

Effects of tobacco abuse on major chromosomal instability in human papilloma virus 16-positive oropharyngeal squamous cell carcinoma

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Abstract. A substantial number of patients with oropharyngeal squamous cell carcinoma (OPSCC) have two oncogenic risk factors: Human papilloma virus (HPV) infection and tobacco use. These factors can be competitive or synergistic at the chromosomal and genomic levels, with strong prognostic and therapeutic implications. HPV16 has been shown *in vitro* to be a high-risk HPV that induces low rates of chromosomal copy number alterations. However, chromosomal instability can be increased by smoking. Evaluating chromosomal instability in HPV-positive patients according to their smoking status is therefore critical for assessing the prognosis and therapeutic

impact. The aim of this study was to assess chromosomal instability in patients with HPV-positive OPSCC according to smoking status. Chromosomal instability was investigated with array-based comparative genomic hybridization (aCGH) in 50 patients with OPSCC. Differences in chromosomal alterations were examined according to the HPV and smoking status of the patients. HPV-positive tumors (24/26 were HPV16-positive) had fewer genomic aberrations ($P=0.0082$) and fewer breakpoints ($P=0.048$) than HPV-negative tumors. We confirmed the association between HPV-positive OPSCC and chromosomal losses at 11q. We verified the association between HPV-negative OPSCC and losses at 3p and 9p and gains at 7q and 11q13. In the patients with OPSCC who were HPV-positive, the total number of chromosomal aberrations per tumor was significantly higher in the group of patients who were smokers ($P=0.003$). However, the cytobands did not differ significantly according to the smoking status. On the whole, the data of this study may help to improve the stratification of HPV-positive OPSCC patients and must be supplemented by next-generation sequencing studies in order to describe the mutational and transcriptomic profiles of such patients according to smoking status.

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Abbreviations: HPV, human papilloma virus; OPSCC, oropharyngeal squamous cell carcinoma; aCGH, array-based comparative genomic hybridization; HNSCC, head and neck squamous cell carcinoma; CNAs, copy number alterations; SNP, single nucleotide polymorphism; DNA, deoxyribonucleic acid; mRNA, messenger ribonucleic acid; FISH, fluorescence *in situ* hybridization; NHIS, National Health Interview Survey; AJCC, American Joint Committee on Cancer; UICC, Union for International Cancer Control; PCR, polymerase chain reaction; MCR, minimum common region; NKK, nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; 4-ABP, arylamine 4-aminobiphenyl; FA, Fanconi anemia; BRCA, breast cancer (gene); NGS, next-generation sequencing

Key words: oropharyngeal squamous cell carcinoma, HPV16, tobacco, aCGH, chromosomal instability

Introduction

In most of the world, high-risk human papilloma viruses (HPVs), particularly HPV16, are the cause of a rapidly growing subset of oropharyngeal squamous cell carcinomas (OPSCCs) mostly located in the tonsils and arising from the crypt epithelium of the palatine and lingual tonsils (1-3). These types of cancer differ markedly from those traditionally induced by tobacco and alcohol abuse, particularly at the biological level (4,5). Specifically, patients with HPV-positive head and neck squamous cell carcinoma (HNSCC) have a survival advantage over their HPV-negative counterparts (6-11). However, HPV-positive OPSCC exhibits significant geographical variations in the incidence rate among patients who are smokers, and the evaluation of the smoking status is not uniform among a series of

OPSCC patients (6-8,12,13). Thus, tobacco use may be a confounding factor in HPV-positive patients with a history of smoking (11,14) and may explain their significantly increased risk of oncological failure (7,8,15-18). The process of tumor transformation from dysplasia into an invasive tumor involves a number of molecular abnormalities. In HNSCC, these abnormalities result from chromosomal instability rather than microsatellite instability (5). If smoking and HPV are distinct risk factors for OPSCC (19), then the nature of the interaction between these two risk factors in mediating HNSCC development remains unclear. As regards the tobacco smoking status, tobacco smoking synergistically increases the risk of HPV-associated OPSCC (19,20); however, the mechanisms through which smoking can biologically affect the development of HPV-related OPSCC remain unclear.

In terms of chromosomal activity, tobacco and HPV may act as either synergistic or competitive factors: Tobacco use may increase chromosomal instability, although the rate of chromosomal alterations is low in HPV-induced cancer, with a higher incidence of the wild-type genome. As regards HPV-induced HNSCC, the HPV16 oncoproteins E6 and E7 have been shown to independently cause structural and numerical chromosomal instability (21,22) by inducing centrosome abnormalities and potentially decreasing the G2-M checkpoint response (4). Nevertheless, *in vitro* experiments have demonstrated that HPV16 is a high-risk HPV that induces one of the lowest rates of chromosomal copy number alterations (CNAs) following keratinocyte immortalization (23). By increasing chromosomal instability and promoting DNA breaks in HPV-positive OPSCC, tobacco smoking may increase HPV integration at fragile sites or 'hotspots' of DNA breakage, which may modify pathways leading to cellular transformation in HPV-positive patients with a previous or current smoking status compared to HPV-positive patients who have never smoked. Mooren *et al* (24) analyzed chromosome stability in 77 tonsillar squamous cell carcinoma samples via fluorescence *in situ* hybridization (FISH). This team studied imbalances in the copy number ratios of chromosomes 1 and 7, as CNAs in these two chromosomes had been detected by FISH in previous studies on head and neck (pre)malignancies (25,26). The authors correlated the chromosome copy number with the HPV16 status and clinicoepidemiological profiles. Other array-based comparative genomic hybridization (aCGH) have studies investigated chromosomal instability in OPSCC according to HPV status, but none to date have considered smoking status (27-29), at least to the best of our knowledge.

