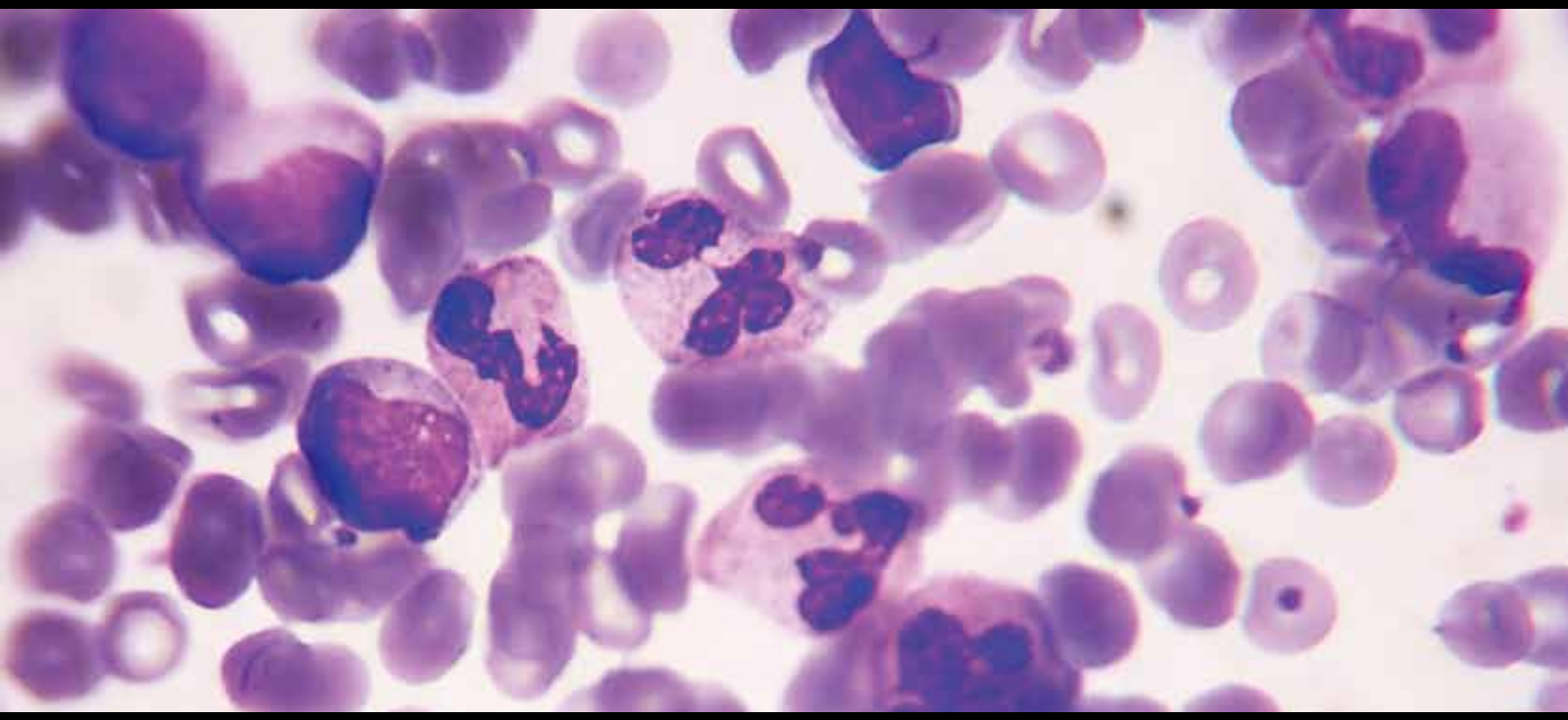
Pathology Research International

Head and Neck Pathology: New Developments in the Diagnosis and Pathogenesis of Head and Neck Tumors

Guest Editors: Stefan E. Pambuccian, Edward B. Stelow, Ioannis G. Koutlas, and Michael J. Thrall



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Editorial

Head and Neck Pathology: New Developments in the Diagnosis and Pathogenesis of Head and Neck Tumors

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In this issue manuscripts are presented that touch on some of the new developments in the diagnosis and pathogenesis of head and neck tumors. The first article by T. Tanaka et al. summarizes our understanding of the development of oral squamous cell carcinoma and discusses possible chemoprevention. As discussed there, the diagnosis of head and neck cancer can be limited as the entirety of the upper aerodigestive tract can be affected by smoking. P. Pujary et al. discuss the use of spectroscopy for the identification of malignant squamous mucosa.

As the incidence of smoking decreases in many parts of the world, there has been a noted decline in the incidence of smoking-related malignancies, including head and neck squamous cell carcinoma. Unfortunately, with this decline, many parts of the world have witnessed a concomitant increase in oropharyngeal squamous cell carcinomas related to high-risk human papillomavirus (HPV) infection. In this issue, Dr. F. Farshadpour et al. show, in a case control study, that oropharyngeal squamous cell carcinomas that develop in nonsmokers who do not abuse alcohol are more likely to be related to HPV infection than those that develop in smokers who do consume alcohol and confirm that the HPV-related tumors have a better prognosis. R. L. Cantley et al. provide a nice review of the various immunohistochemical, in situ hybridization, and molecular studies that can be used to demonstrate HPV infection.

Over the past decade there have been many exciting developments in our understanding of the classification of salivary gland neoplasms and the associated genetic abnormalities. In this issue, M. Shishegar et al. describe a series of salivary gland tumors seen at their institution over a 6-year period. A. F. Costa et al. review the clinicopathologic changes associated with high-grade transformation that can be seen with adenoid cystic carcinomas, acinic cell carcinomas, epithelial-myoepithelial carcinomas, and polymorphous low-grade adenocarcinomas.

Sinonasal intestinal-type adenocarcinomas remain a pathologic enigma, sometimes associated with heavy wood-dust exposure. In the final article in this issue, B. Vivanco et al. review a large series of these tumors and attempt to identify possible precursor lesions for these rare malignancies.

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Research Article

Benign Lesions in Mucosa Adjacent to Intestinal-Type Sinonasal Adenocarcinoma

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Occupational exposure to wood dust is a strong risk factor for the development of intestinal-type sinonasal adenocarcinoma (ITAC); however, knowledge on possible precursor lesions or biomarkers is limited. Fifty-one samples of tumor-adjacent mucosa and 19 control samples of mucosa from the unaffected fossa of ITAC patients were evaluated for histological changes and p53 protein expression. Mild dysplasia was observed in 14%, cuboidal metaplasia in 57%, intestinal metaplasia in 8%, squamous metaplasia in 24%, and cylindrocellular hyperplasia in 53% of cases. P53 immunopositivity was generally weak occurring most frequently in squamous metaplasia. Wood dust etiology did not appear of influence on the histological changes, but p53 showed a tendency for higher positivity. Dysplasia adjacent to tumor was indicative of subsequent development of recurrence. In conclusion, precursor lesions do occur in mucosa adjacent to ITAC. This is clinically important, because it may justify the screening of high-risk individuals such as woodworkers.

1. Introduction

Intestinal-type sinonasal adenocarcinomas (ITACs) are epithelial tumors of the nasal cavities and paranasal sinuses, often related to professional exposure to wood dust. It is a rare tumor representing 8–25% of all malignant sinonasal tumors [1, 2]. According to the WHO histological classification [3], two main categories are recognized: intestinal-type and non-intestinal-type adenocarcinoma. The latter is not related to professional wood dust exposure and is not the subject of this paper. Based on classifications of Barnes and Kleinsasser [2, 4], five pathological types of sinonasal ITAC are distinguished: papillary or papillary tubular cylinder cell I (PTCC-I), colonic (PTCC-II), solid (PTCC-III), mucinous (alveolar goblet and signet ring), and mixed (transitional). The most frequent type is colonic (40%), followed by solid (20%), papillary (18%), and mucinous and mixed type (together 22%) [3].

In the northern part of Spain, the incidence is 0.19 cases/100.000 inhabitants per year [5]. It is located most frequently (85%) in the ethmoid sinus and the upper part of the nasal cavity [6, 7]. Distant or lymph node metastases are exceptional, while local recurrences constitute the main cause of death among patients [8, 9]. The median age of onset lies between 50 and 60 years [4, 10] and in wood dust-related tumors even earlier [10]. Men develop ITAC four times more frequently than women, reflecting the occupational hazard implicated [4].

In the clinic, ITAC often appear as indolent, slow-growing tumors with unspecific unilateral symptoms (occasionally bilateral) normal to this site of origin, such as nasal obstruction, epistaxis, or rhinorrhea [9]. Frequently they are confused with chronic inflammation (rhinitis, sinusitis) or benign tumors. Because of this, diagnosis is often late, with an interval of 6–8 months from the first symptoms to diagnosis. By then the tumor can already be advanced stage

with orbital, intracranial, oral, or facial soft tissue extension. Therefore, there is a need for better ways of prevention and early diagnosis.

Previous reports have focussed on finding precursor lesions in series of sinonasal mucosa samples from persons at high risk of developing ITAC, that is, woodworkers. Aiming to find stronger indications, in this study we analyzed normal sinonasal mucosa of patients who have already developed ITAC. In addition to the histological evaluation, we studied p53 protein expression as a possible marker for early neoplastic transformation.

2. Material and Methods

2.1. Patients and Samples. Fifty-one paraffin tissue samples were taken from mucosa adjacent to the tumor, with presence of both tumor and normal tissues in the same tissue block. Nineteen control samples were obtained from the healthy, unaffected fossa of patients with ITAC. In 9 cases we obtained a sample from both adjacent tissue and the other fossa. All samples were collected from previously untreated patients seen between 1990 and 2009. Informed consent was obtained from all patients, and the study was approved by the ethical committee of our institute.

Of the 51 cases, 1 patient was female and 50 male, with a mean age of 65 years (45–92). Forty-five patients had occupational exposure to wood dust with a median of 35 years (range: 4–55 years), and 27 were tobacco and alcohol users. Fifteen tumors were stage I, five stage II, seventeen stage III, eight stage IVa, and six stage IVb. No patient had metastases at the time of diagnosis. According to the WHO histological classification [3], our series comprised of 7 papillary type or PTCC-I (papillary tubular cylinder cell I), 23 colonic (PTCC-II), 4 solid (PTCC-III), and 17 mucinous type tumors. All patients underwent radical surgery, and in all cases resection margins were free of tumor. Forty of the patients received complementary radiotherapy. Follow-up information was available with a median of 30 months (range: 1–242). The 5-year survival rate was 53%. Twenty-seven patients developed local recurrence, and five other had metastases in the brain. At the time of writing, 26 patients were alive, 21 died of disease, and 4 died of other causes.

Of the 19 controls, 2 patients were female and 17 male, with a mean age of 69 years (45–78). Seventeen had occupational exposure to wood dust with a median of 35 years (range: 1–50 years), and 9 were tobacco and alcohol users.

2.2. Histological Examination. H&E stained paraffin sections were grouped as: (1) mucosa adjacent to tumor and (2) control mucosa from the other unaffected fossa of a patients with ITAC. The surface epithelium of all samples was evaluated for the presence of respiratory epithelium, dysplasia, cuboid metaplasia, squamous metaplasia, and cylindrocellular hyperplasia (including basal cell hyperplasia, mucrosecretory hyperplasia, and transitional type hyperplasia). Intestinal metaplasia was evaluated with the help of cytokeratin 20 immunostaining. The seromucinous glands were analyzed for signs of dysplasia and mucosecretory hyperplasia.

2.3. P53 Immunohistochemistry. Immunohistochemistry was performed on 4 μm paraffin embedded sections with the antibodies anti-p53 clone DO-7 and cytokeratin 20 clone K.208 (DAKO, Glostrup, Denmark) using an automatic staining workstation (Dako Autostainer, Dako Cytomation, Glostrup, Denmark) with the Envision system and diaminobenzidine chromogen as substrate. P53 expression was evaluated both in surface epithelium and in seromucinous glands and scored in 4 categories: 0–10%, 10–25%, 25–50%, and 50–100%.

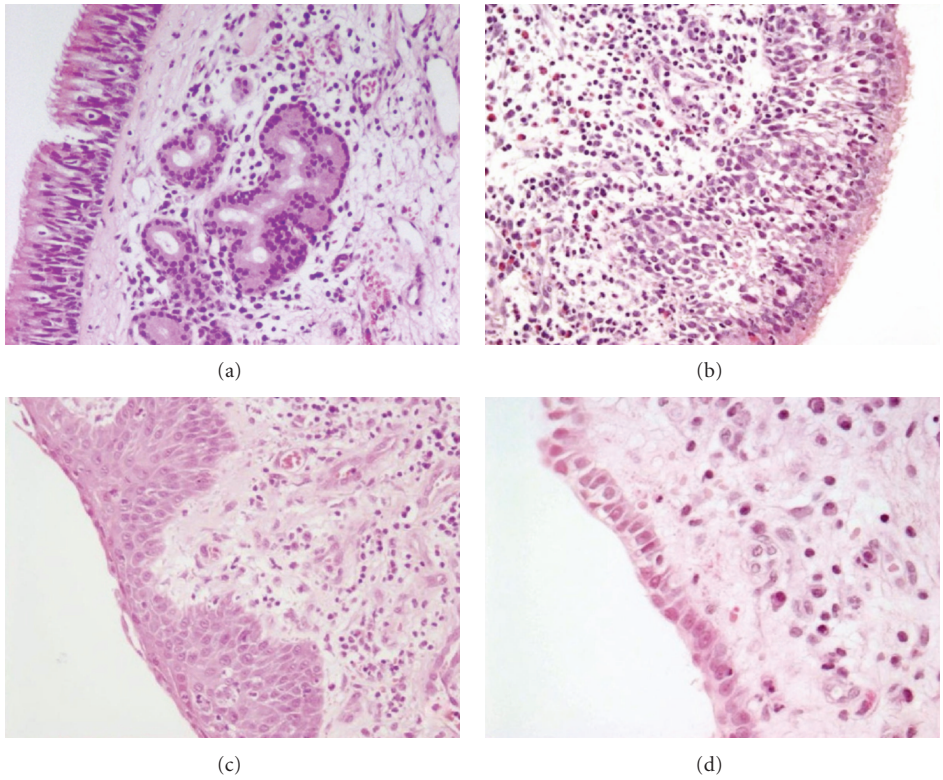
2.4. Statistical Analysis. Possible correlations were statistically analyzed by SPSS 12.0 software for Windows (SPSS Inc. Illinois, USA), using the Fisher Exact and Pearson Chi2 test. Kaplan-Meier analysis was performed for estimation of survival, comparing distributions of survival through the logarithmic range test (log-rank test). P values below .05 were considered significant.

3. Results and Discussion

3.1. Histological Changes. In 6 of 51 (12%) samples, no histological abnormality was observed in the mucosa adjacent to ITAC. Of these 6 cases, 3 ITACs were stage T1, 1 T2, 1 T3, and 1 T4b and concerned 3 colonic and 3 mucinous type tumors. Over half of the cases (24/45) showed more than one histological abnormality.

In the surface epithelium of the remaining 45 samples, we observed mild dysplasia in 7 (16%), cuboid metaplasia in 29 (64%), squamous metaplasia in 12 (27%), and cylindrocellular hyperplasia in 27 (60%) cases (Figure 1). CK20 immunostaining, indicative for intestinal metaplasia (Figure 2), resulted in 4 (9%) positive cases. In the seromucous glands, we detected 10 (20%) cases with mild dysplasia (Figure 3) and 17 (33%) cases with hyperplasia. Dysplasia in the respiratory mucosa correlated with dysplasia in the seromucous glands (Fisher exact chi2: $P = .029$). The 4 cases with CK20 immunopositivity in the surface mucosa also showed positivity in the seromucous glands (Figure 2). Two published studies that evaluated mucosa adjacent to ITAC differ very much, and our data seem to take position in between these two studies. For instance, Valente et al. [11] found no dysplasia and no squamous metaplasia in 15 samples, whereas Wilhelmsson and Lundh [12] reported 73% and 23%, respectively, in 22 samples. Intestinal metaplasia in mucosa adjacent to ITAC, detected in 4/51 of our cases by aid of CK20 immunostaining, has been reported previously in 1/10 [17] and in 4/12 [18] cases.

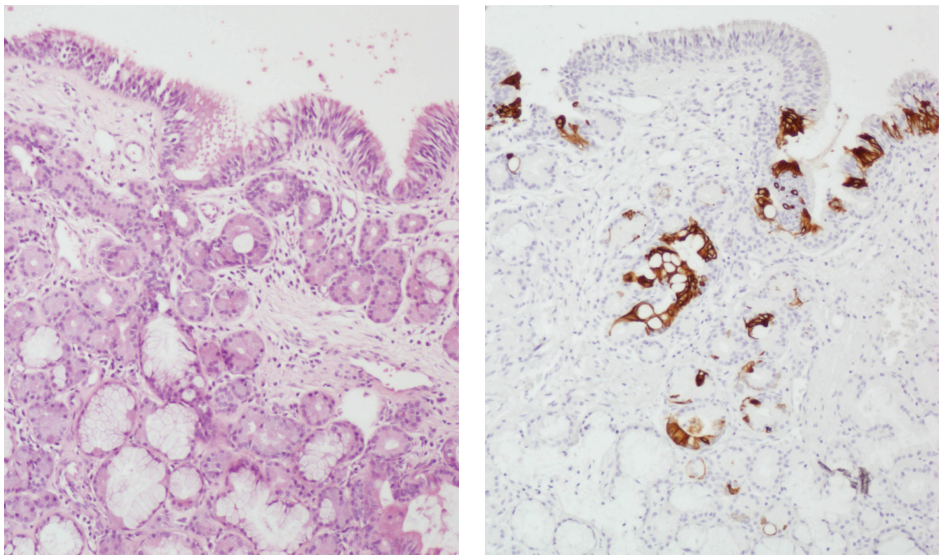
In normal mucosa of the unaffected fossa of ITAC patients, we found 9 of 19 (47%) cases without abnormalities and in general much lower frequencies of histological changes than in the mucosa adjacent to tumor (Table 1). This is similar to the literature concerning mucosa samples obtained from woodworkers who had not developed ITAC, with the exception of squamous metaplasia, which in our



(a) (b)

(c) (d)

Figure 1: Microphotographs of the different histological changes observed adjacent to the tumor. (a) Normal respiratory mucosa; (b) basal cell hyperplasia; (c) squamous metaplasia; (d) cuboid metaplasia. H&E staining, original magnification 200x (a, b, and c) and 400x (d).



(a) (b)

Figure 2: Microphotographs of intestinal metaplasia, both in the surface epithelium and in the seromucous glands. (a) H&E staining. (b) Immunohistochemical staining of CK20, original magnification 200x (a and b).

series was less frequent [11, 13–16] (Table 1). No CK20 positivity or intestinal metaplasia was observed in these 19 control samples, which is in agreement with Palomba et al. who reported complete absence of CK20 in a series of 139 normal mucosa samples from leather workers [15].

Although the number of cases without wood dust etiology was very low, our data suggest that wood dust exposure does not cause specific histological changes, except perhaps for cylindrocellular hyperplasia. This is confirmed by Wolf et al. who studied a large series of samples [13]. On

Table 1: Histological changes in mucosa adjacent to ITAC and in controls and comparison to the literature.

		This study				[11]		[12]	[13]		[14]		[15]		[16]	
	Tissue sample	Adjacent		Control		Adjacent		Adjacent	Mucosa		Mucosa		Mucosa		Mucosa	
	Wood exposure	Yes	No	Yes	No	Yes	No	Yes	Yes	No	Yes	No	Yes*	No	Yes	No
	Number of samples	45	6	17	2	10	5	22	144	31	65	68	139	16	113	54
Surface epithelium	Dysplasia	6 (13%)	1 (17%)	1 (6%)	0	0 (0%)	0 (0%)	16 (73%)	7 (5%)	1 (3%)	0 (0%)	0 (0%)	37 (27%)	0	14 (12%)	1 (2%)
	Cuboidal metaplasia	25 (56%)	4 (67%)	4 (24%)	1 (50%)			19 (86%)	40 (28%)	5 (16%)						
	Squamous metaplasia	10 (22%)	2 (33%)	1 (6%)	0	0 (0%)	0 (0%)	5 (23%)	57 (40%)	16 (52%)	40 (61%)	5 (7%)	90 (65%)	2 (13%)	41 (40%)	17%
	Hyperplasia	26 (58%)	1 (17%)	4 (24%)	1 (50%)				59 (41%)	6 (19%)			30 (22%)	1 (6%)		
	Intestinal metaplasia	3 (7%)	1 (17%)	0	0											
Seromucinous glands	Dysplasia	8 (18%)	2 (33%)	0	0											
	Hyperplasia	14 (31%)	3 (50%)	7 (41%)	0											

*Exposure to leather dust.

Table 2: P53 immunopositivity in mucosa adjacent to ITAC and controls and comparison to the literature.

	This study						[11]					
	Tumor	Tumor	Adjacent	Adjacent	Control	Control	Tumor	Tumor	Adjacent	Adjacent	Control	Control
Wood exposure	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No
Number of samples	45	6	45	6	17	2	10	5	10	5	68	81
Overall	68% ($n = 33$)	27% ($n = 4$)	24% ($n = 22$)	27% ($n = 2$)	35% ($n = 1$)	0%	69%	15–28%	12%	1%	12%	2%
Dysplasia			17% ($n = 1$)	0%	0%	0%						
Metaplasia cuboidal			17% ($n = 1$)	0%	35% ($n = 1$)	0%						
Metaplasia squamous			37% ($n = 6$)	37% ($n = 1$)	0%	0%					28%	8%
Hyperplasia			27% ($n = 2$)	0%	0%	0%						
Respiratory epithelium			21% ($n = 12$)	17% ($n = 1$)	0%	0%						
Seromucinous glands			24% ($n = 22$)	27% ($n = 2$)	NA	NA					12%	1%

P53 immunopositivity is presented as the mean % of positive cells calculated from the cases that showed some positivity (the number of cases is given between brackets). NA: not applicable for lack of representation.

Table 3: Histological changes and p53 expression in relation to adjacent tumor type and tumor T stage.

	Nr	Dysplasia	Cuboid Metaplasia	Squamous Metaplasia	Hyperplasia	p53 Adjacent mucosa				p53 tumor	
						0–10%	10–25%	25–50%	50–100%	<10%	>10%
Papillary	7	1 (17%)	2 (29%)	1 (17%)	5 (83%)	2 (29%)	4 (57%)	1 (14%)	0	1 (14%)	6 (84%)
Colonic	23	5 (22%)	16 (70%)	5 (22%)	5 (22%)	16 (70%)	6 (26%)	1 (4%)	0	3 (13%)	20 (87%)
Solid	4	0	3 (75%)	0	0	2 (50%)	2 (50%)	0	0	0	4 (100%)
Mucinous	17	1 (6%)	8 (47%)	6 (35%)	6 (35%)	7 (41%)	4 (24%)	5 (29%)	1 (6%)	7 (41%)	10 (59%)
T1	15	2 (13%)	9 (60%)	2 (13%)	7 (47%)	11 (73%)	3 (20%)	1 (7%)	0	4 (27%)	11 (73%)
T2	5	1 (20%)	3 (60%)	0	2 (40%)	2 (40%)	2 (40%)	1 (20%)	0	2 (40%)	3 (60%)
T3	17	2 (12%)	9 (53%)	6 (35%)	11 (65%)	10 (59%)	5 (29%)	1 (6%)	1 (6%)	4 (24%)	13 (76%)
T4	14	2 (14%)	8 (57%)	4 (29%)	7 (50%)	4 (29%)	7 (50%)	3 (21%)	0	2 (14%)	12 (86%)

P53 immunopositivity is presented as the number of cases that showed some positivity.

the other hand, some studies reported a higher frequency of squamous metaplasia in woodworkers compared to controls [14–16]. Tobacco smoking did not associate with any of the histological changes. Table 3 shows that the histological changes of the mucosa adjacent to tumor were not related to the histological tumor type or T stage. Only mucosa adjacent to papillary type ITAC seemed to harbour less cuboid metaplasia and more hyperplasia compared to the other three tumor types. Dysplasia was present in 5 of 27 (19%) patients that developed recurrence and only in 2 of 24 (8%) that did not; however, due to the low number of cases, this did not reach statistical significance (Fisher exact chi2 $P = .261$). In addition, all 4 patients with CK20 immunopositivity (i.e., intestinal metaplasia) in the mucosa adjacent to tumor had a tumor recurrence. Neither of the histological aberrations were related to overall or disease-free survival. To our knowledge, the relation between tumor and clinical characteristics and histological changes in mucosa adjacent to the tumor has not been studied previously in the literature.

