to amplify the endogenous control, a fragment of the human gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Samples negative for endogenous control were re-extracted.

2.4. Detection of HPV Genotype. Our study employed the INNO-LiPA HPV Genotyping Extra test (Innogenetics NV, Ghent, Belgium) to detect and genotype HPV DNA, following the manufacturer's instructions. This assay can identify 28 different HPV genotypes, including all known HR-HPV genotypes and probable HR-HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82), as well as several LR-HPV genotypes (6, 11, 40, 43, 44, 54, and 70) and a number of additional types (69, 71, and 74), based on nested PCR amplification of a fragment (65 base pairs) of the L1 region of the HPV genome. Amplified

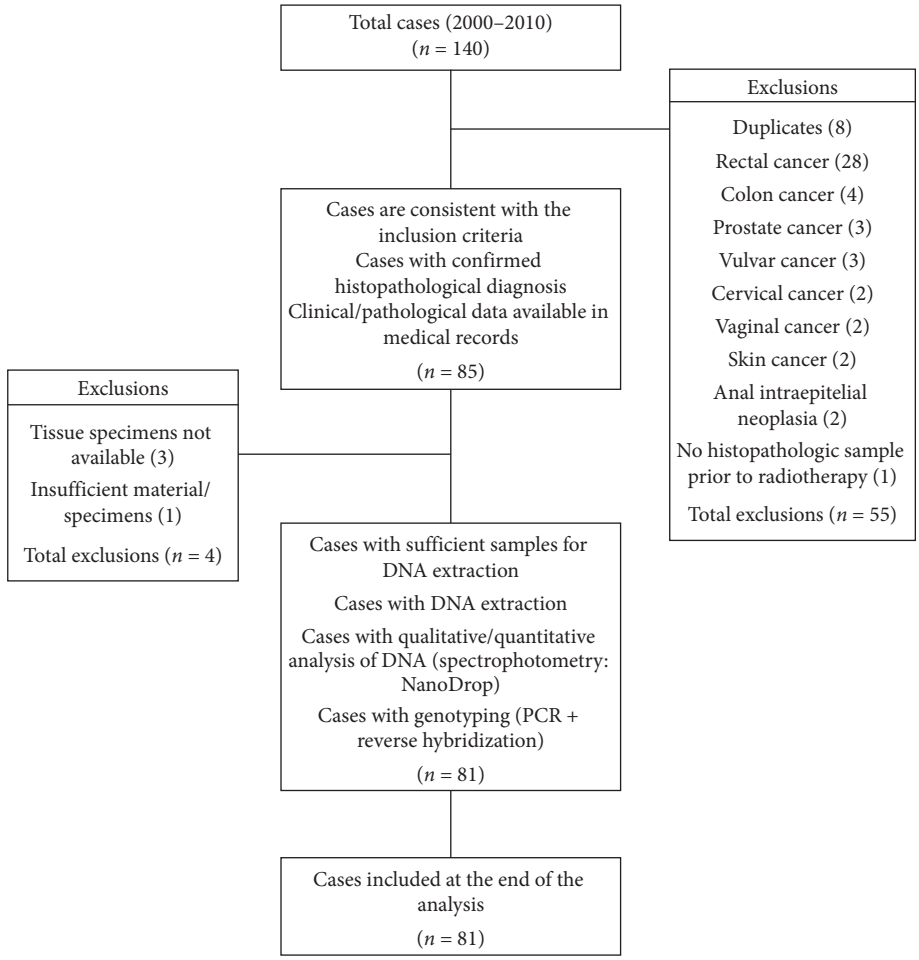


Figure 1: Flowchart of case sample selection.

products were denatured under alkaline conditions and immediately incubated with the test strips in hybridization buffer. The results were visually interpreted by two independent investigators by comparing them with a template provided with the assay. The kit allows simultaneous detection of multiple genotypes in a single sample. Several publications have already proved the performance of the assay in cervical scrapes and in formalin-fixed and paraffin-embedded (FFPE) tissue.

The study employed the INNO-LiPA HPV Genotyping Extra test (Innogenetics NV, Ghent, Belgium) to detect and genotype HPV DNA. This assay is a reverse line hybridization assay validated by several previous studies. The physical state of HPV was not investigated.

In genotyping, only those samples that presented a single genotype of HPV were considered as single infections, and the samples that had more than one HPV genotype were considered multiple infections. In cases of multiple HPV infections with at least one high-risk genotype, the result was considered to be high-risk HPV. In cases that contained only low-risk genotypes, the sample was considered low-risk HPV.

The entire laboratory procedure, from sample handling to HPV detection and genotyping, followed the international standards for HPV testing by the World Health Organization [23].

2.5. Statistical Analysis. Sociodemographic and clinical and pathological data were collected on appropriate forms and transferred to spreadsheets, Microsoft Excel, version 2013. The database was digitized by two independent researchers and compared for data verification and database cleanup. The data were transferred to GraphPad Prism version 4.0 and analyzed using descriptive statistics, in order to generate prevalence estimates with respective confidence intervals.

For the age group, the mean and standard deviation were calculated. In order to evaluate the possible associations between the analyzed variables, a univariate analysis was performed considering the level of significance $p < 0.05$ and chi-square test (χ^2). In order to evaluate the associations between the results obtained for HPV detection and the other variables, odds ratios (OR) were calculated with a 95% confidence interval (CI) and significance level of 5%.

The Kaplan–Meier method was used to calculate survival, and the log-rank test was used to compare survival curves against prognostic factors for anal cancer. Death was considered independent of its cause.

The cases included in the study did not present HIV infection status registered in the patient files, and therefore, these data were not used as a prognostic factor.

3. Results

3.1. Sample Characteristics. Sampling included 81 cases of anal cancer. The characteristics of patients with anal cancer are presented in Table 1.

The majority of the patients were female (63%). Age ranged from 36 to 92 years, and the overall mean age at diagnosis was 61.57 years (±12.73); mean age for women was 62.47 years (±13.01) and for men 60.03 years (±12.30).

Lymph node metastases were reported in 25.9% of patients, and inguinal lymph nodes were the most compromised (data not shown). Distant metastases were described in 8.6% of the group, with the liver and lung being the most affected organs. At the end of 60 months following diagnosis, 55.6% of the patients had reported deaths.

Most of the samples (52%) were diagnosed as anal squamous cell carcinoma, followed by adenocarcinomas (39.5%). The majority of SCC and adenocarcinomas were in T1-T2 stages ($p = 0.01$). The other types of anal cancers were in advanced stages, but without lymph node spread. Both SCCs and adenocarcinomas presented cases with distant metastasis.

3.2. Prevalence of HPV DNA. The prevalence of HPV DNA for the evaluated group was 69.1%. Only 25 patients were negative for HPV DNA. Table 2 presents the prevalence of HPV and its association with the social, demographic, clinical, and pathological characteristics investigated. HPV was more prevalent in women than in men (OR 3.18 95% CI 1.19–8.48). The mean age of the group at diagnosis was similar in HPV-negative (63 years ± 11.6) and HPV-positive patients (61 years ± 13.2). HPV was significantly associated with anal SCC (OR 9.51 95% CI 2.96–30.50) (Table 2).

3.3. HPV Genotype Distribution. Of all patients with anal cancer positive for HPV DNA, 85.7% had a single HPV-type infection, while 14.3% had multiple HPV types. The genotypic distribution of HPV and the presence of single and multiple infections of all genotyped samples are described and presented in Figure 2. The most prevalent genotypes in squamous cell carcinomas and anal adenocarcinomas were HPVs 16, 18, and 33. In the other types, the most common were HPVs 16 and 33 (Table 3).

3.4. Survival. Overall survival at 60 months for patients with anal cancers was 44.3% (Figure 3). The mean follow-up was 31 months (±59.4) with a minimum of 1 month and a maximum of 191 months. The prognostic factors were being female (Figure 4(a)), squamous cell carcinoma (Figure 4(b)), and the presence of distant metastasis (Figure 4(c)). Survival was not influenced by the presence of HPV (Figure 4(d)), lymphatic dissemination ($p = 0.84$), or tumor size ($p = 0.08$); however, all individuals with T3 or larger tumors were deceased after 38 months.

Table 1: Sociodemographic and clinical/pathological characteristics of patients with anal cancers (n 81).

	n	%
Gender		
Female	51	63.0
Male	30	37.0
Age at diagnosis (years)		
<61 years	38	46.9
≥61 years	43	53.1
Marital status		
Single	37	45.7
Married	40	49.4
Ethnicity		
White	29	35.8
Brown (pardo)	48	59.3
Black	4	4.9
Smoker		
Yes	22	27.2
No	54	66.6
Alcohol consumption		
Yes	17	21.0
No	58	71.6
Tumor location		
Anal canal	59	72.8
Anal border	8	9.9
Both	14	17.3
Histological type		
SCC	42	51.9
Adenocarcinoma	32	39.5
Others	7	8.6
Treatment		
Surgery	65	80.2
Radiotherapy	62	76.5
Chemotherapy	51	63.0
No treatment	4	4.9
Size of tumor		
T1-2	58	71.6
T3-4	22	27.2
Not specified	1	1.2
Lymph node metastasis		
Yes	21	25.9
No	60	74.1
Distant metastasis		
Yes	7	8.6
No	74	91.4
Sites of distant metastasis		
Liver	2	2.5
Lung	2	2.5
Bladder	1	1.2
Uterus	1	1.2
Vagina	1	1.2
Death record		
Yes	45	55.6
No	36	44.4

SCC: squamous cell carcinoma. Others: basaloid carcinoma, neuroendocrine, and cloacogenic. Number of patients with data not informed: marital status 4 (4.9%); smoking 5 (6.2%); alcohol consumption 6 (7.4%); and not specified size of tumor 1 (1.2%).

4. Discussion

HPV was present in 69% of anal cancer samples, and HPV 16 was the most prevalent genotype (78.5%), followed by HPV

Table 2: HPV DNA and anal cancers, according to sociodemographic and clinical/pathological characteristics.

Variables	HPV + (*n*)	%	HPV − (*n*)	%	*p*	OR (CI 95%)
Gender						
Female	40	78.4	11	21.6	0.01*	3.18 (1.19–8.48)
Male	16	53.3	14	46.7		
Age at diagnosis (years)						
<61 years	26	68.4	12	31.6	0.89	0.93 (0.36–2.41)
≥61 years	30	69.8	13	30.2		
Marital status						
Single	24	64.9	13	35.1		
Married	29	72.5	11	27.5	0.46	0.70 (0.26–1.84)
Smoker						
Yes	15	68.2	7	31.8	0.97	0.98 (0.33–2.86)
No	37	68.5	17	31.5		
Alcohol consumption						
Yes	11	64.7	6	35.3	0.63	0.76 (0.24–2.39)
No	41	70.7	17	29.3		
Lesion location						
Anal canal	38	64.4	21	35.6	0.55	0.60 (0.11–3.26)
Anal border	6	75.0	2	25.0		
Both	12	85.7	2	14.3		
Histological type						
SCC	37	88.1	5	11.9	0.0001*	9.51 (2.96–30.50)
Adenocarcinoma	14	43.8	18	56.2		
Others	5	71.4	2	28.6		
Size of tumor						
T1-2	41	70.6	17	29.3	0.44	1.50 (0.52–4.25)
T3-4	14	63.6	8	36.4		
Lymph node metastasis						
Yes	14	66.7	7	33.3	0.77	0.85 (0.29–2.48)
No	42	70.0	18	30.0		
Distant metastases						
Yes	5	71.4	2	28.6	1.00	1.13 (0.20–6.25)
No	51	68.9	23	31.1		
Death						
Yes	30	66.7	15	33.3	0.59	0.76 (0.29–2.00)
No	26	72.2	10	27.8		

SCC: squamous cell carcinoma. Others: basaloid carcinoma, neuroendocrine, and cloacogenic; number of patients with data not informed that they were positive for HPV: marital status 3; smoking 4; alcohol consumption 4; and size of tumor not specified 1. *Statistically significant values for $p \leq 0.05$.

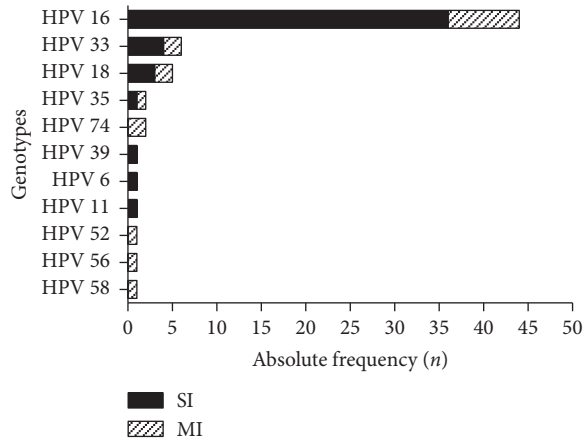


Figure 2: Frequency of 11 HPV genotypes detected in single infection and multiple infections in anal cancers. SI: single infection; MI: multiple infection. Low-risk HPV (LR): 6 and 11. High-risk HPV (HR): 16, 18, 33, 35, 39, 52, 56, 58, and 74.

33 (10.7%) and HPV 18 (8.9%). Although the prevalence of HPV in the anal cancers evaluated was lower than the overall percentage reported in other studies [2, 8, 14, 15, 20–25], it is worth noting that this study included a relatively large number of anal adenocarcinomas (39.5%) and that there are few studies investigating the association of HPV with this

Table 3: Distribution of HPV genotypes according to histological type of anal carcinoma.

HPV	SCC (37/42)		Adenocarcinoma (14/32)		Other carcinomas (5/7)	
	n	%	*n*	%	*n*	%
HPV 16 only	25	67.6	9	64.3	2	40.0
HPV 16 and others	4	10.8	1	7.1	1	20.0
HPV 18 only	2	5.4	1	7.1	0	0.0
HPV 18 and others	0	0.0	0	0.0	0	0.0
HPV 16 and HPV 18	1	2.7	1	7.1	0	0.0
Others single HPV	5	13.5	2	14.3	2	40.0
HPV negative	5	11.9	18	56.3	2	28.6

Other HPV: HPV 6, 11, 33, 35, 39, 52, 56, 58, and 74. Other carcinomas: basaloid carcinoma, neuroendocrine, and cloacogenic.

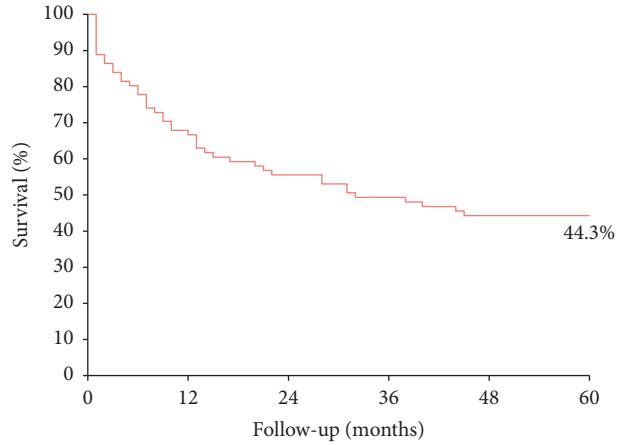


Figure 3: Five-year overall survival for patients with anal cancer (Kaplan–Meier method).

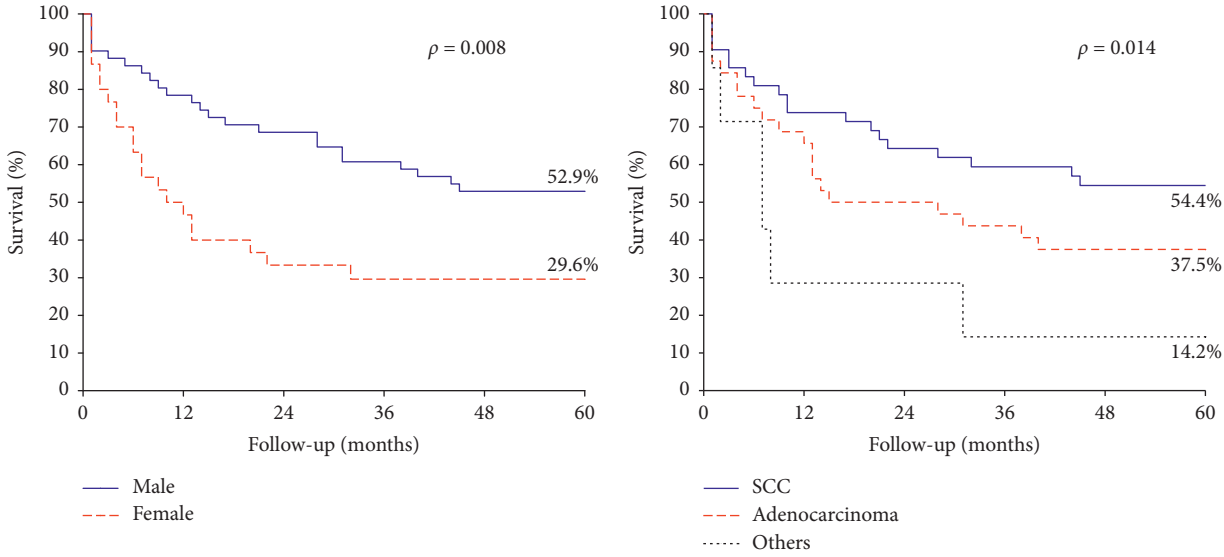


Figure 4: Continued.

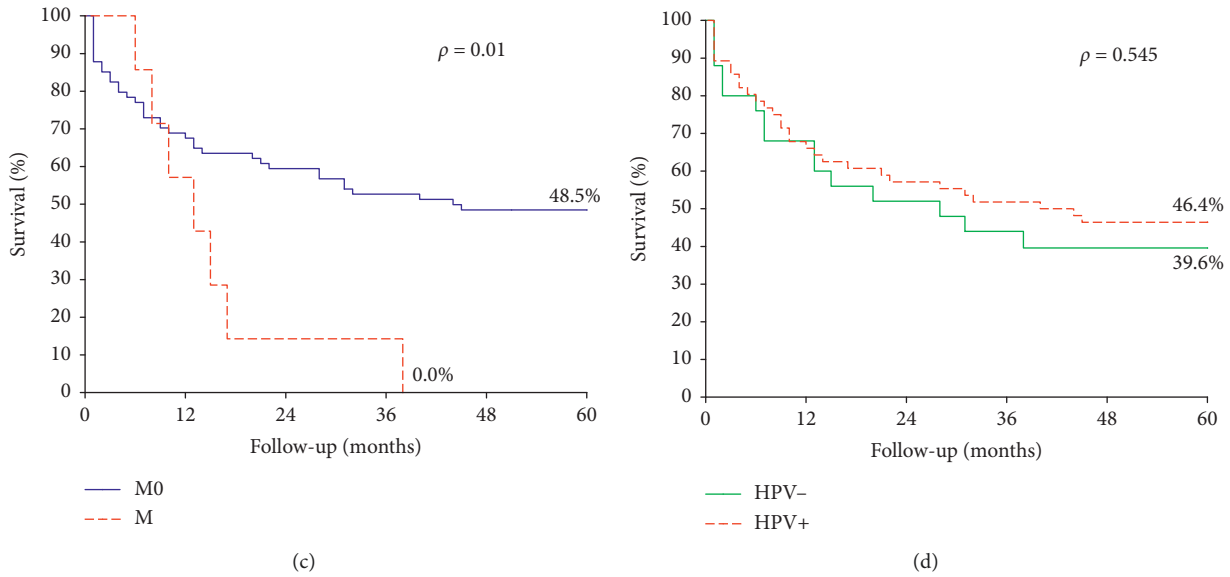


Figure 4: Survival curves for patients with anal cancer according to clinical and pathological characteristics. (a) Gender; (b) tumor histological type; (c) metastasis (M); (d) HPV detection. Others include basaloid carcinoma, neuroendocrine, and cloacogenic.

histologic type of anal cancer [23–26]. SCC is the histological type most associated with the presence of HPV. In our study, the prevalence of the virus in this histological type was 88%, and HPV 16 was present in 82% of the genotyped samples. The high prevalence of HPV in anal SSC has also been observed in 15 other studies, in which the overall prevalence of HPV ranged from 60.6% to 100% [1, 2, 8, 14–16, 20–28]. These large number of SCC cases positive for HPV suggest that virus infection is a necessary cause for this type of anal cancer, as well as cervical cancer, mainly because the transition zone of the anal canal is very similar to the cervical squamocolumnar junction. Because HPV is a virus that is highly tropic in regions covered by squamous epithelium or high proliferative cell activity, these sites become more vulnerable to viral infection [6, 29].

