Pathology & Oncology Research (2019) 25:1047–1058
https://doi.org/10.1007/s12253-018-0462-0

ORIGINAL ARTICLE

Clinical and Molecular Characterization of Surgically Treated Oropharynx Squamous Cell Carcinoma Samples

Ana Carolina de Carvalho[1] · Matias Eliseo Melendez[1] · Cristina da Silva Sabato[3] · Edenir Inez Palmero[1,3] · Lidia Maria Rebolho Batista Arantes[1] · Cristovam Scapulatempo Neto[1,4] · André Lopes Carvalho[1,2]

Received: 4 January 2018 / Accepted: 7 August 2018 / Published online: 11 August 2018

Abstract

A better understanding of the clinical and molecular features of oropharyngeal squamous cell carcinomas (OPSCC) may help in the development of strategies for a better patient management, improving survival rates. This retrospective study conducted a clinical and molecular characterization of surgically treated OPSCC samples. Paraffin-embedded samples from a series of cases were screened for high-risk (HR) human papillomavirus (HPV) infection, methylation of a 5-gene panel, p53 expression, and *TP53* mutation. The study was conducted at Barretos Cancer Hospital. Twenty-five surgically treated OPSCC with available tissue were included in the study. Samples were classified according to HPV status and molecular features and some of these characteristics were associated to clinical data. Twenty percent of the cases were HR-HPV positive and 62.5% presented *TP53* mutations. *DAPK* hypermethylation was associated with HPV status ($p = 0.023$), while methylated *CCNA1* was inversely related to *TP53* mutations in primary tumors ($p = 0.042$) and associated with a better disease-free survival (22.3% vs. 100.0%; $p = 0.028$) and overall survival (8.0% vs. 100.0%; $p = 0.012$). The results show differences regarding molecular and clinical characteristics in the oropharynx cases identified that should be validated in more cases to confirm whether these differences are able to classify patients according to outcome and help in a more thorough patient management.

Keywords Oropharyngeal cancer · Human papillomavirus · Methylation · Mutation · Prognosis

Introduction

Tobacco smoking and alcohol consumption are traditionally the main risk factors for oral cavity and larynx tumors, while oncogenic human papillomavirus infections have been increasingly reported as an etiologic factor for tumors in the oropharynx [1–5]. Despite recent improvements in the epidemiology, pathogenesis and treatment modalities for oropharyngeal squamous cell carcinoma (OPSCC), survival rates are still unsatisfactory [6, 7].

Human papillomavirus-positive (HPV-positive) OPSCC are distinct from HPV-negative OPSCC in many settings [1, 8–14]. In short, these tumors mainly affect a younger population comprised by men, with lower rates of smoking and who commonly present early T stage with advanced nodal disease [1, 8, 9, 10–12, 15].

Traditional treatment for OPSCC tumors includes chemo and radiotherapy-based multimodality therapies [16]. Although HPV-positive patients usually present with advanced stage disease at diagnosis, their prognosis is more favorable regarding both overall and progression free survival [13, 17]. However, most of these studies involved patients undergoing radiotherapy (RT) or chemoradiotherapy (CRT), while few have addressed surgical therapy [10, 13, 18–21].

Currently efforts are being made to better characterize molecular alterations in OPSCC tumors associated with their clinical outcomes so that more tolerable and function-preserving therapies can be safely administrated to those cases

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12253-018-0462-0) contains supplementary material, which is available to authorized users.

✉ Ana Carolina de Carvalho
anacarol.oak@gmail.com

[1] Molecular Oncology Research Center, Barretos Cancer Hospital, Rua Antenor Duarte Vilela, 1331, Barretos, SP 14784-400, Brazil

[2] Department of Head and Neck Surgery, Barretos Cancer Hospital, Rua Antenor Duarte Vilela, 1331, Barretos, SP 14784-400, Brazil

[3] Center of Molecular Diagnosis, Rua Antenor Duarte Vilela, 1331, Barretos, SP 14784-400, Brazil

[4] Department of Pathology, Barretos Cancer Hospital, Rua Antenor Duarte Vilela, 1331, Barretos, SP 14784-400, Brazil

that harbor better-outcome characteristics. On the other hand, traditional intensive treatment can be correctly offered to patients that will more likely have a worse outcome. Therefore, this study aims at identifying molecular alterations that better characterize, together with clinical variables, OPSCC who underwent surgical resection, according to their outcome.

Materials and Methods

Study Population and DNA Extraction

This retrospective study included formalin-fixed paraffin-embedded (FFPE) oropharynx samples from 25 patients surgically treated between 2006 and 2012 at the Department of Head and Neck Surgery of the Barretos Cancer Hospital, Barretos, SP, Brazil. The inclusion criteria were as follows: previously untreated patients with primary OPSCC, submitted to surgery as the first therapeutic modality with curative intent. The use of these samples was approved by the Barretos Cancer Hospital Institutional Review Board. Hematoxylin & eosin sections corresponding to paraffin blocks containing the samples of interest were reviewed by an expert pathologist to confirm the diagnosis and for characterization of the cellular components present in the samples. Scrapings from the region of tissue identified as having at least 80% of tumor cells were processed using QIAamp DNA FFPE Tissue Kit (Qiagen, Germany). DNA was quantified in the Qubit fluorometer (Invitrogen, Carlsbad, CA) and stored at −20 °C until use.

p16 and p53 Immunoexpression

The status of p16 and p53 proteins expression was assessed by IHC using anti-p16 (prediluted, monoclonal mouse anti-human p16INK4A protein, Clone E6H4TM, ready for use, Ventana, Tucson, AZ, USA) and anti-p53 (monoclonal mouse anti-human p53 protein, Clone DO-7, dilution 1:1200, Cell Marque, Rocklin, CA, USA) antibodies in an automated system (Ventana Benchmark ULTRA, Tucson, AZ, USA). Briefly, 4 μm thick sections of formalin-fixed, paraffin-embedded tumor specimens were deparaffinized by heating (75 °C for 4 min). Antigen retrieval was achieved by use of cell conditioning buffer 1 (CC1) at 95 °C for 30 min. Incubation time for anti-p16 and anti-p53 antibody were respectively 20 and 32 min and the reactions were revealed using ultraView Universal DAB Detection Kit polymer amplification system (Ventana Medical Systems, Tucson, AZ, USA) according to manufacturer's instructions. A cervical adenocarcinoma was used as positive control for p16 staining and negative controls were obtained by omitting the primary antibodies. Samples with strong and diffuse nuclear and cytoplasmic in more than 75% of the tumor cells were considered positive for p16 [22, 23]. A colon carcinoma sample with a diffuse nuclear p53 staining was used as a positive control for p53 labelling whilst a breast carcinoma sample was used as a negative control. The slides were evaluated for nuclear expression, and samples with at least 10% of strong nuclear staining [24, 34] were considered positive for p53 expression. All scorings were conducted with no knowledge of clinical characteristics or outcome by a head and neck pathologist (CSN).

TP53 Sequencing

The analysis of *TP53* exons 4 to 9 was performed by PCR followed by direct Sanger sequencing. Briefly, using specific pairs of primers, the target regions were amplified by PCR with an initial denaturation at 96 °C for 15 min, followed by 35 cycles of 96 °C denaturation for 45 s, 60 °C for 45 s and 72 °C elongation for 45 s, and a final elongation at 72 °C for 10 min, in a Verity PCR machine (Applied Biosystems, Carlsbad, California, USA). Amplification of PCR products was confirmed by agarose gel electrophoresis. Sequencing PCR was performed using a Big Dye terminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems) and the ABI PRISM 3500 xL Genetic Analyzer (Applied Biosystems). Data were analysed using SeqScape software v2.7 (Applied Biosystems) and all nucleotide numbers refer to the wild-type genomic DNA sequence of the *TP53* gene as logged in NCBI (NM_000546.5). All *TP53* inactivating variants identified were classified according to their deleterious consequence and clinical effect using the IARC *TP53* database, CLinVar and COSMIC.

