Role of mucosal high-risk human papillomavirus types in head and neck cancers in central India

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Novelty and Impact

This is one of the largest studies performed in India to evaluate the impact of HPV infection on head and neck cancer (HNC) development. We used highly sensitive assays for the detection of HPV DNA and RNA, and compared their positivity with that of $p16^{INK4a}$, a surrogate marker of

HPV infection. Our findings show that the HPV contribution to HNC in this central Indian region is lower than in many developed parts of the world.

Keywords: HPV, head and neck cancer, central India

Abbreviations: HR-HPV: high-risk human papillomavirus; HNC: head and neck cancer; FFPE: formalin-fixed paraffin-embedded; ISH: in situ hybridization; SCC: Squamous Cell Carcinoma; PABAK: Prevalence-Adjusted Bias-Adjusted Kappa

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Abstract (max 250 words)

Mucosal high-risk (HR) human papillomaviruses (HPV) cause a subset of head and neck cancers (HNC). The HPV-attributable fraction of HNC varies substantially between countries. Although HNC has a very high incidence in the Indian sub-continent, information on the contribution of HPV infection is limited. Here, we evaluated the HPV-attributable fraction in HNC (N=364) collected in a central region of India. HNC from three different anatomical subsites were included, namely oral cavity (n=252), oropharynx (n=53) and hypopharynx/larynx (n=59).

In this retrospective study, HPV-driven HNC were defined by presence of both viral DNA and RNA. Overexpression of $p16^{INK4a}$ was also evaluated. HR-HPV DNA was detected in 13.7% of the cases; however only 2.7% were positive for both HPV DNA and RNA. The highest percentage of HPV DNA/RNA double positivity was found in oropharynx (9.4%), followed by larynx (1.7%) and oral cavity (1.6%) (p=0.02). More than half of HPV DNA/RNA-positive cases were $p16^{INK4a}$-negative, while a considerable number of HPV RNA-negative cases were $p16^{INK4a}$-positive (17.9%). HPV16 was the major type associated with HNC (60.0%), although cases positive for HPV18, 35 and 56 were also detected.

Our data indicate that the proportion and types of mucosal HR-HPV associated with HNC in this central Indian region differ from those in other (developed) parts of the world. This may be explained by differences in smoking and/or sexual behaviour compared with North America and northern Europe. Moreover, we show that $p16^{INK4a}$ staining appeared not to be a good surrogate marker of HPV transformation in the Indian HNC cases.

Introduction

With approximately 700,000 incident cases, head and neck cancer (HNC) is the sixth most common malignancy worldwide. Around 380,000 deaths per year are due to HNC[1, 2]. Alcohol consumption, smoking, poor oral hygiene, and genetic features are key risk factors for HNC development[3]. Over the past two decades, it has become clear that a subset of HNC is associated with certain mucosal high-risk (HR) HPV types[3-7], specifically HPV16 (86–95%), while the contribution of the remaining HPV types is unclear[8]. Although the overall incidence of HNC is decreasing in developed countries due to increasing awareness of tobacco and alcohol as risk factors for human carcinogenesis, the proportion of HPV-positive oropharyngeal cancers among HNC has steadily increased in the USA and northern Europe[3, 9-11]. However, epidemiological studies highlighted that the fraction of oropharyngeal cancer associated with HR-HPV types can vary substantially in different countries[3, 12].

The Indian sub-continent has the highest HNC incidence (6.1/100,000 women and 20.9/100,000 men)[1] in the world and accounts for one-third of the world HNC burden. In this geographical area, HNC is the first and third most common malignancy in men and women, respectively[1]. Many etiological factors are involved in HNC development in the Indian population, including alcohol and smoking. The habit of chewing betel quid often mixed with tobacco is widespread in South Asia, and also plays a key role in HNC development[13]. Although age-standardized rates of cervical cancer are among the highest in India[1,2] (22.0/100,000), highlighting the importance of HPV infections in this population, limited information on HPV-associated HNC exists[14-21]. The studies published thus far have been based on a limited number of cases. In addition, HPV positivity was ascertained by PCR-based assays alone. However, HPV DNA detection alone is insufficient proof for viral causality[22-25]. Additional markers, such as viral RNA, antibodies

against the viral oncoproteins and $p16^{INK4a}$ expression as a surrogate for HPV-induced transformation, allow a more precise classification of HNC[8,24,26-29]. Therefore, additional studies applying appropriate molecular methods are required to precisely establish the HR-HPV-attributable fraction for HNC in India.