Unlike SCC, adenocarcinoma of the anus is not intrinsically related to HPV infection. In some studies, this histological type is not analyzed because it is considered an extension of rectal cancer [30]. In this study, the adenocarcinomas included (39.5%) were classified histologically and clinically as primary anal cancers, of which 43.8% were infected by HPV, mainly by high-risk genotypes, such as HPV 16.

Samples from two individuals were classified as low-risk HPV (HPV 6 and HPV 11) during genotyping. These genotypes are responsible for papillomatous lesions and are not considered carcinogenic [29, 31]. It is complicated to define the association of cancer or a precursor lesion with a specific HPV genotype, since the molecular methods used in genotyping generally do not preserve the tissue architecture [22]. Moreover, the PCR method detects the HPV genome in the tumor but does not distinguish whether or not HPV is transcriptionally active, making it impossible to conclude which genotype(s) were actually involved in the carcinogenesis [1, 4, 17].

Multiple infections were observed in 14.3% of patients, a considerably higher value than in the other studies [25, 28, 32, 33]. The genotyping used in this study is based on the technique of reverse hybridization, which in this study identified a larger number of HPV genotypes and the presence of multiple infections. This method uses SPF10 primer oligonucleotides that are able to amplify a broad spectrum of HPV genotypes by virtue of their high sensitivity [2, 13, 32].

As expected, HPV 16 was the most prevalent genotype and was present in 100% of multiple infections. Genotype 16 is considered to be high risk and tends to persist in the host for a longer time [34]. Its oncogenic potential is related to its high expression of the viral oncoproteins E6 and E7, and its ability to integrate the viral genome into host cell DNA [29, 31]. Cell targets of HPV oncoproteins are primarily pRb and p53; however, underlying mechanisms associated with other cellular proteins may occur during carcinogenesis leading to cell cycle progression, evasion of apoptosis, DNA damage, and suppression of the immune response [35].

In the analyzed anal carcinomas, HPV 33 was the second most prevalent genotype (10.7%), followed by HPV 18 (8.92%). The presence of HPVs 16 and 18 is already well established, and both are targets of HPV prophylactic vaccines [13, 36–39]. In our research, only two samples showed both genotypes. The high prevalence of HPV 33 has been found before in other studies, ranging from 2 to 11.9% [20, 24, 25, 27] and suggests that the vaccine might have greater coverage if this genotype were included in the free vaccination program in developing countries such as Brazil, similar to, for example, the introduction of the nonavalent vaccine that includes the genotypes HPVs 16, 18, 31, 33, 45, 52, 58, 6, and 11 [36].

Regarding the analysis by gender, the prevalence of HPV in anal cancers was higher in women (78.4%), as observed in other studies [1, 2, 25, 32, 37]. Persistent long-term HPV

infections or newly acquired infections may contribute to the increased susceptibility in women when associated with hormonal or immunological status changes [1, 2, 32, 37, 40].

The incidence of anal cancer increases with age, and the peak incidence occurs between 50 and 70 years with the mean age at diagnosis being 62 years [2, 32]. In this study, age ranged from 36 to 92 years, and the mean age at diagnosis was 61 years. Similar ages were reported in different studies [2, 13, 25, 26].

Overall survival at 60 months was 44.3%. In the United States, the five-year survival for anal cancer is higher, around 67% [41]. It is important to emphasize that Brazil is still a country with many socioeconomic problems, and access to health services, although free of charge, fails due to delays in patient services, and patients do not always have access to educational information for the prevention and treatment of diseases. In this way, anal cancers end up being stigmatized, surrounded by prejudices. In addition, their symptoms are very similar to common diseases of the anus, making their diagnoses neglected, and as a result, diagnoses are performed in more advanced stages and not always easy to treat [6, 42, 43].

Little is known about the prognostic importance of HPV in anal cancers, and the number of studies that deal with the relationship between survival and HPV infection in anal cancer is low [19, 20, 24, 34, 44]. The presence of the virus as a prognostic factor was investigated in this study; however, the results obtained did not allow a significant conclusion about these variables. Some studies have considered the presence of the virus as an important factor in the prognosis of anal cancer [19, 20, 34]. Biomarkers such as p16 have been investigated for prognostic use in anal intraepithelial neoplasias and anal carcinomas [20, 34, 44].

In this study, gender ($p = 0.008$), histological type ($p = 0.01$), and the presence of distant metastasis ($p = 0.01$) were observed as prognostic factors. Women with anal cancer had a worse prognosis (29.6%) when compared with men (52.9%). These data reflect the need for follow-up of women, not only with the Pap smear, which is intended for the detection of cervical cancer, precancerous lesions, and other genital diseases but also the introduction of anuscopy as a screening method for the detection of anal cancer in women [43].

Regarding histological type, patients with anal adenocarcinoma had shorter survival compared to patients with SCC. Some studies have suggested that patients with anal adenocarcinoma have a worse prognosis, but these studies are limited by their sample size [37, 45]. Although the etiology of adenocarcinoma is very similar to that of SCC, it originates from the glandular tissue generally from the upper part of the anal canal, making it difficult to distinguish it from the adenocarcinoma of the lower rectum [24, 30, 45, 46]. The Franklin study (2016) has shown that survival in patients with anal and rectal adenocarcinomas is significantly worse than in those with anal SCC regardless of the type of treatment, suggesting that adenocarcinomas exhibit unique and aggressive behavior in relation to the site of other carcinomas [45].

It is unclear whether samples negative for HPV DNA are actually negative or whether these cases have actually been triggered by other carcinogens. At the molecular examination, only five of 42 SCC samples were negative for HPV. The rest of the negative anal cancers were adenocarcinomas or cloacogenic, basaloid, and neuroendocrine carcinomas.

The lack of some relevant information in the medical records limited the ability to investigate some variables, such as sexual behavior, which is known to increase the risk of HPV infection [14, 43, 47]. Even so, our data adequately represented the reality of individuals with anal cancer in the Midwestern region of Brazil, in the service area of this referral hospital.

The association of HPV to anal cancer has been demonstrated in this study, especially in SCC. However, the presence of HPV did not influence the prognosis of patients with anal cancer. The most prevalent genotypes were HPVs 16, 33, and 18. Further research on the role of HPV and its genotypes in anal carcinogenesis needs to be planned, and the prognostic aspects of anal cancer need to be better elucidated. Risk groups considered for anal cancer comprised mostly of HIV-positive individuals and men who have sex with men; however, as shown in this study, women need to be included in new public policies of this group.

Data Availability

The medical records, histopathological reports, and protocols of analysis of the sample data used to support the findings of this study are available from the corresponding author upon request or are included within the article. For any additional information, request should be given to larisse.dalla@gmail.com.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors thank Hospital Araújo Jorge and the Pathology Department team. Brian Ream edited this article in English. The authors thank FAPEG, Fundação de Amparo a Pesquisa do Estado de Goiás, for financial support (no. 05/2012) and CAPES, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, for scholarship support.

References

[1] J. R. Daling, M. M. Madeleine, L. G. Johnson et al., "Human papillomavirus, smoking, and sexual practices in the etiology of anal cancer," *Cancer*, vol. 101, no. 2, pp. 270–280, 2004.

[2] L. Abramowitz, A.-C. Jacquard, F. Jaroud et al., "Human papillomavirus genotype distribution in anal cancer in France: the EDiTH V study," *International Journal of Cancer*, vol. 129, no. 2, pp. 433–439, 2011.

[3] M. Plummer, M. Catherine de, J. Vignat, J. Ferlay, F. Bray, and S. Franceschi, "Global burden of cancers attributable to infections in 2012: a synthetic analysis," *The Lancet Global Health*, vol. 4, no. 1, pp. e609–e616, 2016.

[4] 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.

[5] A. H. M. Vento, *Estudo epidemiológico sobre a prevalência nacional de infecção pelo hpv*, PROADI-SUS, Ed., AssociaçãoHospitalar Moinhos de Vento Porto Alegre, Brazil, 1st Edition, 2017.

[6] P. M. Hoff, R. Coudry, and C. M. V. Moniz, "Pathology of anal cancer," *Surgical Oncology Clinics of North America*, vol. 26, no. 1, pp. 57–71, 2017.

[7] F. Bray, J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, and A. Jemal, "Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries," *CA: A Cancer Journal for Clinicians*, vol. 68, no. 6, pp. 394–424, 2018.

[8] C. De Martel, J. Ferlay, S. Franceschi et al., "Global burden of cancers attributable to infections in 2008: a review and synthetic analysis," *The Lancet Oncology*, vol. 13, no. 6, pp. 607–615, 2012.

[9] C. de Martel, M. Plummer, J. Vignat, and S. Franceschi, "Worldwide burden of cancer attributable to HPV by site, country and HPV type," *International Journal of Cancer*, vol. 141, no. 4, pp. 664–670, 2017.

[10] INCA, *Câncer Anal, 2015*, Instituto Nacional do Câncer, Bethesda, MD, USA, 2016, http://www2.inca.gov.br.

[11] INCA, *Estimativas 2012 Incidência de câncer no Brasil*, Instituto Nacional do Câncer, 2011, http://www2.inca.gov.br.

[12] M. T. M. Aguiar, N. C. d. C. Bosso, C. B. Q. d. S. Leal et al., "Clinicopathological aspects and prevalence of human papillomavirus in anal cancer," *Journal of Coloproctology*, vol. 34, no. 2, pp. 76–82, 2014.

[13] L. Alemany, M. Saunier, I. Alvarado-Cabrero et al., "Human papillomavirus DNA prevalence and type distribution in anal carcinomas worldwide," *International Journal of Cancer*, vol. 136, no. 1, pp. 98–107, 2015.

[14] C.-C. Chen and Y.-Y. Chou, "Predictive value of the anal cytology for detecting anal intraepithelial neoplasia or worse: a systematic review and meta-analysis," *Diagnostic Cytopathology*, vol. 47, no. 4, pp. 307–314, 2019.

[15] M. Morton, N. Melnitchouk, and R. Bleday, "Squamous cell carcinoma of the anal canal," *Current Problems in Cancer*, vol. 42, no. 5, pp. 486–492, 2018.

[16] R. D. Cranston, A. Carballo-Diéguez, H. Gundacker et al., "Prevalence and determinants of anal human papillomavirus infection in men who have sex with men and transgender women," *International Journal of STD & AIDS*, vol. 30, no. 2, pp. 154–162, 2019.

[17] R. A. Nelson, A. M. Levine, L. Bernstein, D. D. Smith, and L. L. Lai, "Changing patterns of anal canal carcinoma in the United States," *Journal of Clinical Oncology*, vol. 31, no. 12, pp. 1569–1575, 2013.

[18] A.-B. Moscicki, Y. Ma, J. Jonte et al., "The role of sexual behavior and human papillomavirus persistence in predicting repeated infections with new human papillomavirus types," *Cancer Epidemiology Biomarkers & Prevention*, vol. 19, no. 8, pp. 2055–2065, 2010.

[19] H. Y. Yhim, N. R. Lee, E. K. Song et al., "The prognostic significance of tumor human papillomavirus status for patients with anal squamous cell carcinoma treated with combined chemoradiotherapy," *International Journal of Cancer*, vol. 129, no. 7, pp. 1752–1760, 2011.

[20] E. Serup-Hansen, D. Linnemann, W. Skovrider-Ruminski, E. Høgdall, P. F. Geertsen, and H. Havsteen, "Human papillomavirus genotyping and p16 expression as prognostic factors for patients with American joint committee on cancer stages I to III carcinoma of the anal canal," *Journal of Clinical Oncology*, vol. 32, no. 17, pp. 1812–1817, 2014.

[21] International Agency for Research on Cancer, "World health organization classification of tumours," in *Pathology and Genetics of Tumours of the Digestive System*, R. H. Stanley and A. A. Lauri, Eds., vol. 2, pp. 1–314, International Agency for Research on Cancer, Lyon, France, 2000, http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=11829087&retmode=ref&cmd=prlinks%5Cnpapers3://publication/uuid/C02A82F1-823F-4021-94A7-4D1CE0C4214D%0Ahttp://erj.ersjournals.com/cgi/doi/10.1183/09031936.01.00275301.

[22] M. Steinau, S. S. Patel, and E. R. Unger, "Efficient DNA extraction for HPV genotyping in formalin-fixed, paraffin-embedded tissues," *The Journal of Molecular Diagnostics*, vol. 13, no. 4, pp. 377–381, 2011.

[23] World Health Organization, *Human Papillomavirus Laboratory Manual Human Papillomavirus Laboratory Manual*, vol. 1, WHO Press, Geneva, Switzerland, 1st edition, 2009.

[24] P. S. Ravenda, E. Magni, E. Botteri et al., "Prognostic value of human papillomavirus in anal squamous cell carcinoma," *Cancer Chemotherapy and Pharmacology*, vol. 74, no. 5, pp. 1033–1038, 2014.

[25] A. D. Varnai, M. Bollmann, H. Griefingholt et al., "HPV in anal squamous cell carcinoma and anal intraepithelial neoplasia (AIN): impact of HPV analysis of anal lesions on diagnosis and prognosis," *International Journal of Colorectal Disease*, vol. 21, no. 1, pp. 135–142, 2006.

[26] A. K. Wong, R. C. Chan, N. Aggarwal, M. K. Singh, W. S. Nichols, and S. Bose, "Human papillomavirus genotypes in anal intraepithelial neoplasia and anal carcinoma as detected in tissue biopsies," *Modern Pathology*, vol. 23, no. 1, pp. 144–150, 2010.

[27] S. Ramamoorthy, Y. T. Liu, L. Luo, K. Miyai, Q. Lu, and J. M. Carethers, "Detection of multiple human papillomavirus genotypes in anal carcinoma," *Infectious Agents and Cancer*, vol. 5, no. 1, pp. 1–5, 2010.

[28] S. Valmary-Degano, E. Jacquin, J. L. Prétet et al., "Signature patterns of human papillomavirus type 16 in invasive anal carcinoma," *Human Pathology*, vol. 44, no. 6, pp. 992–1002, 2013.

[29] J. Doorbar, "Host control of human papillomavirus infection and disease," *Best Practice & Research Clinical Obstetrics & Gynaecology*, vol. 47, no. 1, pp. 27–41, 2018.

[30] F. Jin, A. N. Stein, E. L. Conway et al., "Trends in anal cancer in Australia, 1982–2005," *Vaccine*, vol. 29, no. 12, pp. 2322–2327, 2011.

[31] J. Doorbar, N. Egawa, H. Griffin, C. Kranjec, and I. Murakami, "Human papillomavirus molecular biology and disease association," *Reviews in Medical Virology*, vol. 25, pp. 2–23, 2015.

[32] R. Tachezy, T. Jirasek, M. Salakova et al., "Human papillomavirus infection and tumours of the anal canal: correlation of histology, PCR detection in paraffin sections and serology," *APMIS*, vol. 115, no. 3, pp. 195–203, 2007.

[33] N. Ouhoummane, M. Steben, F. Coutlée et al., "Squamous anal cancer: patient characteristics and HPV type distribution," *Cancer Epidemiology*, vol. 37, no. 6, pp. 807–812, 2013.

[34] S. A. Koerber, C. Schoneweg, A. Slynko et al., "Influence of human papillomavirus and $p16^{INK4a}$ on treatment outcome of

patients with anal cancer," *Radiotherapy and Oncology*, vol. 113, no. 3, pp. 331–336, 2014.

[35] N. S. L. Yeo-teh, Y. Ito, and S. Jha, "High-risk human papillomaviral oncogenes E6 and E7 target key cellular pathways to achieve oncogenesis," *International Journal of Molecular Sciences*, vol. 19, no. 6, p. 1706, 2018.

[36] R. J. Hillman, S. M. Garland, M. P. W. Gunathilake et al., "Human papillomavirus (HPV) genotypes in an Australian sample of anal cancers," *International Journal of Cancer*, vol. 135, no. 4, pp. 996–1001, 2014.

[37] F. K. Komlos, B. J. Kocjan, P. Kosorok et al., "Distribution of HPV genotypes in Slovenian patients with anal carcinoma: preliminary results," *Acta Dermatovenerologica Alpina, Pannonica et Adriatica*, vol. 20, no. 3, pp. 141–143, 2011.

[38] R. G. A. Andréia and G. A. e. Silva, "Prevalência de infecção do colo do útero pelo HPV no Brasil: revisão sistemática," *Revista de Saúde Pública*, vol. 44, no. 5, pp. 963–974, 2010.

[39] R. R. Figueiredo Alves, M. D. Turchi, L. E. Santos et al., "Prevalence, genotype profile and risk factors for multiple human papillomavirus cervical infection in unimmunized female adolescents in Goiânia, Brazil: a community-based study," *BMC Public Health*, vol. 13, no. 1, pp. 1–12, 2013.

[40] A. R. G. Ayres, G. A. Silva, M. T. B. Teixeira et al., "Infecção por hpv em mulheres atendidas pela estratégia saúde da família," *Revista de Saúde Pública*, vol. 51, p. 92, 2017.

[41] National Cancer Institute, *Surveillance, Epidemiology, and Results Program U. Seer*, National Cancer Institute, Bethesda, MD, USA, 2018, https://seer.cancer.gov/statfacts/html/anus.html.

[42] D. C. Gilbert, K. Wakeham, R. E. Langley, and C. L. Vale, "Increased risk of second cancers at sites associated with HPV after a prior HPV-associated malignancy, a systematic review and meta-analysis," *British Journal of Cancer*, vol. 120, no. 2, pp. 256–268, 2019.

[43] J. Patel, I. E. Salit, M. J. Berry et al., "Environmental scan of anal cancer screening practices: worldwide survey results," *Cancer Medicine*, vol. 3, no. 4, pp. 1052–1061, 2014.

[44] S. Mai, G. Welzel, M. Ottstadt et al., "Prognostic relevance of HPV infection and p16 overexpression in squamous cell anal cancer," *International Journal of Radiation Oncology Biology Physics*, vol. 93, no. 4, pp. 819–827, 2015.

[45] R. A. Franklin, S. Giri, P. Valasareddy, L. T. Lands, and M. G. Martin, "Comparative survival of patients with anal adenocarcinoma, squamous cell carcinoma of the anus, and rectal adenocarcinoma," *Clinical Colorectal Cancer*, vol. 15, no. 1, pp. 47–53, 2016.

[46] B. E. Hoots, J. M. Palefsky, J. M. Pimenta, and J. S. Smith, "Human papillomavirus type distribution in anal cancer and anal intraepithelial lesions," *International Journal of Cancer*, vol. 124, no. 10, pp. 2375–2383, 2009.

[47] C. Lin, S. Franceschi, and G. M. Clifford, "Human papillomavirus types from infection to cancer in the anus, according to sex and HIV status : a systematic review and meta-analysis," *The Lancet Infectious Diseases*, vol. 18, no. 2, pp. 198–206, 2018.

Hindawi
Journal of Oncology
Volume 2019, Article ID 2128410, 9 pages
https://doi.org/10.1155/2019/2128410

Review Article

The Role of Deubiquitinases in Oncovirus and Host Interactions

Yueshuo Li,[1,2,3] Feng Shi,[1,2,3] Jianmin Hu,[1,2,3] Longlong Xie,[1,2,3] Ann M. Bode,[4] and Ya Cao [1,2,3,5,6,7]

[1]*Key Laboratory of Carcinogenesis and Invasion, Chinese Ministry of Education, Department of Otolaryngology Head and Neck Surgery, Xiangya Hospital, Central South University, Changsha 410078, China*
[2]*Cancer Research Institute and School of Basic Medical Science, Xiangya School of Medicine, Central South University, Changsha 410078, China*
[3]*Key Laboratory of Carcinogenesis, Chinese Ministry of Health, Changsha 410078, China*
[4]*The Hormel Institute, University of Minnesota, Austin, MN 55912, USA*
[5]*Research Center for Technologies of Nucleic Acid-Based Diagnostics and Therapeutics Hunan Province, Changsha 410078, China*
[6]*Molecular Imaging Research Center of Central South University, Changsha 410008, Hunan, China*
[7]*National Joint Engineering Research Center for Genetic Diagnostics of Infectious Diseases and Cancer, Changsha 410078, China*

Correspondence should be addressed to Ya Cao; ycao98@vip.sina.com

Received 11 March 2019; Accepted 18 June 2019; Published 18 July 2019

Guest Editor: Hironori Yoshiyama

Infection-related cancer comprises one-sixth of the global cancer burden. Oncoviruses can directly or indirectly contribute to tumorigenesis. Ubiquitination is a dynamic and reversible posttranslational modification that participates in almost all cellular processes. Hijacking of the ubiquitin system by viruses continues to emerge as a central theme around the viral life cycle. Deubiquitinating enzymes (DUBs) maintain ubiquitin homeostasis by removing ubiquitin modifications from target proteins, thereby altering protein function, stability, and signaling pathways, as well as acting as key mediators between the virus and its host. In this review, we focus on the multiple functions of DUBs in RIG-I-like receptors (RLRs) and stimulator of interferon genes (STING)-mediated antiviral signaling pathways, oncoviruses regulation of NF-κB activation, oncoviral life cycle, and the potential of DUB inhibitors as therapeutic strategies.