Target Gene Selection for Methylation Assays

A total of 5 genes were selected to test for methylation abnormalities. The panel included genes with tumor suppressor activities and reported as targets for epigenetic silencing in different human tumors, therefore, their silencing could contribute to the tumorigenesis process. Among these genes are *CCNA1* and *DAPK* which are involved in cell cycle control and apoptosis and *CDH8, PCDH10* and *TIMP3* which are involved in cell adhesion. Previous studies have shown that the expression of *CCNA1, DAPK,* and *TIMP3* may be affected by aberrant promoter methylation in different types of human malignancies. For *CDH8* and *PCDH10*, our group observed low expression of these genes in a head and neck cancer cell line and the restoration of gene expression upon treatment with 5-azacytidine (unpublished data).

Quantitative Methylation-Specific PCR

Sodium-bisulphite conversion of 1 μg of DNA was performed using the EpiTect Bisulphite Kit (Qiagen, Valencia, CA), following the manufacturer's recommendations. Briefly, incubation of the target DNA with sodium bisulphite resulted in

conversion of unmethylated cytosine residues into uracil, leaving the methylated cytosines unchanged. Converted DNA was used for quantitative methylation-specific PCR analyses (qMSP) as previously described [25] in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, California, USA). Primers and probes for methylated CCNA1, DAPK and TIMP3 were obtained from the literature. For CDH8 and PCDH10, assays were designed in frequently methylated CpG sites in the TCGA database. The gene ACTB was used as an internal control. Primer and probe sequences are provided in Supplementary Table S1. Samples were considered methylated when the amplification of at least two of the triplicates was detected. The percentage of methylation on each sample (PMR) was obtained by the equation: mean number of methylated copies of target gene/average number of copies of ACTB X 100. A cut-off value of ≥0.1% was used to score the samples as positive for all genes. Cut-off values were used to exclude very low-level background readings [26].

Statistical Analysis

Statistical analysis was performed using the software IBM SPSS Statistics 23 for Windows. Categorical variables were compared using Fisher's exact test. Survival curves were calculated by Kaplan-Meier method and differences between groups were compared using the log-rank test. Disease-free survival (DFS) was defined as the interval between the date of initial treatment and the diagnosis of recurrence (local, regional or distant), while the overall survival (OS) interval was defined as the interval between the date of initial treatment and the last follow up visit/information or death. For all analysis, we considered statistical significance when p value ≤0.05.

Results

Patient Characteristics and Clinical Predictors

Clinical and histopathological data of the 25 oropharyngeal cancer patients enrolled in this study are presented in Table 1. Most of the patients profiled in this cohort were male (88.0%) with age ranging from 42 to 77 years (median = 61 years). Tobacco and alcohol consumption was self-reported by 88.0 and 64.0% of the cases, respectively. The expression of p16 protein, commonly used as a surrogate marker for the infection of high-risk HPV, was detected in 5/25 samples (20.0%), which were then considered positive for HPV infection.

The majority of patients had advanced disease at diagnosis (80.0%). Tumor sites within the oropharynx were as follows: 36.0% in the tonsils and base of tongue and 28.0% in the soft palate. All patients underwent surgery as the primary modality of treatment and 84.0% of them underwent postoperative RT: 32.0% had positive surgical margins, 64.0% had compromised dissected lymph nodes and, of these, 62.5% had extracapsular spread.

Table 1 Clinical and pathological data of the patients enrolled in the study

Characteristic	Number of cases (%)
Total patients	25 (100)
Age	59, 61, 42–77 years
Mean, median, range	
< 60 years	12 (48)
> 60 years	13 (52)
Tobacco consumption	
No	3 (12)
Yes	22 (88)
Alcohol consumption	
No	8 (33)
Yes	16 (66)
HPV status	
Negative	20 (80)
Positive	5 (20)
Clinical TNM Stage	
Initial (I/II)	5 (20)
Advanced (III/IV)	20 (80)
Primary tumor site	
Tonsil	9 (36)
Base of tongue	9 (36)
Soft palate	7 (28)
Surgical margins	
Negative	16 (67)
Positive	8 (33)
Pathologic lymph node	
Negative	3 (16)
Positive	16 (84)
Recurrence	
Local	6 (24)
Neck	8 (32)
Distant	2 (8)

The median follow up period for these patients was 26.25 months (range: 1.38 to 72.89 months). Local recurrence occurred in 6 cases (24.0%), neck lymph node metastases occurred in 8 cases (32.0%), while 2 patients had distant metastases (8.0%).

Molecular Evaluation of Tumor Samples

A summary of the clinical and molecular data observed in these samples is presented in Supplementary Table 2.

Sixty-four percent of the samples had methylation of at least one of the genes evaluated. *CCNA1* was found methylated in 16.0% of the cases, *CDH8* in 10.0%, *DAPK* in 32.0%, *TIMP3* in 24.0% and *PCDH10* in 36.0% (Fig. 1).

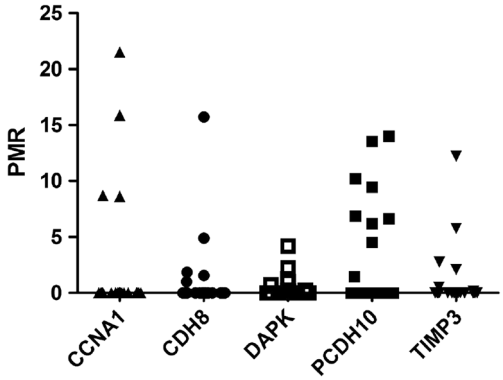

Fig. 1 Scatter plot of the percentage of methylated reference (PMR) of genes *CCNA1*, *CDH8*, *DAPK*, *PCDH10* and *TIMP3* in the samples analyzed in the study

The immunoexpression of p53 was evaluated and 64.0% (16/25) of the samples harbored strong expression of this protein. Regarding *TP53* mutation status, for 1 of the HPV-positive samples, it was not possible to get reliable results since the quality of the DNA was not appropriate. The sequencing results showed that 62.5% (15/24) of the samples had inactivating mutations of this gene. Interestingly, even though 100.0% of the HPV-positive samples were heavy-smokers, only 1 case presented inactivating *TP53* mutation; on the other hand, 13 out of 17 (76.5%) HPV-negative/heavy-smokers were mutated for *TP53*. Table 2 contains the clinically significant genetic variants found in the samples evaluated.

Molecular Profile and Clinical Data Associations

The association between clinical and molecular data was evaluated (Table 3). All patients with positive surgical margins had positive p53 expression ($p = 0.009$); and 90.9% of patients with clinically T1/T2 tumors had unmethylated *PCDH10* ($p = 0.033$). Moreover, all patients with extracapsular spread had unmethylated *CDH8* and *DAPK* ($p = 0.036$ and $p = 0.008$, respectively). The presence of *CCNA1* methylation showed an association with a higher disease-free survival (22.3% vs. 100%; $p = 0.028$; Fig. 2) and an increase in overall survival (8.0% vs. 100.0%; $p = 0.012$; Fig. 3).

Next, the association between molecular variables was evaluated (Table 4). When comparing the methylation data with the presence of mutation in *TP53*, 100.0% of the methylated samples for *CCNA1* and 71.4% of the samples methylated for *DAPK* were wild-type for changes in *TP53* ($p = 0.042$ and 0.061, respectively). *DAPK* methylation was also associated with HPV status: 80.0% of HPV-positive samples presented methylated *DAPK* ($p = 0.023$). Moreover, although not statistically significant, 70.0% of HPV-negative samples and only 25.0% of HPV-positive samples showed inactivating changes ($p = 0.130$). As expected, there was an association between p53 expression and the presence of *TP53* mutation (80.0% of patients with positive expression of the protein had *TP53* inactivating variants; $p = 0.036$).

Discussion

More than 400,000 cases of OPSCC are diagnosed each year worldwide [27], with a world incidence ranging from 7 to 17 cases per 100,000 individuals [27] which is rising in developed countries, particularly in young males [8].