In the context of a recent European and Indian case study (HPV-AHEAD, http://hpv-ahead.iarc.fr), we have collected approximately 3000 formalin-fixed paraffin-embedded (FFPE) HNC tissues in six different urban (n=3) and rural (n=3) hospitals in India, with the aim to determine the HPV-attributed fraction using several laboratory assays. To identify HR-HPV-attributable tumours, a stringent protocol was applied which included the detection of both viral DNA and RNA as well as $p16^{INK4a}$ staining.

In the current report, we describe the first results of a comprehensive histological and molecular assessment of a large number of archived HNC specimens from one rural hospital in central India, using the above mentioned stringent protocol which includes the detection of viral DNA and RNA as well as $p16^{INK4a}$ staining.

Materials and Methods

Specimen collection

We have initially taken into consideration the list of archived material for 2001-2011. We were able to retrieve paraffin embedded tissue blocks for 484 case subjects consecutively diagnosed during mid-2005 to 2011 and archived in a unique centre (Department of Pathology, Mahatma Gandhi Institute of Medical Sciences (MGIMS), Sevagram, Maharashtra, India), with an average of about 70 cases per year. No data on smoking, alcohol consumption or other risk factors were available. Two important criteria were considered for the retrieval of the archived specimens: (i)

only primary cancers at three different anatomical sites (oral cavity, oropharynx and larynx/hypopharynx) were included, and (ii) the specimens were consecutively collected, without the use of any selection criteria other than anatomical site. No potential bias could be identified. The FFPE HNC blocks included squamous cell carcinoma of the oropharynx (International Classification of Diseases for Oncology [ICD-O] C01.9, C02.4, C05.1, C05.2, C09, C10), oral cavity (ICD-O: C02.0–C06.9, excluding C02.4, C05.1, C05.2), the hypopharynx and larynx (ICD-O: C13, C32). Ethical clearance for the investigations reported in this study was obtained from the Institutional Ethical Committees of MGIMS, Sevagram, India and IARC, Lyon, France. Study implied the use of archival material only, and it did not envisage any contact with the patients. Adequate measures to ensure data protection, confidentiality, patients' privacy and anonymization were taken into account. No informed consent was available due to the retrospective design of the study and the large proportion of deceased and untraceable patients. The blocks were used to perform several laboratory assays as described below. Each assay was performed in a single laboratory: (i) the HPV DNA assay at IARC, (ii) the HPV RNA assay at DKFZ, and (iii) the $p16^{INK4a}$/Ki-67 dual staining at Roche mtm laboratories.

Preparation of the tissue sections

FFPE-tissues were in part processed at the International Agency for Research on Cancer, Lyon, France and at the Rajiv Gandhi Centre for Biotechnology Thiruvananthapuram, Kerala, India, following the HPV-AHEAD sectioning protocol. Briefly, depending of the size of the tissue 10 to 31 sections (S) were generated from each FFPE block. The first (S1) and the last (S10 or S31) sections were haematoxylin/eosin stained for histology and used to check for the presence of tumour. S2 and S9 were generated for $p16^{INK4a}$ immunohistochemical staining, while sections 3-

5 (10μ) and 6-8 (10μ) were used for the extraction of RNA and DNA, respectively. To minimize the risk of cross-contamination during sectioning a new blade was used for each FFPE block and the microtome was extensively cleaned after each block with ethanol 70% and DNA Away (Dutscher, Brumath, France). In addition, to monitor possible DNA contamination, after 10 tumour tissue blocks, sections were generated from an empty paraffin block and all were included in the DNA analyses.

Histological analysis

All sections were analysed by the HPV-AHEAD pathology review panel that includes three European (JPB, BLR, FM) and three Indian (TS, RVK, NG) pathologists. An online pathology evaluation form generated by the six pathologists was used. Each pathologist analysed tissues from approximately 80 patients. All sections were re-analysed by a second panel pathologist. Only FFPE blocks where the first and last haematoxylin/eosin sections reflected tumour tissue were included in the study.

HPV DNA genotyping

For DNA extraction three consecutive sections (S6-8) of each block were pooled and incubated in digestion buffer (10mM Tris/HCl pH 7.4, proteinase K 0.5 mg/ml, and Tween 20 0.4%) at 37°C overnight[20]. Water samples were included to monitor possible contaminations during the preparation of DNA.