1. Introduction

About 15-16% of cancer cases are attributable to infection [1]. Viral infection is one of the main risk factors for the development of infection-related cancers. Currently, the known oncogenic viruses include Epstein-Barr virus (EBV) [2–4], Kaposi sarcoma herpes virus (KSHV) [5], human T-cell lymphotropic virus type 1 (HTLV-1) [1, 6], hepatitis B virus (HBV), hepatitis C virus (HCV), human papillomavirus (HPV), and human immunodeficiency virus type 1 (HIV-1) [7]. EBV, also known as human herpes virus 4, was the first virus to be associated with human malignancy. EBV is a double-stranded DNA virus. EBV infects approximately 95% of the world's population, which is the most common and persistent viral infection in humans. HTLV was the first human retrovirus to be identified. About 3–5% of HTLV-1-infected individuals develop adult T-cell leukemia/lymphoma (ATL), which is an aggressive and lethal malignancy with few effective therapeutic options [8]. Hepatocellular cancer (HCC) is the fifth most prevalent malignant tumor and the third leading cause of cancer-related deaths. HCC is a highly lethal cancer and is mainly associated with chronic HBV and HCV infections with about 80% of HCC caused by HBV and HCV infections [9]. Around 5% of global human cancers are caused by HPV [10]. HIV infection increases cancer risk mostly by immunosuppression and chronic immune activation [7] (Table 1).

The fate and function of most proteins depend on post-translational modifications [11]. Ubiquitin is a posttranslational modifier and a key regulatory molecule participating in various cellular activities. Aberrant ubiquitin system activity is linked to many diseases, including cancer [12],

Table 1: Viral caused cancer types.

Virus type	Cancer-related virus	Cancer types	Mechanisms	Ref.
RNA virus	HIV-1	Lymphomas (most EBV-positive), KSHV-caused Kaposi sarcoma, and HPV-associated cervical and Anogenital carcinomas	indirect	[12]
	HTLV-1	Adult T-cell leukemia/lymphoma (ATL)	direct	[6, 11]
	HCV	Hepatocellular cancer, Non-Hodgkin lymphoma (especially B-cell lymphoma)	indirect	[14]
	HBV	Hepatocellular cancer	indirect	[14]
DNA virus	HPV	Cervix, Anal, Vulvar, and Penile cancers, and a subset of head and neck squamous cell carcinomas	direct	[1]
	KSHV	Kaposi sarcoma, primary effusion lymphoma	direct	[10]
	EBV	Nasopharyngeal carcinoma, Gastric cancer, Non-Hodgkin lymphomas (nhls), and Burkitt lymphoma, Nature killer/T-cell lyphoma	direct	[7–9]

infection [13, 14], and neurodegeneration [15]. All viruses need host machinery to maintain infection and replication. Therefore, oncoviruses rely on the ubiquitin system at many levels, and even hijack the ubiquitin system to satisfy their survival needs. Ubiquitination is dynamic and it can be reversed by deubiquitinating enzymes (deubiquitinases or DUBs). This explains why DUBs are the main regulators in the interactions between the virus and its host. Some viruses even encode viral deubiquitinating enzymes to affect multiple host cell processes. However, relevant research findings are very limited. Thus far, identifying and taking full advantage of viral-related DUBs is a continuing challenge [13]. Here, we review current knowledge from both the host and viral points of view, discussing how the DUBs are involved in the viral life cycle and how oncoviruses avoid or utilize the DUBs to satisfy their survival needs.

2. General Functions of DUBs

DUBs maintain ubiquitin system homeostasis by cleaving polyubiquitin chains or completely removing ubiquitin chains from ubiquitinated proteins and then generating and recycling free ubiquitin [16]. Deubiquitination has important functions in regulating the ubiquitin-dependent pathways, including cell cycle regulation, cell death, protein degradation, protein function, gene expression, and signal transduction [17]. Thus far, about 100 DUBs have been identified in six different families and are classified into two categories (Table 2) [18, 19]. Imbalances in DUBs activities are involved in multiple diseases, including cancer, inflammation, neurological disorders, and microbial infections [17]. DUBs, such as A20, OTULIN, and CYLD, mediate NF-κB and cell death to maintain optimal signal transduction and immune homeostasis [20]. Compared with normal cells, cancer cells need elevated synthesis of growth-promoting proteins and protein-degradation capacity to satisfy uncontrolled mitosis. Much research has focused on studying their function and substrates to elucidate the role of DUBs in specific diseases. Abnormal expression of DUBs-encoding genes has been detected in human cancers. A mutant tumor suppressor gene *CYLD* has been identified in familial cylindromatosis and CYLD is downregulated in multiple cancer types [21]. Hajek, *et. al* have identified a distinct subset of HPV-associated head and neck squamous cell carcinomas that have *TRAF3/CYLD* mutations [22]. Multiple oncoviruses utilize these DUBs to edit ubiquitin chains and alter ubiquitin signaling, contributing to virus infection, replication, and pathogenesis. To date, vaccines against HBV and HPV have already begun to decrease the incidence of cancers attributed to these oncoviruses. However, other oncoviruses have no existing vaccines. In addition to prevention by vaccines, targeting the interplay between oncoviruses and their host might give rise to effective and inexpensive treatment strategies with minimal toxicity.

3. DUBs Participate in Antiviral Innate Responses

As the first line of host defense against viral infection, host pattern recognition receptors (PRRs), including RLRs, toll-like receptors (TLRs), and cytosolic dsDNA sensors (such as STING), recognize viral nucleic acids inducing innate immune responses, resulting in the production of type I interferons (IFNs) and proinflammatory cytokines [23, 24]. Using or bypassing host immune signaling is important for

Table 2: DUBs classification.

Categories	Families	DUBs
Cysteine proteases	USP	USP 1-8, USP 9X, USP 9Y, USP 10-16, USP 17 L1, USP 17 L2, USP 18-26, USP 27X, USP 28-54, USP L1, CYLD
	UCH	UCH L1, UCH L3, UCH L5, BAP1
	MJD	ATXN3, ATXN3L, JOSD1, JOSD2
	OTU	OTUB1, OTUB2, OTUD1, OTUD3, OTUD4, OTUD5, OTUD6A, OTUD6B, OTUD7A, OTUD7B, A2O, HIN1L, VCPIP1, TRABID, YOD1
	MINDY	FAM63A, FAM63B, FAM188A, FAM188B
Metalloproteases	JAMM	AMSH, AMSH-LP, BRCC36, COPS5, COPS6, EIF3F, EIF3H, MPND, MYSM1, PSMD7, PSMD14, PRPF8

Six classes of DUBs in the human genome are classified into two categories, cysteine proteases, and metalloproteases. Five classes are cysteine proteases: USP, ubiquitin-specific proteases; UCH, ubiquitin carboxyl-terminal hydrolases; MJD, Machado-Joseph disease protein domain proteases; OTU, ovarian-tumor proteases; MINDY, motif interacting with Ub-containing DUB family. One class is metalloproteases: JAMM, JAMM/MPN domain-associated metallopeptidases.

viruses to successfully establish infection. A thorough understanding of the molecular mechanisms between virus-related deubiquitination and antiviral innate immunity signaling is necessary for the control of infectious diseases and for developing therapeutic targets.

3.1. DUBs Are Involved in RLRs-Mediated Innate Immunity against RNA Oncoviruses. RNA viruses are mainly recognized by RLRs. RLRs recognize viral RNAs through the RNA helicase domain (RLD), and then interact with the mitochondrial antiviral signaling protein, MAVS [25]. The RLRs include retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), which belong to a family of cytosolic host RNA helicases that recognize distinct nonself RNA signatures and trigger innate immune responses against several RNA viral infections. After recognition of viral RNA through the RNA helicase domain (RLD), RIG-I or MDA5 binds to MAVS. The K63-linked polyubiquitination of these adaptors is essential for signal activation. On the other hand, DUBs have also been shown to regulate antiviral innate immunity. Some DUBs negatively regulate the innate immune system to guard against excessive self-destructive immune responses and thus play a critical role in maintaining the balance of the immune system. USP21 [26], USP3 [27], and CYLD [28] negative regulate RIG-I and MDA5 activation by binding to and removing K63-linked polyubiquitin chains. The deubiquitinases OTUB1/2 [29, 30] and MYSM1 [31] inhibit K63-linked ubiquitination of TRAF3/6 and negatively regulate IFNs production. OTUD1 can also remove K48-linked ubiquitination from Smurf1, which targets MAVS for K48-linked ubiquitination and degradation, contributing to the degradation of MAVS [25]. Zhang *et al.* found that RNA viral infection can utilize the OTUD1-Smurf1 axis through the NF-κB signaling pathway to promote down-regulation of the MAVS, TRAF3, and TRAF6 proteins and IFNs production [32]. In addition to the DUBs mentioned above, the host also uses positive regulation of DUBs against viral infection. USP15 reduces the K48-linked ubiquitination of TRIM25 (targeting RIG-I K63-linked ubiquitination and activation) leading to its stabilization [33] and promoting RIG-I activation. USP25 clears virus-triggered K48-linked ubiquitination, promoting the stability of TRAF3 and TRAF6 [34] and positively regulating RNA virus-triggered innate immune responses. USP1 and UAF1 bind to TBK1, remove its K48-linked polyubiquitination, and reverses the degradation process of TBK1. This USP1–UAF1 complex enhances TLR3/4 and RIG-I–induced IFN regulatory factor 3 (IRF3) activation and subsequent IFN-β secretion [35]. These studies indicate that DUBs play a critical role in regulating the virus-triggered RIG-I-like pathway and IFNs production, which are crucial for RNA viruses to establish efficient infection at an early stage (Figure 1).

3.2. DUBs Are Involved in STING-Mediated Innate Immunity against DNA Oncoviruses. Host cells express multiple cytosolic DNA sensors to recognize exogenous viral nucleic acids, such as DAI, DDX41, IFI16, and cyclic GMP-AMP synthase (cGAS). These sensors trigger signaling pathways and activate the adaptor protein stimulator of IFN genes (STING; also known as MITA) to induce the expression of type I IFN [36]. STING is a key adaptor protein for most DNA sensing pathways. Ubiquitination of STING caused by viral infection plays critical roles in virus-triggered signaling [37]. K27- or K63-linked ubiquitination mediated by various E3 ubiquitin ligases, such as TRIM32, AMFR, and INSIG1 [38, 39], is essential for full activation of STING. Double-stranded DNA viruses, such as EBV, use ubiquinase TRIM29 to ubiquitinate and degrade STING, suppressing host innate immunity that leads to the persistence of DNA viral infections [40]. HSV infection can recruit USP21 to STING through p38-mediated phosphorylation of USP21 at Ser538. USP21 deubiquitinates the K27/63-linked polyubiquitin chain on STING, thereby leading to reduced production of type I IFNs [41]. During HTLV-1 and HBV infection, Tax and HBV polymerases decrease the K63-linked ubiquitination of STING and disrupt the interactions between STING and TBK1, which leads to loss of STING function and subsequent impairment of IRF3 activation, IFN-induction, and an antiviral response [42, 43]. In addition, USP13 removes K27-linked polyubiquitin

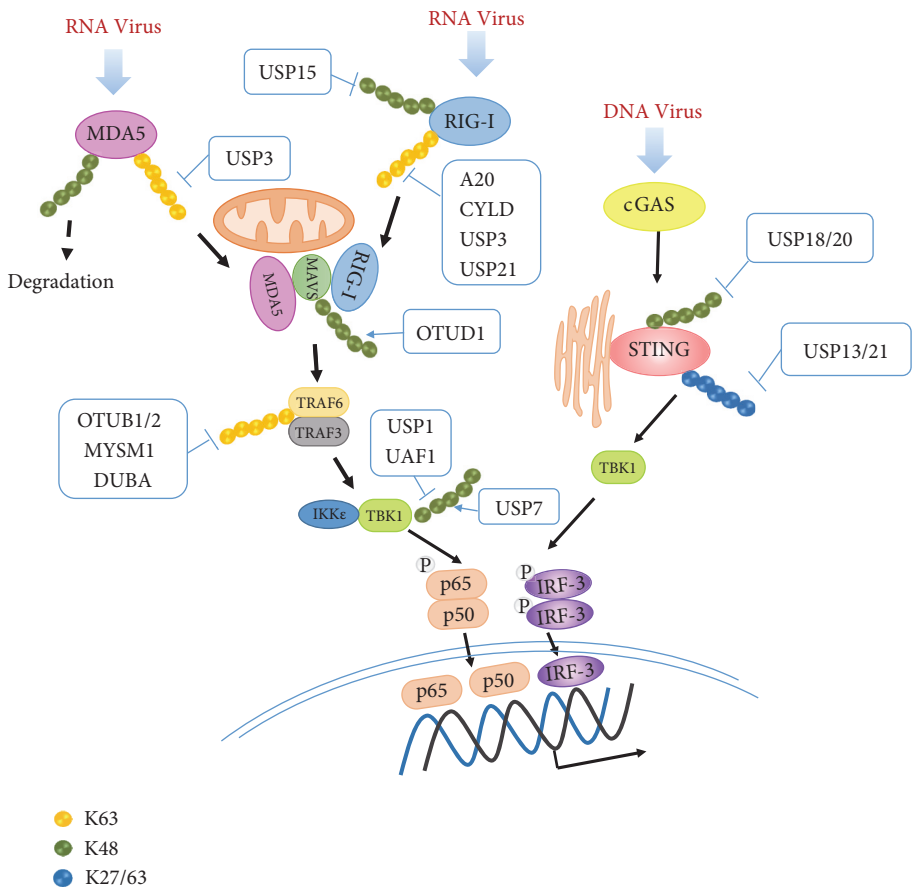


Figure 1: *DUBs participate in antiviral innate immunity.* During virus infection, K63-linked polyubiquitination of RLRs promotes their interaction with MAVS and signal transmission. USP15 inhibits K48-ubiquitination of RNA sensor RIG-I to inhibit RIG-I degradation; A20, CYLD, USP3, and USP21 inhibit K63-ubiquitination of RIG-I to negatively regulate RIG-I activation. USP3 inhibits K63 ubiquitination of MDA5 to inhibit its activation. RIG-I and MDA5 bind to and activate MAVS. Activated MAVS works as a scaffold to recruit various TRAFs, leading to TBK1/IKB kinase Ɛ (IKK-Ɛ)-mediated phosphorylation and nuclear translocation of IRF3 and IRF7, and production of IFNs and OTUD1 stabilizes MAVS by removing K48-ubiquitination. Deubiquitinases OTUB1/2, MYSM1, and DUBA inhibit K63-linked ubiquitination of TRAF3 or TRAF6 and negatively regulate IFNs production. HSV infection can recruit USP21 to deubiquitinate the K27/63-linked polyubiquitin chain on STING. USP13 removes K27-linked polyubiquitin chains from STING and thereby impairs the recruitment of TBK1 to reduce the antiviral immune response against DNA viruses. USP18 recruits USP20 in an enzymatic activity-independent manner and facilitates USP20 to remove K33- and K48-linked ubiquitin chains from STING, thereby preventing degradation of STING caused by DNA virus infection. USP7 interacts with TRIM27 and removes its K48-linked polyubiquitination, promoting the degradation of TBK1. USP1 and UAF1 inhibit K48 polyubiquitin chains to stabilize TBK1 contributing to IFNs production.

chains from STING and then decreases the antiviral immune response against DNA viruses by disrupting the recruitment of TBK1 [44]. To inhibit DNA viral infection, USP18 recruits USP20 in an enzymatic activity-independent manner and facilitates USP20 to remove K33- and K48-linked ubiquitin chains from STING, thereby preventing degradation of STING caused by DNA viral infection [45] (Figure 1). HPV upregulates UCHL1 to clear K63-linked ubiquitin chains from TRAF3, resulting in a lower amount of the downstream signaling complex TRAF3-TBK-1 to suppress the type I IFN pathway [46]. Further research is still needed to find and clarify the functions of DUBs during viral infection. More information will help control infectious diseases and facilitate the development of clinical antiviral therapies.

4. DUBs Regulate Oncovirus Infection and Activation in an NF-κB-Dependent Manner

RLR-, TLR-, and STING-induced innate immune response contribute to activation of NF-κB. NF-κB signaling plays an essential role in immune regulation and its role has been explored in almost all aspects of cellular activity. To achieve successful infection, oncoviruses have developed mechanisms to hijack the NF-κB pathway. Multiple DUBs are key regulators of NF-κB signaling. Several DUBs, such as CYLD and A20, have been extensively studied in the negative regulation of NF-κB signaling. During the viral infection stage, HCV stimulation upregulates A20/ABIN1 expression, thereby suppressing NF-κB activity and leading to inefficient M1 macrophage polarization to promote HCV infection [58].

Table 3: Oncoviruses encoded v-DUBs.

oncovirus	v-DUB	Deubiquitination types	targets	pathways	Ref.
EBV	BPLF1	Lys48- or Lys63-linked polyubiquitin	TRAF6, NEMO, IκBα	Inhibits TLR signaling and NF-κB pathway	[47, 48]
KSHV	ORF64	Lys48- or Lys63-linked polyubiquitin	RIG-I	Inhibits RIG-I-mediated-IFN signaling	[49]

EBV deubiquitinating Enzyme (v-DUB) BPLF1 inhibits TLR signaling through both MyD88- and TRIF-dependent pathways by removing ubiquitin chains from signaling intermediates, such as TRAF6, NEMO, and IκBα [59, 60]. This leads to reduced NF-κB activation and proinflammatory cytokine production in response to EBV and contributes to virus infectivity. During the infection stage, oncoviruses upregulate NF-κB inhibitory DUBs or encode viral DUBs disrupting secretion of antiviral cytokines and interfering with the innate antiviral immune responses by inhibiting NF-κB activation.

NF-κB activation also plays an important role in virus reactivation, replication, and virus-mediated cell transformation. HIV inhibits CYLD to facilitate the NF-κB pathway, playing an important role in HIV reactivation from latency [61]. HTLV-1- encoded Tax inactivates the NF-κB negative regulators, A20 and CYLD, which allows chronic NF-κB activation in HTLV-1-transformed cells [62]. USP20 deubiquitinates TRAF6 and Tax, thus suppressing interleukin 1β (IL-1β)- and Tax-induced NF-κB activation, suggesting USP20 as a key negative regulator of Tax-induced NF-κB signaling [63]. The HPV-encoded E6 protein targets CYLD, resulting in ubiquitination and proteasomal degradation of CYLD to induce NF-κB activation [64]. In keratinocytes, HPV infection inhibits CYLD expression, resulting in enhanced K63-linked polyubiquitination and nuclear translocation of BCL-3, which leads to activation of the NF-κB signaling pathway [65, 66]. Mutation of CYLD in HPV-positive head and neck squamous cell carcinomas (HNSCCs) leads to the activation of NF-κB signaling and maintenance of episomal HPV in tumors. In KSHV-infected primary effusion lymphoma cell lines, KSHV-encoded viral FLICE inhibitory protein (vFLIP) K13 can induce NF-κB activation, which upregulates A20 expression. A20 interacts with K13 and blocks K13-induced excessive NF-κB activation in a negative feedback manner [67, 68]. The regulation of NF-κB signaling by oncoviruses is not only important for the viral life cycle, but also contributes to the development of malignant tumors. Focusing on the role of DUBs in viral biology and NF-κB may contribute to infection-related cancer prevention and treatment.