Therefore, patients with OPSCC with a low rate of genomic alterations for whom efforts to deescalate treatment would be justified, must be differentiated from patients with other HPV-positive OPSCCs resulting from more complex biological drivers, specifically, those due to tobacco/alcohol exposure, for whom conventional treatments would be continued. Thus, this study aimed to evaluate chromosomal instability in patients with OPSCC who are HPV-positive based on smoking status by comparing a group of patients with a strict non-smoking history with a group of patients with HPV-positive OPSCC and a past or present history of smoking regardless of the level of tobacco abuse.

Materials and methods

Patient samples. Tumor samples from 50 patients with OPSCC were retrieved from the Tenon Hospital tissue bank between July, 2008 and July, 2016. All samples were collected prior to treatment. These samples were fresh-frozen tumor specimens (stored at -80°C). The medical files of each patient, including clinical and pathological reports, were prospectively analyzed by the investigators. The study criteria were as follows: A histological diagnosis of OPSCC; the collection of tumor samples before treatment; and the availability of clinicoepidemiological data, HPV status and tobacco use data. Patients were considered non-tobacco smokers if their past consumption was fewer than 100 cigarettes according to the definition of the American National Health Interview Survey (NHIS). TNM classification and tumor stage were assessed according to the 8th edition of the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) staging manual. A separate algorithm for high-risk HPV-associated cancer (distinguishing this etiology from other causes) is one of the major changes in this recent classification (30). Tumors were considered HPV-driven if HPV16/18 DNA was identified in combination with p16 protein overexpression or E6/E7 mRNA identification. Our institutional review board (the CEORL, from the French ENT Society) approved the study protocol, and all patients provided written informed consent.

DNA extraction. Genomic DNA was isolated using a QIAamp® DNA Mini kit (Qiagen) according to the manufacturer's instructions and stored at -4°C.

HPV genotyping. Genomic DNA was isolated using our in-house protocol. Tumor samples were screened by polymerase chain reaction (PCR) followed by hybridization using an INNO-LiPA HPV Genotyping Extra diagnostic assay (Fujirebio Europe N.V.) according to the manufacturer's instructions.

aCGH. DNA isolated from the specimens was fluorescently labeled with the cyanine dye Cy5, and control DNA was labeled with the cyanine dye Cy3 using an Agilent SureTag DNA (Agilent Technologies) labeling kit according to the manufacturer's instructions. Labeled products were cohybridized to a PerkinElmer CGX Oncochip (PerkinElmer) with oligonucleotide coverage targeting 2,410 cancer-relevant regions based on human genome build 19. The microarray design was a 4x180 Custom SurePrint G3 Human CGHplusSNP Microarray 4x180K (AMADID 046984). The hybridization design was a tumor sample versus a systematic commercial normal male reference (Promega G1471; Thermo Fisher Scientific). The average target spacing was 1 probe every 2-5 kb, and the average backbone spacing was 1 probe every 65 kb. CGX oncoarrays also feature backbone single nucleotide polymorphism (SNP) coverage, affording combined CGH/SNP results. Arrays were scanned with an Agilent G2539A DNA Microarray Scanner (Agilent Technologies), and images were processed with Agilent Feature extraction for CytoGenomics and Agilent CytoGenomics Edition 2.7.22.0 software (Agilent Technologies).

Statistical analysis. All data manipulations were performed in the R environment (v3.4). Raw intensities were normalized

according to their dye composition (Ref/Cy3 fitted over Test/Cy5). The data were transformed as $\log_2(\text{Test}/\text{Ref})$ and normalized according to the local GC content through Loess regression. A common ‘wave-effect’ bias track was modeled from the 50 normalized profiles using the *cghseg* package (v1.0.2-1) (31) and subtracted from each profile through Loess regression. The resulting profiles were segmented with the circular binary segmentation (CBS) algorithm implemented in the *DNAcopy* package (v1.42) using the default parameters (32). The segmented profiles were centered using the most centered of the three most populated peaks in the smoothed $\log_2(\text{Test}/\text{Ref})$ distribution density. Aberration levels were called by setting an unsigned $\log_2(\text{Test}/\text{Ref})$ threshold automatically adapted to the internal noise for each profile, which was considered one-fourth of the median value of the absolute differences between consecutive $\log_2(\text{Test}/\text{Ref})$ measurements along the genome. Differential analysis of CGH profile subpopulations was performed using a Wilcoxon rank-sum test for two classes or a Kruskal-Wallis test for more than two classes. The obtained CGH clusters and categorical clinical annotations were compared using Fisher's exact test. All P-values were FDR-adjusted using the Benjamini-Hochberg method. Minimum common regions were identified using ‘region’-level results from *GISTIC2* (v2.0.22) with the default parameters, except for recentering (none performed) (33). Differences in chromosomal alterations were considered significant with P-values <0.05. Differential regions were considered significantly different when their respective raw P-values were <0.001.