HPV typing was detected by a type-specific multiplex genotyping (TS-MPG) assay, which combines multiplex PCR and bead-based Luminex Technology (Luminex Corporation, Austin, TX), as previously described[30, 31]. TS-MPG uses HPV type-specific primers targeting the E7

region of 19 high-risk (HR) or possible/probable HR (pHR) HPV types (HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68a and b, 70, 73 and 82) and two low-risk (LR) HPV types (HPV6 and 11). Detection limits range from 10 to 1000 copies of the viral genome per reaction. Two primers for amplification of the *beta-globin* gene were also included to control for the quality of the template DNA. Slight modifications of the protocol for the amplification of shorter (~100 bp) fragments for ten HPV types (HPV16, 18, 31, 33, 35, 52, 56, 66, 6 and 11), and 117 bp for β-globin, were applied. After PCR amplification, 10 µl of each reaction mixture was analysed by multiplex HPV genotyping (MPG) using Luminex technology (Luminex Corporation, Austin, TX) as described previously[31]. Genotyping controls and DNA preparation were blindly analysed and no sign of contamination of negative controls was detected during the laboratory work. All HPV DNA-positive FFPE specimens and a random subgroup of approximately 10% of HPV DNA-negative specimens were further analysed for the presence of HPV E6*I mRNA and overexpression of the cell cycle inhibitor $p16^{INK4a}$ that is considered a surrogate marker for HPV infection. The 10% of HPV DNA-negative cases were selected randomly and blindly (roughly 1 of every 10 HPV DNA-negative cases), while the study was still anonymized.

HPV RNA analysis

Total RNA was purified from 3 pooled consecutive sections of the same tissue block using the PureLink FFPE Total RNA Isolation Kit (Invitrogen, Carlsbad, CA) as described previously[32]. Extracted RNA was eluted in 50 µl of RNase-free water and stored at –80°C until further use. A DNase I treatment was carried out on the total RNA, following the manufacturer's instructions (Qiagen, Hilden, Germany). RT-PCR was carried out using the QuantiTect Virus Kit (Qiagen),

in a total volume of 25 µl containing 5 µl of 5xQuantiTect Virus Mastermix, 0.25 µl of 100xQuantiTect Virus RT Mix, 0.4 µM of each primer, and 1 µl RNA input as described previously [32]. Reverse transcription was carried out at 50°C for 20 min, directly followed by an initial PCR activation step at 95°C for 5 min in a PCR thermocycler (Mastercycler, Eppendorf AG, Hamburg, Germany). cDNA was amplified in 40 cycles, each including a 15 s denaturation step at 95°C and a 45 s combined annealing/extension step at 60°C. The HPV type-specific E6*I mRNA assays developed for 20 HR/pHR-HPV types[32] were applied for detection of viral transcripts. These assays amplify 65–75 bp HPV and 81 bp ubiquitin C (ubC) cDNA. Analytical sensitivity of each assay is 10 to 100 copies per reaction for 19 HPV types and for ubC and 10,000 copies for HPV70[22]. All HPV DNA-positive specimens and a randomly selected 10% of HPV DNA-negative specimens were analysed for the presence of: (i) HPV16 E6*I mRNA and (ii) ubC mRNA as a cellular mRNA positive control. Tissues positive for DNA of a non-HPV16 type were additionally analysed for E6*I mRNA of this type. Specimens that were HPV E6*I and/or ubC mRNA-positive (RNA+) in RNA analysis were considered RNA valid.

p16^{INK4a} immunohistochemistry

For p16^{INK4a} immunohistochemistry, a dual-immunostaining protocol for the simultaneous immunostaining for both p16^{INK4a} and Ki-67 biomarkers has been applied using the CINtec PLUS kit (Roche mtm laboratories AG, Mannheim, Germany). This dual-staining approach was used to facilitate the identification and assessment of the area of the tissue section comprising the cancer tissue. Slide S2 was simultaneously stained for p16^{INK4a} and Ki-67 using the CINtec PLUS dual-stain kit (Roche mtm laboratories). At an early phase of the HPV-AHEAD study, we performed a comparative analysis on 185 cases where in addition to slide S2 stained with the