5. Oncoviruses Use Host DUBs or Encode v-DUBs to Facilitate Viral Infection and Replication

5.1. EBV. EBV-encoded latent membrane protein 1 (LMP1) is an important tumorigenic protein. Our previous studies have shown that LMP1 rescues p53-induced cell cycle arrest and apoptosis by promoting K63-linked ubiquitination of p53. LMP1 also inhibits cell necroptosis by modulating RIPK1/3(receptor interacting protein kinase 1/3) ubiquitination [69, 70]. LMP1 can also induce the expression of UCH-L1 and it may contribute to viral transformation and the progression of lymphoid malignancies [71, 72]. EBV nuclear antigen 1 (EBNA1) plays important roles in promoting EBV genome replication and persistence, and EBV latent gene expression. EBNA1 interacts with USP7, which is also known as herpes virus associated ubiquitin-specific protease (HAUSP). The EBNA1 and USP7 interaction can promote cell survival and contribute to EBNA1 functions at the EBV oriP and inhibit p53-mediated antiviral responses [73]. The EBV nuclear antigen 3 (EBNA3) family targets and interacts with USP46/USP12 deubiquitination complexes. The complex exhibits DUB activity and contributes to EBNA3-mediated lymphoblastoid cell growth [74]. Besides utilizing host DUBs, EBV can also encode the viral deubiquitinating enzyme, BPLF1, which is an immune evasion gene product that can suppress antiviral immune responses during primary infection [47]. BPLF1 is expressed during the late phase of lytic EBV infection and is incorporated into viral particles. It can eliminate K63- and/or K48-linked ubiquitin chains and act as an active DUB during the productive lytic cycle and EBV infection [48] (Table 3).

5.2. KSHV. KSHV-encoded viral interferon regulatory factor 1 (vIRF1) can bind to USP7 and decrease the deubiquitinase activity of USP7 for stabilizing p53, thereby disrupting the p53 signaling pathway [73]. Latency-associated nuclear antigen (LANA) induces the expression of UCH-L1, which might lead to viral transformation and the progression of lymphoid malignancies [71]. KSHV encoded tegument protein ORF64, which has deubiquitinase activity can inhibit the ubiquitination of RIG-I and suppress RIG-I-mediated IFN signaling. It is necessary for KSHV infection [49] (Table 3).

5.3. HPV. E6 and E7 are the main oncoproteins encoded by HPV. USP11 and USP15 can greatly increase the steady state level of HPV-16 E6 and E7 by reducing their ubiquitination and degradation, thereby increasing the oncogenic potential of HPV [75, 76].

5.4. HIV. HIV-1 Tat is encoded at an early stage after infection and is in charge of enhancing viral production. USP7 and USP47 stabilize the HIV-1 Tat protein by removing its K48 polyubiquitination chain [77]. The stabilization of Tat leads

Table 4: Chemical DUB inhibitors.

DUB Inhibitors(DIs)	target	Cancer types	reference
HBX 41,108	USP5, 7, 8 and UCH-L3	myeloma	[50]
HBX -19,818	USP7	colon carcinoma	[51]
HBX-28,258	USP7	colon carcinoma	[51]
P5091	USP7	myeloma	[50]
P22077	USP7	-	[52]
GW7674	USP1	non-small cell lung cancer	[53, 54]
ML323	USP1 and some DUBs	non-small cell lung cancer and osteosarcoma	[53, 54]
b-AP15 (VLX1500)	UCHL5, USP14 and some DUBs	nonspecific	[55]
WPI 130	USP5/USP9x/USP14/UCHL1/UCHL5	breast cancer	[56, 57]
PR-619	broad-range DUB inhibitor	-	[52]

to enhanced *HIV-1* gene expression, facilitates virus spread, and also reduces immune recognition in HIV-1- expressing cells [78].

5.5. HCV. HCV encodes the core protein and nonstructural (NS) proteins NS3 and NS5A and promotes oncogenic transformation, replication, and virus assembly [9]. Studies show that NS5A binds to the ovarian tumor protein, deubiquitinase 7B (OTUD7B) and enhances OTUD7B DUB activity, which may contribute to viral replication and infection [50].

Oncoviruses utilize host DUBs to stabilize viral proteins, which increases the oncogenic potential of oncoviruses. Oncogenic viral products disturb host cell signaling pathways by enhancing the level of specific DUBs or DUB activity to promote viral genome replication and persistence. One DUB exhibited an opposite role in different oncoviruses, which indicates that if a DUB is used as an antiviral target, the potential effect on other viruses must be considered. Further studies are still needed to describe the detailed mechanisms between DUBs and oncoviruses.

6. DUB Inhibitors (DIs) as Potential Therapeutic Strategies

Inhibition of proteasome deubiquitinating activity is a new cancer therapy. Most DIs are small molecule compounds, exerting their function by suppressing DUB activity. The ubiquitin-specific proteases (USPs) are the largest and the most diverse DUB family and gene mutations, altered activity, or abnormal expression of USPs has been linked to multiple cancer types. USPs attractive are therapeutic targets and interest is growing in the development of enzyme selective or specific chemical inhibitors as antiviral and anticancer agents. The USP7-specific small molecule inhibitors, HBX41, 108, and P5091, induce apoptosis by stabilizing p53 in multiple myeloma cells resistant to conventional bortezomib therapies [55]. b-AP15 inhibits USP14 and UCHL5 and was shown to inhibit tumor growth in multiple solid tumor mouse models and attenuated tumor invasion in acute myelogenous leukemia in *in vivo* models [56]. WPI130 targets USP5, USP9X, and USP14 and inhibits viral progeny production of several RNA viruses, induces apoptosis, and suppresses growth of breast cancer cells [53, 57]. The USP1 inhibitors, GW7647 and ML323, attenuate growth of leukemic cells, non-small-cell lung cancer cells, and osteosarcoma cells [52, 54]. In light of these findings, DIs could be significant as potential therapeutic modalities in the treatment of multiple cancers. Given the multiple functions of DUBs in viral infection, developing inhibitors targeting the functional activities of virus-associated DUBs or virus-encoded DUBs might contribute to the reduction of oncovirus infections and could be used in infection-related cancers as accessory treatments (Table 4).

7. Conclusions and Perspectives

DUBs are central component in the ubiquitin signaling system to modulate proteostasis and have been shown to participate in all aspects of the viral life cycle. To escape from host immune responses, hijacking of the ubiquitin system by viruses continues to emerge as a central theme around virus infection and replication. In this review, we summarized recent studies focusing on the role of deubiquitinases in antiviral immune responses, modulation of the NF-κB pathway, as well as on RNA and DNA oncovirus infection, replication, and pathogenesis. However, the detailed mechanisms between viruses, host, and DUBs are still not clear. As for the potential use of DIs as therapeutic strategies against cancer, many have been identified but none have been used clinically. As a new cancer therapy target, many challenges remain to be addressed for further understanding of DUBs function in order to develop compounds that inhibit or induce their activity to control the pathogenesis of oncoviruses.

Conflicts of Interest

The authors have no conflicts of interest.

Acknowledgments

This study was supported by National Natural Science Foundation of China (81430064, 81602402, and 81672705) and

the College Students' Innovation Project of Central South University (2018zzts234 and 2018zzts230).

References

[1] M. Plummer, C. de Martel, J. Vignat, J. Ferlay, F. Bray, and S. Franceschi, "Global burden of cancers attributable to infections in 2012: a synthetic analysis," *The Lancet Global Health*, vol. 4, no. 9, pp. e609–e616, 2016.

[2] Y. Cao, "EBV based cancer prevention and therapy in nasopharyngeal carcinoma," *Nature Partner Journals Precision Oncology*, vol. 1, no. 1, p. 10, 2017.

[3] A. J. Bass, V. Thorsson, I. Shmulevich et al., "Comprehensive molecular characterization of gastric adenocarcinoma," *Nature*, vol. 513, pp. 202–209, 2014.

[4] F. Suarez and M. Lecuit, "Infection-associated non-Hodgkin lymphomas," *Clinical Microbiology and Infection*, vol. 21, no. 11, pp. 991–997, 2015.

[5] P. S. Moore and Y. Chang, "The conundrum of causality in tumor virology: The cases of KSHV and MCV," *Seminars in Cancer Biology*, vol. 26, pp. 4–12, 2014.

[6] R. L. Siegel, K. D. Miller, and A. Jemal, "Cancer statistics, 2017," *CA: A Cancer Journal for Clinicians*, vol. 67, no. 1, pp. 7–30, 2017.

[7] A. Carbone, E. Vaccher, A. Gloghini et al., "Diagnosis and management of lymphomas and other cancers in HIV-infected patients," *Nature Reviews Clinical Oncology*, vol. 11, no. 4, pp. 223–238, 2014.

[8] C. Giam and O. Semmes, "HTLV-1 infection and adult T-cell leukemia/lymphoma—a tale of two proteins: tax and HBZ," *Viruses*, vol. 8, no. 6, p. 161, 2016.

[9] A. Arzumanyan, H. M. G. P. V. Reis, and M. A. Feitelson, "Pathogenic mechanisms in HBV- and HCV-associated hepatocellular carcinoma," *Nature Reviews Cancer*, vol. 13, no. 2, pp. 123–135, 2013.

[10] C. Casper and C. Fitzmaurice, "Infection-related cancers: prioritising an important and eliminable contributor to the global cancer burden," *The Lancet Global Health*, vol. 4, no. 9, pp. e580–e581, 2016.

[11] X. Liu, F. Shi, Y. Li et al., "Post-translational modifications as key regulators of TNF-induced necroptosis," *Cell Death & Disease*, vol. 7, no. 7, pp. e2293–e2293, 2016.

[12] D. Hoeller and I. Dikic, "Targeting the ubiquitin system in cancer therapy," *Nature*, vol. 458, no. 7237, pp. 438–444, 2009.

[13] M. K. Isaacson and H. L. Ploegh, "Ubiquitination, ubiquitin-like modifiers, and deubiquitination in viral infection," *Cell Host & Microbe*, vol. 5, no. 6, pp. 559–570, 2009.

[14] G. Lopez-Castejon and M. J. Edelmann, "Deubiquitinases: novel therapeutic targets in immune surveillance?" *Mediators of Inflammation*, vol. 2016, Article ID 3481371, 13 pages, 2016.

[15] A. Ciechanover and Y. T. Kwon, "Degradation of misfolded proteins in neurodegenerative diseases: therapeutic targets and strategies," *Experimental & Molecular Medicine*, vol. 47, no. 3, article e147, 2015.

[16] P. Farshi, R. R. Deshmukh, J. O. Nwankwo et al., "Deubiquitinases (DUBs) and DUB inhibitors: a patent review," *Expert Opinion on Therapeutic Patents*, vol. 25, no. 10, pp. 1191–1208, 2015.

[17] R. Pfoh, I. K. Lacdao, and V. Saridakis, "Deubiquitinases and the new therapeutic opportunities offered to cancer," *Endocrine-Related Cancer*, vol. 22, no. 1, pp. T35–T54, 2015.

[18] P. D'Arcy, X. Wang, and S. Linder, "Deubiquitinase inhibition as a cancer therapeutic strategy," *Pharmacology & Therapeutics*, vol. 147, pp. 32–54, 2015.

[19] S. Abdul Rehman, Y. Kristariyanto, S. Choi et al., "MINDY-1 is a member of an evolutionarily conserved and structurally distinct new family of deubiquitinating enzymes," *Molecular Cell*, vol. 63, no. 1, pp. 146–155, 2016.

[20] M. Lork, K. Verhelst, and R. Beyaert, "CYLD, A20 and OTULIN deubiquitinases in NF-κB signaling and cell death: So similar, yet so different," *Cell Death & Differentiation*, vol. 24, no. 7, pp. 1172–1183, 2017.

[21] K. Nikolaou, A. Tsagaratou, C. Eftychi, G. Kollias, G. Mosialos, and I. Talianidis, "Inactivation of the deubiquitinase CYLD in hepatocytes causes apoptosis, inflammation, fibrosis, and cancer," *Cancer Cell*, vol. 21, no. 6, pp. 738–750, 2012.

[22] M. Hajek, A. Sewell, S. Kaech, B. Burtness, W. G. Yarbrough, and N. Issaeva, "*TRAF3/CYLD* mutations identify a distinct subset of human papillomavirus-associated head and neck squamous cell carcinoma ," *Cancer*, vol. 123, no. 10, pp. 1778–1790, 2017.

[23] L. Sun, J. Wu, F. Du, X. Chen, and Z. J. Chen, "Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway," *Science*, vol. 339, no. 6121, pp. 786–791, 2013.

[24] R. Barbalat, S. E. Ewald, M. L. Mouchess, and G. M. Barton, "Nucleic acid recognition by the innate immune system," *Annual Review of Immunology*, vol. 29, pp. 185–214, 2011.

[25] K. M. Quicke, M. S. Diamond, and M. S. Suthar, "Negative regulators of the RIG-I-like receptor signaling pathway," *European Journal of Immunology*, vol. 47, no. 4, pp. 615–628, 2017.

[26] Y. Fan, R. Mao, Y. Yu et al., "USP21 negatively regulates antiviral response by acting as a RIG-I deubiquitinase," *The Journal of Experimental Medicine*, vol. 211, no. 2, pp. 313–328, 2014.

[27] J. Cui, Y. Song, Y. Li et al., "USP3 inhibits type I interferon signaling by deubiquitinating RIG-I-like receptors," *Cell Research*, vol. 24, no. 4, pp. 400–416, 2014.

[28] C. S. Friedman, M. A. O'Donnell, D. Legarda-Addison et al., "The tumour suppressor CYLD is a negative regulator of RIG-I-mediated antiviral response," *EMBO Reports*, vol. 9, no. 9, pp. 930–936, 2008.

[29] S. Li, H. Zheng, A. Mao et al., "Regulation of Virus-triggered Signaling by OTUB1- and OTUB2-mediated Deubiquitination of TRAF3 and TRAF6," *The Journal of Biological Chemistry*, vol. 285, no. 7, pp. 4291–4297, 2010.

[30] Y. Peng, R. Xu, X. Zheng, and V. Thiel, "HSCARG negatively regulates the cellular antiviral RIG-I like receptor signaling pathway by inhibiting TRAF3 ubiquitination via recruiting OTUB1," *PLoS Pathogens*, vol. 10, no. 4, Article ID e1004041, 2014.

[31] S. Panda, J. A. Nilsson, and N. O. Gekara, "Deubiquitinase MYSM1 regulates innate immunity through inactivation of TRAF3 and TRAF6 complexes," *Immunity*, vol. 43, no. 4, pp. 647–659, 2015.

[32] L. Zhang, J. Liu, L. Qian et al., "Induction of OTUD1 by RNA viruses potently inhibits innate immune responses by promoting degradation of the MAVS/TRAF3/TRAF6 signalosome," *PLoS Pathogens*, vol. 14, no. 5, Article ID e1007067, 2018.

[33] E. Pauli, Y. K. Chan, M. E. Davis et al., "The ubiquitin-specific protease USP15 promotes RIG-I-mediated antiviral signaling by deubiquitylating TRIM25," *Science Signaling*, vol. 7, no. 307, p. ra3, 2014.

[34] D. Lin, M. Zhang, M.-X. Zhang et al., "Erratum: Induction of USP25 by viral infection promotes innate antiviral responses by mediating the stabilization of TRAF3 and TRAF6 (Proceedings of the National Academy of Sciences of the United States of America (2015) 112 (11324-11329) DOI: 10.1073/pnas.1509968112)," *Proceedings of the National Acadamy of Sciences of the United States of America*, vol. 112, no. 43, pp. 11324–11329, 2015.

[35] Z. Yu, H. Song, M. Jia et al., "USP1–UAF1 deubiquitinase complex stabilizes TBK1 and enhances antiviral responses," *The Journal of Experimental Medicine*, vol. 214, no. 12, pp. 3553–3563, 2017.

[36] S. Paludan and A. Bowie, "Immune Sensing of DNA," *Immunity*, vol. 38, no. 5, pp. 870–880, 2013.

[37] Q. Chen, L. Sun, and Z. J. Chen, "Regulation and function of the cGAS–STING pathway of cytosolic DNA sensing," *Nature Immunology*, vol. 17, no. 10, pp. 1142–1149, 2016.

[38] Q. Wang, X. Liu, Y. Cui et al., "The E3 Ubiquitin ligase AMFR and INSIG1 bridge the activation of TBK1 kinase by modifying the adaptor STING," *Immunity*, vol. 41, no. 6, pp. 919–933, 2014.

[39] J. Zhang, M. Hu, Y. Wang, and H. Shu, "TRIM32 protein modulates type I interferon induction and cellular antiviral response by targeting MITA/STING protein for K63-linked ubiquitination," *The Journal of Biological Chemistry*, vol. 287, no. 34, pp. 28646–28655, 2012.

[40] J. Xing, A. Zhang, H. Zhang et al., "TRIM29 promotes DNA virus infections by inhibiting innate immune response," *Nature Communications*, vol. 8, no. 1, p. 945, 2017.

[41] Y. Chen, L. Wang, J. Jin et al., "p38 inhibition provides anti–DNA virus immunity by regulation of USP21 phosphorylation and STING activation," *The Journal of Experimental Medicine*, vol. 214, no. 4, pp. 991–1010, 2017.

[42] Y. Liu, J. Li, J. Chen et al., "Hepatitis B virus polymerase disrupts K63-linked ubiquitination of STING to block innate cytosolic DNA-sensing pathways," *Journal of Virology*, vol. 89, no. 4, pp. 2287–2300, 2015.

[43] J. Wang, S. Yang, L. Liu, H. Wang, and B. Yang, "HTLV-1 Tax impairs K63-linked ubiquitination of STING to evade host innate immunity," *Virus Research*, vol. 232, pp. 13–21, 2017.

[44] H. Sun, Q. Zhang, Y. Jing et al., "USP13 negatively regulates antiviral responses by deubiquitinating STING," *Nature Communications*, vol. 8, no. 1, Article ID 15534, 2017.

[45] M. Zhang, M. Zhang, Q. Zhang et al., "USP18 recruits USP20 to promote innate antiviral response through deubiquitinating STING/MITA," *Cell Research*, vol. 26, no. 12, pp. 1302–1319, 2016.

[46] R. Karim, B. Tummers, C. Meyers et al., "Human papillomavirus (HPV) upregulates the cellular deubiquitinase UCHL1 to suppress the keratinocyte's innate immune response," *PLoS Pathogens*, vol. 9, no. 5, Article ID e1003384, 2013.

[47] J. McKenzie, F. Lopez-Giraldez, H. Delecluse, A. Walsh, A. El-Guindy, and P. D. Ling, "The epstein-barr virus immunoevasins BCRF1 and BPLF1 are expressed by a mechanism independent of the canonical late pre-initiation complex," *PLoS Pathogens*, vol. 12, no. 11, Article ID e1006008, 2016.

[48] C. B. Whitehurst, S. Ning, G. L. Bentz et al., "The epstein-barr virus (EBV) deubiquitinating enzyme BPLF1 reduces EBV ribonucleotide reductase activity," *Journal of Virology*, vol. 83, no. 9, pp. 4345–4353, 2009.

[49] K. Inn, S. Lee, J. Y. Rathbun et al., "Inhibition of RIG-I-mediated signaling by kaposi's sarcoma-associated herpesvirus-encoded deubiquitinase ORF64," *Journal of Virology*, vol. 85, no. 20, pp. 10899–10904, 2011.

[50] I. R. Sianipar, C. Matsui, N. Minami et al., "Physical and functional interaction between hepatitis C virus NS5A protein and ovarian tumor protein deubiquitinase 7B," *Microbiology and Immunology*, vol. 59, no. 8, pp. 466–476, 2015.

[51] M. Altun, H. B. Kramer, L. I. Willems et al., "Activity-based chemical proteomics accelerates inhibitor development for deubiquitylating enzymes," *Chemistry & Biology*, vol. 18, no. 11, pp. 1401–1412, 2011.

[52] Q. Liang, T. S. Dexheimer, P. Zhang et al., "A selective USP1–UAF1 inhibitor links deubiquitination to DNA damage responses," *Nature Chemical Biology*, vol. 10, no. 4, pp. 298–304, 2014.

[53] A. Pal and N. J. Donato, "Ubiquitin-specific proteases as therapeutic targets for the treatment of breast cancer," *Breast Cancer Research*, vol. 16, no. 5, p. 461, 2014.

[54] J. Chen, T. S. Dexheimer, Y. Ai et al., "Selective and cell-active inhibitors of the USP1/ UAF1 deubiquitinase complex reverse cisplatin resistance in non-small cell lung cancer cells," *Chemistry & Biology*, vol. 18, no. 11, pp. 1390–1400, 2011.