Results

Patient description. Fifty patients with OPSCC treated between July, 2008 and July, 2016 were available for this study. The median age of the patients was 61 years, and 68% were males (n=34). The main tumor locations were the tonsils (n=35, 70%) and tongue base (n=11, 22%).

The patients were classified into 4 groups as follows: Patients who were HPV-positive, with a positive smoking status (HPV⁺ smoker⁺) (n=16); patients who were HPV-positive, with a non-smoking (HPV⁺ smoker⁻) (n=10); patients who were HPV-negative, with a positive smoking status (HPV⁻ smoker⁺) (n=22); and patients who were HPV-negative, with a non-smoking status (HPV⁻ smoker⁻) (n=2). The cohort consisted of patients with locoregionally advanced oropharyngeal tumors, 16 (32%) who had stage IV disease. The patient characteristics are presented in Table I.

The results of HPV genotyping were as follows: The majority of the positive cases were HPV16 (n=24), 1 case was HPV33 (an HPV⁺ smoker⁺ patient), and 1 case was HPV82 (an HPV⁺ smoker⁺ patient); 24 cases were HPV-negative.

aCGH data. As regards the global aCGH data, acquired genomic CNAs were detected in all samples. The CNA frequency and summed alterations are shown in Fig. 1. The analysis of the minimum common regions (MCRs), which identifies the regions of limited size most likely to have recurrent genes of interest, was conducted in all of our patients. A total of 59 MCRs were identified with 95% confidence (12 gains and 47 losses) and were distributed across all

Table I. Patient characteristics.

Patient characteristics	Number	Percentage
Median age at diagnosis (years)	61	-
Sex		
Male	34	68
Female	16	32
HPV genotyping		
HPV16	24	48
HPV33	1	2
HPV82	1	2
Negative	24	48
HPV and smoking status		
HPV ⁺ smoker ⁺	16	32
HPV ⁺ smoker ⁻	10	20
HPV ⁻ smoker ⁺	22	44
HPV ⁻ smoker ⁻	2	4
Oropharyngeal site		
Tonsils	35	70
Tongue base	11	22
Soft palate	3	6
Other	1	2
T classification ^a		
T1	6	12
T2	18	36
T3	23	46
T4 ^a	3	6
T4 ^b	0	0
N classification ^a		
N0	10	20
N1	23	46
N2	15	30
N3	2	4
M classification ^a		
M0	49	98
M1	1	2
Overall tumor stage ^a		
I	15	30
II	13	26
III	6	6
IV	16	32

^aTNM classification and tumor stage are presented according to the 8th edition of the AJCC/UICC Staging Manual (30). HPV, human papilloma virus.

chromosomes. The gains ranged from 0.15 to 46.4%, and the losses ranged from 0.87 to 65.63%. The percentage of genomic aberration ranged from 4.06 to 81.67%. The median number of breakpoints was 79 (range, 34-474). The size of the aberrations ranged from 7.56 Kb to 35.2 Mb, with a mean of 6.12 Mb (data not shown).