p16^{INK4a}/Ki-67 dual-stain kit, slide S9 of each cases was single-stained only for p16^{INK4a} using the CINtec Histology p16^{INK4a} Histology Kit (Roche mtm laboratories), which has been developed and optimized for use on tissue sections prepared from formalin-fixed, paraffin-embedded specimens. The comparison showed concordant p16^{INK4a} staining results in more than 98% of the cases, indicating that the dual-stain protocol provides comparable p16^{INK4a} staining results as the single-stain protocol, which was optimized for tissue sections. Therefore, we continued the study using the p16^{INK4a}/Ki-67 dual-stain protocol using the CINtec PLUS kit and slide S2 for the whole study. A continuous, diffuse staining for p16^{INK4a} within the cancer area of the tissue sections was considered as positive, while a focal staining or no staining were considered negative. IHC slides were analysed without knowledge of any other clinical information (including HPV DNA and RNA status) by RR, DH and three members of the HPV-AHEAD pathology review panel (JPB, FM and BL). Each staining has been reviewed by at least one researcher and one pathologist. Discrepant cases have been re-checked by a pathologist and final classification of the staining was based on the major consensus of the working group.

Statistical analysis

The primary outcome was HPV-related tumours defined as being HR-HPV DNA- and RNA-positive for the corresponding type, with cases that were HPV DNA- or RNA-negative considered HPV-unrelated. The prevalence of HPV in oropharyngeal, oral cavity and laryngeal SCC cancer patients was compared by subsite and HPV type using the Fisher's exact test. ANOVA test was used to compare mean age by anatomical site The proportion of cancers caused by HPV and 95% confidence interval for the estimate are reported. The concordance between HPV DNA detection, RNA positivity and p16 expression levels was examined by

percent of agreement, kappa statistic (and Prevalence-Adjusted Bias-Adjusted Kappa (PABAK) = 2*observed agreement-1) and McNemar's test p-value. PABAK statistic is a correction of the concordance used when the agreement was high but the kappa was low. All statistical analyses were performed using STATA statistical software, version 13.1 (StataCorp, College Station, TX), and all reported P values are two sided. Statistical significance was set at P less than .05.

Results

Of the 484 patients, two were excluded due to incomplete clinical information and 103 were also excluded since they were diagnosed with cancers of salivary glands (n=4) and sinus (n=27) or the tissue block did not contain cancer tissue (n=72). Of the remaining 379 patients processed for HPV genotyping, 15 extracted DNAs resulted to have poor quality as evidenced by negative ß-globin PCR assay results. Table 1 shows the available information of the final study group of 364 HNC patients, that includes 252 oral cancers, 53 oropharynx and 59 larynx cancers (including hypopharynx, n=18). Mean age at diagnosis was 53.6 years (standard deviation (SD)=12.7), mainly from male (72.3%) patients diagnosed with Conventional Squamous Cell Carcinoma (SCC) (61.0%) between mid-2005 and December 2011. Differences in age, sex and histological diagnosis were observed between HNC sub-sites: cases of oropharyngeal cancer occurred in older patients, affected a higher proportion of men and had less keratinizing and more basaloid histologies than did cases at the other sites. Table 2 shows the HPV DNA, RNA and $p16^{INK4a}$ detection in HNC cases. Of the 364 HNC specimens, 50 (13.7%) contained HPV DNA. HPV16 was the most prevalent type, being present in 36 of the 50 HPV DNA-positive tumours (72.0%), followed by HPV31 (6/50, 12.0%), HPV18 (4/50, 8.0%), HPV35 (2/50, 4.0%) and HPV56 (2/50, 4.0%). No tumour contained DNA from LR-HPV types or from multiple HR-HPV types. Oropharynx showed the highest HR-HPV DNA prevalence (10/53, 18.9%), followed by larynx (10/59, 16.9%) and oral cavity (30/252, 11.9%).