[55] F. Colland, E. Formstecher, X. Jacq et al., "Small-molecule inhibitor of USP7/HAUSP ubiquitin protease stabilizes and activates p53 in cells," *Molecular Cancer Therapeutics*, vol. 8, no. 8, pp. 2286–2295, 2009.

[56] P. D'Arcy, S. Brnjic, M. H. Olofsson et al., "Inhibition of proteasome deubiquitinating activity as a new cancer therapy," *Nature Medicine*, vol. 17, no. 12, pp. 1636–1640, 2011.

[57] J. W. Perry, M. Ahmed, K. Chang et al., "Antiviral activity of a small molecule deubiquitinase inhibitor occurs via induction of the unfolded protein response," *PLoS Pathogens*, vol. 8, no. 7, Article ID e1002783, 2012.

[58] C. Fan, Y. Zhang, Y. Zhou et al., "Up-regulation of A20/ABIN1 contributes to inefficient M1 macrophage polarization during Hepatitis C virus infection," *Virology Journal*, vol. 12, no. 1, 2015.

[59] M. van Gent, S. G. Braem, A. de Jong et al., "Epstein-barr virus large tegument protein BPLF1 contributes to innate immune evasion through interference with toll-like receptor signaling," *PLoS Pathogens*, vol. 10, no. 2, Article ID e1003960, 2014.

[60] C. B. Whitehurst, C. Vaziri, J. Shackelford, and J. S. Pagano, "Epstein-barr virus BPLF1 deubiquitinates PCNA and attenuates polymerase recruitment to DNA damage sites," *Journal of Virology*, vol. 86, no. 15, pp. 8097–8106, 2012.

[61] L. Manganaro, L. Pache, T. Herrmann et al., "Tumor suppressor cylindromatosis (CYLD) controls HIV transcription in an NF-B-dependent manner," *Journal of Virology*, vol. 88, no. 13, pp. 7528–7540, 2014.

[62] R. Pujari, R. Hunte, R. Thomas et al., "Human T-cell leukemia virus type 1 (HTLV-1) tax requires CADM1/TSLC1 for inactivation of the NF-κB inhibitor A20 and constitutive NF-κB signaling," *PLoS Pathogens*, vol. 11, no. 3, Article ID e1004721, 2015.

[63] J. Yasunaga, F. C. Lin, X. Lu, and K. T. Jeang, "Ubiquitin-specific peptidase 20 targets TRAF6 and human T cell leukemia virus type 1 tax to negatively regulate NF-B signaling," *Journal of Virology*, vol. 85, no. 13, pp. 6212–6219, 2011.

[64] J. An, D. Mo, H. Liu et al., "Inactivation of the CYLD deubiquitinase by HPV E6 mediates hypoxia-induced NF-κB activation," *Cancer Cell*, vol. 14, no. 5, pp. 394–407, 2008.

[65] K. Shostak, X. Zhang, P. Hubert et al., "NF-κB-induced KIAA1199 promotes survival through EGFR signalling," *Nature Communications*, vol. 5, no. 1, Article ID 5232, 2014.

[66] K. Shostak and A. Chariot, "EGFR and NF-κB: partners in cancer," *Trends in Molecular Medicine*, vol. 21, no. 6, pp. 385–393, 2015.

[67] H. Matta, R. Gopalakrishnan, V. Punj, H. Yi, Y. Suo, and P. M. Chaudhary, "A20 is induced by kaposi sarcoma-associated herpesvirus-encoded viral FLICE inhibitory protein (vFLIP) K13 and blocks K13-induced nuclear factor-κB in a negative feedback manner," *The Journal of Biological Chemistry*, vol. 286, no. 24, pp. 21555–21564, 2011.

[68] S. Sakakibara, G. Espigol-Frigole, P. Gasperini, T. S. Uldrick, R. Yarchoan, and G. Tosato, "A20/TNFAIP3 inhibits NF-κB activation induced by the Kaposi's sarcoma-associated herpesvirus vFLIP oncoprotein," *Oncogene*, vol. 32, no. 10, pp. 1223–1232, 2013.

[69] L. Li, W. Li, L. Xiao et al., "Viral oncoprotein LMP1 disrupts p53-induced cell cycle arrest and apoptosis through modulating K63-linked ubiquitination of p53," *Cell Cycle*, vol. 11, no. 12, pp. 2327–2336, 2014.

[70] X. Liu, Y. Li, S. Peng et al., "Epstein-Barr virus encoded latent membrane protein 1 suppresses necroptosis through targeting RIPK1/3 ubiquitination," *Cell Death & Disease*, vol. 9, no. 2, 2018.

[71] G. L. Bentz, A. Bheda-Malge, L. Wang, J. Shackelford, B. Damania, and J. S. Pagano, "KSHV LANA and EBV LMP1 induce the expression of UCH-L1 following viral transformation," *Virology*, vol. 448, pp. 293–302, 2014.

[72] L. Li, Q. Tao, H. Jin et al., "The tumor suppressor UCHL1 forms a complex with p53/MDM2/ARF to promote p53 signaling and is frequently silenced in nasopharyngeal carcinoma," *Clinical Cancer Research*, vol. 16, no. 11, pp. 2949–2958, 2010.

[73] S. Chavoshi, O. Egorova, I. K. Lacdao, S. Farhadi, Y. Sheng, and V. Saridakis, "Identification of kaposi sarcoma herpesvirus (KSHV) vIRF1 protein as a novel interaction partner of human deubiquitinase USP7," *The Journal of Biological Chemistry*, vol. 291, no. 12, pp. 6281–6291, 2016.

[74] M. Ohashi, A. M. Holthaus, M. A. Calderwood et al., "The EBNA3 family of epstein-barr virus nuclear proteins associates with the USP46/USP12 deubiquitination complexes to regulate lymphoblastoid cell line growth," *PLoS Pathogens*, vol. 11, no. 4, Article ID e1004822, 2015.

[75] C. Lin, H. Chang, and W. C. Yu, "USP11 stabilizes HPV-16E7 and further modulates the E7 biological activity," *The Journal of Biological Chemistry*, vol. 283, no. 23, pp. 15681–15688, 2008.

[76] R. M. Vos, J. Altreuter, E. A. White, and P. M. Howley, "The ubiquitin-specific peptidase USP15 regulates human papillomavirus type 16 E6 protein stability," *Journal of Virology*, vol. 83, no. 17, pp. 8885–8892, 2009.

[77] A. Ali, R. Raja, S. R. Farooqui, S. Ahmad, and A. C. Banerjea, "USP7 deubiquitinase controls HIV-1 production by stabilizing Tat protein," *Biochemical Journal*, vol. 474, no. 10, pp. 1653–1668, 2017.

[78] C. Setz, M. Friedrich, P. Rauch et al., "Inhibitors of deubiquitinating enzymes block HIV-1 replication and augment the presentation of gag-derived MHC-I epitopes," *Viruses*, vol. 9, no. 8, p. 222, 2017.

Hindawi
Journal of Oncology
Volume 2019, Article ID 8062176, 7 pages
https://doi.org/10.1155/2019/8062176

Research Article

Cervical Human Papillomavirus Prevalence, Genotypes, and Associated Risk Factors among Female Sex Workers in Greater Accra, Ghana

Abdul Rashid Adams,[1] Priscillia Awo Nortey,[2] Benjamin Ansah Dortey,[3] Richard Harry Asmah ,[1] and Edwin Kwame Wiredu [4]

[1]*Department of Medical Laboratory Sciences, School of Biomedical and Allied Health Sciences, College of Health Sciences, University of Ghana, Korle-Bu, Accra, Ghana*
[2]*School of Public Health, College of Health Sciences, University of Ghana, Legon, Accra, Ghana*
[3]*Trauma Hospital, Winneba, Ghana*
[4]*Department of Pathology, School of Biomedical and Allied Health Sciences, College of Health Sciences, University of Ghana, Korle-Bu, Accra, Ghana*

Correspondence should be addressed to Edwin Kwame Wiredu; ekwiredu@chs.edu.gh

Received 7 March 2019; Accepted 14 May 2019; Published 2 June 2019

Guest Editor: Hisashi Iizasa

Cervical cancer is a largely preventable disease mediated by persistent infection with high-risk Human Papillomaviruses (Hr-HPV). There are now three approved vaccines against the most common HPV genotypes. In Ghana, mortality due to cervical cancer is on the rise, due to the absence of an organized and effective cervical cancer prevention and control program. Data on circulating HPV genotypes is important for studying the likely impact of mass introduction of HPV vaccination of the female population before sexual debut. High HPV prevalence has been reported in Female Sex Workers (FSWs), who constitute an important active group for maintenance of HPV in the population. This study was conducted to determine the size of HPV prevalence in this group and to provide information for future assessment of the impact of vaccine introduction in the country. We conducted a cross-sectional study where the snowballing technique was used to identify and select FSW's ≥18 years, operating within suburbs of Greater Accra Region (GAR). A risk factor assessment interview was conducted and cervical swabs were collected for HPV-DNA detection and genotyping by Nested Multiplex PCR. Hundred participants, age ranging from 18 to 45 years, median 24 years, were studied. The prevalence of Cervical HPV was 26%. Eleven genotypes were detected comprising 9 high-risk in order of decreasing prevalence HPV-16 (8%), HPV-35 (5%), HPV-33/39/-68 (3%), HPV-52/51/59 (2%) and HPV-18 (1%) and 2 Low-risk types, HPV-42(3%), and HPV-43 (1%). Three women had HPV types that could not be genotyped by our method. Oral contraceptives use was associated with a reduced chance of HPV infection (P=0.002; OR=0.19, 95% CI 0.07-0.54). This study found a high HPV prevalence among FSWs in the GAR. A high number of Hr-HPV genotypes seen are vaccine preventable, providing additional compelling argument for implementing a national cervical cancer prevention plan including vaccination.

1. Introduction

Globally, cervical cancer remains a primary cause of morbidity and mortality, with estimated 569,847 new cases and 311,365 attributable deaths in 2018 [1, 2]. The highest incidence has been reported in low and middle income countries, particularly in Sub-Saharan Africa where it is the second most common female malignancy [3]. In Ghana, it is likely the commonest cancer among women. Current estimates indicate that every year 3,151 women are diagnosed with cervical cancer and 2,119 die from the disease in the country [4].

Infection of the cervix by a high-risk Human Papillomavirus (HPV), a common sexually transmitted infection, is necessary for the development of cervical cancer [5]

Many studies have found a direct association of HPV infection with sexual behaviour and have indicated that a high number of lifetime partners may lead to a higher transmission of HPV leading to higher cervical cancer rates [6–8].

Female sex workers (FSWs) are a group of females who provide sexual services for economic remuneration. Due to exposure to multiple sexual partners in their occupation, they are prone to various sexually transmitted infections, including HPV. Sexual contact with FSWs plays an important role in HPV transmission and might be a major contributor to the prevalence of HPV and cervical cancer among women in the general population. Also, through the transmission of the virus to their male clients, they increase the risk of penile cancers among these men [9]. Furthermore, due to their likely interaction with foreign clients and sex tourists, they may possibly have a role in the genotype diversity of HPV in the country.

In Ghana, mortality due to cervical cancer is on the rise, most likely due to the lack of an organized prevention and control program. Information on circulating HPV genotypes is crucial for determining the impact of cervical cancer control programmes, including HPV immunization. The high rates of HPV reported among FSWs make them a priority group for study as we seek to characterize the prevalent HPV genotypes in the country in order to predict the likely impact of the current vaccines in reducing the incidence of cervical cancer after introduction of mass female vaccination in the country. The study also looked at other factors that may increase the risk of HPV, contribute to persistence of infection, and/or promote progression of HPV-induced changes in the cervical epithelium. Here, we report the first study of cervical HPV and its associated risk factors among FSW's in Ghana.

2. Methods

2.1. Study Design and Population. This was a cross-sectional study undertaken between February and July 2016 in Greater Accra, one of the ten administrative regions of Ghana (and which houses the national capital) with a predominantly urban population. A risk factor assessment interview was conducted for 100 out of the 109 FSWs who were reached through snowballing, to elicit data on their basic demographics, sexual activities and behaviours (including the age of sexual debut), reproductive history, menarche, sexually transmitted disease (STD) history, screening history, and smoking habit (past and present). Only FSWs of age 18 years and above and had been a sex worker for at least 6 months were included in the study. The Ethics and Protocol Review Committee of School of Biomedical and Allied Health Sciences, University of Ghana, approved this study (SBAHS/10161447/AA/MLS/2015-2016). Participants were fully informed about the purpose, procedures, risks, and benefits of participating in this study and Informed consent was obtained from all subjects.

2.2. Specimen Collection. Following the interviews, a Gynaecologist collected exfoliated cells from the cervix into tubes containing DNAgard™ (Biomatrica, San Diego, CA, USA) for HPV-DNA detection and genotyping. Samples were collected by single use, disposable equipment.

2.3. HPV Testing. HPV detection and typing were carried out by Nested multiplex PCR. [10]. A single consensus forward primer (GP-E6-3F) and two consensus back primers (GP-E7-5B and GP-E7-6B) were used for HPV DNA detection in the first round PCR. The PCR reaction mix of 25μl contained 10X PCR buffer, 2.5mM $MgCl_2$ 200μM of each of the four deoxyribonucleoside triphosphates (dNTP's), 15pmols of each E6/E7 consensus primers, and 1.25 units of Taq polymerase enzyme. Five microliters (5μl) of DNA extracts was used as a template for the amplification reactions using a thermal cycler (Robocycler Gradient 96, Strategene, USA). The cycling parameters for the first round PCR with E63F/E75B/E76B consensus primers were as follows: 94°C for four minutes, followed by 40 cycles of 94°C for one minute, 40°C for two minutes, 72°C for two minutes, and a single final elongation step of 72°C for 10 minutes. In the second round PCR, Primers for the identification of high-risk genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 and low-risk genotypes 6/11, 42, 43, and 44 were used. The primers were used in four cocktails, each containing four to five different primer pairs. Two microliters of first round PCR product, 15 pmols of forward, and reverse primers for genotyping were used. The other parameters remained the same as used in the first round PCR. However, the cycling parameters were as follows: 94°C for four minutes followed by 35 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 45 seconds, and a single final elongation step of 72°C for four minutes [10]. Positive and negative controls were included in each round of amplification.

The amplicons were resolved on 2% agarose gel stained with 0.5μg/ml ethidium bromide. Ten microliters of each sample was added to 2μl of orange G (10X) gel loading dye for the electrophoresis. Hundred base pair DNA molecular weight marker (Sigma, MO, USA) was run alongside the PCR products. The gel was prepared and electrophoresed in 1X TAE buffer using an electrophoresis tank at 80 volts for one hour and the gel photographed over an Ultraviolet (UV) transilluminator [10]. HPV genotypes were identified by comparing the molecular weight of the bands observed to positive control band and or the expected amplicon sizes in each primer cocktail.

2.4. Data Analysis. The data obtained through the questionnaire was checked for accuracy and entered into the computer using Microsoft Excel (2016) Programme and was analysed using SPSS version 20 (IBM Corp. Armonk, New York, USA). Exploratory analysis was first carried out to obtain descriptive statistics. Charts and tables were used to summarize data and display figures where appropriate. The number and proportion of HPV DNA positives and type-specific HPV infection were calculated. To assess the association between HPV DNA positivity and sociodemographic and sexual behavioural factors, odds ratios (ORs) and 95% CIs were calculated using logistic regression. In all statistical considerations a p-value <0.05 was considered statistically significance.

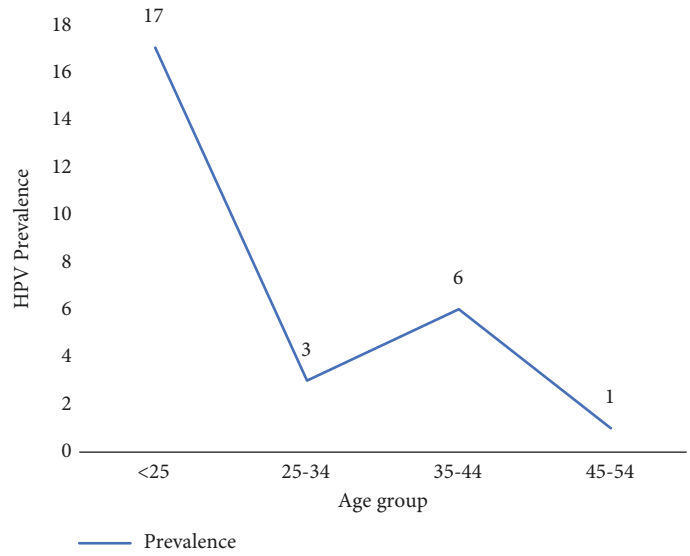


Figure 1: Age-specific HPV prevalence.

3. Results

3.1. Participant Characteristics. A total of 100 FSW, ages ranging from 18 to 45 years with a median age of 24 years, were interviewed. More than half (58%) of them were below 25 years. The level of education among the women was generally low, with the vast majority, 74% (74/100), having at most basic education while 6% (6/100) reported to be illiterates. The median length of sex work was 2 years with a range of 0.5-26 years. About a half (53%) of the sex workers reported engaging in other economic activities (mostly petty trading) while the rest (47%) were full time sex workers. Seventy-seven percent of the subjects who remember their age at sexual debut recalled it to be ≥16 years and 59% of them had at least a child. Condom use always was reported by 82% (82/100) of the study participants whereas oral contraceptive use was reported by 20%. Two (2%) subjects reported to be HIV positive while 14/100 (14%) had had gonorrhoea, 4/100 (4%) syphilis, 3/100 (3%) genital warts, and 2/100 (2%) herpes before. A third (33%) of them had ever smoked cigarette whist 17% are active smokers. Only 9/100 (9%) had ever undergone cervical cancer screening.

3.2. HPV Prevalence and Genotypes. The overall HPV prevalence (any HPV) was 26% (26/100). Eleven genotypes were detected comprising 9 high-risk types and 2 low-risk types. The high-risk HPV types in order of decreasing prevalence were HPV-16 (8%; 8/100), HPV-35 (5%; 5/100), HPV-33/39/68 (3%; 3/100), and HPV-51/52/59 (2%; 2/100) HPV-18 (1%;1/100). HPV 42, (3%; 3/100) and HPV-43 (1%; 1/100) were the low-risk HPV types found. In addition 3% (3/100) had HPV that could not be typed by our method (Table 1). There were 21% single HPV infections (including the three that we could not type) and 5% multiple HPV infections. The 5 multiple infections comprised two each of quadruple- and double-infections and one triple infection. The highest HPV infection rate was among women aged below 25 years with 17% (17/100) (Figure 1). Of the HPV positive women, 73.1% (19/26) were infected with only high-risk types, 3.9% (1/26) were with only low-risk types, and 11.5% (3/26) had both high- and low-risk types.

Table 2 shows the cytological changes found in the high-risk HPV cases detected with 3 cases each of ASCUS, LSIL, and HSIL.

3.3. Risk Factors for HPV Infection. To identify putative risk factors for HPV infection, we performed univariate regression analysis and the results are presented in Table 3. HPV infection was found not to be significantly associated with, age, education, smoking, average number of clients per week, previous sexual disease, condom use, age at sexual debut, and parity. In contrast, the use of oral contraceptives was the only variable that significantly influenced HPV Infection (P=0.002). Female sex workers who used oral contraceptives had 19% reduced odds of having HPV infection compared to those who did not (OR=0.19, 95% CI 0.07-0.54).

4. Discussion

Studies have indicated that a high number of lifetime partners may lead to a higher transmission of HPV [6, 7]. The nature of work conducted by sex workers predisposes them to an increased risk of HPV infection. This study found a crude HPV prevalence of 26% among female sex workers in the Greater Region, which is higher than the WHO estimate of 19.5% HPV Prevalence in women from Western Africa at a given time [3]. This is consistent with existing reports of the elevated HPV prevalence in Female sex workers compared to women in the general population. Similar studies in other African countries have shown much higher prevalence: Madagascar (36.7) among 90 FSWs [11], Senegal (43.5%) among 681 FSWs [12], Tunisia (39.2%) among 51 FSWs [13], Burkina Faso (66.1%) among 360 FSWs [14], Kenya (55.6%); among 789 FSWs [15], South Africa (62.6%), among 99

Table 1: Type-specific HPV prevalence.