3.2. P53 Expression. In general, p53 positivity was weak and occurred in a low percentage of cells (Figure 4), in the mucosa adjacent to ITAC and especially in the mucosa from the other unaffected fossa. In normal respiratory epithelium p53 expression was absent. Among the distinct histological lesions, squamous metaplasia showed the strongest positivity; interestingly dysplasia and cuboid metaplasia demonstrated a very low expression. P53 positivity in the surface epithelium was always accompanied by p53 positivity in the seromucous glands, but there was no correlation with p53 expression in the adjacent tumor (Pearson chi2 $P = .923$). It may be speculated that the relatively low level of p53 positivity in the mucosa adjacent to tumor does not indicate TP53 gene mutation, but rather an upregulation of functional p53 in response to inflammatory signals in the wood dust-exposed sinonasal epithelium.

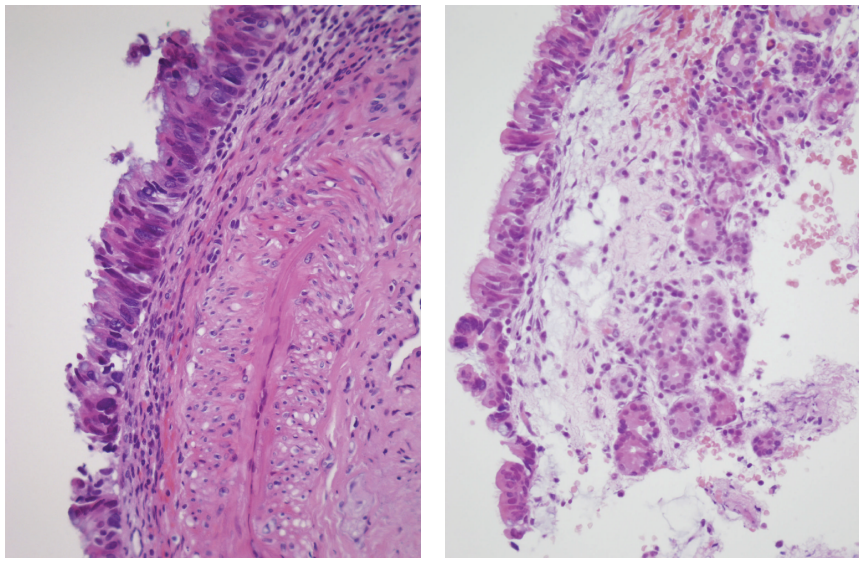
Our data, summarized in Table 2, are similar to a previous study studying ITAC, adjacent mucosa, and wood-exposed controls [11]. In addition, we confirmed a tendency of higher p53 positivity in the samples from patients with wood etiology, but not in the number of samples with some p53 positivity. Studying both ITAC and sinonasal squamous carcinoma, Holmila and coworkers also noted a trend for higher p53 expression in wood dust-related patients [19]. We found no difference in p53 expression between smokers and nonsmokers, and this was also seen by Holmila et al. [19].

When analyzing the p53 results in relation to the adjacent histological tumor type, we noted a higher expression in mucosa adjacent mucinous type ITAC (Table 3). However, p53 expression in the 51 ITAC tumors was different among the four histological subtypes. Finally, p53 positivity in mucosa adjacent to tumor did not correlate to the tumor T stage nor to the development of recurrence during follow-up.

4. Conclusion

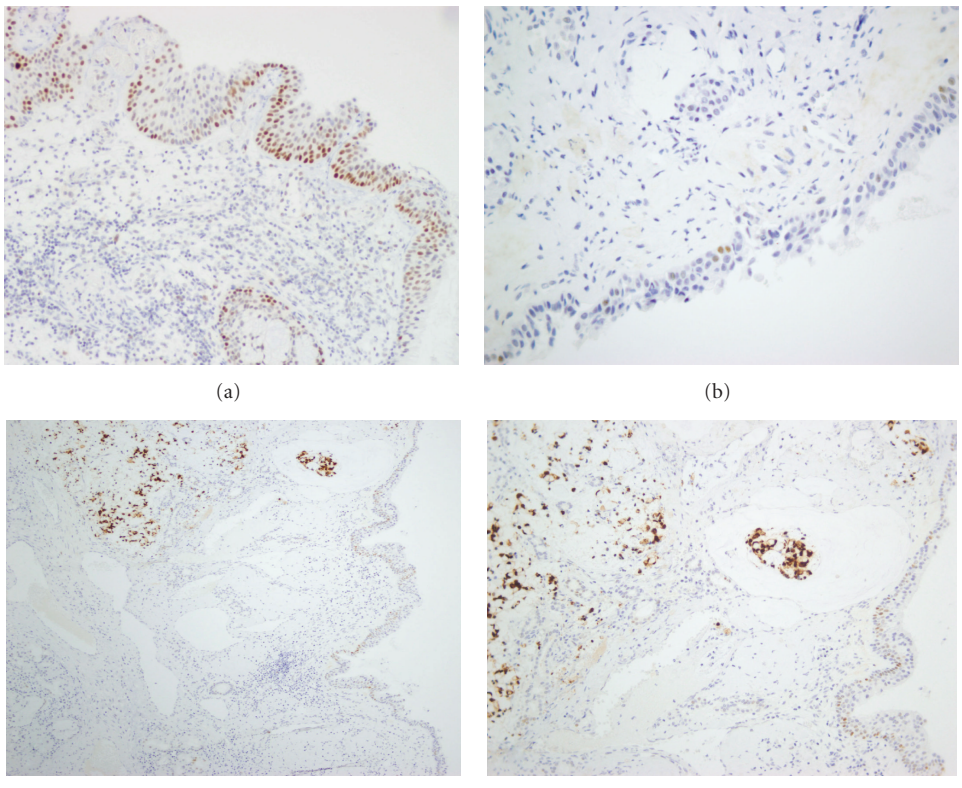
ITAC represents an important occupational health problem with serious consequences, needing better ways of prevention, early diagnosis, and treatment. Despite a clear etiology, it is still unknown how they develop. The present model suggests that inhaled wood dust particles larger than 5 μm become trapped in the mucosa of the middle turbinate and ethmoid [6, 7] and weaken the ciliar function of the nasal cells which prolong their contact with the mucosa and so their possible carcinogenic effects [7]. Since wood dust does not have direct mutagenic properties, it may be hypothesized that prolonged exposure to and irritation by wood dust particles stimulate cellular turn-over by inflammatory pathways. Chronic inflammation is recognized as an important mechanism in tumor initiation and progression in various cancer types, such as colorectal (inflammatory bowel disease), stomach (gastritis), and esophageal carcinoma (Barrett esophagus). Recently, Holmila et al. [19] showed that wood dust-related nasal adenocarcinomas have elevated COX2 levels, indicating a role for chronic inflammation in the tumorigenesis of ITAC. In addition, it has been shown that the TP53 mutation profile found in ITAC fits a causal role for reactive oxygen species, such as generated in chronic inflammatory processes [20]. It will be interesting to further investigate the possible role of chronic inflammation in sinonasal mucosa of woodworkers.

Epithelial cancers are frequently preceded and accompanied by precursor changes in the tissue histology, of which dysplasia is generally believed to be a true premalignant



(a) (b)

Figure 3: Microphotographs of two cases with dysplasia. (a) Mild dysplasia in the surface epithelium. (b) Mild dysplasia both in the surface epithelium and in the seromucous glands. H&E staining, original magnification 400x (a and b).



(a) (b)

(c) (d)

Figure 4: Immunohistochemical analysis of p53 expression. (a) Moderate, diffuse staining in basal and suprabasal cells in squamous metaplasia; (b) weak, focal staining in mild dysplasia; (c) strong staining in a mucinous type ITAC, with weak/moderate staining in adjacent immature metaplasia and seromucous glands; (d) details of (c). H&E staining, original magnification 200x (a, b, and d) and 100x (c).

lesion. In previous studies on normal mucosa of woodworkers, dysplasia was found in around 10% of cases [13–16]. In our study concerning mucosa adjacent to tumor, we had expected to find a higher percentage, but we found a similar number of dysplasias. It may be speculated that dysplasia indeed does precede ITAC in a majority of cases but that it becomes destroyed by the growth of the ITAC tumor mass. However, when we contrasted the presence of dysplasia of stage T1 tumors to larger, later stage tumors, we saw no differences (Table 3). Wilhelmsson et al. observed cuboid metaplasia often together with dysplasia and suggested this to be a precursor to ITAC [12, 21]. We found a high percentage of cuboid metaplasia. In addition, of 5 cases with dysplasia accompanied by cuboid metaplasia, 4 developed a recurrence. Our data may therefore be in accordance with this suggestion. Alternatively, it has been proposed that ITAC, which is predominantly CK20 positive, evolves from intestinal metaplasia [18]. In our series 4 cases had CK20-positive mucosa adjacent to ITAC and all 4 developed a recurrence and may also support this theory. These two notions are not necessarily contradictory. Kennedy et al. hypothesized that normal respiratory mucosa first undergoes cuboid metaplasia, followed by intestinal metaplasia, and then possibly through dysplasia develops into ITAC [17].

Whatever the tumor-initiating causes, cancer arises through a multistep sequence in which histological changes are accompanied by the accumulation of genetic aberrations. The analysis of genetic or epigenetic aberrations in these precursor lesions will give a more definitive insight in their possible role in ITAC development.

Acknowledgments

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Review Article

Oral Carcinogenesis and Oral Cancer Chemoprevention: A Review

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Oral cancer is one of the major global threats to public health. The development of oral cancer is a tobacco-related multistep and multifocal process involving field cancerization and carcinogenesis. The rationale for molecular-targeted prevention of oral cancer is promising. Biomarkers of genomic instability, including aneuploidy and allelic imbalance, are possible to measure the cancer risk of oral premalignancies. Understanding of the biology of oral carcinogenesis will yield important advances for detecting high-risk patients, monitoring preventive interventions, and assessing cancer risk and pharmacogenomics. In addition, novel chemopreventive agents based on molecular mechanisms and targets against oral cancers will be derived from studies using appropriate animal carcinogenesis models. New approaches, such as molecular-targeted agents and agent combinations in high-risk oral individuals, are undoubtedly needed to reduce the devastating worldwide consequences of oral malignancy.

1. Introduction

Head and neck cancer is the sixth most common human cancer [1], representing 3% of all types of cancer. They are located in the oral cavity in 48% of cases, and 90% of these are oral squamous cell carcinoma [2]. They are sometimes preceded by precancerous lesions, such as leukoplakia and erythroplakia. More than 300,000 new cases of oral squamous cell carcinoma are diagnosed annually [3]. Approximately 35,000 new cases are recorded annually in the US [2], 40,000 new cases are recorded in the EU and 10915 new cases in Japan [4]. The most common site for intraoral carcinoma is the tongue, which accounts for around 40% of all cases in the oral cavity proper. Tongue cancers most frequently occur on the posterior-lateral border and ventral surfaces of the tongue. The floor of the mouth is the second most common intraoral location. Less common sites include the gingival, buccal mucosa, labial mucosa, and hard plate.

The incidence of oral cancer has significant local variation. Oral and pharyngeal carcinomas account for up to half of all malignancies in India and other Asian countries, and this particularly high prevalence is attributed to the influence of carcinogens and region-specific epidemiological factors, especially tobacco and chewing betel quid. An increase in the prevalence of oral cancer among young adults is a cause of special concern. There has been a 60% increase in the number of under 40 years olds with tongue cancer over past 30 years. However, little has been published on the etiology and natural history of this increase [5]. Oral malignancy, including tongue cancer, is associated with severe morbidity and long-term survival of less than 50% despite advances in the treatment (surgery, radiation, and chemotherapy) of oral cancer. The survival of the patients remains very low, mainly due to their high risk of developing a second primary cancer. Therefore, the early detection and prevention of oral cancer and premalignancy are quite important [6–10]. This article will focus on the current understanding of oral carcinogenesis for the early detection and prevention of oral malignancy.

2. Oral Carcinogenesis

Oral carcinogenesis is a highly complex multifocal process that takes place when squamous epithelium is affected by several genetic alterations. The use of several molecular

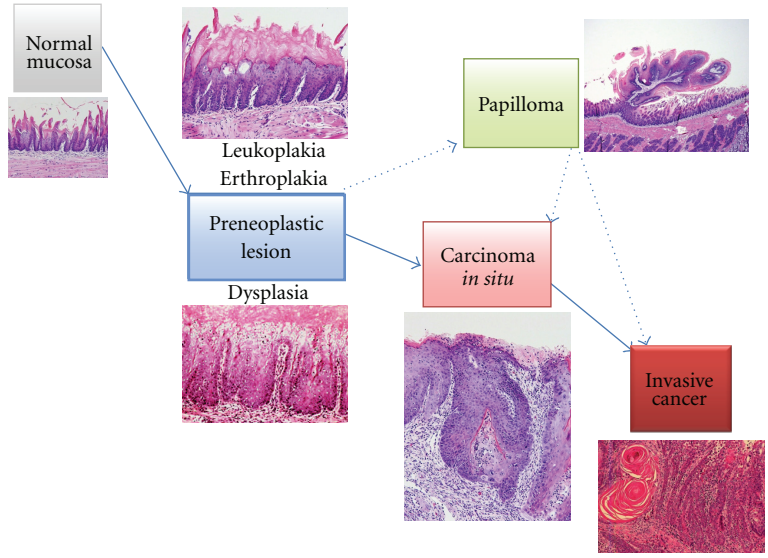

Figure 1: The natural history of oral carcinogenesis.

biology techniques to diagnose oral precancerous lesions and cancer may markedly improve the early detection of alterations that are invisible under the microscope. This would identify patients at a high risk of developing oral cancer [11]. The natural history of oral cancer and sequence of genetic alterations are illustrated in Figure 1. There are several approaches to understanding the molecular basis of oral cancer [12–14]. They include microarray technology, methylation microarrays, gene expression microarrays, array comparative genomic hybridization, proteomics, mitochondrial arrays, and micro-RNA arrays [15]. High-throughput approaches are currently being used to search for oral cancer biomarkers in biofluids, such as saliva and serum [15].

Field cancerization' refers to the potential development of cancer at multiple sites [16, 17]. This has been observed during the development of cancer in the tissues covered with squamous epithelium (head and neck tumor) and transitional epithelium (urothelial carcinoma). It is evident that oral cancer, like carcinomas in other tissues, develops over many years, and during this period, there are multiple sites of neoplastic transformation occurring throughout the oral cavity. "Field cancerization" may also be defined by the expression of mutations in the exons of tumor suppressor genes. One such tumor suppressor gene is *p53*, and mutations of this gene have been observed in various sites of premalignant leukoplakia and carcinoma in the same oral cavity [18]. A reduction in tumor suppressor activity by the gene and the development of mutations in *p53* are associated with smoking and an increased risk for oral carcinoma development [19]. Therefore, multifocal presentations and mutational expressions of tumor suppressor genes may be the consequence of long-term (e.g., 20 ~ 40 years) exposure to various environmental and exogenous factors. The continual presence of mutations may also signify changes in DNA repair and apoptosis, thereby increasing the susceptibility to future transformation. Mutational adaptations that modify the survivability of particular clones of transforming cells may also further enhance the level of resistance to therapeutic control. A recent genetic analysis revealed that cancers developing at distant sites within the oral cavity often are derived from the same initial clone [20]. The multiplicity of the oral carcinogenesis process makes it difficult to interrupt the progression to cancer through the surgical removal of a premalignant lesion.

3. Risk Factors of Oral Cancer

The most important risk factor for the development of oral cancer in the Western countries is the consumption of tobacco [21] and alcohol [22]. Although drinking and smoking are independent risk factors, they have a synergistic effect and greatly increase the risk together. The use of smokeless tobacco products such as gutkha and betel quid in Asian countries [5, 23] is responsible for a considerable percentage of oral cancer cases.

3.1. Genetic. Several studies have reported a significant familial component in the development of oral cancer. The estimates of risk in the first degree relatives of oral cancer patients vary widely and range from 1.1 [24] to 3.8 [25], although some of these cancers refer to head and neck cancer in general. Familial aggregation of oral cancer, possibly with an autosomal dominant mode of inheritance, is observed in a very small percentage of oral cancer patients [26]. Polymorphic variation of genes in the xenobiotic metabolism pathways such as in *CYP1A1* or the genes coding for glutathione *S*-transferase-M1 [27, 28] and *N*-acetyltransferase-2 [29] may be implicated. Individuals that carry the fast-metabolizing alcohol dehydrogenase type 3 (*ADH3*) allele [30] may be particularly vulnerable to the effects of chronic alcohol consumption and could be at increased risk to develop oral cancer [31]. The single

nucleotide polymorphism A/G870 in the *CCND1* gene that encodes Cyclin D is associated with susceptibility to oral cancer. The AA genotype [32] or the GG wild-type genotype [33] may increase risk for oral cancer.

3.2. Inflammation. Cytokines, including interleukins (ILs), tumor necrosis factors (TNFs), and certain growth factors, are an important group of proteins that regulate and mediate inflammation and angiogenesis. Tumor growth, invasion and metastasis are facilitated when there is a deregulation in their production. Genetic association studies suggest a putative correlation between functional DNA polymorphisms in cytokine genes and oral cancer [34]. Increased serum levels of proinflammatory cytokines, interleukin (IL)-1β, IL-6, IL-8, and TNF-α as well as the anti-inflammatory cytokine, IL-10, are seen in patients with oral cancer in comparison to healthy controls. The anti-inflammatory cytokine IL-4 inhibits oral cancer invasion by the downregulation of matrix metalloproteinase-9.

3.3. Infection. Human papillomavirus (HPV), particularly HPV type 16, may be an etiologic factor, especially among persons who do not smoke or drink alcohol [35, 36]. Ang et al. [37] reported that tumor HPV status is a strong and independent prognostic factor for survival among patients with oropharyngeal cancer. They also noted that the risk of death significantly increased with each additional pack-year of tobacco smoking. Although the idea that bacterial infections could lead to oral cancer has been generally discounted, there is an increasing body of evidence to suggest a possible relationship between micro-organisms and the development of oral cancer. The mostnotable example is that of the common pathogenic bacterium *Helicobacter pylori* and its association with gastric cancer. The mouth contains a variety of different surfaces that are home to a huge diversity of micro-organisms, including more than 750 distinct *taxa* of bacteria, thus suggesting that the oral squamous epithelium is constantly exposed to a variety of microbial challenges, on both cellular and molecular levels. It is therefore important to consider how such factors may be related to oral cancer development [38, 39].

3.4. Preneoplasia. There are clinically apparent oral premalignant lesions of oral cancer. They include leukoplakia, erythroplakia, nicotine stomatitis and tobacco pouch keratosis, lichen planus, and submucous fibrosis, [40]. The term "leukoplakia" was first used by Schwimmer in 1877 [41] to describe a white lesion of the tongue that probably represented a syphilitic glossitis. The definition of leukoplakia has often been confusing and controversial. Some clinicians now avoid using this term. The World Health Organization defines leukoplakia as 'a white patch or plaque that cannot be characterized clinically or pathologically as any other disease [42]. Therefore, leukoplakia should be used only as a clinical term. The term has no specific histopathological connotation and should never be used as a microscopic diagnosis. Leukoplakia is a clinical diagnosis of exclusion. Sometimes a white patch is initially believed to represent leukoplakia, but the biopsy reveals another specific diagnosis. These lesions should no longer be categorized as a leukoplakia. Leukoplakia is seen most frequently in middle-aged and older males, with an increasing prevalence with age [43]. Fewer than 1% of males below the age of 30 have leukoplakia, but the prevalence increases to an alarming 8% in men over the age of 70 [43]. The prevalence in females past the age of 70 is approximately 2%t. The most common sites are the buccal mucosa, alveolar mucosa, and lower lip. However, lesions occurring on the floor of mouth, lateral tongue, and lower lip are most likely to show either dysplastic or malignant changes [44].

The term "erythroplasia" originally used by Queyrat [45] to describe a red, precancerous lesion of the penis is used for a clinically and histopathologically similar process that occurs on the oral mucosa. Similar to the definition for leukoplakia, erythroplakia is a clinical term that refers to a red patch that cannot be defined clinically or pathologically as any other condition [42]. This definition excludes inflammatory conditions that may result in a red clinical appearance. Oral erythroplakia occurs most frequently in older males and appears as a red macule or plaque with a soft, velvety texture. The floor of mouth, lateral tongue, retromolar pad, and soft palate are the most common sites of involvement. Often the lesion is well demarcated, but some examples may gradually blend into the surrounding mucosa. Some lesions may be intermixed with white areas (erythroleukoplakia). Erythroplakia is often asymptomatic, although some patients may complain of a sore, burning sensation.

3.5. Tobacco. Nicotine stomatitis is a thickened, hyperkeratotic alteration of the palatal mucosa that is most frequently related to pipe smoking, but milder examples can also develop secondary to cigar smoking or, rarely, from cigarette smoking [42]. The palatal mucosa becomes thickened and hyperkeratotic, sometimes developing a fissured surface. The surface often develops numerous elevations with red centers, which represent the inflamed openings of the minor salivary gland ducts.

Another specific tobacco-related oral mucosal alteration occurs in association with smokeless tobacco use, such as either snuff or chewing tobacco [40]. Such lesions typically occur in the buccal or labial vestibule where the tobacco is held, but they can also extend onto the adjacent gingiva and buccal mucosa. Early lesions show slight wrinkling that disappears when the tissues are stretched. Other lesions may appear as hyperkeratotic, granular patches. Advanced lesions exhibit greatly thickened zones of grayish white mucosa with well-developed folds and fissures. The degree of clinical alteration depends on the type and quantity of tobacco, the duration of tobacco usage, and host susceptibility. Smokeless tobacco keratosis shows microscopic hyperkeratosis and acanthosis of the mucosal epithelium. True epithelial dysplasia is uncommon, and when dysplasia is found, it tends to be mild [46].

3.6. Mutations. Genetic mutations often produce early phenotypic changes that may present as clinically apparent, recognizable lesions. An oral premalignant lesion is an area

of morphologically or genetically altered tissue that is more likely than normal tissue to develop cancer. The reported rates of malignant transformation of leukoplakia range from less than 1% to 18% [47, 48]. There is no accepted method to predict the risk of malignant progression of an individual oral premalignant lesions, but various factors, such as the location within the oral cavity, clinical appearance (homogeneous versus heterogeneous), and the presence of dysplasia are correlated with the risk of progression. The histological finding of dysplasia is strongly associated with an increased rate of invasive cancer development [47]. A velvety reddish mucosal lesion, known as erythroplakia, is associated with a higher rate of cancer development, occurs much less frequently, and is more difficult to detect clinically than oral leukoplakia. Virtually all erythroplakic lesions contain severe dysplasia, carcinoma *in situ*, or early invasive carcinoma at the time of presentation [49]. Formalized classification and staging systems for oral preneoplastic lesions have been proposed [50, 51], and their use is important to facilitate uniform reporting and comparisons of data.

Detection and diagnosis of oral neoplasia has traditionally relied heavily on the clinical experience of the examiners and their ability to recognize often subtle morphologic changes. However, some early malignant lesions are clinically indistinguishable from benign lesions, and some patients develop carcinomas in the absence of clinically identifiable oral premalignant lesions. Furthermore, it can be difficult, even for experts, to determine which oral premalignant lesions are at significant risk to progress to invasive carcinoma. Therefore, an accurate, objective, and noninvasive method to help identify premalignant lesions and to distinguish those at risk of malignant conversion is needed.

4. Biomarkers of Oral Cancer

Biomarkers help in evaluating the preventive measures or therapies and the detection of the earliest stages of oral mucosal malignant transformation. Biomarkers reveal the genetic and molecular changes related to early, intermediate, and late end-points in the process of oral carcinogenesis. These biomarkers will refine the ability to enhance the prognosis, diagnosis, and treatment of oral carcinomas [52]. Genetic and molecular biomarkers will also determine the efficacy and safety of chemopreventive agents. Chemopreventive agents are chemicals of natural or synthetic origin. Unlike other drugs, which do not prevent disease, chemopreventive agents reduce the incidence of diseases such as cancer before clinical symptoms occur. This development is critical for the understanding of early oral mucosal transformation. Biomarkers will also reduce the number of patients and the time for long-term follow-up required to define a significant clinical response to a chemopreventive agent [53, 54]. The markers may therefore clarify the types, doses, frequencies, and regimens to achieve the maximum level of benefit from chemopreventive agents. Decreasing the cost of the clinical trials is another factor that drives the development of biomarkers.