The percentage of HPV-related HNC was 2.7% (10/364) for HPV DNA/RNA positivity and dropped to 1.1% (4/364) when all three markers, i.e., HPV DNA/RNA and $p16^{INK4a}$ were considered. The stratification for HNC subsites and different HPV types is shown in Table 2. The highest percentage of HPV DNA/RNA positivity was found in the oropharynx (five out of

53 HPV DNA-positive cases, 9.4%), comprising three for HPV16, one for HPV35 and one for HPV56. A small percentage of oral cavity (4/252, 1.6%) and laryngeal cancers (1/59, 1.7%) resulted test-positive for both DNA and RNA for HPV16, 18, or 35. Those differences between subsites were statistically significant (p=0.02). We observed that HPV16 and the group of the other mucosal HR-HPV types were similarly represented in oral cavity, being two positive for HPV16 DNA/RNA and two positive for HPV18 or 35 DNA/RNA. The number of HNC cases positive for HPV DNA/RNA and $p16^{INK4a}$ was low (n=4). These cases included three oropharyngeal (HPV16 and HPV56) and one laryngeal (HPV16) cancers (Table 2).

In the concordance analysis including the results for the random sample of 38 HPV DNA-negative cases, we observed a slight concordance between HPV16 DNA with RNA (kappa:0.10; PABAK=0.23; p<0.01),further improved for non-HPV16 types (kappa:0.23; PABAK=0.64; p=0.03), and slight concordance between HPV DNA status with $p16^{INK4a}$ was observed for HPV16 (kappa:0.24; PABAK=0.32; p=0.03) as well as for other HR-HPV types (kappa: 0.01; PABAK=0.41; p=0.92) (Supplementary Table 1). By anatomical subsite, 50.0% (5/10) of the HPV DNA-positive oropharyngeal cases were HPV RNA-positive, while a lower proportion was observed in oral cavity (4/30, 13.3%) and larynx (1/10, 10.0%) (p=0.04). Approximately one third (15/50; 30.0%) of HPV DNA-positive cases were $p16^{INK4a}$-positive, and 7.9% of HPV DNA-negative cases were $p16^{INK4a}$-positive (3/38), regardless of the anatomical subsite (Table 3). In addition, 11 out of 40 (27.5%) HNC cases that were HPV DNA-positive, but RNA-negative, were tested positive by $p16^{INK4a}$ IHC. Proportionally to the number of oral cancers analysed, the majority of these $p16^{INK4a}$-positive HNC were from this anatomical site. Moreover,

six out of 10 HPV DNA/RNA-positive cases (60.0%) were negative for $p16^{INK4a}$, and 14 out of 78 HPV RNA-negative cases (17.9%) were $p16^{INK4a}$-positive (Table 3).

To further evaluate the significance of HPV DNA positivity in HNC specimens, we retrieved multiple FFPE tissue blocks from five HPV DNA-positive and HPV RNA-negative patients, namely one patient with two blocks, three patients with three blocks and one patient with four blocks. The histological analysis confirmed that all FFPE blocks contained cancer tissue. In all cases, we detected HPV DNA only in one specimen for each patient (data not shown).

Discussion

It is now well demonstrated that mucosal HR-HPV types, mainly HPV16, are causally involved in a significant proportion of oropharyngeal cancers and in a much lower extent in a subset of other HNC. However, the contribution of HR-HPV to HNC appears to be subject to geographical variability [6]. Although HNC incidence is very high in India, only a limited number of studies have evaluated the contribution of HPV infection [14-21, 33, 34]. In these studies, the proportion of HPV-positive cases was established only by detecting viral DNA using PCR-based assays, and HPV positivity of HNC cases substantially varied between studies, ranging from 15% to 70% [14-21]. Several independent studies have highlighted that PCR-based assays for the detection of HPV DNA are not sufficiently accurate to establish the viral causality[22-25]. Due to their high sensitivity, these assays detect traces of viral DNA present in the oral cavity, which may not be necessarily directly linked to the carcinogenic process. A recent Indian study also determined the presence of HPV RNA by in situ hybridization[35]. A very low prevalence of HPV-associated HNC (1.8%, 8/427) was detected. This lower percentage of HPV RNA-positive cases in

comparison with our study may be explained by the different sensitivities of the RNA detection assays used in the two studies.