Over the last 20 years, management of oropharyngeal cancer has changed dramatically [28]. Initially, treatment was based on surgery with or without radiation therapy or primary radiation therapy without neck dissection with cumulative 5-

Table 2 List and information about the clinically relevant *TP53* variants found in the samples evaluated this study

Genetic variant	Aminoacid change	Mutation type	ClinVar[a]	COSMIC[b]
c.659A > G	p.Tyr220Cys	Missense	Pathogenic	Pathogenic
c.742C > T	p.Arg248Trp	Missense	Pathogenic	Pathogenic
c.488A > C	p.Tyr163Ser	Missense	n/a	Pathogenic
c.451C > A	p.Pro151Thr	Missense	Pathogenic	Pathogenic
c.535C > T	p.His179Tyr	Missense	Pathogenic	Pathogenic
c.524G > A	p.Arg175His	Missense	Pathogenic	Pathogenic
c.559 + 1G > A	n/a	Intronic (affects splice site)	n/a	n/a
c.742C > T	p.Arg248Trp	Missense	Pathogenic	Pathogenic
c.748C > A	p.Pro250Thr	Missense	n/a	Pathogenic
c.743G > A	p.Arg248Gln	Missense	Pathogenic	Pathogenic
C.779C > T	p.Ser260Phe	Missense	n/a	Pathogenic
c.722C > T	p.Ser241Phe	Missense	Pathogenic	Pathogenic
c.748C > T	p.Pro250Ser	Missense	n/a	Pathogenic
c.839G > A	p.Arg280Lys	Missense	Pathogenic	Pathogenic
c.916C > T	p.Arg306Ter	Missense	Pathogenic	Pathogenic
c.832C > T	p.Pro278Se	Missense	n/a	Pathogenic

[a] ClinVar: www.ncbi.nlm.nih.gov/clinvar/

[b] COSMIC: http://cancer.sanger.ac.uk/cosmic

Table 3 Associations between clinical and molecular data of the 25 OPSCC patients enrolled in the study

	PCDH10 methylation			*TIMP3* methylation			*DAPK* methylation			*CDH8* methylation			*CCNA1* methylation
Characteristic	Negative	Positive	p	Negative	Positive	p	Negative	Positive	P	Negative	Positive	p	Negative
Clinical T stage													
T1/T2	10 (90.9)	1 (9.1)	0.033	9 (81.8)	2 (18.2)	0.661	5 (45.5)	6 (54.5)	0.081	10 (90.9)	1 (9.1)	0.341	8 (72.7)
T3/T4	6 (42.9)	8 (57.1)		10 (71.4)	4 (28.6)		12 (85.7)	2 (14.3)		10 (71.4)	4 (28.6)		13 (92.9)
Clinical N status													
Negative	8 (80.0)	2 (20.0)	0.229	9 (90.0)	1 (10.0)	0.345	6 (60.0)	4 (40.0)	0.667	9 (90.9)	1 (10.0)	0.615	9 (90.9)
Positive	8 (53.3)	7 (46.7)		10 (66.7)	5 (33.3)		11 (73.3)	4 (26.7)		11 (73.3)	4 (26.7)		12 (80.0)
Clinical TNM stage													
Initial (I/II)	5 (100.0)	0 (0.0)	0.123	5 (100.0)	0 (0.0)	1.000	2 (40.0)	3 (60.0)	0.283	5 (100.0)	0 (0.0)	0.544	4 (80.0)
Advanced (III/IV)	11 (55.0)	9 (45.0)		14 (70.0)	6 (30.0)		15 (75.0)	5 (25.0)		15 (75.0)	5 (25.0)		17 (85.0)
Surgical margins													
Negative	11 (68.8)	5 (31.3)	1.000	12 (75.0)	4 (25.0)	1.000	10 (62.5)	6 (37.5)	0.667	12 (75.0)	4 (25.0)	0.631	12 (75.0)
Positive	5 (62.5)	3 (37.5)		6 (75.0)	2 (25.0)		6 (75.0)	2 (25.0)		7 (87.5)	1 (12.5)		8 (100.0)
Pathologic lymph node													
Negative	2 (66.7)	1 (33.3)	1.000	3 (100.0)	0 (0.0)	0.530	2 (66.7)	1 (33.3)	0.530	3 (100.0)	0 (0.0)	1.000	3 (100.0)
Positive	8 (50.0)	8 (50.0)		11 (68.8)	5 (31.3)		13 (81.3)	3 (18.8)		12 (75.0)	4 (25.0)		14 (87.5)
Extracapsular spread													
Negative	2 (33.3)	4 (66.7)	0.608	3 (50.0)	3 (50.0)	0.299	3 (50.0)	3 (50.0)	0.036	2 (33.3)	4 (66.7)	0.008	4 (66.7)
Positive	6 (60.0)	4 (40.0)		8 (80.0)	2 (20.0)		10 (100.0)	0 (0.0)		10 (100.0)	0 (0.0)		10 (100.0)
Perineural infiltration													
Negative	9 (81.8)	2 (18.2)	0.516	9 (81.8)	2 (18.2)	0.516	6 (54.5)	5 (45.5)	0.231	8 (72.7)	3 (27.3)	0.516	8 (72.7)
Positive	2 (50.0)	2 (50.0)		2 (50.0)	2 (50.0)		4 (100.0)	0 (0.0)		4 (100.0)	0 (0.0)		4 (100.0)
Vascular embolization													
Negative	11 (78.6)	3 (21.4)	0.083	11 (78.6)	3 (21.4)	0.083	9 (64.3)	0 (0.0)	1.000	11 (78.6)	3 (21.4)	0.450	11 (78.6)
Positive	0 (0.0)	2 (100.0)		0 (0.0)	2 (100.0)		1 (50.0)	1 (50.0)		1 (50.0)	1 (50.0)		2 (100.0)

	CCNA1 methylation		*TP53* mutation			p53 expression			HPV status		
Characteristic	Positive	p	Negative	Positive	p	Negative	Positive	p	Negative	Positive	p
Clinical T stage											
T1/T2	3 (27.3)	0.288	5 (50.0)	5 (50.0)	0.403	4 (36.4)	7 (63.6)	1.000	7 (63.6)	4 (36.4)	0.133
T3/T4	1 (7.1)		4 (28.6)	10 (71.4)		5 (35.7)	9 (64.3)		13 (92.9)	1 (7.1)	
Clinical N status											
Negative	1 (10.0)	0.626	4 (40.0)	6 (60.0)	1.000	3 (30.0)	7 (70.0)	0.691	9 (90.0)	1 (10.0)	0.615
Positive	3 (20.0)		5 (35.7)	9 (64.3)		6 (40.0)	9 (60.0)		11 (73.3)	4 (26.7)	
Clinical TNM stage											
Initial (I/II)	1 (20.0)	1.000	2 (40.0)	3 (60.0)	1.000	2 (40.0)	3 (60.0)	1.000	4 (80.0)	1 (20.0)	1.000
Advanced (III/IV)	3 (15.0)		7 (36.8)	12 (63.2)		7 (35.0)	13 (65.0)		16 (80.0)	4 (20.0)	

Table 3 (continued)

Surgical margins									
Negative	4 (25.0)	0.262	8 (53.3)	7 (46.7)	0.086	9 (56.3)	7 (43.8)	0.009	13 (81.2)	3 (18.8)	1.000
Positive	0 (0.0)		1 (12.5)	7 (87.5)		0 (0.0)	8 (100.0)		6 (75.0)	2 (25.0)	
Pathologic lymph node											
Negative	0 (0.0)	1.000	1 (33.3)	2 (66.7)	1.000	1 (33.3)	2 (66.7)	1.000	2 (66.7)	1 (33.3)	0.530
Positive	2 (12.5)		6 (37.5)	10 (62.5)		6 (37.5)	10 (62.5)		13 (81.2)	3 (18.8)	
Extracapsular spread											
Negative	2 (33.3)	0.125	3 (50.0)	3 (50.0)	0.607	3 (50.0)	3 (50.0)	0.607	4 (66.7)	2 (33.3)	0.518
Positive	0 (0.0)		3 (30.0)	7 (70.0)		3 (30.0)	7 (70.0)		9 (90.0)	1 (10.0)	
Perineural infiltration											
Negative	3 (27.3)	0.516	6 (60.0)	4 (40.0)	0.559	4 (36.4)	7 (63.6)	1.000	7 (63.6)	4 (36.4)	0.516
Positive	0 (0.0)		1 (25.0)	3 (75.0)		1 (25.0)	3 (75.0)		4 (100.0)	0 (0.0)	
Vascular embolization											
Negative	3 (21.4)	1.000	7 (53.8)	4 (40.0)	0.467	5 (35.7)	9 (64.3)	1.000	10 (71.4)	4 (28.6)	1.000
Positive	0 (0.0)		0 (0.0)	2 (100.0)		0 (0.0)	2 (100.0)		1 (50.0)	1 (50.0)	