HPV type	Single infections	Multiple infections	Total (%)
			n=100(100)
High-risk infections			
16	4	4	8(8)
35	4	1	5(5)
33	2	1	3(3)
39	3	0	3(3)
68	1	2	3(3)
51	2	0	2(2)
52	1	1	2(2)
59	2	0	2(2)
18	0	1	1(1)
sub-total	**19**	**10**	**29**(29)
Low-risk infections			
42	2	1	3(3)
43	0	1	1(1)
sub-total	**2**	**2**	**4**(4)
Un-typeable			
X	3	0	3(3)
Total (LR+HR+X)	**24**	**12**	**36**(36)

Table 2: Cytologic changes in HR-positive individuals.

Cytology results	HR-Genotypes
NILM	16, 18, 68
NILM	16,35,68
NILM	33
NILM	39
HSIL	16,33,68
NILM	52
NILM	35
NILM	16
NILM	16
NILM	16
NILM	39
LSIL	16
LSIL	35
ASCUS	51
HSIL	51
NILM	35
HSIL	39
ASCUS	16,52
NILM	35
LSIL	33
NILM	59
ASCUS	59

FSWs [16]. These variations could be due to difference in sampling strategies and HPV assays employed [17] besides risk factors which are known to vary by region. Though we employed a comparatively more sensitive HPV assay (NMPCR) [10] to the conventional PCR (with either MY09-MY11 or GP5+-GP6+ primers) utilized in some of the studies above, we recorded a lower HPV prevalence compared to those studies. This may likely be due to the fact that 36% of the FSWs from the Burkina Faso study, 35.2 % from the Kenya study, and 50.3% from the South African study were HIV positive compared to the very low 2% in this study. Cervical HPV infections are substantially more common among women infected with HIV, compared with HIV-uninfected women with similar sexual histories due to their impaired immunity and this has been reported by several studies [18–20].

A high number of HPV positive FSWs (17/26), though not statistically significant, were below 25 years. This is consistent with reports in a global review of HPV prevalence among female sex workers [8]. This observation may be linked with acquisition of high rates of HPV following commencement of sex work. Secondly, this may be related to power play as older sex workers are better able to negotiate condom use than younger sex workers, although condom use does not provide full protection from HPV infection. This could also be ascribed to young sex workers enticing more clients than older sex workers culminating in an increased rate of exposure to HPV. It is however worth stating that more than half (58%) of our study subjects were within this age bracket and therefore this age trend could have also been due to this selection bias.

The high-risk HPV genotypes detected in decreasing order of prevalence were 16 (8%), 35 (5%), 33/39/68 (3%), 51/52/59 (2%), and 18 (1%). These genotypes are similar to what is seen in studies in Ghanaian women with and without cervical cancer and elsewhere in Africa but with varying individual genotype prevalence [21–25]. About 50% of

Table 3: Results of univariate logistic regression analysis of risk factors for HPV infection.

Variables	HPV (+)	HPV (-)	OR (95% CI)	P-value
Age(years)				
<25	17	41	0.65 (0.26- 1.67)	0.38
25-34	3	23	3.46 (0.9412.67)	0.06
35-44	6	9	0.46 (0.15-1.46)	0.19
45-54	0	1	-	-
Education				
No formal education	1	5	1.81 (0.20-16.27)	0.60
Basic	18	56	1.38 (0.52-3.71)	0.52
Higher	7	13	0.59 (0.20-1.66)	0.31
Time in prostitution (years)				
<1	6	17	1.45(0.48-4.38)	0.51
1-2	6	27	1.47(0.55-3.94)	0.46
3-4	6	13	0.65(0.21-1.94)	0.44
≥5	8	15	0.58(0.20-.1.65)	0.31
Full time sex worker				
Yes	13	34	0.85 (0.35-2.08)	0.72
Average clients per week				
≤14	14	40	1.01 (0.41-2.71)	0.99
>14	12	34	1.05 (0.43-2.66)	0.92
Contraceptive				
Condom	20	62	1.55 (0.52-4.66)	0.44
Oral contraceptive	11	9	*0.19(0.07-0.54)*	*0.002∗*
Age at sexual debut(yrs.)				
<16	7	16	0.74 (0.27-2.09)	0.58
16-20	19	55	1.07 (0.38-2.93)	0.90
>20	0	3	-	0.99
No. of Children				
0	11	30	0.93 (0.37-2.30)	0.88
1-2	14	36	0.81 (0.33-1.99)	0.65
≥3	1	8	3.03 (0.36-25.47)	0.31
Smoking History				
Current smoking	7	10	0.42 (0.14-1.26)	0.12
Past smoking	9	24	0.91 (0.35-2.32)	0.84
STI in the past				
Yes	9	14	0.44(0.16-1.11)	0.11
Past STI type				
Genital warts	1	1	0.34(0.02-5.68)	0.46
Gonorrhoea	5	9	0.58(0.12-5.68)	0.36
Syphilis	2	2	0.33(0.04-2.50)	0.29
Herpes	1	1	0.34(0.02-5.68)	0.46
HIV	2	0	-	-

high-risk-HPV genotypes detected in this study are covered by the Nona-valent vaccine (Gardasil® 9, Merck) [26] and therefore the introduction of this vaccine and ultimately a national vaccination policy would positively impact cervical prevention efforts in the country. However, the fact that not all high-risk-HPV detected in this and several other studies in Ghanaian women with and without cervical cancer are vaccine types means cervical screening will continue to play an important role in cervical cancer prevention efforts in the country even after the institution of a national vaccination policy. For example, HPV-35, the second most common HPV type found in this study, is not a vaccine type. In another study conducted in Ghana among HIV seropositive and negative women, HPV-35 was the commonest genotype detected and was significantly associated with Squamous intraepithelial lesions [25]. The low-risk-HPV genotypes detected, in decreasing order of prevalence, were 42 and 43. These two genotypes were the two common low-risk types

detected in a study involving pregnant women attending antenatal clinic at the Korle-Bu teaching hospital in Accra [23].

Oral contraceptives use significantly influenced HPV infection (P=0.002), with about 19% decreased chance of HPV infection in oral contraceptive users compared to nonusers (OR=0.19, 95%CI 0.07-0.54). However, a systematic review of 19 epidemiological studies of the risk of genital HPV infection and oral contraceptive use concluded that there was no evidence for a strong positive or negative association between HPV positivity and ever use or long duration use of oral contraceptives [27]. There was lack of significant association of HPV infection with known risk factors such as smoking, age at sexual debut, number of sexual partners, and history of STI. These findings could be due to the small sample size and/or the reliability or otherwise of the information provided by the study subjects. The risk factors assessed in this study relied on self-reported data and therefore prone to both recall and social appeal bias.

This is the first report of cervical HPV and associated risk factors among FSW's in Ghana. Our study also has some limitations: this study was done on a small scale; therefore a larger study with a higher statistical power is needed to determine the extent of HPV infection in this population. Also, the convenience sampling method employed means the prevalence of HPV cannot be generalised to the whole female sex worker population in the Greater Accra.

5. Conclusion

This study found a high HPV prevalence among a cohort of female sex workers in the Greater Accra Region. A high number of the high-risk HPV seen in this population are vaccine preventable, providing additional compelling argument for implementing a national cervical cancer prevention plan including vaccination.

Data Availability

The data used to support the findings of this study are included within the article.

Disclosure

This work received no specific funding and was conducted as part of a Master's degree programme for Abdul Rashid Adams, supervised by Edwin Kwame Wiredu and Priscillia Awo Nortey and as part of fulfilment of obligation to employment by University of Ghana of Edwin Kwame Wiredu, Priscillia Awo Nortey, and Richard Harry Asmah.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Abdul Rashid Adams and Edwin Kwame Wiredu did conceptualization. Abdul Rashid Adams, Priscillia Awo Nortey, Benjamin Ansah Dortey, Richard Harry Asmah, and Edwin Kwame Wiredu were responsible for design, data generation, and data analysis. Abdul Rashid Adams, Richard Harry Asmah and Edwin Kwame Wiredu took care of manuscript Development. All authors critically reviewed and approved the final manuscript.

Acknowledgments

The authors would like to thank all those who agreed to take part in the study, Mr Clement Azigwe, Miss Ramatu, and Sister Janet for their tremendous help in recruiting the study subjects. They are grateful to the management and staff of All Faith Medical Centre, for allowing their facility to be used for sample collection and interviews. They also deeply appreciate the contributions of Mr. Maxwell Akanburichab, Ms. Kathleen Korkor Glover, Mrs. Lois Botwe, and Mr. Bright Katey of all department of Medical Laboratory science, University of Ghana, during the sample collection and interviews.

References

[1] J. Ferlay, M. Ervik, F. Lam et al., *Global Cancer Observatory: Cancer Today*, International Agency for Research on Cancer, Lyon, France, 2018.

[2] L. Bruni, L. Barrionuevo-Rosas, G. Albero et al., "ICO/IARC Information Centre on HPV and Cancer (HPV Information Centre). Human Papillomavirus and Related Diseases in the World," Summary Report 10, 2018.

[3] L. Bruni, L. Barrionuevo-Rosas, G. Albero et al., "ICO/IARC Information Centre on HPV and Cancer (HPV Information Centre). Human Papillomavirus and Related Diseases in the Africa," Summary Report 10, 2018.

[4] L. Bruni, L. Barrionuevo-Rosas, G. Albero et al., "ICO/IARC Information Centre on HPV and Cancer (HPV Information Centre). Human Papillomavirus and Related Diseases in the Ghana," Summary Report 10, 2018.

[5] H. Zur Hausen, "Papillomaviruses in the causation of human cancers—a brief historical account," *Virology*, vol. 384, no. 2, pp. 260–265, 2009.

[6] H. M. Bauer, A. Hildesheim, M. H. Schiffman et al., "Determinants of genital human papillomavirus infection in low-risk women in Portland, Oregon," *Sexually Transmitted Diseases*, vol. 20, pp. 274–278, 1993.

[7] J. W. Sellors, T. L. Karwalajtys, J. Kaczorowski et al., "Incidence, clearance and predictors of human papillomavirus infection in women," *Canadian Medical Association Journal*, vol. 168, no. 4, pp. 421–425, 2003.

[8] M. Soohoo, M. Blas, G. Byraiah, C. Carcamo, and B. Brown, "Cervical HPV infection in female sex workers: A global perspective," *The Open AIDS Journal*, vol. 7, no. 1, pp. 58–66, 2013.

[9] K. L. Stratton and D. J. Culkin, "A contemporary review of HPV and penile cancer," *Oncology*, vol. 30, no. 3, pp. 245–249, 2016.

[10] K. Sotlar, D. Diemer, A. Dethleffs et al., "Detection and typing of human papillomavirus by E6 nested multiplex PCR," *Journal of Clinical Microbiology*, vol. 42, no. 7, pp. 3176–3184, 2004.

[11] J. S. Smith, K. Van Damme, N. Randrianjafisamindrakotroka et al., "Human papillomavirus and cervical neoplasia among female sex workers in madagascar," *International Journal of Gynecological Cancer*, vol. 20, pp. 1593–1596, 2010.

[12] C. L. Langley, E. Benga-De, C. W. Critchlow et al., "HIV-1, HIV-2, human papillomavirus infection and cervical neoplasia in high-risk African women," *AIDS*, vol. 10, no. 4, pp. 413–418, 1996.

[13] E. Hassen, A. Chaieb, M. Letaief et al., "Cervical human papillomavirus infection in Tunisian women," *Infection*, vol. 31, no. 3, pp. 143–148, 2003.

[14] M.-N. Didelot-Rousseau, N. Nagot, V. Costes-Martineau et al., "Human papillomavirus genotype distribution and cervical squamous intraepithelial lesions among high-risk women with and without HIV-1 infection in Burkina Faso," *British Journal of Cancer*, vol. 95, no. 3, pp. 355–362, 2006.

[15] S. M. F. Luchters, D. V. Broeck, M. F. Chersich et al., "Association of HIV infection with distribution and viral load of HPV types in Kenya: A survey with 820 female sex workers," *BMC Infectious Diseases*, vol. 10, no. 1, 2010.

[16] D. J. Marais, E. Vardas, G. Ramjee et al., "The impact of Human Immunodeficiency Virus type 1 status on Human Papillomavirus (HPV) prevalence and HPV antibodies in serum and cervical secretions," *The Journal of Infectious Diseases*, vol. 182, no. 4, pp. 1239–1242, 2000.

[17] F. X. Bosch, Y.-L. Qiao, and X. Castellsagué, "The epidemiology of human papillomavirus infection and its association with cervical cancer," *International Journal of Gynecology & Obstetrics*, vol. 94, 2006.

[18] N. J. Veldhuijzen, S. L. Braunstein, J. Vyankandondera et al., "The epidemiology of human papillomavirus infection in HIV-positive and HIV-negative high-risk women in Kigali, Rwanda," *BMC Infectious Diseases*, vol. 11, pp. 1–14, 2011.

[19] E. N. Fedrizzi, J. K. Laureano, C. Schlup, M. O. Campos, and M. E. Menezes, "Human papillomavirus (HPV) Infection in hiv positive women of florianópolis, state of santa catarina, brazil," *Jornal Brasileiro de Doenças Sexualmente Transmissiveis*, vol. 23, pp. 210–214, 2011.

[20] R. G. Nowak, P. E. Gravitt, C. S. Morrison et al., "Increases in human papillomavirus detection during early HIV infection among women in Zimbabwe," *The Journal of Infectious Diseases*, vol. 203, no. 8, pp. 1182–1191, 2011.

[21] R. K. Ogembo, P. N. Gona, A. J. Seymour et al., "Prevalence of human papillomavirus genotypes among African women with normal cervical cytology and neoplasia: A systematic review and meta-analysis," *PLoS ONE*, vol. 10, Article ID e0122488, 2015.

[22] S. Attoh, R. Asmah, E. K. Wiredu, R. Gyasi, and Y. Tettey, "Human papilloma virus genotypes in Ghanaian women with cervical carcinoma," *East African Medical Journal*, vol. 87, no. 8, pp. 345–349, 2010.

[23] J. Brandful, E. Bonney, R. Asmah, and K. Apea-Kubi, "Oncogenic human papillomavirus (HPV) in women from Ghana," *Journal of Cancer Research and Experimental Oncology*, vol. 6, pp. 31–38, 2014.

[24] A. K. Awua, S. T. Sackey, Y. D. Osei, R. H. Asmah, and E. K. Wiredu, "Prevalence of human papillomavirus genotypes among women with cervical cancer in Ghana," *Infectious Agents and Cancer*, vol. 11, pp. 1–9, 2016.

[25] D. Obiri-Yeboah, P. K. Akakpo, M. Mutocheluh et al., "Epidemiology of cervical human papillomavirus (HPV) infection and squamous intraepithelial lesions (SIL) among a cohort of HIV-infected and uninfected Ghanaian women," *BMC Cancer*, vol. 17, p. 688, 2017.

[26] E. Petrosky, J. A. Bocchini, S. Hariri et al., "Use of 9-valent human papillomavirus (HPV) vaccine: updated hpv vaccination recommendations of the advisory committee on immunization practices," *Morbidity and Mortality Weekly Report (MMWR)*, vol. 64, no. 11, pp. 300–304, 2015.

[27] J. Green, A. B. De Gonzalez, J. S. Smith et al., "Human papillomavirus infection and use of oral contraceptives," *British Journal of Cancer*, vol. 88, no. 11, pp. 1713–1720, 2003.

Hindawi
Journal of Oncology
Volume 2019, Article ID 9173729, 8 pages
https://doi.org/10.1155/2019/9173729

Review Article

Advances in the Management of HPV-Related Oropharyngeal Cancer

F. De Felice,[1] **V. Tombolini**,[1] **V. Valentini**,[2] **M. de Vincentiis**,[2] **S. Mezi**,[3] **O. Brugnoletti**,[2] **and A. Polimeni**[2]

[1]*Department of Radiotherapy, Policlinico Umberto I, "Sapienza" University of Rome, Rome, Italy*
[2]*Department of Oral and Maxillofacial Sciences, Policlinico Umberto I, "Sapienza" University of Rome, Rome, Italy*
[3]*Department of Medical Oncology, Policlinico Umberto I "Sapienza" University of Rome, Rome, Italy*

Correspondence should be addressed to F. De Felice; fradefelice@hotmail.it

Received 28 January 2019; Accepted 13 March 2019; Published 14 April 2019

Guest Editor: Hironori Yoshiyama

Patients with human papillomavirus- (HPV-) related oropharyngeal squamous cell carcinoma (OPSCC) have a better prognosis than HPV-negative OPSCC when treated with standard high-dose cisplatin-based chemoradiotherapy. Consistent with this assertion and due to younger age at diagnosis, novel approaches to minimize treatment sequelae while preserving survival outcomes become of paramount importance. Here, we critically reviewed the evidence-based literature supporting the deintensification strategies in HPV-related OPSCC management, including radiotherapy dose and/or volume reduction, replacement of cisplatin radiosensitising chemotherapy, and the use of transoral surgery. Undoubtedly, further researches are needed before changing the standard of care in this setting of patients.

1. Introduction

Despite oropharyngeal squamous cell carcinoma (OPSCC) representing only 0.9% of all cancer sites, its incidence is rapidly growing worldwide, with an estimated 173,495 new cases in 2018 [1]. The highest incidence rates are seen in the western countries [2]. During the past two decades OPSCC diagnosis increased among men and/or women in different European nations, such as United Kingdom, France, Germany, Denmark, and Sweden [2, 3]. The main reason is oncogenic human papillomavirus (HPV) type 16 infection and nowadays HPV-related OPSCC, primarily located in tonsil and base of tongue, is considered a distinct disease entity [4]. Patients with HPV-related OPSCC have a much better prognosis than those with tobacco/alcohol-driven disease, despite a higher stage at diagnosis due to a typical small primary in the oropharynx with massive regional nodal involvement. Compared with HPV-negative OPSCC, HPV-related OPSCC affects younger patients with a lower comorbidity index, a higher socioeconomic status, and a history of multiple sexual partners and orogenital sexual practice [5]. Intensity modulated radiation therapy with concurrent cisplatin-based chemotherapy represents the standard treatment, when appropriate. This definitive chemoradiotherapy (CRT) approach aims to eradicate tumor cells and minimize both acute and late toxicities. Given the favorable prognosis in a younger patient cohort, novel treatment regimens with the same tumor control and lower toxicity rates are a welcome change.

Here, we presented a critical review of recent advances in the management of HPV-related OPSCC. We focused on the existing literature regarding the proposal applications of radiation therapy and systemic therapy. An assessment of new staging system specifically for HPV-related OPSCC and its development was also reported.

2. Materials and Methods

Key HPV-related OPSCC references were derived from a systematic PubMed query. Articles were obtained using the following combinations of research criteria: "intensity modulated radiotherapy", "imrt", "radiation therapy", "de-intensification", de-escalation", "immune check-point

Table 1: Independent external validation of the 8th edition staging of HPV-related oropharyngeal cancer.

Author	Year of publication	Patients	Primary treatment	5-y OS by 8th edition TNM stage			
				I	II	III	IV
O'Sullivan [6]	*2016*	*1907*	*S: 34; RT: 1873*	*85%*	*78%*	*53%*	*NA*
Haughey [7]	2016	704	S: 704	90%	84%	48%	NA
Cramer [8]	2017	15116	S: 6465; RT: 7841; CHT: 276	87.4%∗	76.6%∗	63.1%∗	20.7%∗
Malm [9]	2017	435	S: 166; RT: 269	92.3%	87.2%	73.6%	40.0%
Porceddu [10]	2017	279	RT: 279	93.6%	81.9%	69.1%	NA

international collaboration on oropharyngeal cancer network for staging (ICON-S) study
∗4-year overall survival
5-y OS: 5-year overall survival; S: surgery; RT: radiotherapy; CHT: chemotherapy; NA: not applicable

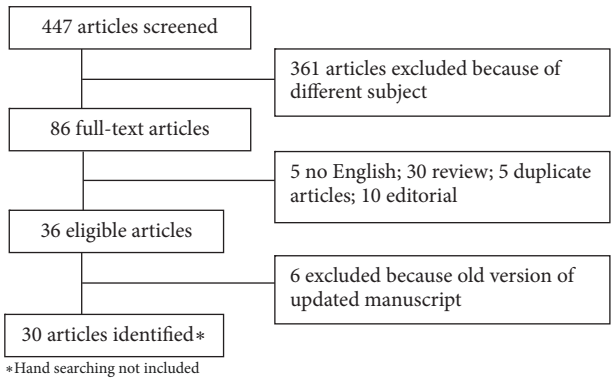


Figure 1: Literature search.

inhibitors", "cetuximab", "cisplatin", "platinum", "toxicity", "quality of life", "chemotherapy", "induction", "treatment", "transoral surgery", "tors", "hpv", "head and neck cancer", "oropharyngeal", "oropharynx" (Figure 1). Hand searching (meeting proceedings of European Society of Medical Oncology, European SocieTy for Radiotherapy & Oncology, American Society for Radiation Oncology and American Society of Clinical Oncology) and clinicaltrials.gov were also used. The last literature search was done in January 2019. Only English written publications were selected. Titles and abstracts of search results were screened to determine eligibility in the manuscript.