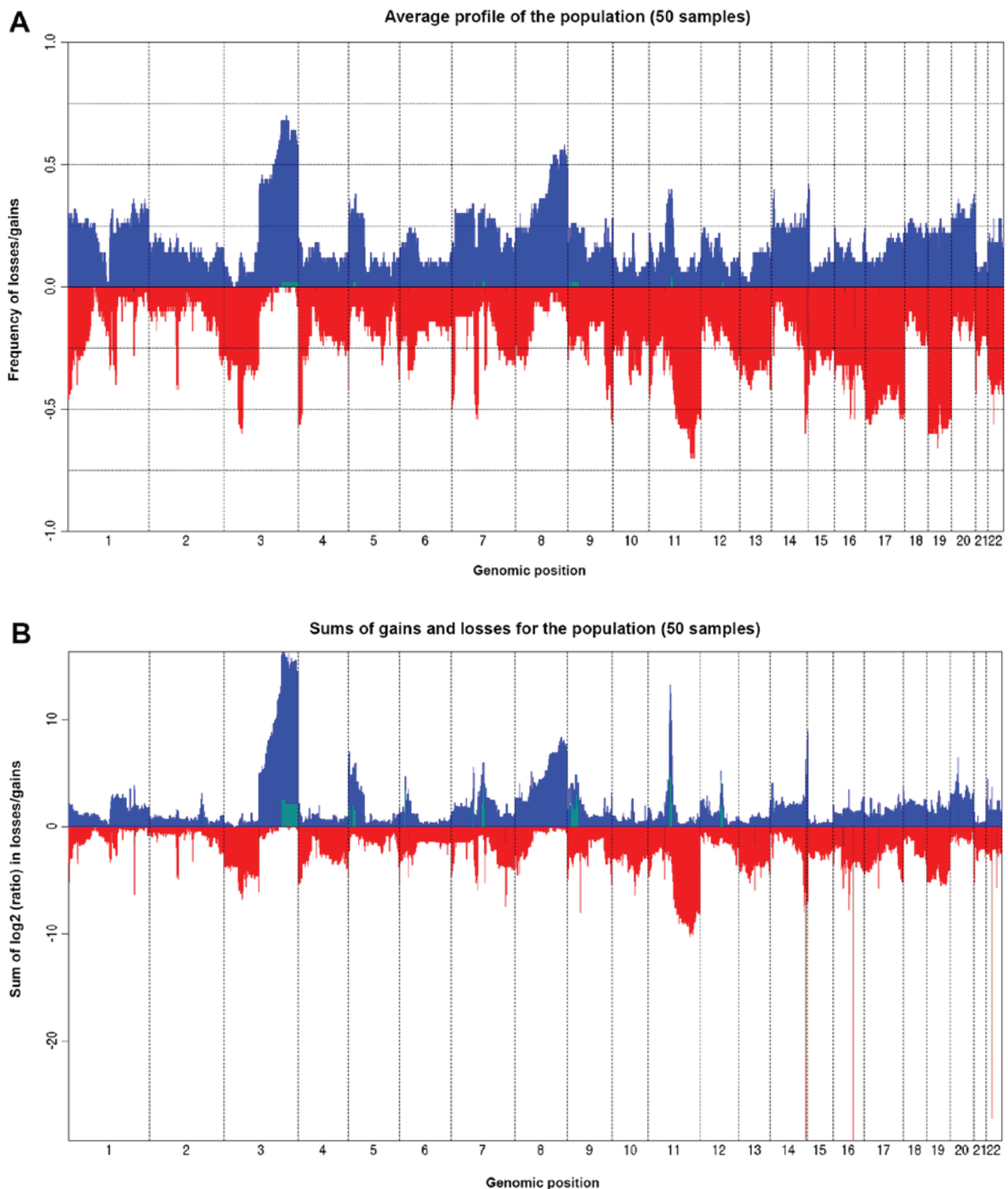


Figure 1. Presentation of chromosomal losses and gains investigated with array-based comparative genomic hybridization (aCGH) in our cohort of 50 patients. (A) Frequency of the chromosomal aberrations, (B) sum of chromosomal aberrations. Chromosomal losses are presented in red and chromosomal gains are presented in blue for chromosomes 1 to 22.

The aCGH results for the number of chromosome alterations by patient group are shown in Table II and Figs. 2-4. Sex was not significantly associated with a variation in either chromosome aberration or breakpoint number (data not shown). A positive HPV status was significantly associated with reductions in chromosomal aberrations ($P=0.0082$) and chromosomal losses ($P=0.048$). No significant difference was observed in chromosomal gains as regards the HPV status. In addition, a positive HPV status was associated with a reduction in instability breakpoints ($P=0.042$) (Fig. 2 and Table II). In patients with OPSCC who were HPV-positive, the total number

of chromosomal aberrations per tumor was significantly lower in the non-smoking patients than in the smoking patients ($P=0.027$) (Fig. 3 and Table II). Furthermore, patients in the HPV⁺ smoker group had significantly fewer chromosomal gains ($P=0.038$), genomic aberrations ($P=0.0097$) and instability breakpoints ($P=0.013$) than their counterparts (HPV⁺ smoker⁺, HPV⁻ smoker⁻ and HPV⁻ smoker⁺ patients) (Fig. 4 and Table II).

Compared with the patients with HPV-negative tumors, HPV-positive patients exhibited significant differences at several chromosomal arms (Fig. 5). HPV-positive patients had

Table II. Comparison of aberrant genome fraction and of instability breakpoints according to HPV and smoking status.

Annotations	HPV ⁺ vs. HPV ⁻	HPV ⁺ according to smoking status	HPV ⁺ smoker vs. others
Chromosomal gain fraction (median ± SD)	HPV ⁻ = 0.20±0.15 HPV ⁺ = 0.13±0.096 P=0.050	HPV ⁺ smoker = 0.12±0.088 HPV ⁺ smoker ⁺ = 0.19±0.097 P=0.15	HPV ⁺ smoker = 0.12±0.088 Others = 0.19±0.13 P=0.038
Chromosomal loss fraction (median ± SD)	HPV ⁻ = 0.26±0.16 HPV ⁺ = 0.19±0.11 P=0.048	HPV ⁺ smoker = 0.17±0.10 HPV ⁺ smoker ⁺ = 0.20±0.10 P=0.36	HPV ⁺ smoker = 0.17±0.10 Others = 0.24±0.14 P=0.092
Chromosomal aberration fraction (median ± SD)	HPV ⁻ = 0.59±0.25 HPV ⁺ = 0.37±0.13 P=0.0082	HPV ⁺ smoker = 0.26±0.13 HPV ⁺ smoker ⁺ = 0.41±0.11 P=0.027	HPV ⁺ smoker = 0.26±0.13 Others = 0.48±0.21 P=0.0097
Instability breakpoints (median ± SD)	HPV ⁻ = 112±105 HPV ⁺ = 70.50±43.70 P=0.042	HPV ⁺ smoker = 54.50±33.36 HPV ⁺ smoker ⁺ = 83.50±46.41 P=0.054	HPV ⁺ smoker = 54.50±33.40 Others = 94±88.40 P=0.013

HPV, human papilloma virus; SD, standard deviation. Significant values are represented in bold font.