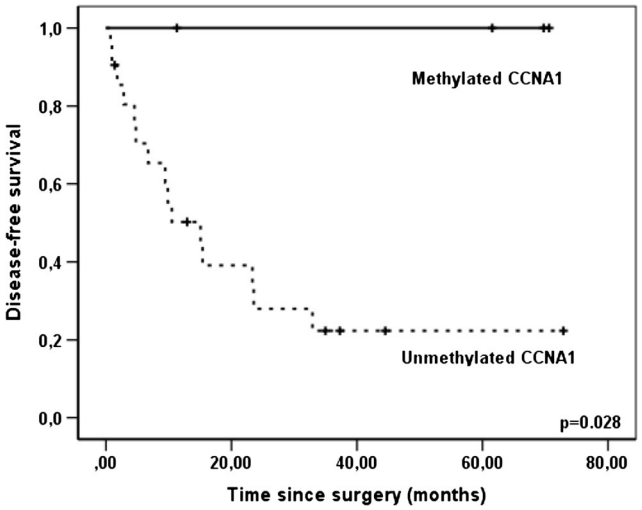

Fig. 2 Kaplan–Meier curve comparing the probability of disease-free survival in patients according to *CCNA1* methylation (5-years disease-free survival: 22.3% vs. 100%; $p = 0.028$)

year survival rates of 43–47% [29]. However, this approach was associated with a 23% rate of severe complication in the primary surgical group and only 6% in the primary radiation group, leading to the assumption that non-operative therapy was superior to operative therapy for OPSCC of all stages [28]. More recently a large meta-analysis comparing primary radiotherapy with chemoradiotherapy concluded an absolute survival benefit of 8.1% after 5 years in OPSCC patients treated with concurrent chemoradiotherapy [30].

Currently, the standard of care of OPSCC is multimodality therapy based on several factors, including clinical stage, individual patient factors such as comorbidities and preferences, and the institutional preference [31]. Data suggest that most institutions prefer organ-preservation protocols in advanced OPSCC

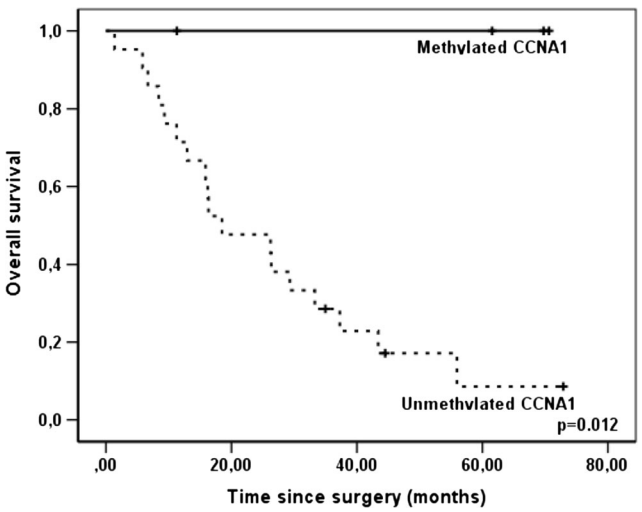

Fig. 3 Kaplan–Meier curve comparing the probability of overall survival in patients according to *CCNA1* methylation (5-years overall survival: 8% vs. 100%; = 0.012)

Table 4 Associations between molecular markers for the 25 OPSCC patients enrolled in the study

		HPV status		*CCNA1* methylation		*CDH8* methylation		*DAPK* methylation		*PCDH10* methylation		*TIMP3* methylation		*TP53* mutation	
		Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
CCNA1 methylation	Negative	17 (85.0)	3 (15.0)												
	Positive	4 (80.0)	1 (20.0)												
	p-value	1.000													
CDH8 methylation	Negative	17 (85.0)	3 (15.0)	17 (85.0)	3 (15.0)										
	Positive	3 (60.0)	2 (40.0)	4 (80.0)	1 (20.0)										
	p-value	0.252		1.000											
DAPK methylation	Negative	16 (80.0)	4 (20.0)	16 (94.1)	1 (5.9)	15 (88.2)	2 (11.8)								
	Positive	1 (20.0)	4 (80.0)	5 (62.5)	3 (37.5)	5 (62.5)	3 (37.5)								
	p-value	0.023		0.081		0.283									
PCDH10 methylation	Negative	13 (65.0)	7 (35.0)	13 (81.2)	3 (18.8)	14 (87.5)	2 (12.5)	10 (62.5)	6 (37.5)						
	Positive	3 (60.0)	2 (40.0)	8 (88.9)	1 (11.1)	6 (66.7)	3 (33.3)	7 (77.8)	2 (22.2)						
	p-value	1.000		1.000		0.312		0.661							
TIMP3 methylation	Negative	16 (80.0)	4 (20.0)	16 (84.2)	3 (15.8)	17 (89.5)	2 (10.5)	15 (78.9)	4 (21.1)	14 (73.7)	5 (26.3)				
	Positive	3 (60.0)	2 (40.0)	5 (83.3)	1 (16.7)	3 (50.0)	3 (50.0)	2 (33.3)	4 (66.7)	2 (33.3)	4 (66.7)				
	p-value	0.562		1.000		0.070		0.059		0.142					
TP53 mutation	Negative	6 (30.0)	14 (70.0)	6 (66.7)	3 (33.3)	6 (66.7)	3 (33.3)	4 (44.4)	5 (55.6)	6 (66.7)	3 (33.3)	6 (66.7)	3 (33.3)		
	Positive	3 (75.0)	1 (25.0)	15 (100.0)	0 (0.0)	13 (86.7)	2 (13.3)	13 (86.7)	2 (13.3)	9 (60.0)	6 (40.0)	12 (80.0)	3 (20.0)		
	p-value	0.130		**0.042**		0.326		0.061		1.000		0.635			
TP53 expression	Negative or weak	7 (77.8)	2 (22.2)	7 (77.8)	2 (22.2)	6 (66.7)	3 (33.3)	4 (44.4)	5 (55.6)	5 (55.6)	4 (44.4)	5 (55.6)	4 (44.4)	6 (66.7)	3 (33.3)
	Positive	13 (81.2)	3 (18.8)	14 (87.5)	2 (12.5)	14 (87.5)	2 (12.5)	13 (81.2)	3 (18.8)	11 (68,8)	5 (31.2)	14 (87.5)	2 (12.5)	3 (20.0)	12 (80.0)
	p-value	1.000		0.602		0.312		0.087		0.671		0.142		**0.036**	

Bold numbers refer to statistically significant p-values

patients, therefore, the majority of publications have focused on patients with OPSCC who received definitive RT [31].

The presence of HPV infection in OPSCC is a major determinant in prognosis. Generally, patients with HPV-positive OPSCC have a better outcome compared with HPV-negative patients [13, 32–34]. At the clinical level, Ang and collaborators were one of the first to show that tumor HPV status is a strong and independent prognostic factor for survival among patients with oropharyngeal cancer [13].

However, this favorable prognosis has been demonstrated in clinical studies that were focused on patients treated by primary radiotherapy (RT) or chemoradiotherapy (CRT). Lee et al., 2016 reported that HPV-positive OPSCC, treated with surgery followed by radiotherapy with or without chemotherapy, showed significantly better 5-year disease-free survival and overall survival than those with HPV-negative tumors. This could be understood in the same context of the previous studies showing an improvement in prognosis of HPV-positive patients treated with primary RT and CRT [13, 33, 35]. Therefore, HPV-positive OPSCC seems to be a distinct type of cancer with a generally better outcome, irrespective of the treatment modality chosen [31].