Biomarkers have been categorized following the recommendation by the Committee on Biological Markers of the National Research Council/National Academy of Sciences [55]. They fall into broad groups that detect exposure, progression, susceptibility to carcinogens, and/or the responses by the target cellular populations [54].

Oral cancer studies have a distinct advantage due the anatomical access to the developing premalignant and malignant lesions. One could readily analyze biopsies of the primary lesion as well as apparently normal mucosal sites to determine the levels of DNA adducts and oral cancer risk. DNA adduct studies and cytogenetic analyses may also provide evidence for altered structure and function of susceptibility sites in the DNA following DNA binding studies of nuclear proteins such as *p53*. Some studies have focused on microscopic cytogenetic and somatic mutation changes as early biologic markers. One of the markers used to define chromosomal aberrations is the staining for micronuclei in exfoliated buccal mucosal cells [56]. Micronuclei have also been used to evaluate the reversal of leukoplakia and the effectiveness of retinoids, carotenoids, and vitamin E [57, 58]. Other methods include the determination of aneuploidy and the assessment of losses and gains of genetic material particularly associated with somatic and sex chromosomes. Other sites of chromosomal aberrations are found in sister chromatid exchanges, and allele typic variations designated by losses on chromosomes 3, 4, 5, 6, 8, 9, 11, 13, 17, and 19.

Some molecular biomarkers with potential diagnostic relevance include DNA content and chromosome polysomy, loss of heterozygosity, nucleolar organizer regions, histo-blood group antigens, proliferation markers, increased epidermal growth factor receptor (EGFR), and decreased expression of retinoic acid receptor-β, *p16,* and *p53* [59, 60]. Although a reliable, validated marker panel for providing clinically useful prognostic information in oral premalignant lesions patients has not yet been established, the advent of high-throughput genomic and proteomic analysis techniques may soon yield major advances toward a prognostically relevant molecular classification system (Table 1).

5. Animal Models for Oral Carcinogenesis

A variety of animals have been used for the study of tumor growth, the process of carcinogenesis, and the prevention/treatment research [8, 61–64]. The continual development of transgenic or knockout mice has improved our understanding of the role of specific genes in tumor growth. The most widely used animal models for oral carcinogenesis are the hamster cheek pouch model [62, 65] and the 4-nitroquinoline 1-oxide- (4-NQO-) induced oral (tongue) carcinogenesis model [8, 61, 66, 67].

DMBA is one of the widely used carcinogens in experimental oral carcinogenesis. Induction of SCC in cheek pouch of hamsters was first described with the aid of three polycyclic aromatic hydrocarbons, such as 7,12-dimethylbenz(*a*)-anthracene (DMBA), 20-methyleholanthrene (20-MC), and 3,4-benzpyrene [68]. A complete carcinogen, DMBA (0.5%), is applied to the hamster cheek pouch three times a week for 16 weeks. All animals exhibit invasive oral squamous cell carcinoma by week 16. Many studies have been conducted using the hamster buccal pouch model and thus elucidated

Table 1: Potential biomarkers for oral carcinogenesis.

Category of biomarkers	Measurements
Genomic	Micronuclei, DNA adduct, DNA content, Chromosomal aberration
Oncogenic	Oncogenic expression, Modified tumor suppressor genes, *Src* genes
Proliferation	Nuclear and cyclin related antigens, Mitotic frequency, Ornithine decarboxylase (ODC), Polyamines
Differentiation	Cytokeratins, Transglutaminase Type I, Transcription factor (AP)-1
Oxidative stress	Glutathione *S*-transferase, Stress proteins (HSPs), Superoxide dismutase
Apoptosis	Bcl-2 family, Chromatin condensation factors, Caspases, Mitochondrial pathway
Immunologic	Various cytokines

an array of changes that are analogous to those observed in human invasive oral carcinoma [62, 65]. These include a mutation in codon 61 of Ha-*ras*, which manifested in an A → T transversion in the second position of codon 61, thus resulting in an amino acid change from glycine to leucine. The expression of c-Ki-*ras* in malignant tumors of the pouch, but not in the normal oral mucosa, is also observed at the very early stages of tumor development [65]. Although the hamster oral tumor model appears to parallel several changes observed in human oral cancer, the hamster still has several areas of uniqueness which must be considered in any evaluation of results from oral carcinogenesis studies. The hamster cheek pouch provides a relatively large surface area of oral mucosa for the development of invasive carcinoma, while the human does not possess this type of mucosal structure. In contrast to humans, mice, or rats, the hamster cheek pouch lacks lymphatic drainage, which thus allows various drugs or molecules to accumulate in the pouch. The Syrian hamster population was also derived from a small breeding pair that resulted in a restricted polymorphism for the antigen recognition region (Ia region) and some of the major histocompatibility K and D regions [69]. In addition, the number of T-cells in the hamster spleen exhibits a lower number/gram weight of the organ in comparison to the mouse or human [69]. The hamster may also respond to antigenic tumor sources with a natural killer macrophage or granulocyte cytotoxicity rather than a T cell response [69]. DMBA and its solvent vehicle (acetone or benzene) are significant local irritants that cause severe inflammatory response, necrosis, and sloughing. Therefore, it is difficult to examine early squamous cell lesions [66, 70, 71]. Neoplasms induced by DMBA in the hamster cheek pouch possess many differences in histological features of differentiated SCC and do not closely resemble the lesions observed in human [72, 73].

The latter animal models for the study of oral carcinogenesis include those in rats and mice using the water soluble carcinogen, 4-NQO. The carcinogen is supplied either in the water (20 ppm) for the rats [66, 71, 74–86] or by painting for the mice [87]. The administration of 4-NQO in drinking water (20 ppm) for 8 weeks in rats and mice produces tongue lesions including squamous cell neoplasms within 32 weeks [83], while topical application of the carcinogen to the mouse palates for up to 16 weeks just like the hamster model develops palate tumors within 49 weeks [87]. The 4-NQO-induced tongue carcinogenesis model is quite useful for investigating oral carcinogenesis and identifying cancer chemopreventive agents, because the most common site for intraoral carcinoma is the tongue and the administration drinking water containing of 4-NQO is a simple and easy method [66, 71, 74–86, 88–96]. Increased levels of polyamine synthesis, as well as nucleolar organizer regions (NORs) with the progression of oral carcinogenesis, have been noted in the rat model [66]. The mouse model with 4-NQO has demonstrated some molecular mimicry of human oral cancers, as is true of the hamster model [87]. A number of chemical carcinogens, including coal tar, 20-MC, DMBA, and 4-NQO, have been used in experimental oral carcinogenesis. However, 4-NQO is the preferred carcinogen apart from DMBA in the development of experimental oral carcinogenesis. 4-NQO is a water soluble carcinogen, which induces tumors predominantly in the oral cavity. It produces all the stages of oral carcinogenesis and several lines of evidences suggest that similar histological as well as molecular changes are observed in the human system. There are several review articles that collate the available information on the mechanisms of action of 4-NQO. In addition, studies have been conducted for the development of biomarkers and chemopreventive agents using 4-NQO animal models [8–10, 61, 66, 67, 74–86].

The complexity and variety of biochemical changes that can increase tumor development is demonstrated in the $p53^{-/-}$ mice [97]. Unfortunately, this model and other genetic mouse models have not been exploited for studying the relationships among chemical oral carcinogenesis, specific genetic defects, and chemoprevention. Genetically altered mouse and rat models have been developed to evaluate molecular-targeted prevention and treatment of oral carcinoma [64]. The *ras*H2 transgenic mouse carcinogenesis model [98] and human c-Ha-*ras* proto-oncogene transgenic rat model [99] have been developed for chemoprevention studies on oral (tongue) carcinogenesis.

6. Chemoprevention

Chemoprevention is the use of natural or synthetic substances to halt, delay, or reverse malignant progression in tissues at risk for the development of invasive cancer [8–10].

Retinoids are the most extensively studied agents for chemoprevention of oral cancer [100]. Administration of 13-*cis*-retinoic acid for only 3 months yields a clinical response rate of 67% versus 10% for placebo. However, the toxicity is considerable, and there is a very high rate of relapse within 3 months of stopping treatment. Subsequent studies with retinoids in patients with oral premalignant lesions have confirmed clinical and pathologic response rates, though toxicities remain a concern [101]. However, translational studies show that molecular abnormalities persist in some patients with a complete clinical and pathologic response to retinoid therapy [102], suggesting that cancer development may be delayed rather than prevented by these agents. Other agents that have been assessed in clinical trials to evaluate the chemoprevention activity in oral leukoplakia patients include vitamin E [52], Bowman-Birk inhibitor concentrate (BBIC) derived from soybeans [103], curcumin [104], and green tea polyphenol epigallocatechin-3-gallate. Small clinical trials using oral BBIC have revealed no significant toxicity and a 32% response rate [103].

Attention is currently focused on the development of agents targeted to specific steps in the molecular progression from normal to oral premalignancy and to invasive carcinoma. Examples of molecularly targeted agents that have shown promise *in vitro*, in animal models, or in early clinical trials include cyclooxygenase- (COX-) 2 inhibitors and epidermal growth factor receptor (EGFR) inhibitors [105–107]. Data from several sources suggest that the cyclooxygenase pathway is a good target for oral cancer prevention. COX-2 is overexpressed in head and neck squamous carcinoma [108], and COX-2 inhibitors prevent oral cancer development in animal models [109]. A randomized placebo-controlled trial of the COX-2 inhibitor ketorolac administered as an oral rinse in oral leukoplakia patients revealed that the treatment is well tolerated but does not result in a greater clinical response than placebo [110]. However, an analysis of the results of this trial is somewhat confounded by the high response rate (32%) in the placebo arm and difficulty in determining whether topical delivery of the agent allowed penetration to the damaged cells. The future of COX-2 inhibitors as chemoprevention agents will also depend on determining the extent of risk for cardiac toxicities associated with this class of agents. The EGFR is also a promising molecular target for intervention in oral malignant progression [105–107]. EGFR is a receptor tyrosine kinase that is overexpressed in oral dysplasia and invasive cancer and associated with poor prognosis in patients with head and neck squamous carcinoma [111, 112]. EGFR inhibitors, alone or in combination with chemotherapy and radiotherapy, show activity against head and neck squamous carcinoma in clinical trials and are generally well tolerated [113]. Evidence suggests that combination therapy targeting COX-2 and EGFR may be efficacious [107, 114]. Although chemoprevention appears to be a promising approach to managing oral premalignancy, prospective clinical trials using specific agents, and strong corollary translational and laboratory investigations, are needed to evaluate clinical, histological, and molecular efficacy. It may be possible and necessary to individualize medical therapy to specific genetic abnormalities detected within the oral mucosa.

7. Conclusion

Human oral cancer is the sixth largest group of malignancies worldwide. Seventy percent of oral cancers appear from premalignant lesions. The process of formation of oral cancer results from multiple sites of premalignant change in the oral cavity (field cancerization). Animal models are now being widely used for the development of diagnostic and prognostic markers. The appearance of these premalignant lesions is one distinct feature of human oral cancer. There is currently a dearth of biomarkers to identify which of these lesions will turn into malignancy. Regional lymph node metastasis and locoregional recurrence are the major factors responsible for the limited survival of patients with oral cancer. The paucity of early diagnostic and prognostic markers strongly contributes to the higher mortality rates. Determining high- and low-risk populations by measuring reliable biomarkers is expected to contribute to achieving a better understanding the dynamics and prevention of oral cancer development. The quantitation of genetic and molecular changes and the use of these changes as markers for the detection and prevention of early premalignant change require the harvesting of tissues and cells. Promising technologies are being rapidly developed to assist in the identification of an abnormal oral mucosa, noninvasive and objective diagnosis and the characterization of identified mucosal lesions, and in the therapies for patients with oral cancer. Undoubtedly, the prevention or reduction in the use of tobacco products and alcohol consumption would have a profound influence on the incidence of oral cancer. Chemoprevention also has an impact on the development of malignant changes in the oral mucosa. Prevention through chemoprevention and/or the use of systemic medications is an extensively studied strategy and continues to hold promise as a way of diminishing the morbidity and mortality associated with this malignancy.

Abbreviations

BBIC: Bowman-Birk inhibitor concentrate
COX: Cyclooxygenase
DMBA: 7,12-dimethylbenz(*a*)anthracene
EGFR: Epidermal growth factor receptor
HPV: Human papillomavirus
IL: Interleukin
20-MC: 20-methylcholanthrene
NORs: Nucleolar organizer regions
4-NQO: 4-nitroquinoline 1-oxide
RAR: Retinoid acid receptor
TNF: Tumor necrosis factor.

Conflict of Interests

The authors declared that there is no conflict of interest.

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Review Article

Ancillary Studies in Determining Human Papillomavirus Status of Squamous Cell Carcinoma of the Oropharynx: A Review

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Squamous cell carcinoma (SCC) of the oral cavity and pharynx represents the sixth most common form of malignancy worldwide. A significant proportion of these cases are related to human papillomavirus (HPV) infection. In general, HPV-associated SCC is more commonly nonkeratinizing and poorly differentiated, whereas non-HPV-associated SCC is typically keratinizing and moderately differentiated. Nevertheless, significant overlap in morphology is seen between these two forms of SCC. The purpose of this paper is to highlight the utility of ancillary studies in the establishment of HPV status of oropharyngeal SCC, including p16 immunohistochemistry, high-risk HPV in situ hybridization, polymerase chain reaction, and newer HPV detection modalities.

1. Introduction

Malignancy of the oral cavity and pharynx constitutes the sixth most common form of malignancy worldwide [1]. In the US, approximately 36,540 cases and 7880 deaths occur per annum [2]. Greater than 90% of these malignancies are squamous cell carcinomas (SCC) [1]. Alcohol and both smoked and smokeless tobacco use are associated with increased risk of developing malignancy of the oral cavity and pharynx [3]. Studies have found a synergism between heavy smoking and heavy alcohol use, with a reported 30-fold increase in risk. As rates of tobacco use have declined, so have rates of oral cavity carcinoma [3].

More recently, human papilloma virus (HPV) infection has been implicated as a major etiologic agent for SCC development [3–12]. HPV consists of a family of encapsulated DNA virus containing over 100 genotypes [4]. High-risk genotypes, most commonly types 16 and 18, are associated with increased risk of squamous cell carcinoma in a number of locations, including cervix, vulva, anus, and oropharynx [4–8]. In contrast to the declining rates of tobacco and alcohol-associated oral cavity carcinomas, the incidence SCC of the oropharynx is increasing, in particular in the base of tongue and tonsils [3, 6–9]. This increased incidence is thought to reflect an increase in HPV-associated SCC. Patients with HPV-associated SCC tend to be younger, more frequently white, and more frequently male compared to those with non-HPV associated SCC [3]. As with cervical SCC, oropharyngeal SCC appears to be associated with sexually transmitted HPV, as high-risk sexual behaviors, including a high lifetime number of sexual partners and younger age at first intercourse, increase the risk [3, 10].

Evidence suggests that there is a causal association between HPV infection and SCC of the oropharynx, with molecular characteristics that distinguish it from non-HPV-related SCC, including alterations of p16 and c-myc expression [13–15]. The protein p16, a cyclin-dependent kinase inhibitor, is frequently utilized as a surrogate marker of HPV infection. Increased nuclear expression of p16 is seen with downregulation of its regulator, Rb protein, as occurs in functional inactivation of Rb by HPV E7 protein [3–5, 16]. Reflecting the differences in pathogenesis, histologic

distinctions between HPV and non-HPV-associated SCC are often appreciable. Despite having a better prognosis, HPV associated lesions tend to be nonkeratinizing, poorly differentiated lesions (Figure 1(a)), whereas non-HPV-associated lesions are generally moderately differentiated and keratinizing (Figure 2(a)) [3, 4, 13, 17]. Nonetheless, significant overlap is seen, and both HPV and non-HPV associated tumors frequently demonstrate intermediate features, such as nonkeratinizing tumors with areas of obvious squamous differentiation [13].

Distinction between HPV- and non-HPV-related SCC is important in relation to clinical outcome. A study by Ang et al. found three-year survival rate of 82.4% for HPV positive tumors versus 57.1% for HPV negative tumors [18]. A number of additional studies have demonstrated similar outcomes [8, 11, 13, 19–22]. The effect appears unrelated to the particular treatment regimen, as the prognosis has been better for patients treated with radiotherapy [11, 19], concomitant chemotherapy and radiotherapy [11, 18], and surgery alone [20, 21]. Further, the favorable outcome of HPV-associated SCC calls into question the necessity of aggressive postoperative treatment in these cases [22]. In the future, it is possible that treatment strategies may target specific molecular pathways that differ between HPV and non-HPV-associated SCC, further increasing the importance of this distinction.

Despite the importance of establishing the HPV status of SCC, no consensus has been reached on the optimal way to identify HPV-associated SCC [11]. The focus of this paper is the use of ancillary studies in the distinction between HPV positive and negative SCC, including immunohistochemical (IHC) staining for p16, HPV polymerase chain reaction (PCR) testing, and HPV in situ hybridization (ISH) analysis, and newer techniques that are currently under investigation.

2. Immunohistochemical Staining for p16

IHC staining for p16 is frequently used as a surrogate marker of HPV infection. It has the advantage of being easy to perform on formalin-fixed, paraffin-embedded (FFPE) tissue, and monoclonal antibodies against p16 are commercially available. HPV protein E7 binds to Rb, a negative regulator of p16 expression. Thus, HPV infection leads to increased nuclear p16 expression. As a result, IHC staining for p16 has a sensitivity approaching 100% for detecting HPV-associated SCC (Figures 1(b) and 2(b)) [13, 15].

However, p16 is overexpressed in a subset of tumors apparently lacking evidence for the presence of HPV DNA [4, 13, 22, 23]. Of note, Chernock et al. found that among cases of nonkeratinizing, poorly differentiated SCC of the oropharynx, p16 positivity by IHC staining was present in 100% of cases compared to just 69% positivity for HPV by in situ hybridization [13]. Among these p16 positive tumors, no difference in overall or disease-specific survival was found between those that were HPV positive and HPV negative [13]. Similarly, Lewis et al. found in a series of 239 cases of oropharyngeal SCC that 187 were positive for p16 by immunohistochemical stain [24]. Among these 187 cases, 26 (13.9%) were negative for HPV by both ISH and PCR (using SPF10-INNO primers). In addition, there was no difference in outcome between the p16 positive, HPV positive tumors and the p16 positive, HPV undetectable tumors [24]. In contrast; however, a recent study by Thavaraj et al. using a different set of PCR primers (GP5+/GP6+) from those in the Lewis study found that only 2 out of 142 (1.4%) p16 positive tonsillar SCC were negative for HPV by both PCR and ISH [25].

It is possible, then, that there is a subset of non-HPV-associated tumors with histologic phenotype, molecular characteristics, and prognosis similar to HPV-associated SCC. The percentage of these p16 positive, HPV negative tumors varies significantly between the Lewis and Thavaraj studies. Whether this represents differences in sensitivities of the HPV tests used or true differences in HPV prevalence in different populations is not definitely clear.

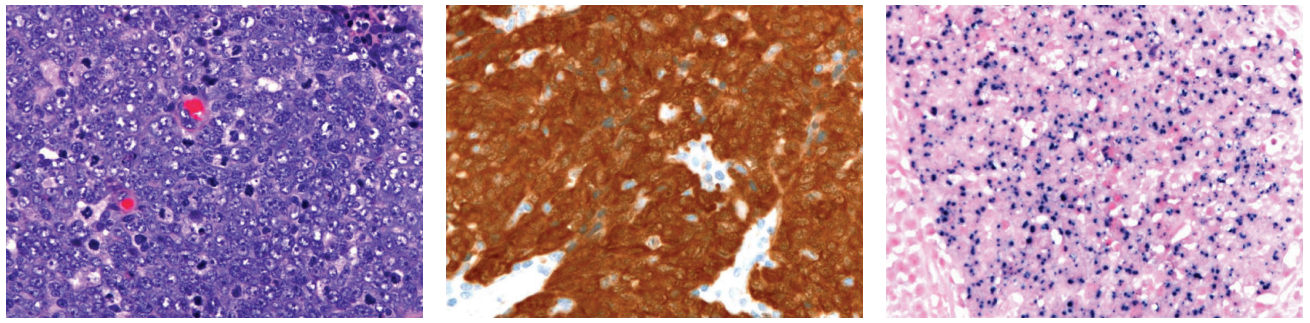
In any event, p16 positivity is a sensitive marker for nonkeratinizing, poorly differentiated yet prognostically favorable SCCs. While p16 may not be a specific marker of HPV infection, it can provide important prognostic information, and future therapies aimed at targeting this pathway of HPV tumorigenesis may well be effective in treating p16 positive, HPV negative SCC.

3. High Risk HPV In Situ Hybridization

ISH testing for HPV has the benefit of being the only molecular method allowing for direct identification of HPV in topographical relation to the pathologic lesion in tissue (Figures 1(c) and 2(c)) [26]. Unlike other direct detection methods for HPV that are performed in solutions or on solid supports, ISH occurs in the nuclei of infected cells by way of chromogen or fluorescent labeled complimentary nuclei acid probes against either DNA or mRNA [4, 26, 27]. Dot-like or punctuate positivity on microscopic examination indicates integration of the viral genome into the host cell genome, whereas diffuse staining indicates the presence of episomal DNA [4, 26–28].

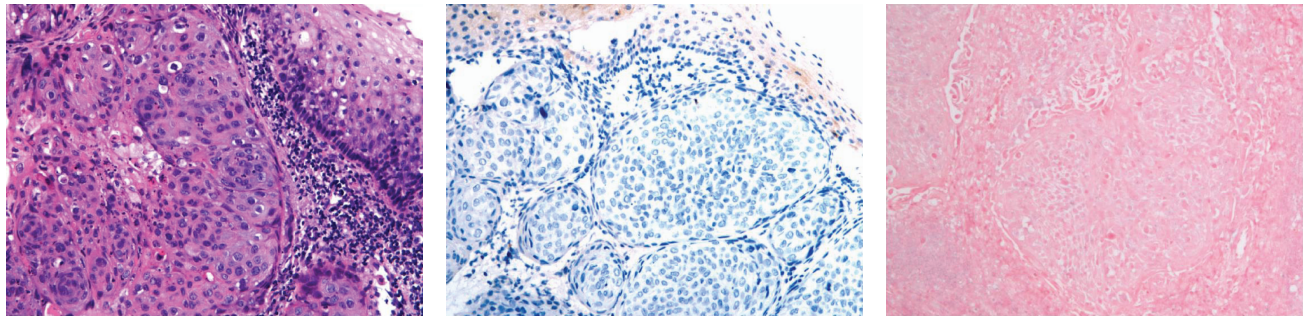
Numerous technically validated HPV ISH assays are commercially available, most containing a cocktail of probes targeting multiple types of HPV. Though probes for individual types can be used if subtyping is clinically relevant, HPV subtype 16 is by far the most commonly found in oropharyngeal SCC [5, 7, 10, 26]. The commercially available tests include INFORM HPV, Zytofast HPV probe, HPV OncoTect Test Kit, and GenPoint HPV Biotinylated DNA Probe [26]. These tests have demonstrated similar specificity in HPV detection of cervical specimens [26], but to our knowledge, comparisons of the commercially available tests in HPV detection of oropharyngeal lesions have not been performed.

The most common technical difficulties experienced with ISH are background and an absence of signal [27]. Background, defined as nonspecific binding of a probe to nontarget molecules, can be managed by decreasing the concentration of the probe or optimizing the posthybridization wash [26]. Absence of signal can be related to insufficient



(a) (b) (c)

Figure 1: Poorly-differentiated squamous cell carcinoma lacking clear evidence of squamous differentiation (hematoxylin and eosin) (a). In the oropharynx, these are typically HPV-associated neoplasms. Immunohistochemical stain demonstrates diffuse nuclear and cytoplasmic staining for p16 (b), while in situ hybridization highlights the presence of HPV DNA.



(a) (b) (c)

Figure 2: Moderately differentiated squamous cell carcinoma (hematoxylin and eosin) (a) lacking evidence of HPV infection by p16 immunohistochemical stain (b) or in situ hybridization (c). Though well and moderately-differentiated lesions tend to be negative for HPV, and poorly-differentiated lesions are typically HPV positive, there is significant morphologic overlap between HPV positive and negative tumors.

protease digestion, denaturing temperatures below 95°C, and an insufficient number of copies of the target DNA in the cell [27–29]. Approximately 10 to 20 copies of the target DNA per cell are required for detection by standard ISH techniques [28, 29].