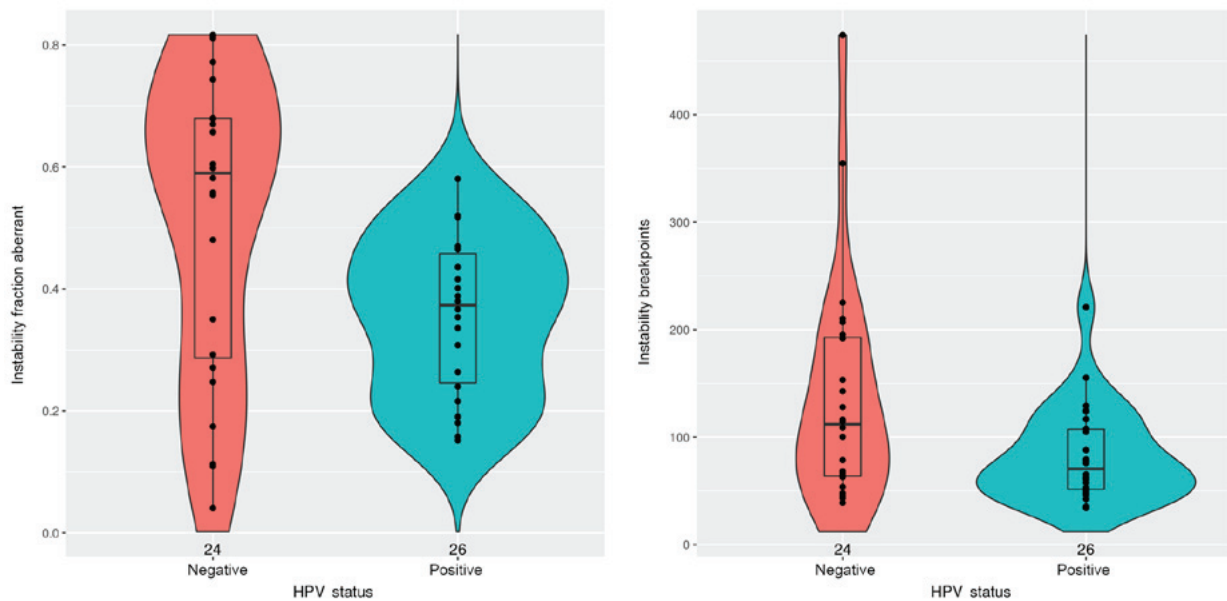


Figure 2. Genomic aberrations and breakpoints in our cohort of 50 patients based on their human papilloma virus (HPV) status. On the violin plots, the black and bold lines represent the median. The width of the colored shape represents the density. HPV-negative patients are shown in the red plot, and HPV-positive patients are shown in the green plot.

fewer chromosomal losses at 3p (3p13, 3p14 and 3p21) (P<0.03), fewer chromosomal gains at 7p (7p11 and 7p14) (P<0.03), fewer chromosomal gains at 7q21 (P<0.03) and fewer chromosomal losses at 9p (9p13 and 9p21) (P<0.03). Furthermore, HPV-positive tumors exhibited more chromosomal gains at 18q21 (P<0.03).

Many differences in chromosomal aberrations were observed on chromosome 11: In total, 36 cytobands differed between the HPV-negative and HPV-positive tumors. HPV-positive tumors had significantly more losses at some 11q cytobands (11q14, 11q21, 11q22, 11q23, 11q24 and 11q25), while HPV-negative tumors had significantly more gains at 11q13 (both P<0.03). No difference was found at the other

chromosome arms between HPV-positive and HPV-negative tumors (data not shown).

Finally, no significant differences were observed in the various cytobands at these chromosomal arms between the patients with HPV-positive tumors with a smoking and non-smoking status (data not shown).