3. Results

3.1. New Classification System for HPV-Related Oropharyngeal Cancer. The 8th edition American joint committee on cancer (AJCC) tumor, lymph node, and metastasis (TNM) staging manual on OPSCC introduced significant modifications from the prior 7th edition [11]. HPV-related OPSCC—based on the overexpression of the cyclin-dependent kinase p16—was part of a separate section. It specifically resulted in a change of T and N categories, due to the important need to discriminate between the different stage groups compared to OPSCC associated to other causes. HPV-related OPSCC clinical (c) T classification no longer included a cT4b category, because 5-year overall survival was similar for patients classed as cT4a and cT4b according to 7th edition TNM staging system [6]. N classification, both clinical and pathological (p), represented the main change from the tobacco/alcohol-driven OPSCC. Because cN1, cN2a, and cN2b (7th edition TNM) cohorts had similar impact on 5-year survival, they were grouped as one cN1 category, including ≥ 1 ipsilateral lymph nodes, none larger than 6 cm whereas cN2c was reserved for contralateral or bilateral lymph nodes, none larger than 6 cm, and cN3 included ≥ 1 lymph nodes larger than 6 cm. The combination of cT and cN into stages—stage I (cT1-2 cN0-1), stage II (cT1-2 cN2 or cT3N0-2), and stage III (cT4 or cN3)—depicted an adequate discrimination in HPV-related OPSCC prognosis groups. Interestingly only distant metastatic disease (M1) was considered stage IV.

The rationale for these changes is based on the international collaboration on oropharyngeal cancer network for staging (ICON-S) multicentre cohort study, including 1907 patients with HPV-related OPSCC from seven institutions across Europe and North America [6]. Several independent external validations have been proposed [7–10]. Details are listed in Table 1. Results showed similar or even better 5-years overall survival rates weighed against the ICON-S study. Globally, these cohorts confirmed that the new classification in HPV-related OPSCC provided better survival discrimination across the different stage categories compared to the 7th edition TNM. Several considerations should be addressed. Firstly, this favorable effect could be mainly driven by the high treatment strategy (surgery and/or CRT). An illustrative example included cT2cN1 disease, now stage I (8th edition TNM) and previously stage III (7th edition TNM). Therefore, it remains unknown whether the high survival rate observed in HPV-related OPSCC patients represents an effective good prognosis factor or merely reflects an overtreatment in this population. Secondly, other factors, such as age, smoke, and alcohol, may potentially even better stratify this setting of patients.

pN categories focused only on number of positive lymph nodes, using a cut-off of 4 to discriminate between pN1 (≤ 4 positive lymph nodes) and pN2 (> 4 positive lymph nodes). Pathologic data emerged from surgical HPV-related OPSCC cohort of 704 patients from five cancer centers [7]. It should be noted that the presence of bilateral/contralateral lymph nodes had prognostic impact (p=0.049) in the univariate analysis for overall survival, as well as extranodal extension (ENE) having a positive trend (p=0.060). An

external validation, based on 3745 patients from the national cancer database (NCDB), concluded that ENE could play a prognostic role in HPV-related OPSCC [12]. Results showed a significant negative ENE effect ($p < 0.001$) on survival. But this effect remained statistically significant when stratified by N-stage only for pN1 disease. Further studies with large cohort of patients are necessary to validate these pathological changes. But, again, maybe, to improve discrimination between pathological groups, more factors, such as bilaterally/contralaterally and ENE, should be considered.

3.2. Radiation Therapy in HPV-Related Oropharyngeal Cancer. When appropriate, definitive cisplatin-based CRT using intensity modulated technique (IMRT) is the standard of care in OPSCC. But this approach has drawbacks in terms of toxicity and subsequent patient quality of life (QoL). Considering the good prognostic value of HPV-driven disease, novel treatment paradigms have been proposed in HPV-related OPSCC. These treatment strategies include (i) radiation dose deescalation, (ii) radiation volume deescalation, (iii) induction response-based therapy, (iv) transoral surgery and deintensification of adjuvant treatment. The joint aim is to determine whether a less intensive regimen could minimize toxicity while maintaining similar cure rates.

Radiation Dose Deescalation. Late RT-related toxicity represents a significant burden to OPSCC survivors, because it negatively impacts on their QoL and their ability to function in society. The dose delivered to surrounding tissues plays a crucial role in the development of late toxicity. A dose-effect relationship between dose exposure—maximum dose (Dmax) and/or mean dose (Dmean) and/or percentage of volume receiving x Gy (Vx)—of a specific organ at risk (OAR) and development of its related toxicity has been well established. For instance, a Dmean greater than 50 Gy to pharyngeal constrictor muscles, a Dmean greater than 26 Gy to parotid gland, and a V50 greater than 40.5% to mandible can, respectively, cause moderate to severe swallowing impairment, xerostomia, and osteoradionecrosis [13–15]. Ideally each OAR in the head and neck region should receive a low dose exposure to reduce the risk of RT-induced toxicity. But, in OPSCC, RT with curative intent requires large treatment fields and high doses to be effective. Traditionally the total dose delivered to eradicate clinical and subclinical disease is 70 Gy (2 Gy per fraction) and 50 Gy (2 Gy per fraction), respectively. Therefore it is not always feasible to respect all OARs dose constraints, especially for those structures in close proximity to burden tumor, such as dysphagia-related structures, parotid gland, and mandible. Given the IMRT technical ability (that permits including OARs in the optimization process) and the low incidence of regional failures in the elective volume (that receives a prophylactic dose of 50 Gy), deintensification RT strategies could result in toxicity reduction without compromising survival outcomes [16, 17]. Radiation dose deescalation strategies are currently under investigation. A phase III randomized clinical trial was performed to evaluate the dose reduction effect on late toxicity and regional tumor control in head and neck cancer patients [18]. Independently of HPV status, 200 patients with head and neck carcinoma were randomized to the standard dose of 50 Gy versus the experimental dose of 40 Gy prescribed to the elective nodal volumes. Primary end-point was dysphagia at 6 months of follow-up. Results showed a trend to less dysphagia ($p = 0.02$) and less salivary gland toxicity ($p = 0.01$) at 6 months without differences in overall, disease-free, and disease-specific survival, as well as local, regional, and distant control. But absolute numbers of regional recurrences and distant metastases were too small to draw definitive conclusions on the safety of dose deescalation to 40 Gy to the elective nodal volume. For sure it represents an interesting approach especially in the context of HPV-related OPSCC, due to the long life expectancy of a patient once his cancer is cured.

A parallel between HPV-related OPSCC and HPV-related anal canal carcinoma could be even more interesting. In fact, these two malignancies presented similar tumor histology and viral etiology. A main consideration can be made in the context of organ preservation strategy, using combined CRT modality. In anal canal carcinoma, a total dose of 59.4 Gy (1.8 Gy per fraction) with concurrent chemotherapy is recommended to assure a curative intent [19]. Therefore it could be reasonable to prescribe a lower radiation dose (≤ 60 Gy) plus concomitant chemotherapy in the treatment of HPV-related OPSCC. It might result in similar clinical outcomes decreasing toxicity rates. Evidence is accumulating that radiation dose deescalation can refer to primary tumor target volume [20]. In a phase II trial, 43 favorable risk HPV-related OPSCC patients were treated with IMRT to a total dose of 60 Gy (2 Gy per fraction) plus concomitant weekly cisplatin (30 mg/m^2 per week). Compared to standard CRT regimen, radiation dose was reduced by 16% (70 to 60 Gy) and cumulative chemotherapy dosage was reduced by 60% (300 mg/m^2 to 180 mg/m^2). Primary end-point was pathological complete response (pCR) based on biopsy of the primary site and a limited or selective neck dissection of pretreatment positive lymph node regions. This allowed for a more patient safety standpoint due to authors being worried for detrimental outcomes of deintensified strategy. The pCR rate was 86% with relatively decreased toxicity. Globally results were encouraging, but a randomized clinical trial to make a direct comparison to standard regimen is paramount to assess the real impact of deintensified CRT on both long-term tumor control and toxicities.

Recently, the Memorial Sloan-Kettering Cancer Center group performed a pilot study to test hypoxia imaging—^{18}F-fluorodeoxyglucose (^{18}F-FDG) and dynamic ^{18}F-fluoromisonidazole (^{18}F-FMISO) positron emission tomography (PET)—as selection criteria for radiation dose deescalation to gross nodal disease in HPV-related OPSCC patients [21]. Stages III-IVb HPV-related OPSCC (7th edition) patients without pretreatment hypoxia or with resolution of hypoxia within 1 week of treatment on intratreatment ^{18}F-FMISO PET received a 10 Gy dose reduction (from 70 Gy to 60 Gy) to either the primary site and/or lymph node(s). Of the 33 patients enrolled, 10 patients (30%) met the criteria for radiation dose deescalation. At a median follow-up of 32 months, the 2-year locoregional control,

overall survival, and distant metastasis-free survival were 100%, 100%, and 97%, respectively, with minimal toxicity. This approach emphasized the potential role of ^{18}F-FMISO PET to guide therapeutic decisions, but further studies are necessary.

Several studies attested the high radiosensitivity of HPV-related OPSCC, reporting comparable clinical outcomes in patients with HPV-positive head and neck cancer treated with definitive RT alone instead of standard CRT [22–24]. The reported influence of tumor HPV-status on RT responsiveness should be considered in radiation dose deescalation strategies, even though exactly how to individualized treatment remains uncertain. In this context, the NRG Oncology cooperative group is leading a randomized phase II trial (NCT02254278) to test exclusive modestly reduced-dose IMRT (60 Gy, 2.4 Gy per fraction) versus CRT (weekly 40 mg/m^2 cisplatin and 60 Gy, 2 Gy per fraction) in 296 planned patients with cT1-2, cN1-2b, or cT3, N0-2b (7th edition) HPV-related OPSCC and a lifetime cumulative smoking history < 10 pack-years [25].

Radiation Volume Deescalation. Several investigators assumed that limited radiation to the ipsilateral neck without compromising locoregional control could be feasible in selected patients also in the HPV era [26–30]. In general, elective neck irradiation is not recommended if subclinical disease risk is < 10%, due to RT morbidity [29]. Compared to bilateral irradiation, unilateral neck irradiation permitted to better spare OARs and reduce the risk of RT-related side effects, such as xerostomia, improving patients' QoL [26]. A recent publication showed that ipsilateral RT continued to be safe and contralateral neck failure remained low for patients with cT1-2 cN0-2b (7th edition) HPV-related tonsillar cancer [30]. With regard to control of lymphatic spread, a careful case selection—well-lateralized lesion, without extension to soft palate or tongue base, without muscle involvement or any suspicion of deeper penetration, and no contralateral neck lymph node metastasis— become essential. Prospective clinical trials addressing the suitability of ipsilateral radiation in HPV-related OPSCC are warranted to confirm the efficacy of this approach. Restaging of HPV-related OPSCC series according to 8th edition TNM and reevaluation of previous treatment indications could result in a change of the therapeutic strategies for HPV-related OPSCC. Probably radiation volume deescalation is only imaginable in low-risk HPV-positive patients.

Induction Response-Based Therapy. Different groups have pursued an approach of radiation dose deescalation following the use of induction chemotherapy [31–35].

The Optima trial was a phase II deescalation study designed for patients with HPV-related OPSCC [31]. Induction chemotherapy was adopted to identify favorable patients to apply significantly lower (chemo) radiation doses than standard CRT. Patients were classified as low-risk (≤ T3, ≤ N2b, ≤ 10 pack-year history) and high-risk (T4 or ≥ N2c or >10 pack-year history). They received induction chemotherapy, including 3 cycles of carboplatin (AUC 6) and nab-paclitaxel (100 mg/m^2). Based on response to induction treatment, locoregional therapy was stratified as (i) low-dose RT alone to 50 Gy (2 Gy per fraction) in low-risk patients with ≥ 50% response, (ii) low-dose CRT to 45 Gy (1.5 Gy twice-daily fraction and paclitaxel, 5-fluorouracil, and hydroxyurea) in low-risk patients with 30-50% response or high-risk patients with ≥ 50% response, (iii) standard-dose CRT to 75 Gy (1.5 Gy twice-daily fraction and paclitaxel, 5-fluorouracil, and hydroxyurea) in poor responders. Primary site biopsy and neck dissection were performed only after deescalated (C)RT for pathologic confirmation. The primary endpoint was 2-year progression-free survival (2-y PFS). With a median follow-up of 29 months, the 62 patients enrolled achieved excellent 2-y PFS rates (95% for low risk patients, 94% for high risk patients). Severe acute toxicity, including oral mucositis, skin dermatitis, and PEG-tube requirement, was significantly lower with deescalated treatment. These results compare favorably to the historical control and justified the evaluation of this strategy in a larger comparative trial. But it should be noticed that standard-dose CRT scheme—1.5 Gy twice-daily fraction and paclitaxel, 5-fluorouracil, and hydroxyurea—differed from the standard of care cisplatin-based CRT treatment.

Similarly, the ECOG-ACRIN Cancer Research Group trial evaluated induction chemotherapy (cisplatin, paclitaxel, and cetuximab) followed by concurrent cetuximab and RT to 54 Gy (2 Gy per fraction), complete responders, or 69.3 Gy (2.1 Gy per fraction), no-complete responders, in HPV-related OPSCC patients [32]. The primary end-point was 2-y PFS. Globally, 80 patients were evaluated. After a median follow-up of 35.4 months, 2-y PFS was 80% in cohort with clinical complete response. Interestingly, treatment failures occurred within 2 years after accrual and were recorded on patients with a > 10 pack-year smoking history. Significantly fewer patients treated with dose deescalation had difficulty swallowing solids or impaired nutrition.

Another ongoing US single-arm phase II trial investigated whether weekly paclitaxel CRT with radiation dose deescalation would maintain survival outcomes while improving functional outcomes [33]. After two cycles of paclitaxel/carboplatin-based induction chemotherapy, complete or partial responders received 54 Gy (2 Gy per fraction) and those with less than partial or no responses received 60 Gy (2 Gy per fraction). The primary endpoint was 2-y PFS. A total of 45 patients with stages III-IV (7th edition) HPV-related OPSCC were enrolled. Median follow-up was 30 months and 2-y PFS rate was 92% with an acceptable toxicity profile.

The Quarterback is an active phase III trial that directly compared a radiation dose deescalation to the standard of care in HPV-positive patients [34]. After 3 cycles of docetaxel cisplatin and 5-fluorouracil induction chemotherapy, patients with a clinical or radiographic complete/partial response are randomized to receive a reduced (56 Gy) or standard (70 Gy) dose RT with weekly carboplatin. A total of 365 patients with advanced HPV-related oropharynx cancer, nasopharynx cancer, or unknown primary are planned to determine the comparative rate of PFS at 3 years. Preliminary results—based on 23 patients enrolled and 20 randomized–have been presented at ASCO meeting in 2017 and the 2-y PFS rates were

87.5% for those patients receiving standard dose and 83.3% for those patients receiving dose deescalation [35].

Globally, all these studies indicated that HPV-related OPSCC could be successfully treated with a sequential treatment strategy of induction chemotherapy followed by radiation dose deescalation preserving both clinical and functional outcomes. Definitive phase III randomized clinical trials adopting the 8th edition TNM classification and standard of care treatment arm are paramount to confirm these results, define appropriate candidates, and alter standard clinical practice. Surely, independently of radiation treatment modalities—dose deescalation, volume deescalation, and following induction chemotherapy—the high-quality RT is paramount to guarantee reliable treatment outcome.

Transoral Surgery and Deintensification of Adjuvant Treatment. Adjuvant (C)RT dose reduction following primary transoral surgery is also being proposed as an alternative deescalation treatment strategy for HPV-related OPSCC. Its main advantage is the proper adjuvant treatment based on objective criteria driven by pathologic staging. To our knowledge, there are as yet no published prospective randomized data on this topic, but several clinical trials are ongoing [36–41]. Actually, the Mayo Clinic group presented at ASTRO 2017 meeting the results of the phase II MC1273 trial but full-text is still not available [36]. This study included patients with HPV-related OPSCC and ≤ 10 pack-year smoking history. Following surgery with negative margins, patients with ≥T3, ≥N2, lymphovascular invasion, or perineural invasion received 30 Gy (1.5 Gy twice daily fraction) with concomitant docetaxel. In case of evidence of extracapsular spread, patients received the same treatment plus a simultaneous integrated boost to nodal levels with extracapsular spread to 36 Gy (1.8 Gy twice daily fraction). Results showed a locoregional control rate (95%) comparable to historical controls. No patients required feeding tube. Based on these data, a phase III multicenter study (DART-HPV trial) has been designed and is actively accruing [37]. A total of 214 are planned. Patients are randomized to receive deescalated adjuvant docetaxel-based CRT (30 Gy in 1.5 Gy fractions twice daily in intermediate risk patients or 36 Gy in 1.8 Gy fractions twice daily in high risk patients) versus standard of care treatment with weekly cisplatin 40 mg/m^2 concomitant to RT to 60 Gy delivered in 2 Gy per fraction. Primary end-point is adverse events rate at 2 years.

The ECOG-ACRIN Cancer Research Group designed a phase II trial for stages III-IVb HPV-related OPSCC [38]. cN0 patients are not eligible. Based on their risk status —low risk: no adverse pathological features, intermediate risk: T1-3, N2a-2b, perineural and/or vascular invasion or close margins, and high risk: positive margins and/or extracapsular spread—patients are assigned to (i) transoral robotic surgery (TORS) alone (low risk), (ii) TORS and low-dose RT, 50 Gy 2 Gy per fraction (intermediate risk), (iii) TORS and standard dose RT, 60 Gy 2 Gy per fraction (intermediate risk), and (iv) TORS and standard dose weekly platinum-based CRT, 66 Gy 2 Gy per fraction (high risk). Patients classified as intermediate risk are randomized to low-dose or standard dose treatment arm. Primary end-point is 2-y PFS.

In the ADEPT trial, HPV-related OPSCC patients received either RT alone (60 Gy, 2 Gy per fraction) or weekly cisplatinum-based CRT (60 Gy, 2 Gy per fraction) after margin-clearing TORS of their T1-4a oropharynx primary (7th edition) and a neck dissection with extracapsular spread in their lymph nodes [39]. Primary end-points were 5-year disease-free survival and 5-year locoregional control.

The primary outcome of the prospective randomized PATHOS study is to improve patient-reported swallowing outcome testing adjuvant dose deescalation RT in order to continue to a phase III noninferiority study with overall survival as the primary end-point [40]. Patients with stage T1-3, N0-2b (7th edition) HPV-related OPSCC, are enrolled. Following surgery and based on pathological risk factors for recurrence, patients will receive (i) no adjuvant treatment, (ii) randomization to adjuvant RT to 60 Gy (2 Gy per fraction) or 50 Gy (2 Gy per fraction), and (iii) randomization to adjuvant weekly cisplatin-based CRT to 60 Gy (2 Gy per fraction) or RT alone to 60 Gy (2 Gy per fraction).

An interesting approach was proposed by the Memorial Sloan Kettering Cancer Center [41]. Investigators conducted a pilot study using ^{18}F- FMISO PET to identify HPV-related OPSCC patients eligible for adjuvant dose deescalation. Patients received surgery to primary tumor only, whereas lymph nodes were evaluated by ^{18}F- FMISO PET. Patients without hypoxia or with resolution at intratreatment ^{18}F-FMISO PET received 30 Gy (2 Gy per fraction) to the tumor bed and neck with 2 cycles of concurrent high-dose cisplatin or carboplatin/5-FU. Patients with persistent hypoxia received standard CRT up to 70 Gy. Neck dissection was performed 4 months after CRT. In total 19 patients were enrolled and 15 patients were deescalated to 30 Gy. Globally, 18 out of 19 patients (95%) remain disease free. A multicenter trial to validate these pilot results is ongoing.