In our study, we could not observe a significant change in survival rates between HPV-positive and HPV-negative cases treated with surgery (DFS: 32.4% vs. 40.0%, $p = 0.555$; OS: 19.3% vs. 30.0%, $p = 0.330$). However, a positive association may have been hindered due to the small number of cases evaluated. Previous studies report that despite the overall good prognosis for HPV-positive OPSCC, some aggressive subtypes have been described, characterized by distant spread [36] and advanced nodal stage [37] which are associated with a poor outcome [31]. In this cohort of HPV-positive cases, 20.0% (1/5) of HPV-positive had distant metastases, in comparison to only 5.0% (1/20) of HPV-negative cases; and 80.0% (4/5) versus 55.0% (11/20) had positive dissected lymph nodes, respectively. Moreover, in spite of the very small number of positive cases for HPV (20.0%), all of these cases were heavy-tobacco smokers. A retrospective analysis by Ang et al., stratified 433 patients with OPSCC according to different risk-of-death and found that HPV-positive/smokers patients had intermediate survival rates in comparison to HPV-positive/non-smokers and HPV-negative/smokers [13]. Therefore, even though HPV is a very relevant prognostic factor in OPSCC, when heavy tobacco smoking is also present in this cohort, viral infection status has a smaller influence in prognosis and a subset of these patients seem to have an outcome more related to tobacco. Moreover, the results here presented regarding HPV-status and survival come from the evaluation of a small number of cases and cannot be expanded to the entire population of HPV-positive/smokers patients. More cases should be evaluated in order to allow a unbiased comparison to previous studies.

Recently, a few studies characterizing the spectrum of molecular alterations in head and neck tumors by whole-exome sequencing in a large number of samples were published, enabling a better understanding of the molecular alterations that play a role in the head and neck carcinogenesis [38–41]. A high level of intertumoral heterogeneity was observed and confirmed the biological complexity commonly associated with these tumors [42]. The favorable prognosis in HPV-positive OPSCC seems to be more related to the distinct molecular pathways implicated in HPV-associated HNSCC, characterized by the integration of DNA from high-risk HPV serotypes into the host genome, causing the constitutive expression of E6 and E7 viral oncogenes and promotion of DNA replication and degradation of p53 and pRb [42]. Moreover, HPV-driven tumors are more likely to harbor typically wild type *TP53* status and, as a consequence, more genetic stability. A common finding in HNSCC exome sequencing studies is that HPV-negative tumors have a higher mutation burden than HPV-positive. A study performed a comprehensive view of genetic alterations in 32 HNSCC and found obvious differences in the genetic landscapes of HPV-associated and HPV-negative head and neck squamous cell carcinomas (HNSCC), with far fewer genes mutated per tumor in the HPV-associated tumors regardless of smoking status. Moreover, *TP53* mutations were not found in any of the HPV-associated tumors, while present in 78% of HPV-negative ones [41]. In the same year, another study performed whole-exome sequencing in 74 tumor-normal pairs and reported again that the mutation rate of HPV-positive tumors was approximately half of that found in HPV-negative HNSCC and once more observed an inverse correlation between HPV status and *TP53* mutation [39]. On the other hand, The Cancer Genome Atlas Network published the results of the molecular profile of 279 HNSCC samples and, in contrast with previous reports, the mutation rates did not differ by HPV status. However, the different profile previously found was confirmed at the molecular level, with frequent helical domain mutations of the oncogene PIK3CA in HPV-positive cases and *TP53* mutations and *CDKN2A* inactivation in HPV negative cases comprehensive [43]. All these data suggest that in HPV-negative tumors, the absence of the oncogenic effect from HPV oncoproteins requires the accumulation of multiple genetic aberrations to allow malignant transformation [42].

In the present study, clinical features and molecular characteristics related to the methylation and mutation status of genes important in the carcinogenesis were evaluated in order to make possible the determination of a group of factors that help in the classification of a population of surgically treated OPSCC patients.

Cell-cycle is one of the key cellular processes that is commonly deregulated in cancer cells to overcome senescence and to obtain limitless replicative potential [44]. *TP53* is a well-established tumor suppressor gene involved in the regulation

of the cell cycle and that is targeted by mutations in HNSCC [45]. Somatic mutations in *TP53* are found in 60–80% of HNSCC cases and are usually associated to tobacco-driven tumors [46–48]. In the present study, 62.5% of the OPSCC samples evaluated harbored somatic *TP53* mutations and as expected, there was a good correlation between *TP53* mutations and p53 expression (80.0% of positive cases were also mutated for *TP53*). *TP53* mutations are uncommon (0%–3%) in HPV-positive primary HNSCC [13, 49–51]. Even though all HPV positive cases in this study were heavy-smokers, only 1 had mutated *TP53*, and other 2 cases harbored strong p53 expression. The patient with positive expression and mutated *TP53* (62-year old male), had a very poor outcome and died shortly after the treatment of a local recurrence (9 months overall survival). Leemans et al., hypothesized that *TP53* mutation in a subset of HPV-positive tumors may be associated with tobacco use, occur later in tumor evolution, and reflect poorer prognosis [45]. The patient with negative *TP53* mutation and positive expression (62-year old male) had a second primary tumor in the oral tongue (HPV-negative) and distant metastases in the lung. In this case, even though the HPV status was positive this tumor was very likely to be caused by tobacco smoking through a p53-independent pathway. For the last positive case for protein expression, the *TP53* mutational status could not be evaluated since the quality of the DNA was not appropriate, however, this 48-years old male was alive and disease-free at the last follow-up (60 months). Therefore, we could hypothesize that this is a truly HPV-induced tumor.

Additionally, when considering all patients, despite of HPV status, patients with positive surgical margins had positive p53 expression in the primary tumors. This finding agrees with previous studies that found *TP53*-mutated DNA and or p53 expression in tumor and surgical margins to be related to the presence of precursor lesions and locoregional treatment failure [47, 52, 53].

Among the common molecular alterations in the carcinogenesis of head and neck tumors, silencing of genes by methylation of their promoter region is a more frequent event than inactivation of genes by mutation. Protocadherins constitute the largest subgroup in the cadherin superfamily of cell adhesion molecules. *PCDH10* was found methylated and silenced in nasopharyngeal carcinoma and several other carcinomas, but not in normal tissues. The reversion of *PCDH10* transcriptional silencing suppressed tumor cell growth, migration, invasion and colony formation [54]. In the present study, the majority of patients with clinically T1/T2 tumors had unmethylated *PCDH10*, therefore agreeing with the reported results showing that the expression of this protein suppresses tumor growth.

Death-associated protein kinase 1 (DAPK-1) is a positive mediator of gamma-interferon induced programmed cell death [55]. Loss of its expression via promoter hypermethylation has been associated with the formation of metastases and advanced disease stages in multiple cancer types, including head and neck cancers [56]. *CDH8* codes for a type II classical cadherin from the cadherin superfamily, integral membrane proteins that mediate calcium-dependent cell-cell adhesion, no previous studies evaluated the role of this gene in head and neck carcinogenesis. Therefore, the finding that all patients with extracapsular spread had unmethylated *CDH8* and *DAPK* ($p = 0.036$ and $p = 0.008$, respectively) agrees with previous data on *DAPK* and also with the described function of *CDH8*.

Several articles have explored the association between HPV status in head and neck tumors and methylation status [57–59]. The analysis of the 25 cases of oropharyngeal tumors of this cohort, of which 20.0% are HPV-positive, showed a significant association between the presence of *DAPK* methylation and the status of HPV infection. A recent study evaluated the profile of gene-methylation of head and neck tumors and found a significant higher methylation of *DAPK*, among other genes, in HPV-positive cases compared to HPV-negative [60], however, this study only evaluated a few oropharynx cancer cases, no other study reports an association with specifically oropharyngeal tumor samples. Moreover, a trend toward association of this gene with wild-type *TP53* was observed, suggesting a relationship with HPV status, since HPV-positive samples often show wild-type *TP53*.

The presence of *CCNA1* gene methylation in the samples evaluated showed an statistically significant association with better DFS and OS, suggesting that this alteration seems to induce advantageous changes in the genomic safeguarding leading to a better prognosis in this group of patients. Kostareli and colleagues evaluated the protein expression of ccna1 in 81 HNSCC samples and found an association between the expression of this gene ($p < 0.001$), the presence of HPV16 ($p = 0.001$) and a worse recurrence-free survival ($p = 0.002$) [61]. Therefore, considering that the presence of *CCNA1* methylation leads to the silencing of this gene in HPV-positive samples, and Kostareli found that the presence of CCNA1 protein expression is related to a worse prognosis, our results are consistent. In another study, *CCNA1* methylation was present in 45.0% of the evaluated HNSCC samples, and showed an association with absence of mutation in *TP53* [62]. We also observed a significant association between methylated *CCNA1* and wild-type *TP53* suggesting that a better prognosis in patients with *CCNA1* methylation may be related to the presence of wild-type *TP53*, independent of HPV status and smoking.