Discussion

The question of whether the confounding factors of tobacco and/or alcohol abuse can act as cofactors and/or effect modifiers in either the risk or the etiology of HPV-related OPSCC remains unanswered. Anantharaman *et al* (34) demonstrated

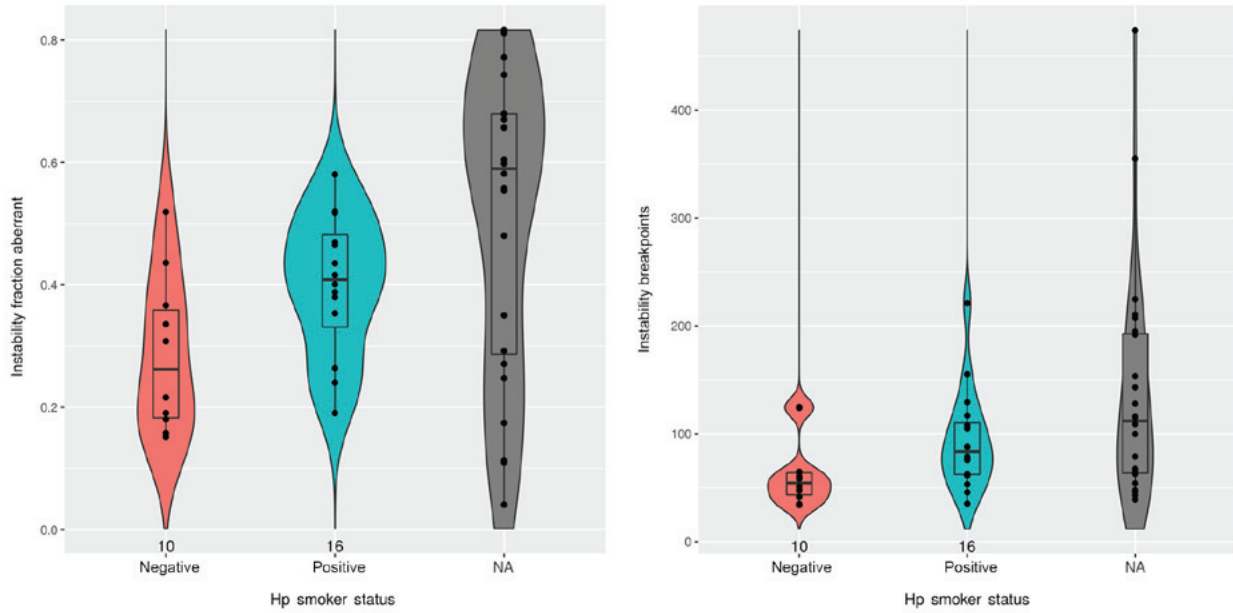


Figure 3. Genomic aberrations and breakpoints in human papilloma virus (HPV)-positive patients according to their smoking status. On the violin plots, the black and bold lines represent the median. The width of the colored shape represents the density. The red plots represent HPV-positive (Hp) non-smoking patients. The green plots represent HPV-positive smoking patients. The gray plots represent HPV-negative non-smoking patients with even greater instability.

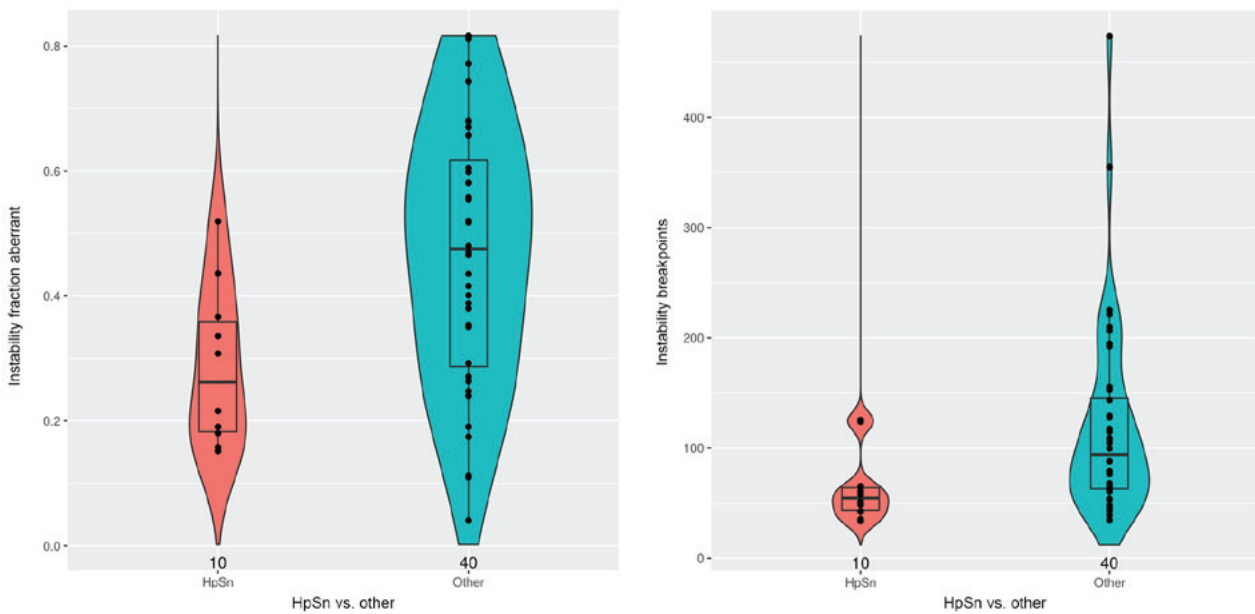


Figure 4. Comparison of genomic aberrations and instability breakpoints in human papilloma virus (HPV)-positive and non-smoking patients versus other patients. On the violin plots, the black and bold lines represent the median. The width of the colored shape represents the density. The red plots represent HPV-positive non-smoking (HpSn) patients. The green plots represent other patients.