The sensitivity of the assay is increased by signal enhancement techniques. One such technique is tyramide signal amplification, which has been shown to have a 10 to 100-fold increase on sensitivity [28]. In this system, peroxidase-conjugated streptavidin is applied to DNA-DNA hybridization mixture, followed by incubation with biotinylated tyramide. Peroxidase-conjugated streptavidin is then applied, and lastly, the chromogenic substrate diaminobenzidine is added [29]. Using an enzymatic amplification procedure such as this one allows a low copy number of a nucleic acid sequence to be identified. Such techniques have increased the sensitivity of ISH to the extent that it can detect as little as one to two copies of DNA per cell [4, 29].

Nevertheless, the sensitivity of ISH appears to be less than that seen in PCR analysis, as a metaanalysis by Termine et al. found HPV in 39.9% of cases by PCR compared to 29.8% by ISH [30]. However, due to the nature of the probe for specific viral nucleic acid sequences, ISH is highly specific for HPV infection, markedly more so than p16 immunohistochemical staining [28]. Based on the differences in sensitivity and specificity between tests, some authors have recommended two-tiered systems of HPV detection, such as the use of p16 immunohistochemistry as a screening tool and ISH as a confirmatory test [28, 31].

4. Polymerase Chain Reaction Detection of HPV

PCR is a process in which a signal sequence of DNA or mRNA is amplified several orders of magnitude through several rounds of denaturing at high temperature (~95°C), annealing of complimentary oligonucleotide primers at a lower temperature (~55°C), and DNA replication at an intermediate temperature (~72°C) by a heat-resistant DNA polymerase. In theory, it can be used to detect as few as one copy of a DNA sequence, making it a highly sensitive detection assay [4].

Material for PCR can be obtained from FFPE tissue by scraping tissue from a tissue block, digesting, centrifuging, and using the resultant supernatant for PCR studies [32]. In addition, samples can be obtained for direct PCR analysis via fresh tissue from oral biopsies [1]. In general, PCR for HPV

is more sensitive on fresh frozen tissue compared to FFPE tissue [27].

PCR has the advantages of being highly sensitive for HPV detection, widely available, and cost effective. However, standard PCR techniques have a number of drawbacks. PCR has lower specificity than ISH and is technically cumbersome to perform [27]. In contrast to ISH, it does not allow distinction between HPV that is present in the neoplastic cells and HPV that is present in surrounding nonneoplastic epithelium or stroma, nor can it distinguish between episomal and integrated HPV DNA [4, 26, 27]. In addition, while primers targeting the conserved L1 region are commonly employed, this region may be deleted during viral integration, potentially reducing the sensitivity [27, 32, 33]. However, Agoston et al. found that PCR directed at L1 was more sensitive than PCR directed at the obligate virulence factor E7 (90.2% compared to 72.5%), suggesting that loss of L1 is not seen in a significant number of cases and thus likely does not have a major influence on sensitivity of HPV detection [33].

Several PCR amplification techniques are commercially available. These PCR screening assays commonly have primers designed to amplify a region of DNA that is present in multiple HPV types (most commonly within the highly conserved L1 gene) [4, 33]. Since most commercially available PCR kits use consensus sequences from multiple HPV subtypes, specific typing is generally not possible through PCR alone. Among the more commonly commercially available primer sets are PGMY09/11, GP5+/GP6+, and SPF10 LiPA [34]. All three target sequences within the L1 gene though they are of varying length (450 basepairs, 140 basepairs, and 65 basepairs, resp.). Targeting shorter stretches of DNA generally results in higher sensitivity on FFPE tissue, as DNA fragmentation often occurs during extraction from the archived tissue [34]. Thus, the GP5+/GP6+ and SPF10 primers are more ideal for use in FFPE tissue from surgical specimens. As noted previously, the Lewis et al. study found HPV positivity by PCR in 86% of p16 positive SCC [24], while the Thavaraj et al. study found HPV positivity by PCR in 99% of p16 positive SCC [25]. The notable difference between these studies was the use of SPF10 primers in the former and GP5+/GP6+ in the latter. Differences in sensitivities between the different primer sets could explain this discrepancy. However, to our knowledge, no study has been done to directly compare the sensitivities of these two primer sets in detecting HPV in oropharyngeal SCC.

The use of real-time PCR has also been assessed in HPV detection. Real-time PCR allows for quantification of target DNA via colorimetric markers that accumulate during PCR amplification, allowing a mechanism of identification of HPV DNA as well as an estimation of viral load [4, 23]. This quantitative approach may allow for identification of more clinically relevant high viral loads, and, when targeted against mRNA, provides evidence of active gene transcription [27]. However, real-time PCR does not differentiate between integrated and episomal DNA [23].

Recent studies have looked into the ability of PCR to distinguish between episomal and integrated HPV DNA. The HPV gene for E2 protein is a common break site prior to viral integration into the host genome [4]. E2 protein is a regulator of E6 and E7 proteins, and its gene disruption results in upregulation of these tumorigenic factors [35]. When E2 is disrupted, PCR with primers designed to amplify the entire E2 gene will fail [4]. Thus, comparing PCR amplification of the E2 gene with a gene known to rarely be disrupted during integration (such as the E6 gene) can suggest whether the viral DNA is integrated or not, as the amplification ratio of E2 to E6 would be lower in integrated HPV compared to episomal HPV [4, 20, 36]. However, HPV DNA breakpoints are known to be variable, so E2 disruption is not necessarily seen in all integrated cases, limiting the sensitivity of this technique [4, 37]. Further, it is known that intact episomal E2 may be present even when integrated E2 is disrupted in cases of SCC of the cervix, potentially further reducing the sensitivity [38].

Finally, there are a number of commercially available assays for the detection of HPV by PCR by reverse transcriptase PCR. These kits target mRNA of the oncogenic E6 and E7 proteins. Thus, they have the advantage of detecting transcriptionally active HPV [4, 26]. It has the disadvantage of being time consuming and technically difficult. Further, performance of reverse transcriptase PCR is generally better on fresh tissue than FFPE tissue [4].

Overall, PCR is a reliable, sensitive marker of HPV DNA. Nonetheless, ISH still has a number of advantages over PCR, including higher specificity, the ability to reliably distinguish episomal from integrated HPV DNA, and the ability to localize HPV to the area of neoplasia.

5. Additional Techniques for HPV Detection

Over the past two decades, a technique has been developed for combining PCR and ISH, referred to as PCR in situ hybridization (PISH) [27, 39, 40]. In this case, PCR is performed using typical PCR reagents performed on FFPE tissue slides [27, 39]. The slide is then washed, dehydrated in alcohol, and dried. The PCR products present on the slide are then hybridized with specific DNA probes in the same manner that standard ISH is performed [39]. PISH can be utilized to perform PCR for HPV on intact tissue preparations of SCC followed by in situ hybridization detection, thus combining the sensitivity of PCR with the tissue localization of ISH [27, 39]. Studies looking at HPV detection rates in cervical invasive and in situ SCC have found significantly higher detection rates with PISH compared to ISH alone [39–42]. However, to date, no studies have looked at the utility of PISH in detecting HPV in oropharyngeal SCC.

Another hybridization technique, coined hybrid capture II (HC-II) has been developed and utilized in the detection of HPV. This is an FDA-approved method for HPV detection in cervical pap smears, and studies have demonstrated its utility in demonstrating the presence of HPV in lesions of the cervix and oropharynx [1, 26, 43, 44]. Suspicious lesions in the oropharynx are sampled by brush [1]. DNA is extracted from the exfoliated cells, denatured, and converted to single-stranded form [1, 26]. RNA probes against individual

HPV subtypes—typically as a cocktail of multiple high-risk types—are then hybridized in solution [1]. These DNA-RNA hybrids are put in microwell plates coated with anti-DNA-RNA hybrid antibodies. The immobilized complex is then reacted with antibodies conjugated to alkaline phosphatase, and cleavage of an added chemiluminescent substrate is measured by emitted light [1]. The intensity of the light emitted allows for an estimation of the viral load. Chaudhary et al. found increased sensitivity for HC-II compared to PCR in the detection of HPV in oropharyngeal SCC [1]. This test has the advantage of allowing HPV testing without the need for biopsy. However, because the reaction occurs in solution, it does not allow for localization of HPV to a histological area of interest. In addition, the high-risk probe cocktail typically used has been shown to detect at least 28 non-targeted HPV types, including many low-risk HPV types, creating the potential for false positives [26].

While IHC staining against p16 is frequently used as a surrogate marker of HPV, to date, IHC staining against specific HPV proteins has generally not been performed. Nevertheless, the development of IHC stains against the oncogenic E6 and E7 proteins would have a number of potential advantages over other HPV detection methods. It would have the ability to prove that HPV DNA is being expressed and directly demonstrate that important HPV oncogene proteins are present [4]. Development of reliable antibodies against E6 and E7 protein could be an excellent means for HPV detection in the future.

6. Conclusion

A number of tests for the detection of HPV in oropharyngeal SCC are available, each possessing its own strengths and weaknesses. At the present time, IHC staining for p16 and PCR for HPV appear to be the most sensitive markers of HPV, while ISH confers the greatest specificity. For most clinical laboratories, the combination of a sensitive test (e.g., p16 IHC) and a specific test (e.g., ISH) allows for the best potential to accurately establish the presence or absence of HPV in a given case of SCC.

The study by Thavaraj et al. utilizes an algorithm originally developed by Weinberger et al. [25, 45] in which oropharyngeal SCCs are classified first by p16 status and then by HPV status (either by PCR or ISH). In this way, SCCs are categorizing as p16−/HPV− (Class I), p16−/HPV+ (Class II), p16+/HPV+ (Class III), or p16+/HPV− (Class IV). Of note, when PCR was used to assess HPV status, 9% of cases fell into the Class II category (p16−/HPV+) compared to just 1% when ISH was used [25]. Given the fact that HPV PCR is known to lack specificity relative to ISH (89% specificity in the study by Smeets et al.), HPV positivity in the absence of p16 positivity by IHC may represent false positivity [23, 25]. In contrast, when ISH was used to assess HPV status, 11% of cases fell into the Class IV category (p16+/HPV−) compared to just 2% by PCR. Since ISH is known to lack sensitivity relative to PCR (86% sensitivity in the study by Smeets et al.), it is reasonable to assume that some of these Class IV cases represent false negatives [23, 25].

Given the relative frequency of discordant p16 and HPV results (i.e., Class II or Class IV) when using p16 in conjunction with a single HPV test, Thavaraj et al. suggest a three-tiered algorithm. Tumors are still categorized as p16 positive or negative by IHC. Those that are negative are then assessed for HPV by ISH and ultimately categorized accordingly as Class I (p16−/HPV−) or Class II (p16−/HPV+). Likewise, tumors that are p16 positive are initially assessed for HPV by ISH, and if ISH is positive, the tumor is categorized as Class III (p16+/HPV+). However, if the tumor is negative by ISH, a confirmatory PCR test is performed. If the tumor is still negative by PCR, it is classified as Class IV (p16+/HPV−). On the other hand, if the tumor is positive for HPV by PCR, the tumor is considered Class III (p16+/HPV+) despite the negative ISH result.

In their study, this three-tiered approach resulted in just a small minority of cases falling under one of the discordant categories (1% class II, 1% class IV), with concordance of p16 and HPV results in 97% (35% class I, 62% class III). This study suggests that with the three most commonly utilized tests, HPV status can be confidently determined in the vast majority of cases of oropharyngeal SCC.

In the future, more recently applied molecular technologies, such as PISH and HC-II, may offer even more accurate diagnosis of HPV in the clinical laboratory, and development of IHC against important viral proteins may ultimately provide the optimal test for active HPV genomic transcription and translation in SCC. Nonetheless, using current ancillary tests in combination with clinical clues and morphology, such as the presence of nonkeratinizing, poorly differentiated lesions, HPV status can be accurately assessed in the vast majority of cases of oropharyngeal SCC.

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Clinical Study

Salivary Gland Tumors in Maxillofacial Region: A Retrospective Study of 130 Cases in a Southern Iranian Population

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Tumors of the salivary glands are uncommon head and neck neoplasia. We conducted a retrospective study of 392 cases over the last 6 years in Shiraz, south of Iran, to investigate the clinicopathological features of these tumors in Iranian population. The age of the patients ranged from 8 to 85 years, with the mean age 44.57 ± 14.65 years and male-to-female (M : F) ratio was 1.02 : 1. For benign tumors, there was a propensity towards females, whereas the malignant tumor was more common in males. The ratio of benign tumors to malignancies was 2.19 : 1. Pleomorphic adenoma (PA) was the most common tumor and accounted for 85% of all benign tumors, followed by Warthin's tumor (8.6%). Of the 125 malignancies, adenoid cystic carcinoma (40%), mucoepidermoid carcinoma (24%) and invasive squamous cell carcinoma (16%) were the most common histological types. Most of the salivary gland tumors (75%) originated from major salivary glands and the remained (25%) originated from minor glands. The parotid gland was the most common site both in benign and malignant tumors. Most of our findings were similar to those in the literature, with some variations. The salivary tumors slightly predominated in males. Adenoid cystic carcinoma and mucoepidermoid carcinoma constituted the most common malignancies.

1. Introduction

Salivary gland tissues are diffusely distributed in the upper aerodigestive tract. The parotid, submandibular, and sublingual glands are the major salivary glands. Minor salivary glands are present in many sites, such as the lips, gingiva, cheek, palate, tongue, oropharynx, paranasal sinuses, and parapharyngeal space. Salivary gland tumors are relatively uncommon lesions accounting for 3–6% of all head and neck neoplasms [1]. The global incidence of these tumors is 0.4–13.5 per 100,000 persons annually [2–4]. These neoplasms composed heterogeneous groups of tumors with variable histological pictures. The site, patient age, and sex distributions of different types of salivary gland neoplasms vary with race and geographic location. The incidence of these tumors is different in between geographic areas and ethnic groups [2, 3, 5].

In the English literature, there is little report [1] on salivary gland tumors in Iranian population. The aim of this study was to analyze the relative frequency, location, patient sex, and age of salivary gland tumors in the southern Iranian population over the last 6 years.

2. Material and Methods

This study included patients with primary epithelial salivary gland neoplasms between 2004 to 2009, who underwent operations in the Department of Maxillofacial Surgery, Khalili Hospital, Shiraz. Hematoxylin-eosin- (H&E-) stained slides of all cases were reviewed by two pathologists based

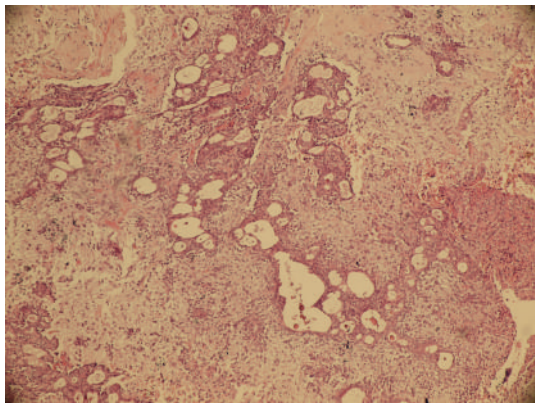

Figure 1: Benign mixed tumor (pleomorphic adenoma) with a biphasic admixture of epithelium and stroma (H&E ×200).

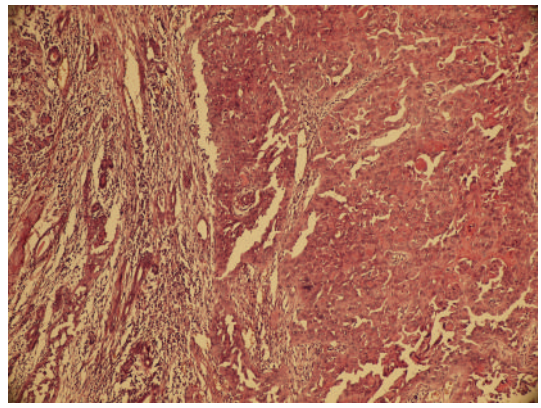

Figure 2: High-grade mucoepidermoid carcinoma composed of squamous with few intermediate and clear cells (H&E ×200).

on the 2005 World Health Organization classification of head and neck tumors criteria [6]. Information regarding age, gender, and anatomical location of the tumors was collected from the patients' hospital records. This research was approved by the Ethics Committee of Shiraz University of Medical Sciences. The data were analyzed for their distribution of patient's sex and age and anatomical location of tumors.

3. Results

3.1. Histological Types. 392 patients underwent operations for salivary gland tumors during this period. Among them, 267 (68.2%) were benign and 125 (31.8%) were malignant. The ratio of benign tumors to malignancies was 2.19 : 1. The distribution of histological patterns by anatomical locations for benign and malignant salivary tumors is shown in Table 1 and Figure 1, respectively.

Pleomorphic adenoma (PA) was the most common tumor and accounted for 85% (227/267) of all benign tumors (Figure 1), followed by Warthin's tumor (23/267, 8.6%). Myoepithelioma, basal-cell adenoma, and oncocytoma accounted for 4.5% (12/267), 1.1% (3/267) and 0.7% (2/267) of benign tumors, respectively (Table 1).

Of the 125 malignancies, adenoid cystic carcinoma (ACC, 50/125, 40%) (Figure 2), mucoepidermoid carcinoma (MEC, 30/125, 24%), (Figure 3), and squamous cell carcinoma (SCC, 20/125, 16%) were the most common histological types, followed by acinic cell carcinoma (6/125, 4.8%), adenocarcinoma, not otherwise specified (NOS) (5/125, 4%) and epithelial-myoepithelial carcinoma (4/125, 3%). Carcinoma ex-pleomorphic adenoma (2/125, 1%), salivary duct carcinomas (2/125, 1%), polymorphous low-grade adenocarcinoma (2/125, 1%) and basal-cell adenocarcinoma (2/125, 1%) were rare tumors. Twenty-two cases of squamous cell carcinoma (22/125, 17%) was reported in this series that all of them were direct invasion from overlying skin or metastatic to intraparotid lymph nodes (Table 1).

Table 1: Location and histological types of benign and malignant salivary glands tumors.

Tumor type	Number (%)	Major salivary gland ($n = 297$)	Minor salivary gland ($n = 95$)
Pleomorphic adenoma	227 (58)	188	39
Warthins' tumor	23 (6)	23	0
Myoepithelioma	12 (3)	7	5
Basal-cell adenoma	3 (0.7)	3	0
Oncocytoma	2 (0.6)	2	0
Mucoepidermoid carcinoma	30 (8)	26	4
Adenoid cystic carcinoma	50 (13)	14	36
Adenocarcinoma NOS	5 (1)	0	5
Acinic cell carcinoma	6 (1.5)	5	1
carcinoma ex-pleomorphic adenoma	2 (0.6)	0	2
Epithelial-myoepithelial carcinoma	4 (1)	4	0
salivary duct carcinoma	2 (0.6)	2	0
Polymorphous low-grade adenocarcinoma	2 (0.6)	1	1
Basal-cell adenocarcinoma	2 (0.6)	0	2
Squamous cell carcinoma (Invasion from overlying skin)	22 (5)	22	0

3.2. Locations. Most of the salivary gland tumors (297/392, 75%) originated from major salivary glands and the remained (95/392, 25%) originated from minor glands mainly located in the palate and lips. The parotid gland was the most common site both in benign (175/297, 59%) and malignant (56/125, 45%) tumors (Figure 2). Most of the tumors in the minor salivary glands were malignant rather than benign (53/34) and the palate was the most frequent location for minor gland tumors. Among benign tumors, almost all Warthin's tumors (100%), oncocytomas (100%),

Table 2: Comparison of reported distribution of salivary gland tumors in south of Iran and other countries.

	Total	Benign	Malignant	PA	ACC	MEC	Reference
Current study	392	267 (68)	124 (32)	227 (58)	50 (13)	30 (8)	—
Iran, west	130	89 (68)	41 (32)	85 (65)	3 (2)	15 (11)	[1]
China	6982	4743 (68)	2239 (32)	3281 (47)	681 (10)	673 (10)	[4]
UK	741	481 (65)	260 (35)	329 (44)	62 (8)	85 (11)	[7]
Italy	454	405 (89)	49 (11)	287 (63)	8 (2)	15 (3)	[8]
Jordan	221	151 (68)	70 (32)	139 (63)	12 (5)	38 (17)	[2]
Congo	275	180 (65)	95 (35)	152 (55)	44 (16)	22 (8)	[9]
Brazil	496	335 (68)	161 (32)	269 (54)	39 (8)	67 (14)	[10]
Uganda	268	145 (54)	123 (46)	107 (40)	36 (13)	25 (9)	[11]
USA	218	198 (90)	20 (9)	137 (62)	0 (0)	20 (9)	[12]
Bratislava	767	649 (85)	118 (15)	550 (71)	65 (8)	53 (6)	[13]
Nigeria	78	44 (56)	34 (43)	37 (49)	1 (1)	18 (23)	[14]
Sri Lanka	713	356 (50)	356 (50)	274 (38)	96 (13)	154 (22)	[15]
India	684	422 (62)	262 (38)	588 (86)	123 (18)	171 (25)	[16]

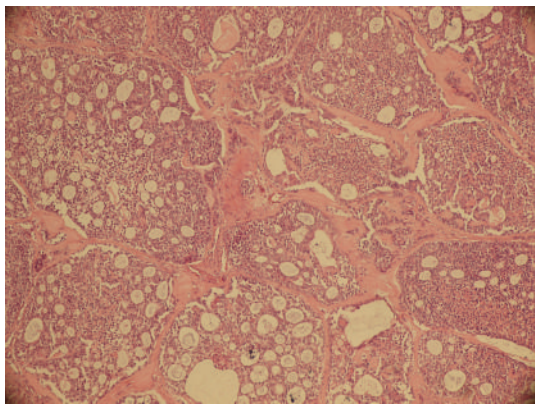

Figure 3: Adenoid cystic carcinoma, nests of cells of rather bland appearance are arranged concentrically around gland-like spaces (H&E ×200).

basal-cell adenomas (100%), and most myoepitheliomas (60%) were located in the parotid gland.

Among the malignancies, ACC and MEC were the most common types. ACC more frequently occurred in minor salivary glands, but the MEC was more cited in major salivary glands. Neither malignant nor benign tumors possessed a dominance of left- or right-side involvement. Bilateral involvement was not present in this study.

3.3. Age and Sex. Among 392 patients with salivary gland tumors, 198 were male, and 194 were female; the male-to-female (M : F) ratio was 1.02 : 1. For benign tumors, there was a propensity towards females (130/137, M : F = 0.9 : 1), whereas the malignant tumors were more common in males (68/57, M : F = 1.1 : 1), (Figure 2).

The age of the patients in this study ranged from 8 to 85 years, with the mean ± SD = 44.57 ± 14.65 years. The peak incidence for both benign and malignant salivary gland tumors was the fifth decade of life (Figure 3).

The peak incidence of PA is the fourth to sixth decades. Warthin's tumor is more prevalent in the 5-6th decade, and the oncocytoma and basal-cell adenoma are more common in fourth decade of life. For malignant tumors, the highest incidences of MEC and ACC were all in the fifth to sixth decade of life.

The total number of tumors (benign and malignant) occurring in young people under 20 years was 31, representing 8% of all tumors. In this age group, benign tumors were predominant (Figure 3), and PA was the most common type of tumor (23/31, 74%), followed by ACC (4/31, 12%). The ACC was the most common type of malignant tumors in this age group.

4. Discussion

Khalili Hospital is the largest referral hospital for maxillofacial tumors in the south of Iran, and many salivary gland tumors are treated in this hospital. In this study, benign salivary gland comprised 68% of all salivary tumors and predominated in major glands, similar to the rates reported by authors in the west of Iran, China, Jordan, UK, USA, India, Brazil, Nigeria, Congo, Uganda, Bratislava, and Sri Lanka [1, 2, 4, 7, 9–12, 14] (Table 2). In all these reports from different countries, benign tumors accounted for more than 50% of all salivary tumors, suggesting that benign tumors are predominant in salivary gland tumors worldwide.

In this study, PA was the most common type of salivary gland tumor (58%). This was consistent with other reports from different parts of the world, which have considered prevalence rates for PA between 40–65% (Table 2). The majority of PA was in major salivary glands. This finding was similar to a WHO report [6], in which approximately 80% of all PAs were occurred in the parotid gland, and 10% developed in the various minor glands.