Our study has several limitations, being the small population size the most remarkable one. Because there is a preference in this institution to treat oropharynx tumor patients with organ preservation protocols, we were only able to include a small number of cases, restricted to patients that had other comorbidities that discouraged the choice for chemoradiation

treatment and patients who preferred to undergo surgery. Therefore, studies evaluating surgically-treated oropharynx tumors cohorts tend to have smaller number of cases. The differential of our study is the fact that these rare samples were broadly evaluated at the molecular level through the assessment of several markers. Moreover, it is well-known that in Brazil the prevalence of HPV-positive cases among oropharynx cancer tumors is low [63, 64], therefore even though only 20.0% of the cases were positive, the cohort evaluated in this study was enriched for HPV-positive cases.

In conclusion, even though all samples analyzed were of the oropharynx, several differences regarding molecular and clinical characteristics were identified. This scenario raises questions about the influence of HPV and tobacco in these patients exposed to both etiological factors and about the tumor heterogeneity within the group of tumors comprised by the oropharynx. Knowing that some patients with HPV-positive OPSCC remain at risk of poor outcome, more cases should be evaluated in order to assess whether the differences identified are able to classify these patients according to their outcome and help in a more thorough patient management.

Acknowledgements This study was funded by São Paulo Research Foundation (FAPESP), grant # 2015/01286-0 and National Counsel of Technological and Scientific Development (CNPq), grant # 445.053/2014-3. ACC was recipient of scholarship from São Paulo Research Foundation (FAPESP). ALC has a National Counsel of Technological and Scientific Development (CNPq) scholarship.

References

1. Gillison ML, D'Souza G, Westra W, Sugar E, Xiao W, Begum S, Viscidi R (2008) Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. J Natl Cancer Inst 100(6):407–420. https://doi.org/10.1093/jnci/djn025
2. Marur S, D'Souza G, Westra WH, Forastiere AA (2010) HPV-associated head and neck cancer: a virus-related cancer epidemic. Lancet Oncol 11(8):781–789. https://doi.org/10.1016/S1470-2045(10)70017-6
3. Marklund L, Hammarstedt L (2011) Impact of HPV in oropharyngeal Cancer. J Oncol 2011:509036. https://doi.org/10.1155/2011/509036
4. Gillison ML, Chaturvedi AK, Anderson WF, Fakhry C (2015) Epidemiology of human papillomavirus-positive head and neck squamous cell carcinoma. J Clin Oncol Off J Am Soc Clin Oncol 33(29):3235–3242. https://doi.org/10.1200/JCO.2015.61.6995
5. Kreimer AR, Clifford GM, Boyle P, Franceschi S (2005) Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer Epidemiol Biomarkers Prev 14(2):467–475. https://doi.org/10.1158/1055-9965.EPI-04-0551
6. Zhen W, Karnell LH, Hoffman HT, Funk GF, Buatti JM, Menck HR (2004) The National Cancer Data Base report on squamous cell carcinoma of the base of tongue. Head Neck 26(8):660–674. https://doi.org/10.1002/hed.20064
7. Lybak S, Ljokjel B, Haave H, Karlsdottir A, Vintermyr OK, Aarstad HJ (2017) Primary surgery results in no survival benefit compared to primary radiation for oropharyngeal cancer patients stratified by high-risk human papilloma virus status. Eur Arch Otorhinolaryngol 274(1):477–487. https://doi.org/10.1007/s00405-016-4203-2
8. Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, Jiang B, Goodman MT, Sibug-Saber M, Cozen W, Liu L, Lynch CF, Wentzensen N, Jordan RC, Altekruse S, Anderson WF, Rosenberg PS, Gillison ML (2011) Human papillomavirus and rising oropharyngeal cancer incidence in the United States. J Clin Oncol Off J Am Soc Clin Oncol 29(32):4294–4301. https://doi.org/10.1200/JCO.2011.36.4596
9. Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, Zahurak ML, Daniel RW, Viglione M, Symer DE, Shah KV, Sidransky D (2000) Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst 92(9):709–720
10. Mellin H, Friesland S, Lewensohn R, Dalianis T, Munck-Wikland E (2000) Human papillomavirus (HPV) DNA in tonsillar cancer: clinical correlates, risk of relapse, and survival. Int J Cancer 89(3):300–304
11. Hafkamp HC, Manni JJ, Haesevoets A, Voogd AC, Schepers M, Bot FJ, Hopman AH, Ramaekers FC, Speel EJ (2008) Marked differences in survival rate between smokers and nonsmokers with HPV 16-associated tonsillar carcinomas. Int J Cancer 122(12):2656–2664. https://doi.org/10.1002/ijc.23458
12. Goldenberg D, Begum S, Westra WH, Khan Z, Sciubba J, Pai SI, Califano JA, Tufano RP, Koch WM (2008) Cystic lymph node metastasis in patients with head and neck cancer: an HPV-associated phenomenon. Head Neck 30(7):898–903. https://doi.org/10.1002/hed.20796
13. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, Westra WH, Chung CH, Jordan RC, Lu C, Kim H, Axelrod R, Silverman CC, Redmond KP, Gillison ML (2010) Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med 363(1):24–35. https://doi.org/10.1056/NEJMoa0912217
14. Gillison ML, Zhang Q, Jordan R, Xiao W, Westra WH, Trotti A, Spencer S, Harris J, Chung CH, Ang KK (2012) Tobacco smoking and increased risk of death and progression for patients with p16-positive and p16-negative oropharyngeal cancer. J Clin Oncol Off J Am Soc Clin Oncol 30(17):2102–2111. https://doi.org/10.1200/JCO.2011.38.4099
15. Deschler DG, Richmon JD, Khariwala SS, Ferris RL, Wang MB (2014) The "new" head and neck cancer patient-young, nonsmoker, nondrinker, and HPV positive: evaluation. Otolaryngol Head Neck Surg 151(3):375–380. https://doi.org/10.1177/0194599814538605
16. Dansky Ullmann C, Harlan LC, Shavers VL, Stevens JL (2012) A population-based study of therapy and survival for patients with head and neck cancer treated in the community. Cancer 118(18):4452–4461. https://doi.org/10.1002/cncr.27419
17. Huang SH, Xu W, Waldron J, Siu L, Shen X, Tong L, Ringash J, Bayley A, Kim J, Hope A, Cho J, Giuliani M, Hansen A, Irish J, Gilbert R, Gullane P, Perez-Ordonez B, Weinreb I, Liu FF, O'Sullivan B (2015) Refining American Joint Committee on Cancer/Union for International Cancer Control TNM stage and prognostic groups for human papillomavirus-related oropharyngeal carcinomas. J Clin Oncol 33(8):836–845. https://doi.org/10.1200/JCO.2014.58.6412
18. Lindel K, Beer KT, Laissue J, Greiner RH, Aebersold DM (2001) Human papillomavirus positive squamous cell carcinoma of the oropharynx: a radiosensitive subgroup of head and neck carcinoma. Cancer 92(4):805–813
19. Dahlstrand HM, Dalianis T (2005) Presence and influence of human papillomaviruses (HPV) in tonsillar cancer. Adv Cancer Res 93:59–89. https://doi.org/10.1016/S0065-230X(05)93002-9