that smoking was consistently associated with an increased risk of OPSCC regardless of the HPV status, suggesting that smoking and HPV16 may act as independent risk factors. The population-level burden of HPV-positive oropharyngeal cancers is significantly higher among ever-smokers than never-smokers in the US, although HPV-related OPSCC is characterized as a disease of never-smokers due to the high HPV prevalence in OPSCC cases among never-smokers (35). Therefore, the biological mechanisms underlying the interrelation between high-risk HPVs, particularly HPV16, and smoking toxicity can be questioned. To date, three

studies have compared mutations in HPV-driven OPSCCs according to tobacco consumption (36-38). However, only one of these studies found statistically significant differences in five mutated genes between smokers and non-smokers (38). Additional studies with a meaningful number of genes and a large patient population seem necessary in order to further describe the mutational landscape of HPV-positive OPSCC according to the smoking status of patients.

Mooren *et al* (24) studied chromosomal stability in 77 tonsillar squamous cell carcinomas and found that the disomy of chromosomes 1 and 7 was associated with HPV16-positive

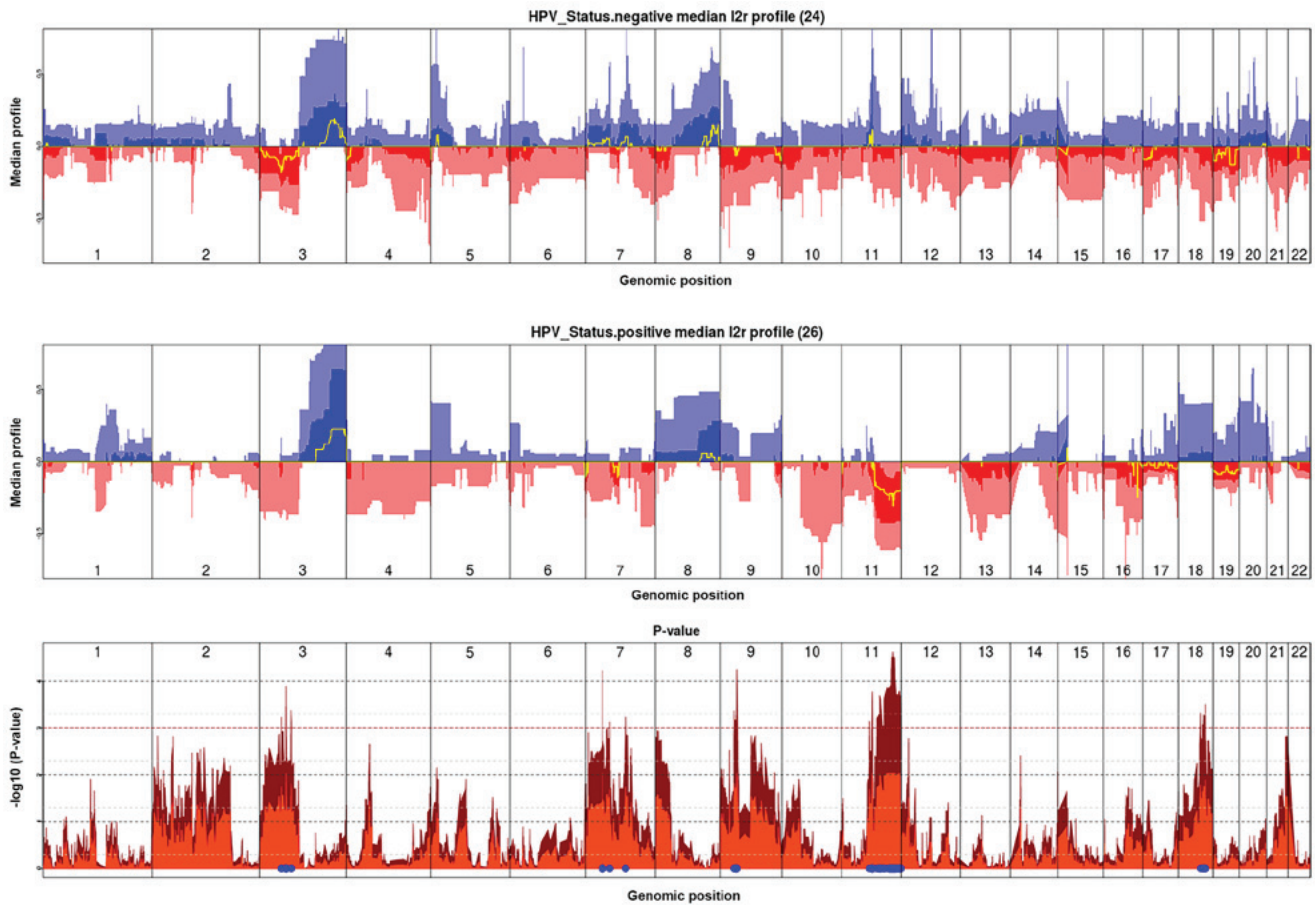


Figure 5. Comparison of chromosomal losses and gains investigated with array-based comparative genomic hybridization (aCGH) according to the human papilloma virus (HPV) status of the patients. The comparison plots are presented with the following conventions: The upper panel shows the median profile of chromosomal gains (in blue) and chromosomal losses (in red) for each autosome in HPV-negative patients. The middle panel shows the median profile of chromosomal gains (in blue) and chromosomal losses (in red) for each autosome in HPV-positive patients. The median $\log_2(\text{ratio})$ profile of each subclass is presented as a yellow line, with an opaque box indicating the 1st and 3rd quartiles and a transparent box displaying the 5th and 95th centiles. In the bottom panel, the significance of the tests is expressed as the $-\log_{10}(\text{P-value})$. The significance threshold is denoted as a red line (when within the data range). Significant regions are marked with blue dots at the bottom of the plot.

tumors ($P=0.002$) and that aneusomies significantly correlated with tobacco and alcohol consumption ($P=0.001$ and $P=0.016$, respectively). Moreover, the authors observed greater chromosome instability and poorer prognoses in 13 of 32 HPV-positive patients, 10 of whom were smokers (24). The authors recommended that the chromosomal ploidy status should be considered a prognostic factor in HPV-positive tonsillar squamous cell carcinoma (24).

Previous aCGH studies have identified differences in the distributions of DNA gains and losses between HPV-positive and HPV-negative samples, with more chromosomal alterations in the HPV-negative samples; however, they did not consider the smoking history of the patients (27-29). To the best of our knowledge, this study is the first to use global aCGH covering 2,410 targeted cancer-relevant regions based on human genome build 19 in OPSCC considering both the HPV and smoking statuses of patients. Our aim was to determine whether chromosomal alterations are more important in HPV-positive OPSCCs for smoking patients than non-smoking patients and whether these alterations may be a possible mechanism affecting the prognosis of such patients. We clearly demonstrated that chromosomal instability is substantially increased in smoking patients with HPV-positive