The second most common benign tumor in this study, Warthin's tumor, comprised 23% of all salivary tumors,

which was less than the prevalence in Denmark and parts of Pennsylvania (about 30% of parotid tumors) [17, 18]. This tumor was rare in African populations [11]. Most of these tumors occurred in males (78%), and the M : F ratio was 3.6 : 1. Previous studies mentioned an increasing incidence of Warthin's tumor in females during the past 50 years, and the M : F ratio changed from 10 : 1 to 1.2 : 1, which may be related to the increased numbers of female smokers [6, 17]. In this study the majority of patients with Warthin's tumor had a history of tobacco smoking.

The reported frequencies for malignant salivary gland ranged were between 10–46%, and the MEC was the most common malignant tumor, with a prevalence ranging from 4–12% [6] (Table 2). Our data showed that malignancies comprised 32% of all salivary gland, and the ACC was the most common one (13%). The overall incidence of malignant tumors was similar to those reported from west of Iran and other countries [1, 2, 4, 7, 9–12, 14]. The higher prevalence of ACC was near the reported incidence from China and Congo [4, 9], but in the west of Iran, China, Jordan, UK, USA, India, Brazil, Nigeria, Uganda, and Bratislava the frequency of MEC was higher than that of ACC, and MEC was the most common malignancy (Table 2). These findings suggest a geographic variation in the frequencies of ACC and MEC. However, our report was different from Buchner et al. findings. They studied relative frequency of intraoral minor salivary gland tumors in northern California, USA. MEC was the most common (21.8%), followed by PLGA (7.1%) and ACC (6.3%) [19]. According to WHO classification of salivary gland tumors 2005, PLGA is the second more common malignant tumor of minor salivary gland, being surpassed only by MEC.

Most studies [6, 7, 10] revealed that the occurrence of salivary gland tumors was slightly higher in females. In the present study, males were slightly more affected (M : F = 1.02 : 1) like the finding in previous study [4]. The reason for this was the significant male predominance for Warthin's tumor and SCC.

In summary, this study was an epidemiological analysis of salivary gland tumors in the south Iranian population. Most of the findings about the distribution of histological type, age, and sex in this population were similar to those reported in the literature. However, there were few racial and geographic variations in the frequency and distribution of tumors between this study and other populations. PA was the most common benign and ACC ranked as the most common malignant salivary gland tumors followed by MEC. The overall occurrence of salivary gland tumors was slightly higher in males.

The reason for these differences remains unclear. Therefore, more research on this field is greatly encouraged.

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Research Article

Human Papillomavirus and Oropharyngeal Squamous Cell Carcinoma: A Case-Control Study regarding Tobacco and Alcohol Consumption

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We aimed to determine the role of HPV in the pathogenesis and outcome of oropharyngeal squamous cell carcinoma (OSCC) in lifelong nonsmoking and nondrinking patients. A case-case analysis was performed to compare the presence of HPV-DNA in tumor cells of 16 nonsmoking and nondrinking with 16 matched smoking and drinking patients (matching criteria: age at incidence, gender, tumor sublocation, tumor stage). HPV was detected using 2 PCR tests, FISH analysis, and $p16^{INK4A}$ immunostaining. Nonsmoking and nondrinking patients had more HPV-positive tumors than smoking and drinking patients ($n = 12$; 75% versus $n = 2$; 13%; $P < 0.001$). All HPV-positive tumors showed $p16^{INK4A}$ overexpression, and 1 HPV-negative tumor had $p16^{INK4A}$ overexpression, ($P < 0.001$). Overall survival and disease-specific survival were higher for HPV-positive compared to HPV-negative cases ($P = 0.027$, $P = 0.039$, resp.). In conclusion, HPV is strongly associated with OSCC of nonsmoking and nondrinking patients. Specific diagnostic and therapeutic actions should be considered for these patients to achieve a better prognosis.

1. Introduction

The most important risk factors for developing head and neck squamous cell carcinoma in the Western countries are consumption of tobacco and alcohol [1]. However, there is a small population of nonsmoking and nondrinking patients with head and neck squamous cell carcinoma, so other risk factors may be important [2]. Substantial evidence has shown that oncogenic human papillomavirus (HPV), which is the primary cause of uterine cervical cancer, is etiologically involved in the development of head and neck squamous cell carcinoma [3–10].

It is estimated that up to 15–20% of all head and neck squamous cell carcinomas are associated with high-risk HPV infection [3–10]. This prevalence varies broadly, depending on the sublocation of the tumor, the studied population, the detection method, and the type of specimen used [4–10]. The highest rates of HPV-DNA (up to 70%) have been found in oropharynx squamous cell carcinomas (OSCCs), especially the tonsils. HPV type 16 has been detected in 90–95% of HPV-related OSCC, HPV-18 in some cases, and HPV type 31, -33, and -35 in considerably less cases [7, 9–13].

In the pathogenesis of HPV-related cancer, integration of the viral genome into the cellular DNA and, as a result,

upregulation of the viral oncoproteins E6 and E7 seem to be crucial events. These oncoproteins subsequently cause dysfunction of amongst others tumor suppressor proteins, p53 and pRb, respectively, leading to cell proliferation, impaired apoptosis, and ultimately chromosome instability [14].

Immunohistochemical detection of $p16^{INK4A}$ overexpression, a product of tumor suppressor gene CDKN2A, has been associated with HPV-related head and neck squamous cell carcinoma and in some studies used as a surrogate biomarker for HPV detection [5, 15, 16]. Recent studies have characterized a subset of HPV-related OSCC in which $p16^{INK4A}$ overexpression predicts the presence of oncogenic HPV infection and identifies those with a better prognosis [17, 18]. Moreover, deletion of the CDKN2A locus together with functional inactivation of the tumor suppressor protein $p16^{INK4A}$ have been detected in head and neck squamous cell carcinoma without a relationship with HPV infection [19, 20].

HPV-positive head and neck squamous cell carcinomas are predominantly poorly differentiated and show a characteristic basaloid morphology in comparison with HPV-negative tumors [4, 6]. Furthermore, patients with HPV-positive tumors are less likely to consume large amounts of tobacco and alcohol [9, 15, 21, 22] and seem to have a better response to radiotherapy and a favorable survival rate [4, 11, 18, 23, 24]. So there are signs that these tumors form a separate entity within the heterogeneous group of head and neck squamous cell carcinomas.

The correct determination of HPV's involvement in the pathogenesis and prognosis of OSCC is dependent on several patient- and tumor-related cofactors, such as tobacco and alcohol use, TNM stage, and treatment modality. Although most investigators have found a trend between HPV and lesser amount of tobacco and alcohol use, the definitions of the used amounts are not always clear. Furthermore, to date, no matched analysis with smoking and drinking patients has been performed. In addition, previous studies have often used only one assay to determine the biological association of HPV infection with tumorigenesis.

In this study, we aimed to determine the role of HPV in carcinogenesis and disease outcome for nonsmoking and nondrinking patients with OSCC. Therefore, we performed a case-case study of a well-defined population of 16 nonsmoking and nondrinking and 16 matched, smoking and drinking patients with OSCC for the presence of HPV DNA and overexpression of biomarker $p16^{INK4A}$. The presence of HPV DNA was analysed using three different methods, that is, fluorescence in situ hybridization (FISH) and two polymerase-chain-reaction- (PCR-) based assays (Amplicor and Linear Array HPV detection kits).

2. Material and Methods

Patients were selected from a database at the University Medical Center Utrecht, in which all patients with newly diagnosed head and neck squamous cell carcinoma are prospectively registered. Between 1980 and 2004, 4607 patients were entered in the database. This database contains information on patient characteristics, risk factors, tumor classification, treatment modalities, and follow-up data including number of recurrences and subsequent primary tumors. Patients were classified as nonsmoking and nondrinking ($n = 198$), when they had no history of smoking tobacco and alcohol consumption. Patients were classified as smoking and drinking ($n = 2181$), when they actively smoked tobacco and consumed alcohol. Former smokers or drinkers were not included. All patients were treated according to institutional protocols, and final decision was made in consultation with the patient. Follow-up time (in months) was considered from the date of diagnosis (i.e., first proven biopsy) to the date of death or date of last followup (January 1, 2009). Seventeen nonsmoking and nondrinking patients with a primary head and neck squamous cell carcinoma located in the oropharynx (ICD codes 141.0, 145.3, 145.4, 146.0, 146.1, 146.2, 146.3, and 146.6) were found in the database of which 16 were selected because of absence of tumor tissue in 1 case. These patients were matched with smoking and drinking patients on gender, age (±5 years), sublocation of tumor, and tumor stage. A case-case analysis was performed to compare the prevalence of HPV DNA and overexpression of $p16^{INK4A}$ in both groups.

2.1. Tissue Specimens. 32 formalin-fixed, paraffin-embedded (FFPE) tumor tissue blocks from either biopsy or surgical resection specimens were obtained. Two experienced head and neck pathologists (JAK, PS) examined H&E-stained slides to select the areas in which tumor cells were present and evaluated the morphological appearances. Both pathologists were blinded to the smoking and drinking status. Tumor grade was recorded as well, moderate, or poor according to the criteria of the World Health Organization [25]. In addition, tumors were assessed for the absence or presence of hyperkeratosis, vasoinvasive and perineural growth, and typical basaloid features, that is, small, dark cells with scant cytoplasm, hyperchromatic nuclei, marked mitotic activity, a predominant lobular pattern of growth, and the absence of prominent keratinisation [26].

2.2. HPV Analysis

2.2.1. DNA Isolation and PCR Analysis. For DNA extraction tumor, areas from FFPE slides were isolated by microdissection. After deparaffinization, the tissue fragments were digested in 150 μL 50 mM Tris/HCL (pH 8.0) 0,5% (v/v) Tween-20 with proteinase K (final concentration 2 mg/mL). After 1 hour incubation at 56°C, the lysates were boiled to inactivate the proteinase K and subsequently centrifuged. Supernatants were transferred into clean eppendorf tubes and directly used for PCR. PCR was performed using the Amplicor HPV Test kit (Amplicor HPV Amplification kit: 03610799 190, Amplicor HPV Detection kit: 03610799 190, Amplicor HPV Controls Kit: 03610756 190; Roche, Basel, Sz) as well as the Linear Array HPV Genotyping Test (Linear Array HPV Genotyping Kit: 03378179 190, Linear Array HPV Detection Kit: 208693; Roche). Both tests were carried out according to the manufacturer's recommended protocol including positive and negative controls. The Amplicor test is a qualitative in vitro test which uses amplification of target

DNA by PCR and nucleic acid hybridization for the detection of high-risk HPV DNA genotypes (i.e., HPV types 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66, and 68). It uses primers to define a sequence of nucleotides within the L1 region of the HPV genome that is 150 base-pair (bp) long. This test also features a concurrent isolation and amplification of the human β-globin gene to assess DNA integrity for each tested specimen. The Linear Array test uses the same detection technique; however, it targets an HPV genome sequence of 450 bp and is able to detect high-risk (same types as mentioned above) as well as low-risk HPV-DNA (i.e., HPV types 6, 11, 40, 42, 43, and 44).

2.2.2. FISH. FISH was performed on 4 μm thick tissue sections as described previously [5, 15]. Briefly, sections were deparaffinized, pretreated with 85% formic acid/0.3% H_2O_2, 1 M NaSCN, and 4 mg/mL pepsin in 0.02 M Hcl, postfixed in 1% formaldehyde in PBS, dehydrated in an ethanol series, and hybridized with a digoxigenin-labeled HPV 16-specific probe (PanPath, Amsterdam, The Netherlands) according to the manufacturer's instructions. After hybridization, the preparations were washed stringently in 50% formamide, 2×SSC, pH 7.0 at 42°C (2 times 5 min). The probes were detected by application of mouse antidigoxin (Sigma, St. Louis, MO), peroxidase-conjugated rabbit antimouse IgG, and peroxidase-conjugated swine antirabbit IgG (both Dako; Glostrup, Dk) and visualized by a peroxidase reaction using rhodamine-labeled tyramide. Preparations were mounted in Vectashield (Vector Laboratories, Burlingame, Calif, USA) containing 4,6-diamidino-2-phenyl indole (DAPI; Sigma: 0.2 ug/mL). Microscope images were recorded with the Metasystems Image Pro System (black and white CCD camera; Sandhausen, Germany) mounted on top of a Leica DM-RE fluorescence microscope equipped with DAPI and rhodamine filters. Evaluation of nuclear hybridization signals was performed by two investigators (FF and EJMS) according to the previously described criteria [15]: punctate and/or diffuse signals throughout the nucleus indicating integrated and episomal HPV DNA, respectively, and granular FISH pattern if >1 nuclear signals, varying significantly in size and intensity, were observed. Control hybridizations were performed as described previously [15].

2.2.3. Immunohistochemical Detection of P16^{INK4A}. 4 μm thick tissue sections were deparaffinized with xylene and rehydrated by serial ethanol dilutions. Endogenous peroxidase activity was blocked by incubation for 30 minutes with 0.3% (v/v) H_2O_2 in methanol followed by antigen retrieval by boiling in 0.01 M sodium citrate buffer pH 6 for 15 minutes in a microwave oven. Slides were then incubated with a p16^{INK4A}-specific primary mouse monoclonal antibody (Neomarkers, Fremont, USA) and diluted 1 : 160 for one hour at room temperature followed by a secondary visualisation reagent for 45 minutes (Powervision Goat-anti-Mouse/Rabbit/Rat labelled with horseradish peroxidase, ImmunoLogic, ImmunoVision Technologies, Brisbane, USA). After each incubation step, slides were washed in phosphate-buffered saline containing 3% (w/v) BSA. Peroxidase activity was visualized by incubation with diaminobenzidine/H_2O_2, and cell nuclei were counterstained with hematoxylin. All p16^{INK4A}-positive cases were assessed for nuclear and/or cytoplasmic staining pattern. The staining patterns were scored semiquantitatively for the percentage of p16^{INK4A}-positive tumor cells. The sections were graded as positive (+) when at least 75% of the tumor cells showed p16^{INK4A} positivity and as negative (−) when no staining was visible. Only one case (Table 2) showed 25% p16^{INK4A}-positive tumor cells and was considered as ±.

2.2.4. Statistics. The association between HPV status and other variables was tested using Chi-square and Fisher's exact test. Disease-specific survival (i.e., death due to primary tumor, tumor recurrence, or subsequent primary tumor) and overall survival (i.e., mortality due to all causes) were determined for HPV-positive and HPV-negative cases, nonsmoking and nondrinking and smoking and drinking groups, and for cases with and without p16^{INK4A} overexpression using a univariate approach (i.e., Kaplan-Meier) method as patients were matched on possible confounding factors. Estimated survival curves were compared using log-rank test. A P value ≤ 0.05 was considered statistically significant.

3. Results

Sixteen nonsmoking and nondrinking patients with OSCC were matched with 16 smoking and drinking patients according to the above-mentioned criteria. The smoking and drinking patients used the following amounts of tobacco and alcohol at the time of diagnosis: 2–4 units of alcohol/day ($n = 10$), 5–9 units of alcohol/day ($n = 4$), and >9 units of alcohol/day ($n = 2$); ≤20 cigarettes/day ($n = 5$) and >20 cigarettes/day ($n = 11$). For 2 nonsmoking and nondrinking patients, the best possible match was disease stage IVA instead of III. The incidence dates ranged from 1980 to 2005. Table 1 summarizes the basic clinical characteristics of all cases.

HPV status for all cases was determined using two PCR-based test kits and FISH analysis (Table 2). The Amplicor PCR test showed 12 HPV-positive and 15 HPV-negative cases and was in 5 cases inconclusive due to negative β-globin gene results. The Linear Array PCR test showed 7 HPV-positive and 19 HPV-negative cases, and 6 cases that were inconclusive due to negative β-globin gene results. The FISH analysis revealed 12 HPV 16-positive cases of which 1 with a very low signal intensity (4B), and 19 cases without a detectable signal and 1 case which was inconclusive due to insufficient tissue material. Eight of the FISH-positive cases showed punctate signals in the tumor cell nuclei indicating integrated HPV DNA, and 4 showed granular nuclear staining. Based on these outcomes (see also discussion), we determined the HPV status as follows: 12 of 16 nonsmoking and nondrinking cases (75%) had a positive HPV status versus 2 of 16 smoking and drinking controls (12.5%, $P < 0.001$, Tables 2 and 3).

Immunohistochemical analysis for biomarker p16^{INK4A} was detected as shown in Tables 2 and 3. p16^{INK4A} overex-

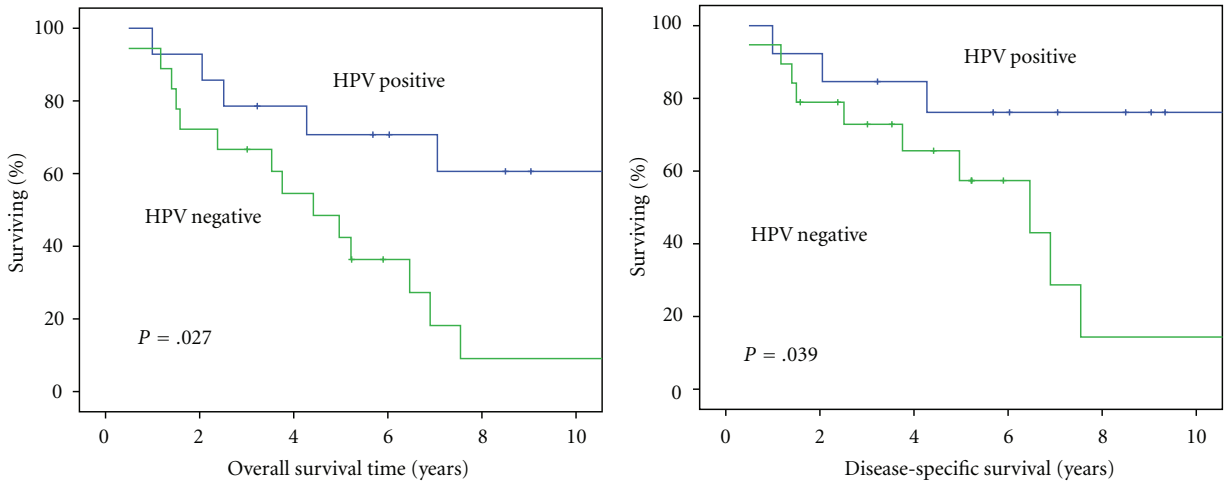

Figure 1: (a) Overall survival for HPV-positive compared to HPV-negative cases. (b) Disease-specific survival for HPV-positive compared to HPV-negative cases.

pression (at least 75% of cells with positive staining) was found in 14 cases (44%), in 1 case (1B) 25% of cells stained positive (3%) and 17 cases (53%) were negative. All positive cases had strong nuclear as well as cytoplasmic staining except case 16B which showed predominantly cytoplasmatic staining. All HPV-positive cases had $p16^{INK4A}$ overexpression, whereas 17 of 18 HPV-negative cases had no detectable $p16^{INK4A}$ ($P < 0.001$, Table 3).

The associations between HPV status and tumor subsite, T- or N-classification, tumor stage, year of initial diagnosis, treatment, and vasoinvasive and perineural growth were not significant (Table 3). In contrast, HPV-positive tumors showed significantly less often keratinisation ($P = 0.025$) and more often basaloid features ($P = 0.039$, Table 3). Tumor recurrence was found in 3 HPV-positive (2 locoregional and 1 distant) and 3 HPV-negative cases (all locoregional) and in 2 nonsmoking and nondrinking and 4 smoking and drinking patients. Second primary tumor was found in 1 HPV-positive (in the oral cavity) and 4 HPV-negative cases (1 oral cavity, 3 oropharynx, and 1 lung) and in 1 nonsmoking and nondrinking patient and 5 smoking and drinking patients (Table 3, no significant correlations).

3.1. Survival Data. Follow-up time ranged from 5.9 to 182.1 months. Median follow-up time was 61.1 months. The 5-year overall and disease-specific survival for all cases was 53% and 64%, respectively. Cause of death in 20 deceased patients was as follows: due to primary tumor ($n = 4$; 1 nonsmoking and nondrinking, 3 smoking and drinking), other causes ($n = 7$; 4 nonsmoking and nondrinking, 3 smoking and drinking) of which 5 cardial and 2 pulmonary disease, recurrent disease ($n = 6$; 2 nonsmoking and nondrinking, 4 smoking and drinking), and second primary tumor ($n = 3$ smoking and drinking). For HPV-positive and HPV-negative cases, the 5-year overall survival was 71% and 42%, and 5-year disease-specific survival was 76% and 57%, respectively. Overall and disease-specific survival were both significantly higher for HPV-positive compared to HPV-negative cases ($P = 0.027$, $P = 0.039$, resp., Figure 1), for nonsmoking and nondrinking patients compared to the smoking and drinking counterparts ($P = 0.037$, $P = 0.013$, resp.) and for cases with $p16^{INK4A}$ overexpression compared to those without detectable $p16^{INK4A}$ overexpression ($P = 0.028$, $P = 0.030$, resp.).

Table 1: Basic characteristics of all cases.

	Nonsmoking and nondrinking	Smoking and drinking
	n	*n*
Gender		
Male	3	3
Female	13	13
Age at tumor incidence (years)		
Mean	64.8	63.0
Range	45–83	50–78
Tumor stage		
II	3	3
III	6	4*
IVA	7	9*
Year of initial diagnosis		
1982–1986	2	1
1987–1991	2	3
1992–1996	6	5
1997–2001	2	5
2002–2006	4	2
Tumor location (ICD-code)		
Base of tongue (141.0)	6	6
Tonsil (146.0)	5	5
Tonsillar fossa (146.1)	3	3
Vallecula (146.3)	2	2

* Best possible match for 2 cases was stage IVA instead of III.

Table 2: HPV and $p16^{INK4A}$ results of all cases.

Case-case	$p16^{INK4A}$ overexpression	HPV			Final HPV outcome
		PCR (Amplicor)	PCR (Linear Array)	FISH	
1A*	+	Present	HPV-33/52,33,35,58	Absent§	Positive
1B†	±	Present	Absent	Absent	Positive
2A	+	Present	Absent	Present	Positive
2B	+	Present	HPV-16	Present	Positive
3A	+	Present	HPV-16	Present	Positive
3B	−	Absent	Absent	Absent	Negative
4A	+	Present	HPV-16	Present	Positive
4B	−	Absent	Absent	Present‖	Negative
5A	+	Present	Absent	Present	Positive
5B	−	Absent	Absent	Absent	Negative
6A	+	Present	HPV-16	Present	Positive
6B	−	Absent	Absent	Absent	Negative
7A	+	Present	HPV-16	Present	Positive
7B	−	Absent	Absent	Absent	Negative
8A	+	Present	HPV-16	Present	Positive
8B	−	Absent	Absent	Absent	Negative
9A	+	NO‡	NO‡	Present	Positive
9B	−	Absent	Absent	Absent	Negative
10A	+	Present	NO‡	NO‡	Positive
10B	−	Absent	Absent	Absent	Negative
11A	+	Present	NO‡	Present	Positive
11B	−	Absent	Absent	Absent	Negative
12A	+	NO‡	NO‡	Present	Positive
12B	−	NO‡	NO‡	Absent	Negative
13A	−	Absent	Absent	Absent	Negative
13B	−	Absent	Absent	Absent	Negative
14A	−	Absent	Absent	Absent	Negative
14B	−	Absent	Absent	Absent	Negative
15A	−	Absent	Absent	Absent	Negative
15B	−	Absent	Absent	Absent	Negative
16A	−	NO‡	NO‡	Absent	Negative
16B	+	NO‡	Absent	Absent	Negative

* A: nonsmoking and nondrinking.
† B: smoking and drinking.
‡ Not obtained (for PCR tests, for example, due to a negative β-globin PCR).
§ HPV-16-specific FISH probe.
‖ poor signal.

4. Discussion

To date, this study is the first that analyses the role of HPV in the pathogenesis and clinical behavior of OSCC in nonsmoking and nondrinking patients in comparison with matched smoking and drinking patients. HPV was strongly associated with OSCC in the absence of tobacco and alcohol use. HPV was found in 86% of the nonsmoking and nondrinking patients compared to 22% of the smoking and drinking patients. Our results are consistent with other studies although they mostly have shown this association separately in a group of nonsmokers or in a group of nondrinkers. Lindel et al. found HPV in 62 percent of nonsmokers and 38 percent of nondrinkers with oropharyngeal tumors [9]. Tachezy et al. demonstrated HPV-positive oropharynx and oral cavity tumors in all nonsmokers and 69% of nondrinkers, and in a recent study, nonsmoking and nondrinking patients with OSCC were reported to be 6.1 times more likely to be infected with high-risk HPV [22, 27]. One could hypothesize that smoking and drinking are independent risk factors and that the effect of HPV is enriched in the absence of these risk factors [28]. Increasing

Table 3: Characteristics of all cases according to HPV status.