20. Shimura E, Hama T, Suda T, Ikegami M, Urashima M, Kojima H (2017) The presence of HPV DNA in neck lymph node metastasis correlates with improved overall survival of patients with oropharyngeal cancer undergoing surgical treatment. Oncology 92(2):87–93. https://doi.org/10.1159/000452420
21. Weinberger PM, Yu Z, Haffty BG, Kowalski D, Harigopal M, Brandsma J, Sasaki C, Joe J, Camp RL, Rimm DL, Psyrri A (2006) Molecular classification identifies a subset of human papillomavirus–associated oropharyngeal cancers with favorable prognosis. J Clin Oncol Off J Am Soc Clin Oncol 24(5):736–747. https://doi.org/10.1200/JCO.2004.00.3335
22. Begum S, Gillison ML, Ansari-Lari MA, Shah K, Westra WH (2003) Detection of human papillomavirus in cervical lymph nodes: a highly effective strategy for localizing site of tumor origin. Clin Cancer Res 9(17):6469–6475
23. Singhi AD, Westra WH (2010) Comparison of human papillomavirus in situ hybridization and p16 immunohistochemistry in the detection of human papillomavirus-associated head and neck cancer based on a prospective clinical experience. Cancer 116(9):2166–2173. https://doi.org/10.1002/cncr.25033
24. Keswani RN, Noffsinger A, Waxman I, Bissonnette M (2006) Clinical use of p53 in Barrett's esophagus. Cancer Epidemiol Biomarkers Prev 15(7):1243–1249. https://doi.org/10.1158/1055-9965.EPI-06-0010
25. Rettori MM, de Carvalho AC, Bomfim Longo AL, de Oliveira CZ, Kowalski LP, Carvalho AL, Vettore AL (2013) Prognostic significance of TIMP3 hypermethylation in post-treatment salivary rinse from head and neck squamous cell carcinoma patients. Carcinogenesis 34(1):20–27. https://doi.org/10.1093/carcin/bgs311
26. Brabender J, Usadel H, Danenberg KD, Metzger R, Schneider PM, Lord RV, Wickramasinghe K, Lum CE, Park J, Salonga D, Singer J, Sidransky D, Holscher AH, Meltzer SJ, Danenberg PV (2001) Adenomatous polyposis coli gene promoter hypermethylation in non-small cell lung cancer is associated with survival. Oncogene 20(27):3528–3532. https://doi.org/10.1038/sj.onc.1204455
27. Chaturvedi AK, Anderson WF, Lortet-Tieulent J, Curado MP, Ferlay J, Franceschi S, Rosenberg PS, Bray F, Gillison ML (2013) Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. J Clin Oncol Off J Am Soc Clin Oncol 31(36):4550–4559. https://doi.org/10.1200/JCO.2013.50.3870
28. Howard J, Masterson L, Dwivedi RC, Riffat F, Benson R, Jefferies S, Jani P, Tysome JR, Nutting C (2016) Minimally invasive surgery versus radiotherapy/chemoradiotherapy for small-volume primary oropharyngeal carcinoma. Cochrane Database Syst Rev 12:CD010963. https://doi.org/10.1002/14651858.CD010963.pub2
29. Parsons JT, Mendenhall WM, Stringer SP, Amdur RJ, Hinerman RW, Villaret DB, Moore-Higgs GJ, Greene BD, Speer TW, Cassisi NJ, Million RR (2002) Squamous cell carcinoma of the oropharynx: surgery, radiation therapy, or both. Cancer 94(11):2967–2980. https://doi.org/10.1002/cncr.10567
30. Blanchard P, Hill C, Guihenneuc-Jouyaux C, Baey C, Bourhis J, Pignon JP (2011) Mixed treatment comparison meta-analysis of altered fractionated radiotherapy and chemotherapy in head and neck cancer. J Clin Epidemiol 64(9):985–992. https://doi.org/10.1016/j.jclinepi.2010.10.016
31. Lee J, Chang JS, Kwon HJ, Kim SH, Shin SJ, Keum KC (2016) Impact of p16 expression in oropharyngeal cancer in the postoperative setting: the necessity of re-evaluating traditional risk stratification. Jpn J Clin Oncol 46(10):911–918. https://doi.org/10.1093/jjco/hyw099
32. Dahlstrom KR, Calzada G, Hanby JD, Garden AS, Glisson BS, Li G, Roberts DB, Weber RS, Sturgis EM (2013) An evolution in demographics, treatment, and outcomes of oropharyngeal cancer at a major cancer center: a staging system in need of repair. Cancer 119(1):81–89. https://doi.org/10.1002/cncr.27727
33. D'Souza G, Kreimer AR, Viscidi R, Pawlita M, Fakhry C, Koch WM, Westra WH, Gillison ML (2007) Case-control study of human papillomavirus and oropharyngeal cancer. N Engl J Med 356(19):1944–1956. https://doi.org/10.1056/NEJMoa065497
34. O'Sullivan B, Huang SH, Siu LL, Waldron J, Zhao H, Perez-Ordonez B, Weinreb I, Kim J, Ringash J, Bayley A, Dawson LA, Hope A, Cho J, Irish J, Gilbert R, Gullane P, Hui A, Liu FF, Chen E, Xu W (2013) Deintensification candidate subgroups in human papillomavirus-related oropharyngeal cancer according to minimal risk of distant metastasis. J Clin Oncol Off J Am Soc Clin Oncol 31(5):543–550. https://doi.org/10.1200/JCO.2012.44.0164
35. Rischin D, Young RJ, Fisher R, Fox SB, Le QT, Peters LJ, Solomon B, Choi J, O'Sullivan B, Kenny LM, McArthur GA (2010) Prognostic significance of p16INK4A and human papillomavirus in patients with oropharyngeal cancer treated on TROG 02.02 phase III trial. J Clin Oncol Off J Am Soc Clin Oncol 28(27):4142–4148. https://doi.org/10.1200/JCO.2010.29.2904
36. Huang SH, Perez-Ordonez B, Weinreb I, Hope A, Massey C, Waldron JN, Kim J, Bayley AJ, Cummings B, Cho BC, Ringash J, Dawson LA, Siu LL, Chen E, Irish J, Gullane P, Hui A, Liu FF, Shen X, Xu W, O'Sullivan B (2013) Natural course of distant metastases following radiotherapy or chemoradiotherapy in HPV-related oropharyngeal cancer. Oral Oncol 49(1):79–85. https://doi.org/10.1016/j.oraloncology.2012.07.015
37. McIlwain WR, Sood AJ, Nguyen SA, Day TA (2014) Initial symptoms in patients with HPV-positive and HPV-negative oropharyngeal cancer. JAMA Otolaryngol Head Neck Surg 140(5):441–447. https://doi.org/10.1001/jamaoto.2014.141
38. Lui VW, Hedberg ML, Li H, Vangara BS, Pendleton K, Zeng Y, Lu Y, Zhang Q, Du Y, Gilbert BR, Freilino M, Sauerwein S, Peyser ND, Xiao D, Diergaarde B, Wang L, Chiosea S, Seethala R, Johnson JT, Kim S, Duvvuri U, Ferris RL, Romkes M, Nukui T, Kwok-Shing Ng P, Garraway LA, Hammerman PS, Mills GB, Grandis JR (2013) Frequent mutation of the PI3K pathway in head and neck cancer defines predictive biomarkers. Cancer Discov 3(7):761–769. https://doi.org/10.1158/2159-8290.CD-13-0103
39. Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, Kryukov GV, Lawrence MS, Sougnez C, McKenna A, Shefler E, Ramos AH, Stojanov P, Carter SL, Voet D, Cortes ML, Auclair D, Berger MF, Saksena G, Guiducci C, Onofrio RC, Parkin M, Romkes M, Weissfeld JL, Seethala RR, Wang L, Rangel-Escareno C, Fernandez-Lopez JC, Hidalgo-Miranda A, Melendez-Zajgla J, Winckler W, Ardlie K, Gabriel SB, Meyerson M, Lander ES, Getz G, Golub TR, Garraway LA, Grandis JR (2011) The mutational landscape of head and neck squamous cell carcinoma. Science 333(6046):1157–1160. https://doi.org/10.1126/science.1208130
40. Pickering CR, Zhang J, Yoo SY, Bengtsson L, Moorthy S, Neskey DM, Zhao M, Ortega Alves MV, Chang K, Drummond J, Cortez E, Xie TX, Zhang D, Chung W, Issa JP, Zweidler-McKay PA, Wu X, El-Naggar AK, Weinstein JN, Wang J, Muzny DM, Gibbs RA, Wheeler DA, Myers JN, Frederick MJ (2013) Integrative genomic characterization of oral squamous cell carcinoma identifies frequent somatic drivers. Cancer Discov 3(7):770–781. https://doi.org/10.1158/2159-8290.CD-12-0537
41. Agrawal N, Frederick MJ, Pickering CR, Bettegowda C, Chang K, Li RJ, Fakhry C, Xie TX, Zhang J, Wang J, Zhang N, El-Naggar AK, Jasser SA, Weinstein JN, Trevino L, Drummond JA, Muzny DM, Wu Y, Wood LD, Hruban RH, Westra WH, Koch WM, Califano JA, Gibbs RA, Sidransky D, Vogelstein B, Velculescu VE, Papadopoulos N, Wheeler DA, Kinzler KW, Myers JN (2011) Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. Science 333(6046):1154–1157. https://doi.org/10.1126/science.1206923
42. Mountzios G, Rampias T, Psyrri A (2014) The mutational spectrum of squamous-cell carcinoma of the head and neck: targetable