OPSCC. HPV-positive tumors have fewer genomic aberrations and breakpoints than HPV-negative tumors. Furthermore, among the HPV-positive OPSCC patients, the total number of chromosomal aberrations per tumor was significantly lower in non-smoking patients than in smoking patients. As regards the cytobands in the present study, HPV-negative tumors had significantly more losses at 3p and 9p and gains at 7q and 11q13 than HPV-positive tumors, confirming the results of previous studies (27-29). Moreover, we observed that HPV-negative tumors had more chromosomal gains at 7p. We described 11q losses in patients with HPV-positive OPSCC, similar to the study of Dahlgren *et al* (27), and we also described 18q21 gains in these patients.

This study confirmed that chromosomal alterations are more important in patients with HPV-positive (particularly HPV16-positive) OPSCC and a history of smoking. As our data were not verified by FISH, we cannot exclude the possibility that tri- or tetraploid tumors with no unbalanced rearrangements appeared normal. However, no differences were found in the cytobands between smoking and non-smoking patients with HPV-positive OPSCC. Nevertheless, we did not identify a clear chromosomal pattern that could differentiate these two groups of tumors, which may be due to the random distributions of chromosomal gains and

losses or low discrimination (regarding the number of pack-years or a possible duration of smoking cessation of longer than ten years) within the smoking group. Although the association between tobacco abuse and chromosomal instability has been well established, specifically in lung cancer (39), the mechanisms through which smoking induces chromosomal damage remain unclear. Tobacco smoke contains >60 carcinogens, including polycyclic aromatic hydrocarbons, aromatic amines and nitrosamines (40). Among these carcinogens, nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NKK) and arylamine 4-aminobiphenyl (4-ABP) increase chromosomal instability (41-43). In addition, cigarette smoke has been shown to suppress the activity of the Fanconi anemia (FA)/BRCA pathway, which is essential for the maintenance of chromosomal stability (44). Speculation has arisen regarding the possible role of smoking abuse in the integration of HPV DNA into the host genome, specifically by altering DNA breakage hotspots, for example, common fragile sites.

In conclusion, in this study, we found that chromosomal instability was more important in patients with HPV16-positive OPSCC and a history of smoking. This finding may help to improve the stratification of HPV-positive OPSCC patients, particularly if these data are complemented by next-generation sequencing (NGS) studies on large gene panels to establish the mutational and transcriptomic profiles of these tumors according to patient smoking status.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Author's contributions

AV, RL, JLStG, BB and SA were involved in the acquisition of the data and the study design. AV, RL, JLStG, BB and SA were involved in the writing of the manuscript. AV and SH performed the DNA extraction and aCGH experiments. BJ performed the statistical analysis. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This research was performed in accordance with the Declaration of Helsinki. Our institutional review board (the CEORL, from the French ENT Society) approved the study protocol, and all patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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