	HPV *n* (%)		
Variable	Positive ($n = 14$)	Negative ($n = 18$)	*P* value
Tobacco and Alcohol			*<0.001*
Nonsmoking and nondrinking	12 (86)	4 (22)	
Smoking and drinking	2 (14)	14 (78)	
$p16^{INK4A}$ overexpression			*<0.001*
+	13 (93)	1 (6)	
−	0	17 (94)	
±	1 (7)	0	
Tumor location (ICD-code)			*NS**
Base of tongue (141.0)	4 (29)	8 (45)	
Tonsil (146.0)	4 (29)	6 (33)	
Tonsillar fossa (146.1)	4 (29)	2 (11)	
Vallecula (146.3)	2 (15)	2 (11)	
Tumor			*NS*
T1	3 (21)	1 (6)	
T2	6 (43)	7 (39)	
T3	3 (21)	6 (33)	
T4	2 (15)	4 (22)	
			NS
T1-T2	9 (65)	8 (45)	
T3-T4	5 (35)	10 (55)	
Nodal involvement			*NS*
N0	4 (29)	7 (39)	
N1	3 (21)	4 (22)	
N2	7 (50)	7 (39)	
Stage			*NS*
II	2 (15)	4 (22)	
III	5 (35)	5 (28)	
IVA	7 (50)	9 (50)	
Year of initial diagnosis			*NS*
1982–1986	1 (7)	2 (11)	
1987–1991	2 (15)	3 (17)	
1992–1996	6 (43)	5 (28)	
1997–2001	2 (15)	5 (28)	
2002–2006	3 (21)	3 (17)	
Treatment modality			*NS*
Radiotherapy	6 (43)	6 (33)	
Chemotherapy + radiotherapy	2 (15)	0	
Surgery + radiotherapy	5 (35)	9 (50)	
Surgery	0	2 (11)	
Chemotherapy	0	1 (6)	
Supportive	1 (7)	0	
Tumor grade			*NS*
Moderate	7 (50)	4 (22)	
Poor	7 (50)	14 (78)	

Table 3: Continued.

	HPV n (%)		
Variable	Positive ($n = 14$)	Negative ($n = 18$)	P value
Perineural growth			*NS*
Yes	4 (29)	2 (11)	
No	10 (71)	16 (89)	
Vasoinvasive growth			*NS*
Yes	3 (21)	1 (6)	
No	11 (79)	17 (94)	
Keratinization			*0.025*
Yes	3 (21)	11 (61)	
No	11 (79)	7 (39)	
Basaloid features			*0.039*
Yes	9 (65)	5 (28)	
No	5 (35)	13 (78)	
Tumor recurrence			*NS*
Yes	3 (21)	3 (17)	
No	11 (79)	15 (83)	
Second primary tumor			*NS*
Yes	1 (7)	4 (22)	
No	13 (93)	14 (78)	

* Nonsignificant.

evidence shows a particular risk factor profile for HPV-related head and neck squamous cell carcinoma with not only less consumption of tobacco and alcohol but also a different sexual behavior and higher use of marijuana in mostly younger patients (<55 years [28]) compared to non-HPV-associated head and neck squamous cell carcinoma [21, 29, 30]. We do not have patient data regarding sexual behavior and use of drugs in our studied population.

Additional characteristics of our studied HPV-positive tumors included the presence of basaloid features and lack of keratinisation which has been reported by previous studies [4, 6]. Likewise in this study as well as numerous other studies, HPV-related tumors proved to be associated with not only a better overall survival but also a better disease-specific survival [4, 9, 16, 23, 24]. The underlying mechanism for this prognostic effect of HPV is unclear. Although only one HPV-positive case had a second primary tumor compared to three HPV-negative cases, this difference was not significant. Also no correlation was found between recurrent disease or different treatment modalities and HPV positivity. Nevertheless, a better response on treatment like an increased sensitivity for radiotherapy possibly due to remaining amounts of p53 function in HPV-associated tumors might also explain the favorable prognosis. Worden et al. found induction chemotherapy followed by chemoradiotherapy to be an effective treatment in especially HPV-positive OSCC [31]. So it seems important to recognize patients with HPV-related head and neck squamous cell carcinoma to customize therapeutic decisions. Moreover, combination of HPV with recently identified prognostic indicators such as loss of chromosome 16q and the presence of $p21^{CIP1/WAF1}$ or nuclear survivin expression holds further promise to select patients for this purpose [19].

We also found better overall and disease-specific survival for nonsmoking and nondrinking cases and those with $p16^{INK4A}$ overexpression compared to their counterparts. We consider these results to be related to HPV positivity. In another recent study by our studygroup regarding disease outcome for all head and neck squamous cell carcinoma in our center, we found no difference in survival between those who smoke and drank and those who did not [32].

Some controversy exists concerning the most reliable way to determine biologically relevant HPV infection in FFPE tissue. Therefore, it has been proposed to use at least more than one method to identify a firm association of the virus with the tumor cells. Most studies agree upon the use of the surrogate marker $p16^{INK4A}$ followed by a HPV-specific test, such as HPV DNA PCR [16, 18], HPV E6 RT-PCR [17], or HPV FISH [15, 33]. We used four methods to detect the HPV status, that is, $p16^{INK4A}$ immunostaining, PCR using two different test kits, and FISH analysis, which strongly correlated with each other. In 4 cases (9A, 12A, 12B, and 16A), the β-globin gene could not be amplified by both PCR tests; hence, the FISH data were used to determine the HPV status, which corresponded with the presence of $p16^{INK4A}$ overexpression in case of HPV positivity. Nevertheless, also some discrepancies were found between the different tests used. In cases 5A and 2A, the Amplicor test was positive for HPV, whereas the Linear Array test was negative, probably due to the large fragments that need to be amplified in the latter assay. As a consequence, the Amplicor and the FISH results were used to proof HPV positivity for these cases.

In case 1A, PCR revealed the presence of HPV DNA of types 33/52, 33, 35, and 58 with corresponding $p16^{INK4A}$ overexpression, which explains the negative outcome of the HPV type 16-specific FISH analysis. Only in cases 1B and 4B, FISH analysis did not correlate with PCR and $p16^{INK4A}$ immunostaining, and in these cases, we decided to consider a positive $p16^{INK4A}$ and PCR status as signs for HPV positivity. However, the opposite may also be true as one considers the very high sensitivity of HPV DNA PCR, which may lead to false-positive results [34], as well as the fact that $p16^{INK4A}$ can be overexpressed without the presence of HPV, for example, case 16B and a study by Hafkamp et al. [15]. On the other hand, the $p16^{INK4A}$ staining pattern in case 16B was purely cytoplasmic in contrast to the other $p16^{INK4A}$-positive cases in which cytoplasmic and nuclear pattern was seen. This may point to other reasons than HPV for upregulation of this biomarker. Furthermore, the results as mentioned in Table 3 and survival curves would not be affected by opposite results of cases 1B and 4B.

We conclude that HPV is strongly associated with oropharyngeal tumors, especially in lifelong nonsmokers and nondrinkers. With better and more valid detection techniques, it is likely that these patients will be recognized as a specific entity within the heterogeneous group of head and neck cancer. Diagnostic and therapeutic actions will then be more focussed on this distinct group and may lead to better prognosis.

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Research Article

Raman Spectroscopic Methods for Classification of Normal and Malignant Hypopharyngeal Tissues: An Exploratory Study

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Laryngeal cancer is more common in males. The present study is aimed at exploration of potential of conventional Raman spectroscopy in classifying normal from a malignant laryngopharyngeal tissue. We have recorded Raman spectra of twenty tissues (aryepiglottic fold) using an in-house built Raman setup. The spectral features of mean malignant spectrum suggests abundance proteins whereas spectral features of mean normal spectrum indicate redundancy of lipids. PCA was employed as discriminating algorithm. Both, unsupervised and supervised modes of analysis as well as match/mismatch "limit test" methodology yielded clear classification among tissue types. The findings of this study demonstrate the efficacy of conventional Raman spectroscopy in classification of normal and malignant laryngopharyngeal tissues. A rigorous evaluation of the models with development of suitable fibreoptic probe may enable real-time Raman spectroscopic diagnosis of laryngopharyngeal cancers in future.

1. Introduction

"Hypopharyngeal," also known as "laryngopharyngeal," cancers are tumors of a subsite of the upper aerodigestive tract within the group of head and neck malignancies. The hypopharynx is the region between the oropharynx and the esophageal inlet. Approximately 7% of all cancers of the upper aerodigestive tract are of hypopharyngeal origin [1]. Incidence of these cancers seems to be four to five times less common compared to laryngeal cancers. All pharyngeal subsites accounted for approximately 1,24,000 cancer cases worldwide in 2002 [1]. India has the second largest population in the world with predominant oral, pharyngeal, and oesophageal cancers among females and laryngeal cancers among males [2, 3]. This is attributed to intake of various tobacco products like "paan." Smoked tobacco and slaked lime in paan are said to have synergistic carcinogenic effect in the upper aerodigestive tract [4]. Hypopharyngeal cancers are usually squamous cell carcinomas (SCCs) and are notorious as they usually present in advanced primary disease with or without nodal metastasis. The reconstruction after wide surgical resection in such cases is challenging and may increase morbidity and mortality. Hence early diagnosis is essential. A Contrast-enhanced computed tomography (CT) or a magnetic resonance imaging (MRI) of the head and neck is the mainstay initial radiological evaluation of these cancers [5]. PET scan is the latest imaging technique emerged to detect residual, recurrent tumors or secondaries. Due to occasional false positive results in cases of active inflammation or infection, this technique is also eventually dependant on biopsy for confirmation. Presently, rigid endoscopy and biopsy as mandatory as histopathology is the current gold standard for tissue diagnosis. The clinicians are dependant on skilled pathologist for accurate diagnosis. Moreover, the tissue sample may be inadequate or the pathologist may request deeper "repeat" multiple tissue biopsy. In anticipation of the biopsy report, patient may lose three to four days before active intervention of treatment. Only the gross manifestation of tissue changes arouse suspicion making assessment by hypopharyngoscopy under general

anaesthesia mandatory. This subjects the patient to possibility of excess bleeding or anaesthesia-related complications especially in elderly patients and/or postoperative pain while swallowing. The tissue biopsy is especially challenging in irradiated cases wherein frank growth (residual or recurrent) may be obscured due to induration or Edema. The other modalities of tissue diagnosis may be particularly necessary as confirmation in false positive interpretation [6] of malignancies. Hence, it is crucial to depend on alternative methods to (1) confirm malignancy, (2) detect latent or early mitotic changes before gross appearance of abnormal tissue, and (3) extend its application to *in vivo* or *in situ* conditions.

Optical spectroscopic methods such as autofluorescence [7–9], Fourier transform infrared (FTIR) [10, 11], and Raman [12] have been the other methods of detection of malignancies. These optical methods attribute noninvasiveness unlike a painful biopsy with no prerequisite for staining or sample preprocessing. All are amenable to multivariate statistical tools for easy analysis.

Among the above-mentioned optical methods, fluorescence and FTIR are more popular due to simple instrumentation. Raman spectroscopy offers distinct advantages compared to other popular optical techniques. This is because less harmful near-infrared radiation is used for excitation with easy extraction of information due to distinct and sharp spectral features. The water content in tissues may not deter precision in diagnosis for *in vivo* and *in situ* future applications.

The shortcoming of fluorescence technique is that it may require an experienced ENT specialist to detect laryngeal cancer *in vivo* and it has had low specificity in tissue diagnosis. The method of diagnosis by contact endoscopy for preoperative screening of laryngeal malignancy also has limitation in its application. It allows assessment of only the superficial layers of epithelium [13].

The mode of diagnosis by tissue analysis using Raman spectroscopy has been proved to be a useful tool in classifying oral [14–17], brain [18], breast [19–21], cervical [22, 23], ovarian [24], nasopharyngeal [25], laryngeal [26–28], gastrointestinal tract [29–33], and skin [34] malignancies.

There are only three series of laryngeal and one study of nasopharyngeal cancers reported [25–28] so far. The significance of diagnosing hypopharyngeal cancers early is evident by the fact that these present worst prognosis especially because most of them present in advanced stages. With application of lasers in head and neck surgeries, a precise and optimum excision of a localized hypopharyngeal lesion is possible with good long-term prognosis. This highlights emphasis on early detection of hypopharyngeal tissue malignancy. Raman spectroscopy methodologies are ideal tools for noninvasive screening of population due to it's suitability for *in situ* and *in vivo* measurements. Since no spectroscopy study of hypopharyngeal cancers has been reported in the literature to date, we have carried out an exploratory conventional *ex vivo* Raman spectroscopy study of hypopharyngeal squamous cell carcinoma. We found that conventional Raman spectroscopy, unlike microscopy, probes larger areas thus provides representative spectra. Conventional Raman studies of *ex vivo* tissues have been an exploratory approach before the eventual *in vivo* applications. In this exploratory study, patients with histopathologic evidence malignancy involving the free border of aryepiglottic fold were selected and compared with the other normal subsite. The findings of the study are discussed in the present paper.

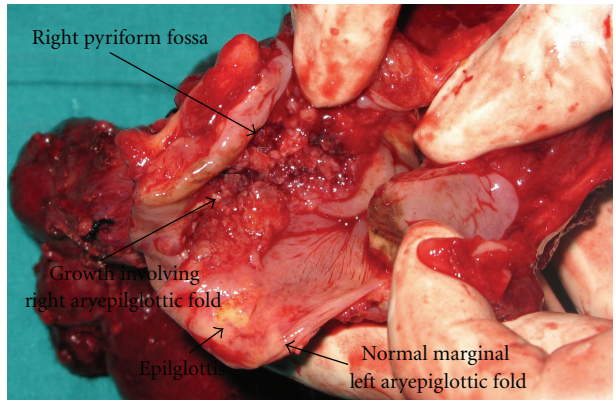

Figure 1: Pictorial presentation of gross laryngeal specimen after excision showing marginal zone formed by the upper margin of the aryepiglottic fold (AEF). The right AEF (big spot) showing ulceroproliferative growth and left AEF (small spot) that appears normal are indicated.

2. Materials and Methods

In total, twenty tissue samples were studied comparing the malignant tissue site with the corresponding normal site in each patient from January 2007 to December 2007. Ten patients with age range 43 years to 75 years and male to female ratio of 9 : 1 were considered for biopsy. Patients with unilateral marginal zone or the aryepiglottic fold malignancy were chosen because it is a transition area from laryngeal mucosa to hypopharyngeal mucosa. This is also known as "laryngopharynx" and is representative of the upper aerodigestive tract histologically. The marginal zone on the other side was grossly free of lesion as it appeared as soft and supple tissue. This study was approved for one year by the Manipal University Ethical Clearance Committee.

The biopsy specimens were taken from the growth and the corresponding normal side (Figure 1). These were put in individual saline bottles and delivered to the laser spectroscopy department. All the specimens were snap frozen in liquid nitrogen and passively thawed before subjected to Raman studies. A total of twenty samples were subjected to this study. A mirror image of all biopsy specimens were also sent for confirmative histopathology. Histopathologically, the ten malignant specimens were diagnosed to be squamous cell carcinoma (six patients had moderately differentiated while two patients each had infiltrating type and poorly differentiated carcinoma).

3. Laser Raman Spectroscopy

Raman spectra were recorded using the setup which was assembled by us [14, 15, 20, 22, 24, 31, 33]. In brief, this

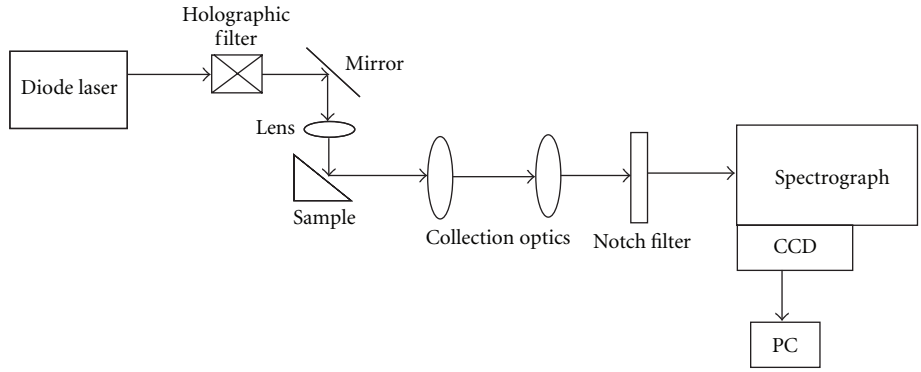

Figure 2: Schematic of the Raman instrumentation.

instrumentation employed diode laser (SDL-8530 785 nm, 100 mW) for excitation and HR 320 spectrograph (600 g/mm blazed at 900 nm) and spectrum one liquid N_2-cooled CCD for dispersion and detection of Raman signals. The Rayleigh scattering was filtered out using holographic filter (HSBF-785.0; Kaiser Optics). A schematic of the Raman instrumentation is presented in Figure 2. More than six spectra were recorded in each tissue. Each spectrum was acquired for 30 seconds and averaged over 20 accumulations. These experimental settings were kept constant during the study. Samples were kept moist in saline during spectral acquisition. The recorded spectra were postcalibrated with a cubic fit to known frequencies of Tylenol.

4. Data Analysis

The spectra were baseline corrected, smoothened, calibrated using diode adjust algorithms in Grams 32 (Galactic Industries corporation, USA) [35] and normalized over δCH_2 band. The preprocessed spectra were then subjected to Principle Component Analysis (PCA), a known data reduction technique where huge spectral data are decomposed into small independent variables known as "factors" and contributions of these factors were called "scores." Spectral data Analysis was carried out over entire region as well as several selected short regions besides derivatives of the same regions for standardization purposes. Total percentage variance, eigenvalues, and factor profiles were employed for standardization of PCA. Trail runs were carried out using 20, 15, 12, and 9 factors. In our analysis spectral range of 900–1750 cm^{-1} with 9 factors gave optimum results. Further data analysis was carried out under these conditions. Analysis was carried out in unsupervised and supervised modes. In the unsupervised approach, scores of factor were used as discriminating parameter whereas, in the supervised mode, Mahalanobis Distance and spectral residuals were used as discriminating parameters [35, 36]. We have also explored match/mismatch "limit test" approach which is known to bring out objective and unambiguous discrimination [14, 15, 20, 22, 24, 31, 33]. The flow chart of the study design is shown in Figure 3.

5. Results and Discussion

Mean Raman spectra of normal and malignant hypopharyngeal tissues are shown in Figure 4. On cursory examination, mean normal spectrum exhibits weak 1650 cm^{-1}, δCH_2 band at around 1445 cm^{-1}, sharp peaks at 1304 and 1277 cm^{-1}, and a broad peak at 1085 cm^{-1}. These spectral features indicate abundance of lipids in normal hypopharyngeal tissues. On the other hand, mean malignant spectrum, distinguished by broad and strong amide I at around 1655 cm^{-1}, red shifted δCH_2 at around 1449 cm^{-1}, broad amide III, and sharp peak at 1004 cm^{-1} suggest increased protein content with respect to normal tissues. We have observed similar features of abundance of lipids and proteins in normal and malignant oral tissue spectra, respectively [14–17].

For better correlation of spectral and biochemistry, the difference spectrum was computed by subtracting mean normal spectrum from mean malignant spectrum as shown in Figure 5. All the negative peaks (917, 983, 1072, 1302, 1440 cm^{-1}) seen in Figure 5 were contributed by normal spectrum attributable to lipids whereas all positive peaks (949, 1004, 1127, 1238, 1340, 1643 cm^{-1}) were from malignant spectrum which could be assigned to proteins. Besides high protein content, spectral features of the mean malignant tissue spectrum also indicate the presence of additional biomolecules like DNA (1340 cm^{-1}) and variations in secondary structure of the protein as indicated by amide I and III bands [37, 38]. We have also verified heterogeneity of spectra among same class of tissues, for example, normal and malignant tissues, by computing mean and standard deviation of normal as well as malignant spectra as illustrated in Figure 6. In Figure 6, the mean and standard deviation spectra indicate very minor heterogeneity and minor intensity differences.

It is well known that there are several multivariate statistical methods available for the spectroscopist for data mining. We have opted PCA for spectral data analysis in order to discriminate malignant from normal tissue types. In our method of PCA, the mean of all samples in the data set is first formed. The differences of this mean from each sample are calculated to give the variations of each sample

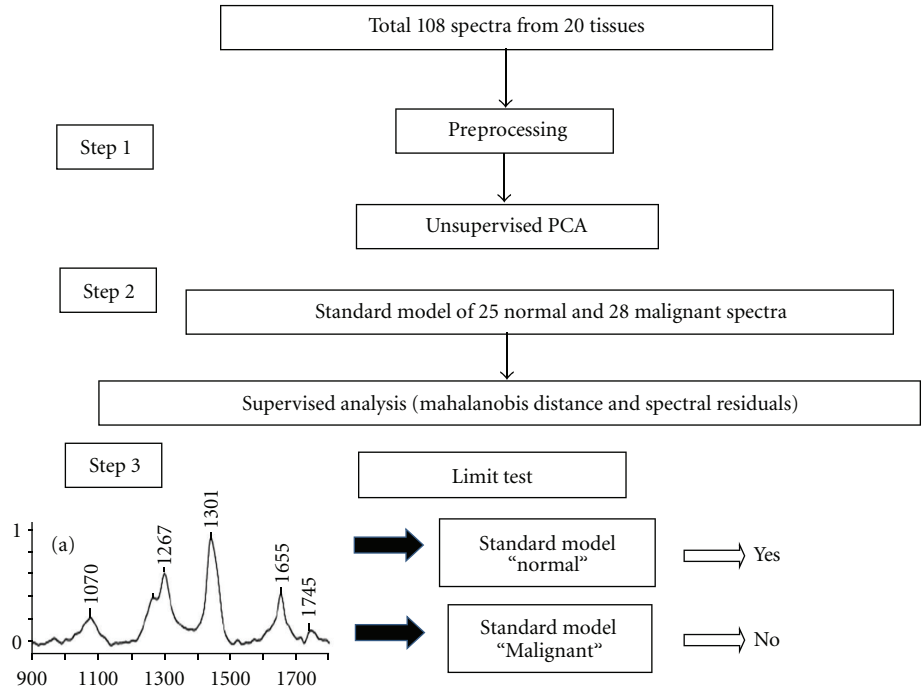

Figure 3: Flow chart of the study design.

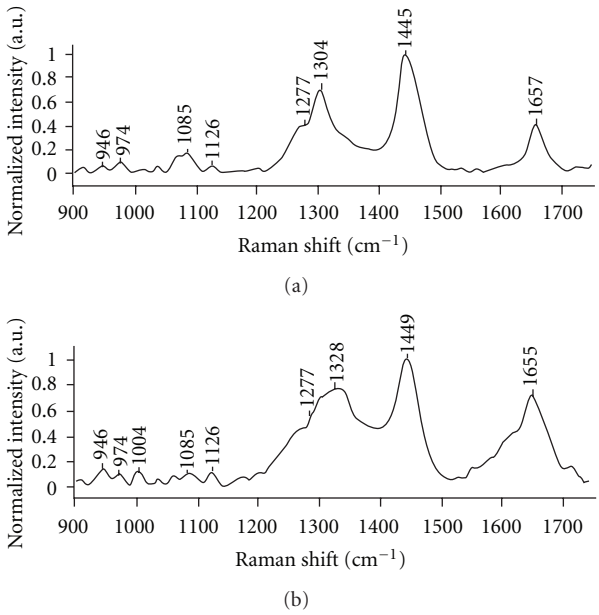

Figure 4: Mean spectra of (a) normal and (b) malignant hypopharyngeal tissues.