In summary, waiting for definitive results of the proposed trials, no firm conclusions can be drawn. We agree with the principle of pathological risk and functional imaging assessment to guide treatment deescalation decisions.

3.3. Systemic Therapy in HPV-Related Oropharyngeal Cancer. Efforts to minimize acute and late toxicity of primary CRT in HPV-related OPSCC patients also include systemic therapy. The options are (i) replacing cisplatin with the epidermal growth factor receptor (EGFR) inhibitor cetuximab and (ii) replacing cisplatin with immune check-point inhibitors.

Replace Cisplatin with Cetuximab. Cetuximab is an IgG1 monoclonal antibody against the EGFR approved by the US Food and Drug Administration in 2006 due to its proven survival benefit (median survival from 29.3 months to 49 months) without increasing the common toxic effects compared to RT alone in locally advanced head and neck cancer (IMCL-9815 trial) [42]. The updated data of IMCL-9815 trial for subgroup analyses of patient and tumor factors suggested a potential increased survival benefit from cetuximab in those patients with early T stage and advanced N stage OPSCC, age < 65 years, and high performance status [43]. Importantly, the IMCL-9815 trial was not powered for this subgroup analysis. Therefore these data could be ascribable to chance,

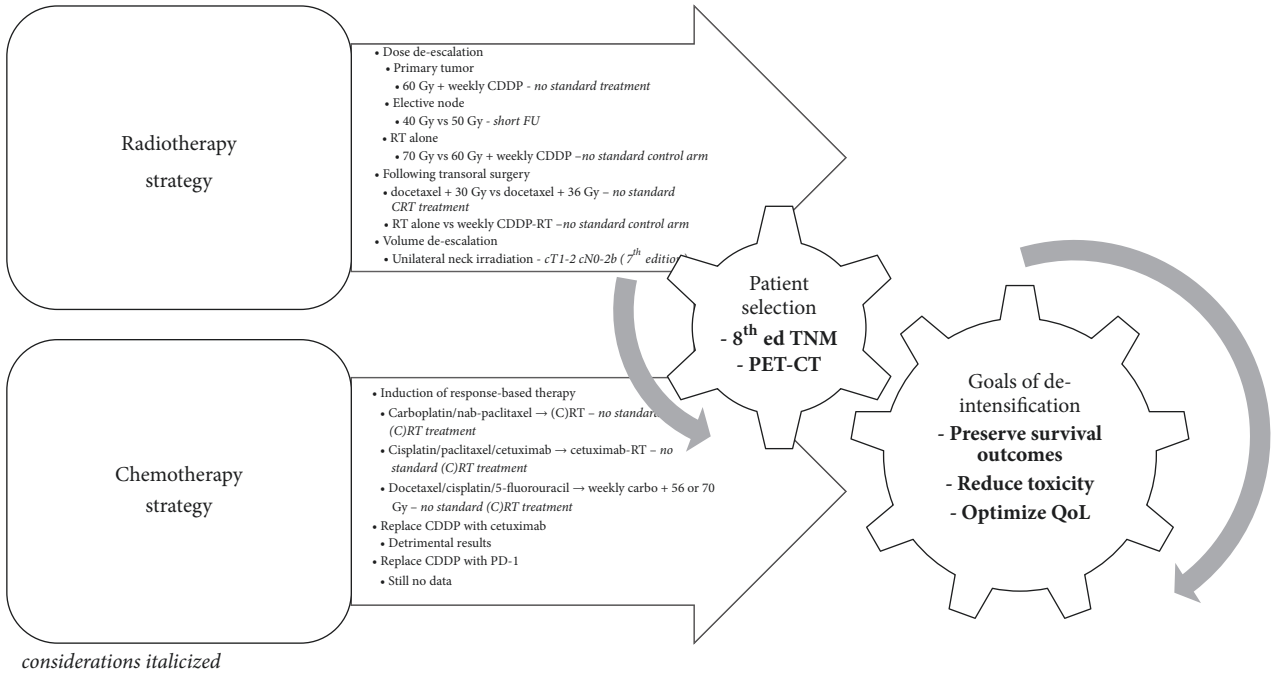


Figure 2: Deintensification strategies.

but it should be noted that these characteristics are common to patients with HPV-related disease and this finding has encouraged research groups to test the use of cetuximab in these patients. Two randomized noninferiority trials, the De-ESCALaTE HPV trial and the RTOG 1016 trial, proposed cetuximab for treatment deescalation strategy in HPV-related OPSCC [44, 45]. The aim was to reduce standard cisplatin-based CRT toxicity profile while preserving survival efficacy. Final data analyses were published online in November 2018. Contrary to expectations, replacing cisplatin with cetuximab demonstrated a significantly detrimental impact on survival end-points, in both trials. In light of these results, RT plus cetuximab cannot be considered a deescalation strategy to reduce toxicity while maintaining survival in patients with HPV-related OPSCC. Cisplatin-based CRT remains the standard of care.

Replace Cisplatin with Immune Check-Point Inhibitors. During the past few years, there has been an exciting development of immunotherapy, especially check-point inhibitors in different human malignancies, including head and neck cancer [46]. The immune check-point inhibitors represent a successful immunotherapeutic approach, due to their peculiar ability to target lymphocyte receptors, as opposed to target therapy, such as cetuximab, that act directly on the tumor cells [47]. They mainly include antiprogrammed death-1 (PD-1) antibody and anticytotoxic T lymphocyte associated antigen 4 (CTLA-4) antibody. Nivolumab and pembrolizumab are both anti-PD-1 antibody and are recommended as categories 1 and 2a, respectively, in recurrent and/or metastatic head and neck cancer (nonnasopharyngeal cancer) if disease progresses on or after platinum-based chemotherapy [4]. Based on phase III CheckMate 141 study (nivolumab) and phase Ib KEYNOTE-012 trial (pembrolizumab), deintensification by replacing cisplatin with immune check-point inhibitors could represent a promising strategy to achieve optimum disease control with minimal long-term toxicities in HPV-related OPSCC with favorable risk disease.

A phase II study with safety lead-in has been designed to test safety, tolerability, and efficacy of anti-CTLA4 (ipilimumab) and anti-PD-1 (nivolumab) in combination with RT up to 60 Gy (2 Gy per fraction) in patients with 8th edition stages T1N2, T2N1-2, and T3N0-2 HPV-related OPSCC [48]. This study is not yet recruiting.

[To note, the potential role of RT combination with these agents has recently been proposed in patients with HPV-related OPSCC with smoking status > 10 pack-years, stage T1-2N2b-N3, or ≤ 10 pack-years, stages T4N0-N3 or T1-3N3 [49]. The aim is to test the safety of nivolumab added to several CRT regimens, including weekly cisplatin, high-dose cisplatin, cetuximab, or IMRT alone. Final data collection for primary outcome measures is estimated in March 2019.]

Enrolment in current trials of RT plus immune check-point inhibitors in this patient population should be strongly encouraged where possible.

4. Conclusions

At present, HPV-related OPSCC can be considered a distinct disease primarily as a consequence of its anatomical location and its viral aetiology. Its optimal treatment approach is still not well-defined. For sure, HPV-related OPSCC is extremely sensitive to radiation exposure and patients generally are complete responders and long-term survivors. Therefore over the years scientific interest has shifted to new stratagems to potentially improve functional outcomes. Figure 2

summarizes the main deintensification strategies, based upon the published literature discussed above. We believe that Figure 2 could add value to the indirect comparisons of these methods. It must be appreciated that its bullet points are suggestions to standardize protocols and develop a gold-standard assessment panel. In fact, an important question is how to best implement both intradisciplinary and interdisciplinary into the current HPV-related OPSCC management. Actually, the vast majority of clinical trial is testing different approaches. Thus, in the coming years, there will be a big data disorder that could delay the expected change in the standard of care. It should emphasize the importance of a trial design and the value to compare what is already conformed to the standard. In addition, accurate patient selection should be critical to optimal implementation of a new strategy. Research groups should endeavor to consider such observations to implement and optimize clinical results. At present no changes in HPV-related OPSCC management should be made outside clinical trials.

Conflicts of Interest

The authors report no conflicts of interest.

References

[1] F. Bray, J. Ferlay, I. Soerjomataram, R. L. Siegel, L. A. Torre, and A. Jemal, "Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries," *CA: A Cancer Journal for Clinicians*, vol. 68, no. 6, pp. 394–424, 2018.

[2] A. K. Chaturvedi, W. F. Anderson, J. Lortet-Tieulent et al., "Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers," *Journal of Clinical Oncology*, vol. 31, no. 36, pp. 4550–4559, 2013.

[3] E. P. Simard, L. A. Torre, and A. Jemal, "International trends in head and neck cancer incidence rates: Differences by country, sex and anatomic site," *Oral Oncology*, vol. 50, no. 5, pp. 387–403, 2014.

[4] "National Comprehensive Cancer Network (NCCN). Guidelines Head and Neck Cancers, Version 2.2018," http://www.nccn.org.

[5] F. Kamangar, G. M. Dores, and W. F. Anderson, "Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world," *Journal of Clinical Oncology*, vol. 24, no. 14, pp. 2137–2150, 2006.

[6] B. O'Sullivan, S. H. Huang, J. Su et al., "Development and validation of a staging system for HPV-related oropharyngeal cancer by the International Collaboration on Oropharyngeal cancer Network for Staging (ICON-S): a multicentre cohort study," *The Lancet Oncology*, vol. 17, no. 4, pp. 440–451, 2016.

[7] B. H. Haughey, P. Sinha, D. Kallogjeri et al., "Pathology-based staging for HPV-positive squamous carcinoma of the oropharynx," *Oral Oncology*, vol. 62, pp. 11–19, 2016.

[8] J. D. Cramer, K. E. Hicks, A. W. Rademaker, U. A. Patel, and S. Samant, "Validation of the eighth edition american joint committee on cancer staging system for human papillomavirus-associated oropharyngeal cancer," *Head & Neck*, vol. 40, no. 3, pp. 457–466, 2018.

[9] I.-J. Malm, C. J. Fan, L. X. Yin et al., "Evaluation of proposed staging systems for human papillomavirus-related oropharyngeal squamous cell carcinoma," *Cancer*, vol. 123, no. 10, pp. 1768–1777, 2017.

[10] S. V. Porceddu, R. Milne, E. Brown et al., "Validation of the ICON-S staging for HPV-associated oropharyngeal carcinoma using a pre-defined treatment policy," *Oral Oncology*, vol. 66, pp. 81–86, 2017.

[11] W. M. Lydiatt, S. G. Patel, B. O'Sullivan et al., "Head and Neck cancers—major changes in the American Joint Committee on cancer eighth edition cancer staging manual," *CA: A Cancer Journal for Clinicians*, vol. 67, no. 2, pp. 122–137, 2017.

[12] K. Y. Zhan, A. Eskander, S. Y. Kang et al., "Appraisal of the AJCC 8th edition pathologic staging modifications for HPV-positive oropharyngeal cancer, a study of the national cancer data base," *Oral Oncology*, vol. 73, pp. 152–159, 2017.

[13] F. De Felice, M. de Vincentiis, V. Luzzi et al., "Late radiation-associated dysphagia in head and neck cancer patients: evidence, research and management," *Oral Oncology*, vol. 77, pp. 125–130, 2018.

[14] C. M. Nutting, J. P. Morden, K. J. Harrington et al., "Parotid-sparing intensity modulated versus conventional radiotherapy in head and neck cancer (PARSPORT): a phase 3 multicentre randomised controlled trial," *The Lancet Oncology*, vol. 12, no. 2, pp. 127–136, 2011.

[15] F. De Felice, C. Thomas, V. Patel et al., "Osteoradionecrosis following treatment for head and neck cancer and the effect of radiotherapy dosimetry: the Guy's and St Thomas' Head and Neck Cancer Unit experience," *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*, vol. 122, no. 1, pp. 28–34, 2016.

[16] S. Nuyts, M. Lambrecht, F. Duprez et al., "Reduction of the dose to the elective neck in head and neck squamous cell carcinoma, a randomized clinical trial using intensity modulated radiotherapy (IMRT). Dosimetrical analysis and effect on acute toxicity," *Radiotherapy & Oncology*, vol. 109, no. 2, pp. 323–329, 2013.

[17] F. De Felice, C. Thomas, S. Barrington, A. Pathmanathan, M. Lei, and T. G. Urbano, "Analysis of loco-regional failures in head and neck cancer after radical radiation therapy," *Oral Oncology*, vol. 51, no. 11, pp. 1051–1055, 2015.

[18] D. Nevens, F. Duprez, J. F. Daisne et al., "Reduction of the dose of radiotherapy to the elective neck in head and neck squamous cell carcinoma; a randomized clinical trial. Effect on late toxicity and tumor control," *Radiotherapy & Oncology*, vol. 122, no. 2, pp. 171–177, 2017.

[19] "National Comprehensive Cancer Network (NCCN). Guidelines Anal Carcinoma," Version 2.2018, http://www.nccn.org.

[20] B. S. Chera, R. J. Amdur, J. Tepper et al., "Phase 2 trial of de-intensified chemoradiation therapy for favorable-risk human papillomavirus-associated oropharyngeal squamous cell carcinoma," *International Journal of Radiation Oncology • Biology • Physics*, vol. 93, no. 5, pp. 976–985, 2015.

[21] N. Lee, H. Schoder, B. Beattie et al., "Strategy of using intratreatment hypoxia imaging to selectively and safely guide radiation dose de-escalation concurrent with chemotherapy for locoregionally advanced human papillomavirus–related oropharyngeal carcinoma," *International Journal of Radiation Oncology • Biology • Physics*, vol. 96, no. 1, pp. 9–17, 2016.

[22] A. M. Chen, T. Zahra, M. E. Daly et al., "Definitive radiation therapy without chemotherapy for human papillomavirus-positive head and neck cancer," *Head & Neck*, vol. 35, no. 11, pp. 1652–1656, 2013.

[23] P. Lassen, J. G. Eriksen, S. Hamilton-Dutoit, T. Tramm, J. Alsner, and J. Overgaard, "Effect of HPV-associated p16INK4A expression on response to radiotherapy and survival in squamous cell carcinoma of the head and neck," *Journal of Clinical Oncology*, vol. 27, no. 12, pp. 1992–1998, 2009.

[24] P. Lassen, J. G. Eriksen, A. Krogdahl et al., "The influence of HPV-associated p16-expression on accelerated fractionated radiotherapy in head and neck cancer: Evaluation of the randomised DAHANCA 6&7 trial," *Radiotherapy & Oncology*, vol. 100, no. 1, pp. 49–55, 2011.

[25] https://clinicaltrials.gov/NCT02254278.

[26] A. P. Jellema, B. J. Slotman, P. Doornaert, C. R. Leemans, and J. A. Langendijk, "Unilateral versus bilateral irradiation in squamous cell head and neck cancer in relation to patient-rated xerostomia and sticky saliva," *Radiotherapy & Oncology*, vol. 85, no. 1, pp. 83–89, 2007.

[27] S. M. Jackson, J. H. Hay, A. D. Flores et al., "Cancer of the tonsil: the results of ipsilateral radiation treatment," *Radiotherapy & Oncology*, vol. 51, no. 2, pp. 123–128, 1999.

[28] K. Kagei, H. Shirato, T. Nishioka et al., "Ipsilateral irradiation for carcinomas of tonsillar region and soft palate based on computed tomographic simulation," *Radiotherapy & Oncology*, vol. 54, no. 2, pp. 117–121, 2000.

[29] B. O'Sullivan, P. Warde, B. Grice et al., "The benefits and pitfalls of ipsilateral radiotherapy in carcinoma of the tonsillar region," *International Journal of Radiation Oncology • Biology • Physics*, vol. 51, no. 2, pp. 332–343, 2001.

[30] S. H. Huang, J. Waldron, S. V. Bratman et al., "Re-evaluation of ipsilateral radiation for T1-T2N0-N2b tonsil carcinoma at the princess margaret hospital in the human papillomavirus era, 25 years later," *International Journal of Radiation Oncology • Biology • Physics*, vol. 98, no. 1, pp. 159–169, 2017.

[31] T. Y. Seiwert, C. C. Foster, E. A. Blair et al., "OPTIMA: a phase II dose and volume de-escalation trial for human papillomavirus-positive oropharyngeal cancer," *Annals of Oncology*, vol. 30, no. 2, pp. 297–302, 2019.

[32] S. Marur, S. Li, A. J. Cmelak et al., "E1308: Phase II trial of induction chemotherapy followed by reduced-dose radiation and weekly cetuximab in patients with HPV-associated resectable squamous cell carcinoma of the oropharynx- ECOG-ACRIN cancer research group," *Journal of Clinical Oncology*, vol. 35, no. 5, pp. 490–497, 2017.

[33] A. M. Chen, C. Felix, P.-C. Wang et al., "Reduced-dose radiotherapy for human papillomavirus-associated squamous-cell carcinoma of the oropharynx: a single-arm, phase 2 study," *The Lancet Oncology*, vol. 18, no. 6, pp. 803–811, 2017.

[34] https://clinicaltrials.gov/NCT01706939.

[35] H. Rainey, E. Roy, I. Selkridge et al., "Standard of care vs reduced-dose chemoradiation after induction chemotherapy in HPV+ oropharyngeal carcinoma patients," *Journal of Clinical Oncology*, vol. 35, no. 15, pp. 6069-6069, 2017.

[36] D. Ma, K. Price, E. Moore et al., "Two-year results for MC1273, a phase 2 evaluation of aggressive dose de- escalation for adjuvant chemoradiation in HPV+ oropharynx squamous cell carcinoma (OPSCC)," *International Journal of Radiation Oncology • Biology • Physics*, vol. 99, no. 5, p. 1320, 2017.

[37] https://clinicaltrials.gov/NCT02908477.

[38] https://clinicaltrials.gov/NCT01898494.

[39] https://clinicaltrials.gov/NCT01687413.

[40] W. Owadally, C. Hurt, H. Timmins et al., "PATHOS: A phase II/III trial of risk-stratified, reduced intensity adjuvant treatment in patients undergoing transoral surgery for Human papillomavirus (HPV) positive oropharyngeal cancer," *BMC Cancer*, vol. 15, no. 602, 2015.

[41] N. Riaz, E. Sherman, N. Katabi et al., "A personalized approach using hypoxia resolution to guide curative-intent radiation therapy dose-reduction to 30 Gy: a novel de-escalation paradigm for HPV-associated oropharynx cancers treated with concurrent chemoradiation therapy," *International Journal of Radiation Oncology • Biology • Physics*, vol. 99, no. 2, p. S136, 2017.

[42] J. A. Bonner, P. M. Harari, J. Giralt et al., "Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck," *The New England Journal of Medicine*, vol. 354, no. 6, pp. 567–578, 2006.

[43] J. A. Bonner, P. M. Harari, J. Giralt et al., "Radiotherapy plus cetuximab for locoregionally advanced head and neck cancer: 5-year survival data from a phase 3 randomised trial, and relation between cetuximab-induced rash and survival," *The Lancet Oncology*, vol. 11, no. 1, pp. 21–28, 2010.

[44] H. Mehanna, M. Robinson, A. Hartley et al., "Radiotherapy plus cisplatin or cetuximab in low-risk human papillomavirus-positive oropharyngeal cancer (De-ESCALaTE HPV): an open-label randomised controlled phase 3 trial," *The Lancet*, vol. 393, no. 10166, pp. 51–60, 2019.

[45] M. L. Gillison, A. M. Trotti, J. Harris et al., "Radiotherapy plus cetuximab or cisplatin in human papillomavirus-positive oropharyngeal cancer (NRG Oncology RTOG 1016): a randomised, multicentre, non-inferiority trial," *The Lancet*, vol. 393, no. 10166, pp. 40–50, 2019.

[46] F. De Felice, A. Polimeni, V. Valentini et al., "Radiotherapy controversies and prospective in head and neck cancer: a literature-based critical review," *Neoplasia*, vol. 20, no. 3, pp. 227–232, 2018.

[47] F. De Felice, C. Marchetti, I. Palaia et al., "Immune check-point in cervical cancer," *Critical Review in Oncology/Hematology*, vol. 129, pp. 40–43, 2018.

[48] https://clinicaltrials.gov/NCT03799445.

[49] https://clinicaltrials.gov/NCT02764593.