genetic events and clinical impact. Ann Oncol 25(10):1889–1900. https://doi.org/10.1093/annonc/mdu143
43. Cancer Genome Atlas Network (2015) Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature 517 (7536):576–582. https://doi.org/10.1038/nature14129
44. Dakubo GD, Jakupciak JP, Birch-Machin MA, Parr RL (2007) Clinical implications and utility of field cancerization. Cancer Cell Int 7:2. https://doi.org/10.1186/1475-2867-7-2
45. Leemans CR, Braakhuis BJ, Brakenhoff RH (2011) The molecular biology of head and neck cancer. Nat Rev Cancer 11(1):9–22. https://doi.org/10.1038/nrc2982
46. Poeta ML, Manola J, Goldwasser MA, Forastiere A, Benoit N, Califano JA, Ridge JA, Goodwin J, Kenady D, Saunders J, Westra W, Sidransky D, Koch WM (2007) TP53 mutations and survival in squamous-cell carcinoma of the head and neck. N Engl J Med 357(25):2552–2561. https://doi.org/10.1056/NEJMoa073770
47. van Houten VM, Tabor MP, van den Brekel MW, Kummer JA, Denkers F, Dijkstra J, Leemans R, van der Waal I, Snow GB, Brakenhoff RH (2002) Mutated p53 as a molecular marker for the diagnosis of head and neck cancer. J Pathol 198(4):476–486. https://doi.org/10.1002/path.1242
48. Balz V, Scheckenbach K, Gotte K, Bockmuhl U, Petersen I, Bier H (2003) Is the p53 inactivation frequency in squamous cell carcinomas of the head and neck underestimated? Analysis of p53 exons 2-11 and human papillomavirus 16/18 E6 transcripts in 123 unselected tumor specimens. Cancer Res 63(6):1188–1191
49. Kutler DI, Auerbach AD, Satagopan J, Giampietro PF, Batish SD, Huvos AG, Goberdhan A, Shah JP, Singh B (2003) High incidence of head and neck squamous cell carcinoma in patients with Fanconi anemia. Arch Otolaryngol Head Neck Surg 129(1):106–112
50. Hopkins J, Cescon DW, Tse D, Bradbury P, Xu W, Ma C, Wheatley-Price P, Waldron J, Goldstein D, Meyer F, Bairati I, Liu G (2008) Genetic polymorphisms and head and neck cancer outcomes: a review. Cancer Epidemiol Biomarkers Prev 17(3):490–499. https://doi.org/10.1158/1055-9965.EPI-07-2714
51. Smith RB, Sniezek JC, Weed DT, Wax MK (2007) Utilization of free tissue transfer in head and neck surgery. Otolaryngol Head Neck Surg 137(2):182–191. https://doi.org/10.1016/j.otohns.2007.04.011
52. Koch WM, Brennan JA, Zahurak M, Goodman SN, Westra WH, Schwab D, Yoo GH, Lee DJ, Forastiere AA, Sidransky D (1996) p53 mutation and locoregional treatment failure in head and neck squamous cell carcinoma. J Natl Cancer Inst 88(21):1580–1586
53. Graveland AP, Golusinski PJ, Buijze M, Douma R, Sons N, Kuik DJ, Bloemena E, Leemans CR, Brakenhoff RH, Braakhuis BJ (2011) Loss of heterozygosity at 9p and p53 immunopositivity in surgical margins predict local relapse in head and neck squamous cell carcinoma. Int J Cancer 128(8):1852–1859. https://doi.org/10.1002/ijc.25523
54. Ying J, Li H, Seng TJ, Langford C, Srivastava G, Tsao SW, Putti T, Murray P, Chan AT, Tao Q (2006) Functional epigenetics identifies a protocadherin PCDH10 as a candidate tumor suppressor for nasopharyngeal, esophageal and multiple other carcinomas with frequent methylation. Oncogene 25(7):1070–1080. https://doi.org/10.1038/sj.onc.1209154
55. Don KR, Ramani P, Ramshankar V, Sherlin HJ, Premkumar P, Natesan A (2014) Promoter hypermethylation patterns of P16, DAPK and MGMT in oral squamous cell carcinoma: a systematic review and meta-analysis. Indian J Dent Res 25(6):797–805. https://doi.org/10.4103/0970-9290.152208
56. Sanchez-Cespedes M, Esteller M, Wu L, Nawroz-Danish H, Yoo GH, Koch WM, Jen J, Herman JG, Sidransky D (2000) Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. Cancer Res 60(4):892–895
57. Noorlag R, van Kempen PM, Moelans CB, de Jong R, Blok LE, Koole R, Grolman W, van Diest PJ, van Es RJ, Willems SM (2014) Promoter hypermethylation using 24-gene array in early head and neck cancer: better outcome in oral than in oropharyngeal cancer. Epigenetics 9(9):1220–1227. https://doi.org/10.4161/epi.29785
58. van Kempen PM, Noorlag R, Braunius WW, Stegeman I, Willems SM, Grolman W (2014) Differences in methylation profiles between HPV-positive and HPV-negative oropharynx squamous cell carcinoma: a systematic review. Epigenetics 9(2):194–203. https://doi.org/10.4161/epi.26881
59. Sartor MA, Dolinoy DC, Jones TR, Colacino JA, Prince ME, Carey TE, Rozek LS (2011) Genome-wide methylation and expression differences in HPV(+) and HPV(−) squamous cell carcinoma cell lines are consistent with divergent mechanisms of carcinogenesis. Epigenetics 6(6):777–787
60. Choudhury JH, Ghosh SK (2015) Promoter hypermethylation profiling identifies subtypes of head and neck cancer with distinct viral, environmental, genetic and survival characteristics. PLoS One 10(6):e0129808. https://doi.org/10.1371/journal.pone.0129808
61. Kostareli E, Holzinger D, Bogatyrova O, Hielscher T, Wichmann G, Keck M, Lahrmann B, Grabe N, Flechtenmacher C, Schmidt CR, Seiwert T, Dyckhoff G, Dietz A, Hofler D, Pawlita M, Benner A, Bosch FX, Plinkert P, Plass C, Weichenhan D, Hess J (2013) HPV-related methylation signature predicts survival in oropharyngeal squamous cell carcinomas. J Clin Invest 123(6):2488–2501. https://doi.org/10.1172/JCI67010
62. Tokumaru Y, Yamashita K, Osada M, Nomoto S, Sun DI, Xiao Y, Hoque MO, Westra WH, Califano JA, Sidransky D (2004) Inverse correlation between cyclin A1 hypermethylation and p53 mutation in head and neck cancer identified by reversal of epigenetic silencing. Cancer Res 64(17):5982–5987. https://doi.org/10.1158/0008-5472.CAN-04-0993
63. Perdomo S, Martin Roa G, Brennan P, Forman D, Sierra MS (2016) Head and neck cancer burden and preventive measures in Central and South America. Cancer Epidemiol 44(Suppl 1):S43–S52. https://doi.org/10.1016/j.canep.2016.03.012
64. Lopez CR, Antoni MH, Pereira D, Seay J, Whitehead N, Potter J, O'Sullivan M, Fletcher MA (2013) Stress management, depression and immune status in lower income racial/ethnic minority women co-infected with HIV and HPV. J Appl Biobehav Res 18(1):37–57. https://doi.org/10.1111/jabr.12003