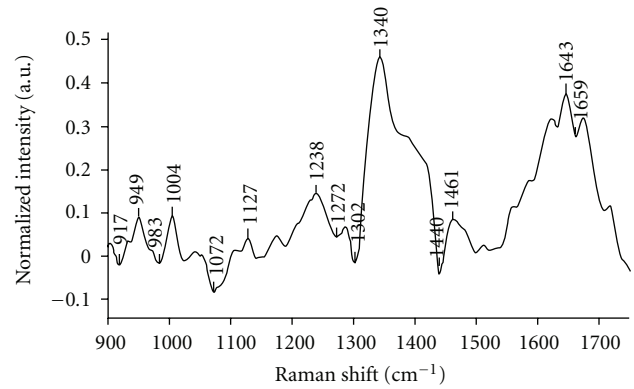

Figure 5: Difference spectrum of mean malignant minus mean normal spectrum.

from the mean. With n samples, each having p data points, we thus get an [n × p] matrix of these variations. Because all the samples contain more or less the same components (e.g., lipids, proteins, and collagen) the large amount of data can be represented by a much smaller set of components and their contributions to each spectrum depending on their concentrations. In matrix language this implies that the [n × p] matrix of variations discussed above is highly redundant. It will have only a few eigenvectors (principal components), and the eigenvalues of these will rapidly come down to almost zero after the first few. Solving the eigen value-eigen vector problem will give us the principal components (factors), % variance (contribution of the factors to the variations in the data set), and scores of factors for each sample. The scores for a given sample correspond to the contribution of each principal component to the variation of that sample from the mean. It is therefore possible to simulate the observed spectrum of any sample by multiplying the eigen vectors with their respective scores for that sample and adding these products to the mean of the data set.

As described above, in PCA large amount of spectral data is expressed by independent variables called eigenvectors, factors, or principal components and their scaling constants,

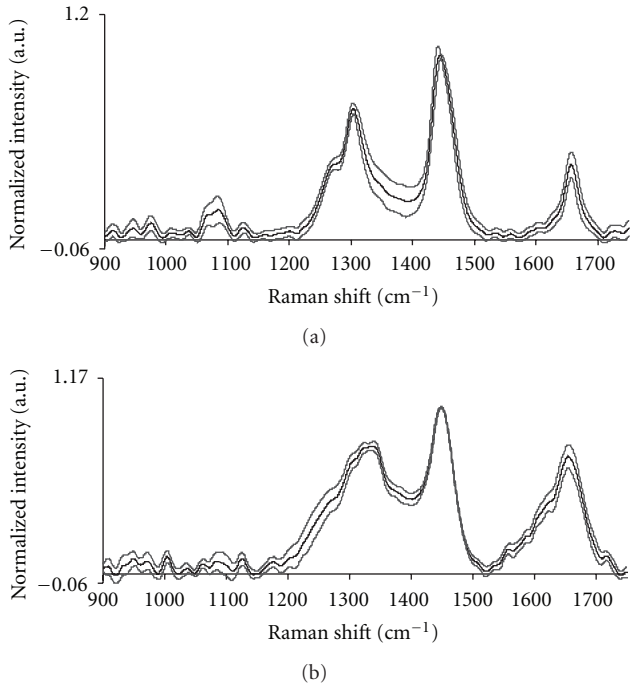

Figure 6: Mean and standard deviation spectra of hypopharyngeal tissues. (a) Normal. (b) Malignant.

scores. Scores of factors are often used as parameters to achieve objective discrimination. As mentioned earlier in Section 4, analysis was carried out in two different approaches: (1) unsupervised analysis, (2) supervised analysis. We have successfully tested these approaches in our earlier Raman spectroscopic studies of cervix, oral, and breast cancers [14, 15, 20, 22, 24, 31, 33].

In the first approach a total of 108 spectra from normal and malignant hypopharyngeal tissue were ascertained for unsupervised classification. Profiles of the factor loadings are shown in Figure 7. The first five factors contribute 94% of variance, and the last two account for noise. There is clustering of normal and malignant spectra based on score of factor 1, as shown in Figure 8. The scores of factor 1 for normal spectra were generally positive for malignant and negative for normal tissues with a mean standard deviation of 0.011 ± 0.05 and -0.06 ± 0.03, respectively. Mean and standard deviation values of normal and malignant spectra of score of factor 2 were 0.02 ± 0.05 and -0.01 ± 0.12. A minor overlap is present between clusters up to ± 1 standard deviation, which indicates a sensitivity and specificity of 75%.

Analysis by score of factors may give a clear classification of tissues for discrimination; however this approach of classification is somewhat cumbersome and tedious because diagnosis of a sample needs entire analysis to be repeated along with new spectra. Moreover, it may be of limited practical utility for the end-users, clinicians, since a visual decision-making is involved in the case of borderline samples. In view of these considerations, we have developed a second method using multiple discriminating parameters to give a better and objective diagnosis. For this, like in any analytical technique where standards with calibration curves are used for routine analysis, spectra of a set of clinically/pathologically diagnosed samples can be used as a *standard calibration set*. This standard calibration set can be subjected to PCA to derive parameters which will be highly characteristic for any sample of that type. Any *test sample* can then be included in the set, and the corresponding parameters for the test sample can be compared to the mean parameters for the set to decide whether the test sample belongs to that set and, if so, with what statistical probability. We have thus several statistical parameters available for decision-making in PCA, especially when standard calibration sets are used. In this mode besides scores of factor, PCA provides other discriminating parameters of classification such as Mahalanobis distance [35, 36] (a measure of proximity of two spectra) and spectral residuals (squared error sum of difference between recorded and simulated spectrum). Hence the supervised mode which provides multiple discriminating parameters is better suited for objective diagnosis by spectroscopy methods, especially for clinical conditions. In this analysis certain certified samples were used to develop standard sets. A given spectra was compared with these sets to decide whether it belongs to the standard set with the statistical probability of inclusion. If Mahalanobis distance of the test spectra has values more than three, compared to the training sets it had a probability of 0.5% or less of being grouped as the same class. The Mahalanobis distance [35] is normally expressed in units of standard deviation and expressed as

$$D^2 = (S_{\text{test}})M^{-1}(S_{\text{test}})'. \quad (1)$$

In the previous equation, S_{test} is the vector of the scores and the sum of squared spectral residuals for a given test sample, where

$$M = S'S/(n-1). \quad (2)$$

"S" contains the corresponding parameters for the calibration set (n standards).

In our study, we have selected 25 normal and 28 malignant spectra randomly based on a score of factor 1 and histopathological certification. The consistency of the standard sets was verified by rotating spectra from training sets and comparing them against both training sets. The spectra corresponding to same class of training sets procure lower Mahalanobis distance and spectral residues and vice versa. As an example, results obtained against a malignant training set were shown in Figure 9(a). The mean Mahalanobis distance of normal and malignant spectra were 15.1 ± 8.13 and 0.93 ± 0.61, respectively. The mean spectral residual values for normal and malignant tissues were 48.11 ± 24.23 and 3.52 ± 2.99, respectively.

These standard sets were further evaluated by spectra that were not involved in training sets wherein the test spectra were compared against both the training sets. A good discrimination was achieved, for example, as shown in Figure 9(b) of results obtained against the normal training set. The mean Mahalanobis distance of normal and malig-

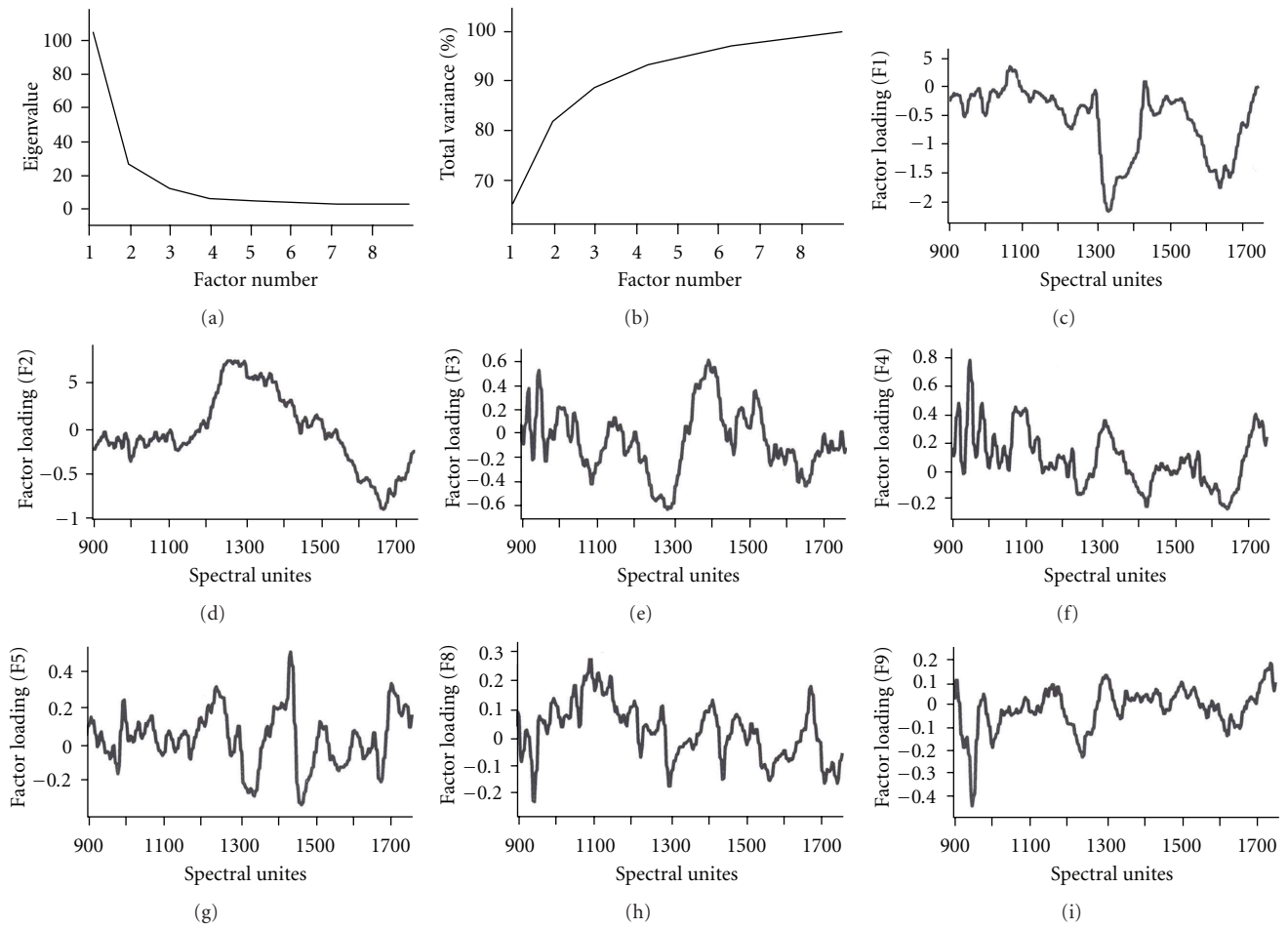

Figure 7: PCA of normal and malignant larynx tissue (a) eigen value, (b) total % variance, (c) loadings of factor 1, (d) loadings of factor 2, (e) loadings of factor 3, (f) loadings of factor 4, (g) loadings of factor 5, (h) loadings of factor 8, (I) loadings of factor 9.

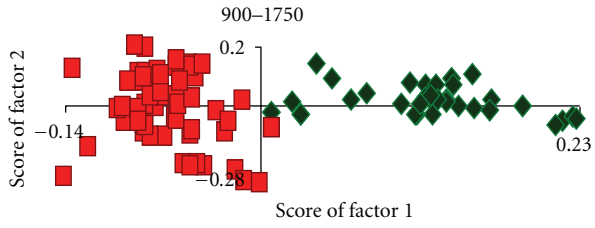

Figure 8: Unsupervised analysis of hyphoharyngeal spectra. ◊ Normal. ■ Malignant.

nant were 3.37 ± 2.47 and 32.05 ± 12.8, respectively.

The approach of computing mahalanobis distance and spectral residuals is further extended to multiparametric "limit test" approach in order to achieve objective and unambiguous discrimination. This is a typical match/mismatch approach against a standard set. A given spectra was compared with fixed values of inclusion/exclusion criteria for analysis of Mahalanobis distance, spectral residuals, and scores of factors. Based on these values of a given spectrum being within or without the set limits, the spectrum was

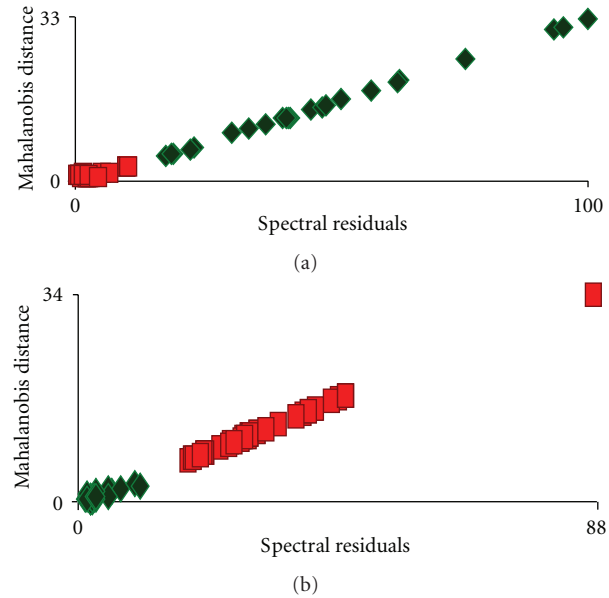

Figure 9: Supervised analysis of hyphoharyngeal tissue spectra. ◊ Normal. ■ Malignant. (a) Verification of the standard sets against malignant standard set. (b) Evaluation of the standard sets against normal standard set.

Table 1: Limit test approach against normal standard set (1–48 normal, 49–108 malignant).

Sample Number	Match	Limit test
1	POSSIBLE	PASS (PP?#)
2	POSSIBLE	PASS (PP?#)
3	YES	PASS (PPP#)
4	YES	PASS (PPP#)
5	YES	PASS (PPP#)
6	POSSIBLE	PASS (PP?#)
7	YES	PASS (PPP#)
8	YES	PASS (PPP#)
9	YES	PASS (PPP#)
10	YES	PASS (PPP#)
11	YES	PASS (PPP#)
12	YES	PASS (PPP#)
13	POSSIBLE	PASS (PP?#)
14	POSSIBLE	PASS (PP?#)
15	YES	PASS (PPP#)
16	YES	PASS (PPP#)
17	YES	PASS (PPP#)
18	POSSIBLE	PASS (PP?#)
19	YES	PASS (PPP#)
20	YES	PASS (PPP#)
21	YES	PASS (PPP#)
22	YES	PASS (PPP#)
23	POSSIBLE	PASS (PP?#)
24	YES	PASS (PPP#)
25	POSSIBLE	PASS (PP?#)
26	YES	PASS (PPP#)
27	POSSIBLE	PASS (PP?#)
28	YES	PASS (PPP#)
29	POSSIBLE	PASS (PP?#)
30	YES	PASS (PPP#)
31	YES	PASS (PPP#)
32	POSSIBLE	PASS (PP?#)
33	YES	PASS (PPP#)
34	YES	PASS (PPP#)
35	YES	PASS (PPP#)
36	YES	PASS (PPP#)
37	YES	PASS (PPP#)
38	YES	PASS (PPP#)
39	POSSIBLE	PASS (PP?#)
40	POSSIBLE	PASS (PP?#)
41	YES	PASS (PPP#)
42	POSSIBLE	PASS (PP?#)
43	YES	PASS (PPP#)
44	YES	PASS (PPP#)
45	YES	PASS (PPP#)
46	YES	PASS (PPP#)
47	POSSIBLE	PASS (PP?#)
48	POSSIBLE	PASS (PP?#)

Table 1: Continued.

Sample Number	Match	Limit test
49	NO	FAIL (FFF#)
50	NO	FAIL (FFF#)
51	NO	FAIL (FFF#)
52	NO	FAIL (FFF#)
53	NO	FAIL (FFF#)
54	NO	FAIL (FFF#)
55	NO	FAIL (FFF#)
56	NO	FAIL (FFF#)
57	NO	FAIL (FFF#)
58	NO	FAIL (FFF#)
59	NO	FAIL (FFF#)
60	NO	FAIL (FFF#)
61	NO	FAIL (FFF#)
62	NO	FAIL (FFF#)
63	NO	FAIL (FFF#)
64	NO	FAIL (FFF#)
65	NO	FAIL (FFF#)
66	NO	FAIL (FFF#)
67	NO	FAIL (FFF#)
68	NO	FAIL (FFF#)
69	NO	FAIL (FFF#)
70	NO	FAIL (FFF#)
71	NO	FAIL (FFF#)
72	NO	FAIL (FFF#)
73	NO	FAIL (FFF#)
74	NO	FAIL (F?F#)
75	NO	FAIL (FFF#)
76	NO	FAIL (FFF#)
77	NO	FAIL (FFF#)
78	NO	FAIL (FFF#)
79	NO	FAIL (FFF#)
80	NO	FAIL (PFF#)
81	NO	FAIL (PFF#)
82	NO	FAIL (FFF#)
83	NO	FAIL (FFF#)
84	NO	FAIL (FFF#)
85	NO	FAIL (FFF#)
86	NO	FAIL (FFF#)
87	NO	FAIL (F?F#)
88	NO	FAIL (FFF#)
89	NO	FAIL (FFF#)
90	NO	FAIL (FFF#)
91	NO	FAIL (P?F#)
92	NO	FAIL (P?F#)
93	NO	FAIL (FFF#)
94	NO	FAIL (FFF#)
95	NO	FAIL (FFF#)
96	NO	FAIL (FFF#)
97	NO	FAIL (FFF#)

Table 1: Continued.

Sample Number	Match	Limit test
98	NO	FAIL (F?F#)
99	NO	FAIL (FFF#)
100	NO	FAIL (FFF#)
101	NO	FAIL (FFF#)
102	NO	FAIL (FFF#)
103	NO	FAIL (FFF#)
104	NO	FAIL (FFF#)
105	NO	FAIL (FFF#)
106	NO	FAIL (FFF#)
107	NO	FAIL (PFF#)
108	NO	FAIL (FFF#)

labeled as "Yes/possible/pass (match)" or "No/fail (mismatch)" respectively. In this analysis, as an example, a normal spectrum should show "Yes/possible/pass" when compared to a normal standard set and "No/fail" with other standard sets and vice versa. Since the spectra are matched against all the standard sets, a reasonable and objective discrimination is achieved before concluding the type of the tissue. All malignant and nonmalignant spectra show "match" and "no match", respectively, when compared with a malignant standard set (Table 1). In this table, spectra 1–48 were normal tissue spectra, and spectra 49–108 were of malignant tissues. Efficacy of this approach was demonstrated in our earlier Raman studies of oral, breast, cervix, stomach, and colon cancers [14, 15, 20, 22, 24, 31, 33].

The results obtained in this pilot study provide reliable evidence on Raman spectroscopic discrimination of malignant hypopharyngeal tissues from normal. The limit test approach is significant in early clinical diagnosis as a clinician or technician can match a recorded spectrum with the training sets once they are developed for different pathological conditions aiding easy objective decisions, which is the ground stone for attempting curative treatment plan.

The future lies in designing a fibre probe tissue interface obtaining calibrated intensity information and depth ranging information. Raman probes may be designed to eliminate scattering distortion while providing the endoscopic images of the chemical and/or morphological properties of the tissue to complement tissue diagnosis on immediate basis during surgery or a diagnostic procedure.

6. Conclusion

Tobacco chewing and smoking is rampant and hazardous in an already rapidly increasing population. This doubles the need and effort to make early, easy, and immediate detection of malignant changes of the abused and vulnerable hypopharyngeal tissues. Though there are various methods to detect cancerous tissue, each has a drawback that may be overcome by expanded study of an alternative modality of tissue diagnosis such as conventional Raman spectroscopy. Spectral signatures were characterized by variations in the protein and lipid content at biomolecular level. Discriminating parameters scores of factor, Mahalanobis distance, spectral residuals provided clear classification between normal and malignant tissue types. Further the "limit test" approach also provided unambiguous and objective discrimination, which is more user-friendly and adaptable to routine clinical practice as it requires a minimally trained person and even a clinician and technician can come to a conclusion before taking a decision.

However, a confirmed application of Raman spectroscopy technique will come to force following prolonged prospective study and introducing endoscopy friendly Raman probes.

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Review Article

Current Concepts on Dedifferentiation/High-Grade Transformation in Salivary Gland Tumors

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The concept of dedifferentiation had previously been used in salivary gland carcinomas. Recently, the term "high-grade transformation" was introduced for adenoid cystic carcinoma, acinic cell carcinoma, epithelial-myoepithelial carcinoma, and polymorphous low-grade adenocarcinoma and may better reflect this phenomenon, although transformation into moderately differentiated adenocarcinoma (i.e., not "high grade") has also been described. Among the immunohistochemical markers, Ki-67 seems to be the only one that can help distinguish between the conventional and transformed components; however, the combination of morphological criteria is still sovereign. The overexpression of p53 was observed in the transformed component in all tumor types studied, despite few cases having been demonstrated to carry mutations or deletions in TP53 gene. Genetic studies in salivary gland tumors with dedifferentiation/high-grade transformation are rare and deserve further investigation. This paper aims at providing an overview on the recent concepts in histopathological classification of salivary gland tumors, complemented by immunohistochemical and genetic findings.

1. Introduction

The concept of dedifferentiation was first proposed by Dahlin and Beabout in 1971 [1], when they described dedifferentiated chondrosarcoma as a distinct clinicopathologic entity characterized by a low-grade chondrosarcoma juxtaposed to a histologically different high-grade sarcoma [1]. Lately, dedifferentiation has been recognized in a variety of salivary gland carcinomas, including adenoid cystic carcinoma [2], mucoepidermoid carcinoma [3], myoepithelial carcinoma [4], epithelial-myoepithelial carcinoma [5], and acinic cell carcinoma [6].

Dedifferentiation is the progression of cells towards a less differentiated state in which the original line of differentiation is no longer evident [7]. The term dedifferentiation might not be properly used in epithelial tumors, especially when the dedifferentiated component is still recognizable as carcinoma or adenocarcinoma [8]. Recently, Seethala et al. introduced the term "high-grade transformation" for adenoid cystic carcinomas. This term better reflects the fact that the dedifferentiated component often maintains some features of the original tumor, such as glandular differentiation [8–10]. In recent studies, our group and others have demonstrated that adenoid cystic carcinomas can also undergo transformation to adenocarcinomas which are not poorly differentiated, suggesting that also the term "high-grade transformation" may not be adequate, at least in the case of adenoid cystic carcinoma [11, 12].

Although considerable progress has been made in elucidating the genetic events that underlie the progression of many malignancies, those involved in salivary gland tumors are still poorly understood and the relationship between histological progression and genetic events is not well defined. The general theory of monoclonal evolution assumes that the mutational complexity of a tumor increases with time and, therefore, tumor genomes with the fewest chromosome aberrations contained the earliest mutations in tumor progression [13, 14]. In contrast, high-resolution comparative genomic hybridization (CGH) microarrays have been used to study the genome structure of heterogeneous breast

tumors and shown that they progress by different genomic rearrangement patterns [15]. Thus, the genomic heterogeneity can be ascribed to genetically distinct subpopulations, which contain a set of common mutations (early events) that are inherited and persistent throughout their evolution, while events unique to the profiles are late [16]. However, genetic alterations cannot solely explain the histological heterogeneity in tumors. Epigenetic alterations, such as DNA hypomethylation in tumor cells, cause chromatin decondensation and chromosomal rearrangements that may result in chromosomal instability. Moreover, DNA hypermethylation of CpG islands near the promoter regions silences specific genes including tumor suppressor genes in cooperation with histone modification [17]. Therefore, the histological heterogeneity could also involve modifications of epigenetic switch.

This paper will give an overview on the recent concepts in histopathological classification of salivary gland tumors in which dedifferentiation/transformation has been described: adenoid cystic carcinoma, acinic cell carcinoma, epithelial-myoepithelial carcinoma, polymorphous low-grade adenocarcinoma, mucoepidermoid carcinoma, and myoepithelial carcinoma. Below follows a one-by-one description of these tumors, and emphasis will be placed on immunohistochemical and genetic findings.

2. Clinical and Morphological Features

Since dedifferentiation/transformation is extremely rare, there are few data to establish how the prognosis compares to that of their conventional counterparts. Several clinical features have proven to be relevant, such as lymph node metastasis in adenoid cystic carcinoma and acinic cell carcinoma [8, 9], recurrence in polymorphous low-grade adenocarcinoma and mucoepidermoid carcinoma [3, 18], and metastasis in epithelial-myoepithelial carcinoma [10].

The majority of *adenoid cystic carcinoma (AdCC)* with transformation occurs during the sixth decade or later and most commonly involves the sinonasal mucoserous glands, palate, and submandibular glands. This tumor shows a slight male predominance, unlike conventional AdCC, and is often detected at an advanced stage due to extraglandular or bone involvement. One of the most important clinical features is the high propensity for lymph node metastasis (57% versus 5–25% in conventional AdCC), suggesting that this tumor should be also placed in the high-risk category for neck dissection [8, 19]. Until the time of writing, the literature revealed a total of 36 cases [2, 11, 12, 20–28]. The median survival of the largest reported series of AdCC with transformation, in which all cases were poorly differentiated carcinomas, was estimated at 12 months [8]. However, AdCC with transformation into moderately differentiated adenocarcinoma seems to present a slower course (in some cases comparable to conventional AdCC), in contrast to AdCC with transformation into poorly differentiated carcinoma which usually shows a more aggressive clinical course [11, 12]. Seethala et al. were the first to establish morphological criteria for differentiating AdCC with high-grade transformation [8]. At least three major criteria are required, proliferation of tumor cells with at least a focal loss of myoepithelial cells surrounding tumor nests, nuclear size at least 2-3 times the size of tubular/cribriform AdCC nuclei, thickened irregular nuclear membranes, and prominent nucleoli in a majority of cells. The squamous areas, micropapillary, and the loss of myoepithelial differentiation are considered unique morphological findings in the area transformed [8]. Based on the degree of gland formation (differentiation), cellular pleomorphism, and mitotic activity, Bonfitto et al. and Costa et al. classified the transformed components into moderately differentiated adenocarcinomas (when at least 2/3 of the transformed component presented gland formation) and poorly differentiated carcinomas (gland formation was scarce or absent) [11, 12]. These morphological features are observed in Figure 1. The literature does not suggest a minimum percentage of the transformed component [8].

The first *acinic cell carcinoma (AcCC)* with dedifferentiation/high-grade transformation of salivary gland was reported by Stanley et al., in 1988 [6]. Thirty-five cases have been described in the literature [6, 9, 29–41] and most of them showed poor clinical outcome, but in 3 cases it was described to remain unclear [29, 42, 43]. All cases reported to date were of parotid gland origin with involvement of both the superficial or deep lobes. Especially those AcCCs in the deep parotid lobe have been associated with a poor clinical outcome [35]. The median age of 58 years is higher than that reported for conventional AcCC, 44 years [34]. In contrast with its conventional counterpart, AcCC dedifferentiation/high-grade transformation shows a slight male predominance, high recurrence rate, and high propensity for cervical lymph node metastasis, suggesting a role for neck dissection in management of patients [9, 35].

Dedifferentiated AcCC (Figure 2) generally shows conventional low-grade AcCC juxtaposed with high-grade carcinoma, which may be either poorly differentiated adenocarcinoma or undifferentiated carcinoma. Both solid and microcystic patterns of AcCC have been described in the low-grade component. The high-grade component generally shows a population of anaplastic cells with abundant cytoplasm, large polymorphic nuclei, and loss of acinar differentiation. Furthermore, comedonecrosis and vascular and perineural invasion are typically observed in AcCC [6, 9, 35, 36, 38].

Epithelial-myoepithelial carcinoma (EMC) is a biphasic tubular neoplasm of clear myoepithelial cells surrounding small lumina lined by ductal epithelial cells. Typically, this is a low-grade malignancy that mainly occurs in the parotid gland and exhibits distinctive subtypes including tubular, papillary, cystic, and solid patterns [44]. In EMC, myoepithelial as well as ductal epithelial cells can transform into a high-grade carcinomatous component [10] which has gone under a variety of terminologies such as high-grade carcinoma [45, 46], dedifferentiated [5, 47–50], myoepithelial anaplasia [48], and myoepithelial carcinoma arising in EMC [2]. Recently the term "EMC with high-grade transformation" has been proposed for the all lesions where a more aggressive carcinoma is observed regardless whether it originated as a gradual transition or an abrupt transformation of the ductal or myoepithelial component [10]. The reasons for the adoption of this term were the difficulties in defining criteria for the cellular classification of the high-grade component,

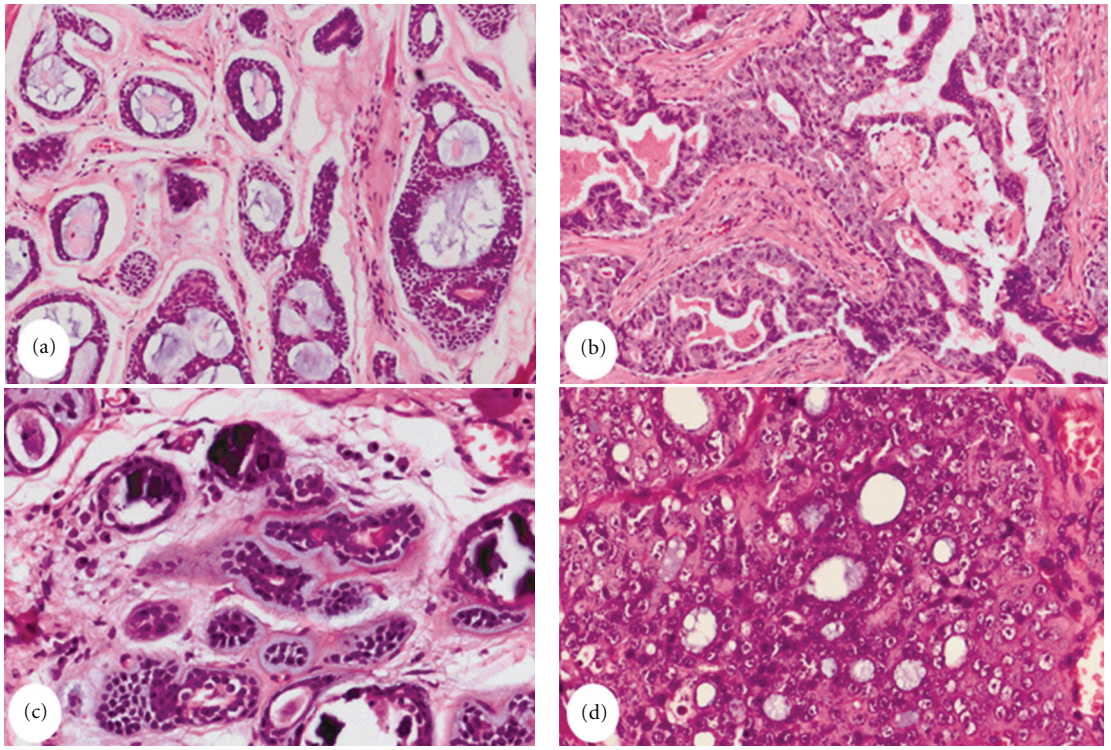

Figure 1: Adenoid cystic carcinoma with transformation to a moderately differentiated adenocarcinoma (a) and (b) and to poorly differentiated carcinoma (c) and (d). (a) and (b) H&E original magnification 200x and (c) and (d), 400x.

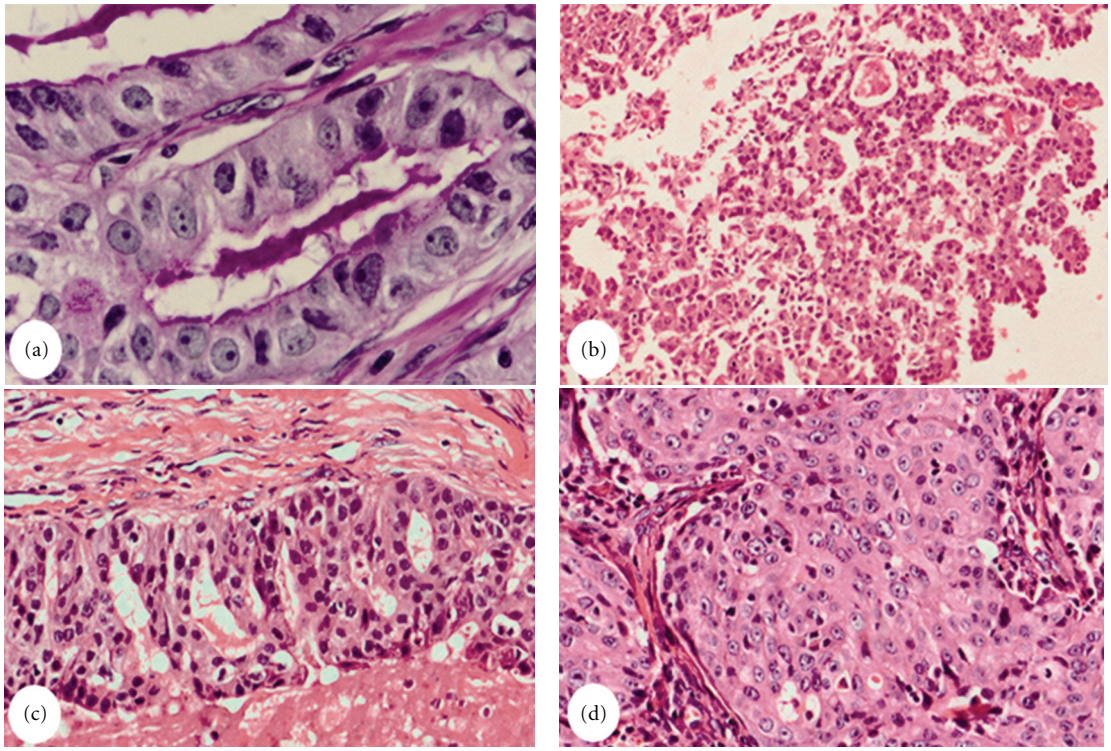

Figure 2: Acinic cell carcinoma with high-grade transformation to undifferentiated carcinoma. (a) and (b); (c) Conventional component; (d) high-grade transformed component. (a) PAS original magnification 1000x; (b) H&E original magnification 100x; (c) and (d), H&E original magnification 400x.

the possibility that some tumors may have features of both cell types, and the fact that these lesions uniformly show worse prognosis than typical EMC.

A common feature in many of transformed EMC also is a history of indolent growth prior to the development of transformation [50]. Patients with EMC containing high-grade transformation were older than conventional EMC patients (mean 75.9 years) and most commonly involved the parotid glands, with frequent extraglandular extension. Of all 17 cases reported in the literature, 61.5% were female versus 38.5% males. They appeared more aggressive than conventional EMC, mainly due to high propensity for lymph node and distant metastasis, prominent infiltrative growth pattern, and higher proliferative activity. However, little follow-up information is available, with a mean of 27.6 months (range: 3 to 72 months). The worse prognosis suggests the need for wider excision, neck dissection, and adjuvant radiotherapy [5, 10, 45–50].

Five cases of *polymorphous low-grade adenocarcinoma (PLGA)* with transformation to high-grade carcinoma described in the literature showed significant similarities in the morphology and in the origin, minor salivary gland of palate. These cases underwent transformation to poorly differentiated adenocarcinomas characterized by a predominantly solid and cystic growth pattern, nuclear atypia with prominent nucleoli and foci of necrosis [18, 51, 52] Lloreta et al. described one more case originating in the nasal cavity and adjacent sinuses with extensive areas of undifferentiated carcinoma consisting of compact epithelial cell nests with central necrosis [53]. The histological transformation seems to have occurred after a protracted clinical course with multiple recurrences, a late phenomenon in tumor progression [18, 52]. The possible role of radiation therapy as an initiator of this transformation may have been important in the three of the five published cases of transformation in PLGA [51, 52]. Despite the fact that high-grade transformation is recognized as an event with a more aggressive clinical course, only one case of PLGA in the literature died in consequence of the disease [53] and none showed metastasis thereafter [18, 51, 52].

The only two cases reported of dedifferentiated *mucoepidermoid carcinoma (MEC)* in the literature both presented a biphasic histology comprising a high-grade component and a low grade component separated by a transition zone [3, 54]. The high-grade component exhibited solid nests and sheet-like growth patterns, without glandular or cystic structures. Sheets of undifferentiated anaplastic or sarcomatoid growth with marked pleomorphism, frequent mitoses (>50%), and extensive necrosis were also observed (Figure 3) [3, 54].

The first case described by Nagao et al. concerned a 55-year-old man with a parotid gland tumor. Despite two recurrences within a short period after surgery, the patient remained alive during the next 10 years [3]. The second case of an 11-year-old girl located in trachea, conversely, rapidly metastasized to pleura, mediastinal lymph nodes, abdominal wall and vertebral bones leading to death in <3 months from diagnosis [54]. With few cases in the literature, it is difficult to establish clinical correlations in relation to the conventional MEC, but dedifferentiated MEC seems to reach the same broad age range as the conventional counterpart.

One case of dedifferentiated *myoepithelial carcinoma (MCa)* was described by Ogawa et al. in 2003 [4]. Two histologically distinct neoplastic cell populations were observed in the multinodular tumor of parotid gland. The first population was diagnosed as low-grade MCa and occupied more than 80% of the tumor. The second population consisted of polygonal or short spindle cells with pleomorphism as well as infiltration and high mitotic rate suggesting undifferentiated carcinoma. Moreover, these tumor cells lost the immunohistochemical characteristics of myoepithelial differentiation. A 59-year-old man presented first recurrence in the primary site after 5 months and a second one was observed 4 months later, although radiation therapy was used. The patient is alive and metastasis was not recognized [4].

3. Immunohistochemical Profile

3.1. Myoepithelial Markers. Myoepithelial cells exhibit dual epithelial and smooth muscle characteristics and traditionally are stained with antibodies against myoid proteins, such as α-smooth muscle actin (α-SMA), muscle-specific actin (HHF35), vimentin, or calponin. Recently, p63 has become a popular marker for abluminal cells (basal cells and myoepithelial cells) [55–57]. In tumors with myoepithelial component, such as AdCC and EMC, the participation of myoepithelial cells in the dedifferentiated/high-grade transformed areas seems to differ markedly. In AdCC, the loss of myoepithelial component has been used as one of the major criteria to identify the transformed areas [8]. Thus the expression of p63, α-SMA, and calponin should be absent or at least focal in the high-grade component [8]. In contrast, in EMC the myoepithelial participation appears to be important in the high-grade component [10], although the cells show a heterogeneous expression of the myoepithelial markers (Table 1). S-100 protein and p63 have been found to be diffusely or focally positive in the transformed component in many dedifferentiated/high-grade transformed EMC, whereas α-SMA was rarely detected and calponin negative [5, 10, 45, 46, 50]. It should be emphasized that neoplastic myoepithelial cells present a great plasticity in terms of immunoprofile and in EMC, even the conventional tumors show differences regarding the expression of myoepithelial markers [48]. In these, p63 expression is more frequently encountered than SMA.

3.2. Other Markers. The immunoprofile of the dedifferentiation/high-grade transformation in salivary gland tumors has been evaluated in many studies (Table 1), however, with variable and therefore inconclusive results, possibly due to the small number of cases. The marker that best distinguished between the transformed and the conventional components was Ki-67, since, in all salivary gland tumors studied, an increased proliferation index was detected in the transformed component when compared to the conventional counterpart [2, 3, 5, 8, 9, 11, 12, 18, 21, 22, 25, 27, 28, 36, 45, 48, 49, 54]. However, a distinct cut-off for the proliferation index that could identify the transformed component has

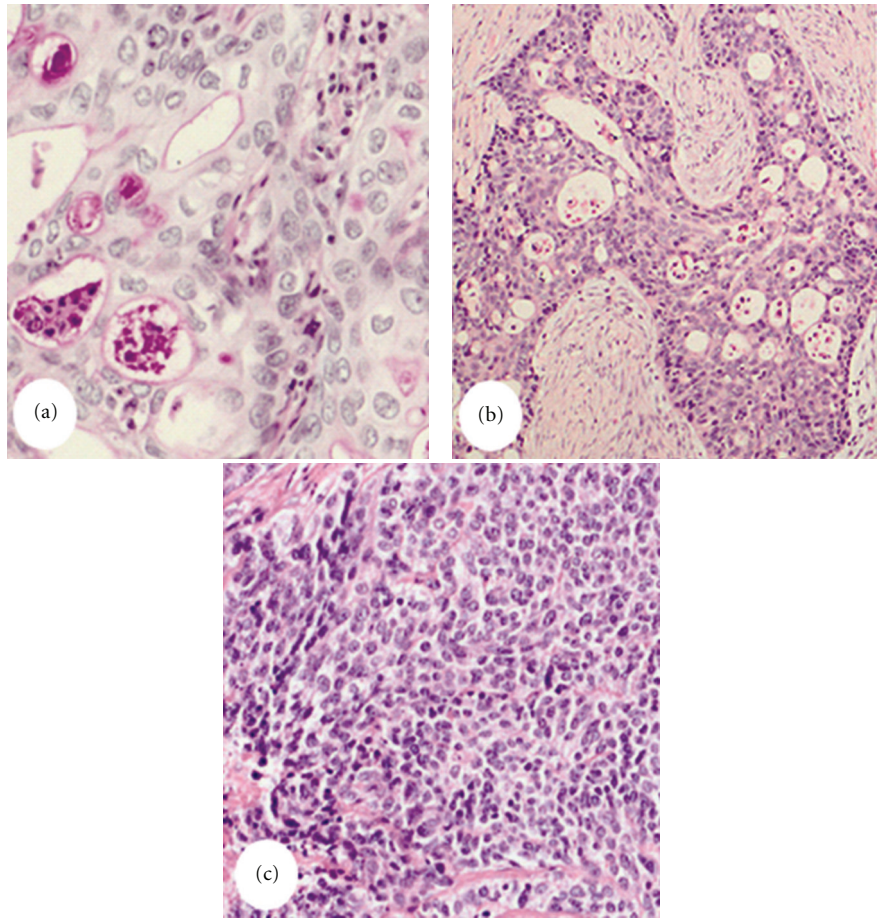

Figure 3: Mucoepidermoid carcinoma with dedifferentiation to undifferentiated carcinoma. (a) and (b), Conventional component; (c) dedifferentiated component. (a) PAS original magnification 400x; (b) H&E original magnification 100x; (c) H&E original magnification 200x.

yet to be established and probably it is variable among the different types of tumors. The expression of p53 in the transformed areas showed, in most of the cases and in most of the salivary tumor types, higher levels than in the conventional areas. Nevertheless, Di Palma et al. (1999) and Henley et al. (1997) showed negative expression of this protein in the dedifferentiated/high-grade component of AcCC [35, 36]. Fonseca et al. and Sarode et al. also showed lack of specificity to p53 in both components of their cases of EMC [49, 50]. These inconsistent results suggest that TP53 alteration is not the only mechanism for transformation in salivary gland tumors but may indicate a poor prognosis, similar to what is known for conventional AdCC [58]. Cyclin D1 is an important regulator of the G1 phase of cell cycle [59]. Positive expression was observed in dedifferentiated/transformed and conventional area of AdCC, AcCC, and EMC [5, 8, 9, 21, 25, 28]. However, in the MEC, Subramaniam et al. did not find positive expression in the dedifferentiated area [54]. The precise mechanisms responsible for the observed cyclin D1 overexpression in dedifferentiated/high-grade transformed salivary gland tumors (Table 1) are not fully established; a role in dedifferentiation of AdCC has been suggested in early studies [2, 21]. Gene amplification of cyclin D1 might contribute, as has been described in conventional AdCC [60].

Many other markers such as b-catenin [9], E-cadherin [27], pRB [28], BCL2 [18], and glucose transporters (GLUT) have been studied in individual tumors and deserve to be analyzed in other salivary gland tumors with dedifferentiation/high-grade transformation. GLUT1 has been considered a key molecule regulating the transport and metabolism of glucose. Overexpression of GLUT1 has been correlated with poor prognosis, tumor aggressiveness, and lymph node metastases [61]. Bonfitto et al. showed increased expression of GLUT1 in the transformed area when compared to conventional area of AdCC, suggesting a change in metabolic state of cancer cells imposing an increased utilization of energy. However, the authors did not find any correlation between GLUT1 expression and clinical outcome [11]. In summary, the immunohistochemical differences between the conventional and transformed areas require further studies.

4. Molecular Profile

Studies on the genetic changes in salivary gland tumors with dedifferentiation/high-grade transformation are rare. Only

Table 1: Immunoprofile of dedifferentiated/high-grade transformed areas in salivary gland tumors.

Antibodies	Immunoprofile of high-grade transformed areas					
	AdCC	AcCC	EMC	PLGA	MEC	MCa
Proliferative antigen						
Ki-67	HI	HI	HI	HI	HI	HI
Cytokeratins						
AE1-AE3	+	+/*F*	+	+	+/−	*F*
CAM 5.2	+	NA	+/*F*	+	−	NA
34bE12	+	NA	*F*	NA	NA	NA
CK 5/6	NA	−	+	NA	NA	NA
CK 7	NA	−	+/−	+	NA	NA
CK 14	−/*F*	−	−/*F*	NA	NA	NA
CK 20	NA	−	NA	−	NA	NA
Myoepithelial cell						
S-100	+/*F*/−	+/−	+/*F*/−	+/−	−	−
α-SMA (alfa-smooth muscle actin)	−/*F*	−	−/*F*	−	−	−
p63	−/*F*	NA	+/*F*/−	+/−	NA	NA
Vimentin	NA	+	+/−	+/−	NA	+
Calponin	*F*	−	−	−/*F*	NA	NA
HHF35 (muscle-specific actin)	−/*F*	NA	−	NA	NA	NA
Desmin	NA	NA	−	−	NA	NA
Cell cycle control						
p53	HI/+	HI/−	+/−	HI	HI/*F*	+
Cyclin D1	+/*F*	HI	+	NA	NA	+
Membrane receptors						
C-kit (CD117)	+/−	−	NA	NA	−	NA
HER2/neu (c-erbB2)	+/−	+	−	+	−	NA
EMA (epithelial membrane antigen)	+	NA	+	+	+	NA
Structural proteins						
GFAP (glial fibrillary acidic protein)	−/*F*	NA	−	+/−	−	NA
Cell Adhesion proteins						
CEA (carcinoembryonic antigen)	−/*F*	NA	+	+/−	−	NA
Steroid receptor						
Androgen receptor	−	−	NA	+/*F*	NA	NA

+: positive expression; −: negative expression; *F*: focal expression; NA: not available; HI: higher index than in conventional area; AdCC: adenoid cystic carcinoma; AcCC: acinic cell carcinoma; EMC: epithelial-myoepithelial carcinoma; PLGA: polymorphous low-grade adenocarcinoma; MEC: mucoepidermoid carcinoma; MCa: myoepithelial carcinoma.

few cases have been demonstrated to carry mutations or deletions in TP53 gene by loss of heterozygosity (LOH), polymerase chain reaction (PCR), and microarray comparative genomic hybridization (CGH) [9, 12, 21, 28, 35, 36]. However, p53 positive immunostaining is often indicative of mutations in TP53 and was observed in the transformed component in all tumor types studied [2–5, 8, 9, 11, 12, 18, 21, 22, 25, 27, 28, 54]. Therefore, the evidence suggests that p53 abnormalities may be implicated in the process of dedifferentiation, although its real importance in this process should be clarified by further molecularstudies.

Fluorescence in situ hybridization (FISH) analysis did not demonstrate gene amplification in the transformed area with mild overexpression of HER-2/neu protein in

acinic cell carcinoma [9]. DNA content has been studied only in dedifferentiated/high-grade transformed AcCC and EMC. Aneuploid AcCCs were associated with poor clinical outcome whereas no aneuploid tumor was found in the EMC group [31, 35, 36].

Using a high-resolution microarray CGH analysis in AdCC with high-grade transformation, a correlation between the number of chromosomal aberrations and the degree of gland differentiation of the transformed component was found. The AdCC with transformation in moderately differentiated carcinomas carried one single abnormality, whereas the AdCC with transformation in poorly differentiated carcinomas showed a higher number of alterations. These findings suggest that the AdCC with high-grade transformation may not necessarily reflect a more advanced stage of tumor progression, but rather a transformation to another histological form, which encompasses a wide spectrum of carcinomas in terms of aggressiveness [12]. A comparison of the microarray CGH results of the transformed and the conventional components in two cases of AdCC with high-grade transformation (unpublished data on cases in [12]) showed identical genetic profiles. A search in the literature shows that this finding is not unusual. Among others, examples are dedifferentiated liposarcomas and biphasic carcinosarcomas [62, 63]. This indicates that the genetic abnormalities have been acquired early in tumorigenesis, or at least before the start of the phenotypic change. It may be speculated that the genetic changes not detectable by microarray CGH analysis such as mutation or epigenetic silencing underlie the phenotypic change.

In conclusion, the dedifferentiation/high-grade transformation in salivary gland tumors seems to be a more complex process than simple progression through histological grades.

Currently, the most useful tool in identifying the transformed component is still a combination of morphological criteria aided by Ki67 expression analysis.

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