Here, we have evaluated the contribution of HPV to HNC development in a central Indian region by detecting HPV DNA and RNA, as well as by determining the positivity for $p16^{INK4a}$. Fourteen percent of HNC contained HR-HPV DNA, but only 3% were positive for both HPV DNA and RNA. Thus, the HNC fraction attributable to transforming HPV infections in this Indian region appeared to be considerably lower compared to various other geographical regions worldwide[6]. In agreement with previous studies in other countries and continents, the highest percentage of HPV DNA and RNA double positivity was found in the oropharynx (9.4%). Regarding the other anatomical sites, only a small fraction of oral cavity and laryngeal cancers were double-positive for HPV DNA and RNA, (1.6 % and 1.7%, respectively). HPV16 and the group of the other mucosal HR-HPV types were similarly represented in oral cavity cancers. It remains to be evaluated whether these findings reflect possible differences in the carcinogenic process. We assessed ICD-O classification of all HPV-positive tumours of oral cavity cancer, and inferred that potential subsite misclassification could be clearly excluded since all tumours were classified as malignant neoplasms of upper gum (C03.0, n=1; C03.9, n=1), and of cheek mucosa (C06.0, n=1; C06.8, n=1).

The analysis of additional FFPE cancer blocks from five patients originally classified as HPV DNA-positive/HPV RNA-negative revealed that HPV DNA was only present in one specimen for each patient, indicating that HPV DNA was not homogeneously distributed in all cancer tissue, and its presence may be due to infection in other sites of the oral cavity. These findings are in agreement with previous studies that highlighted the limitations of HPV DNA detection alone as proof for viral causality[22-25].

A limitation of our study is the lack of information on other HNC risk factors that are well known to be present in India, such as alcohol consumption, smoking and use of smokeless tobacco products, e.g. pan masala, gutka, or zarda[13]. This limitation was mainly due to the fact that the study implied the retrieval of HNC specimens from the pathology archive, which was often not associated with detailed clinical information. We are currently performing prospective studies in this and other Indian regions to determine the possible interaction of HPV infection and other risk factors in HNC development. Another limitation of the study was the limited number of oropharyngeal cancers analysed. This was mainly due to the fact that only a small fraction of HNC occurs in the oropharynx in central India and a vast majority of available archived HNC tissue specimen was from the oral cavity.

$p16^{INK4a}$ staining appeared not to be a good surrogate marker of HPV transformation in these Indian HNC cases. More than half of HPV DNA/RNA-positive cases were $p16^{INK4a}$-negative, and a considerable number of HPV RNA-negative cases were $p16^{INK4a}$-positive. The first finding especially contrasts with the scenario observed in several European studies. In the Netherlands an assay algorithm for archival FFPE samples, consisting of double positivity after $p16^{INK4a}$ immunostaining followed by a PCR-based HPV DNA detection test was shown to be valid to identify HPV RNA-positive cases[27, 28]. An Italian study reported a fair agreement between HPV16 RNA positivity and $p16^{INK4a}$ overexpression in oropharyngeal cancer ($k = 0.61$)[36]. This difference between the various studies might be explained by the fact that certain populations are heavily exposed to additional carcinogens, which may lead to alterations of $p16^{INK4a}$ expression independently of the presence of HPV infection. Regarding the HPV DNA and RNA negatives but $p16^{INK4a}$ positives, a similar percentage of false HPV-positive cases has been reported in other studies, including a recent worldwide HNC study[37].

The results of this study warrant additional analyses to evaluate whether there might be geographical differences in $p16^{INK4a}$ positivity, which if confirmed in larger studies may impact the universal adoption of test algorithms for the classification of HNC that include $p16^{INK4a}$ staining.

In conclusion, our study provides novel insights on the contribution of mucosal HR-HPV types in the development of HNC and highlights important differences between published data from developed parts of the world and our own data from a rural hospital in central India.

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Declaration of Interests

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RR and SR are employees of Roche Diagnostics, a company that commercializes in vitro diagnostic tests used in this study.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *International journal of cancer Journal international du cancer* 2015;**136**: E359-86.

2. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer; 2013. Available from http://globocan.iarc.fr.

3. Marur S, D'Souza G, Westra WH, Forastiere AA. HPV-associated head and neck cancer: a virus-related cancer epidemic. *The Lancet Oncology* 2010;**11**: 781-9.

4. Gillison ML, Alemany L, Snijders PJ, Chaturvedi A, Steinberg BM, Schwartz S, Castellsague X. Human papillomavirus and diseases of the upper airway: head and neck cancer and respiratory papillomatosis. *Vaccine* 2012;**30 Suppl 5**: F34-54.

5. Gillison ML, Castellsague X, Chaturvedi A, Goodman MT, Snijders P, Tommasino M, Arbyn M, Franceschi S. Eurogin Roadmap: comparative epidemiology of HPV infection and associated cancers of the head and neck and cervix. *International journal of cancer Journal international du cancer* 2014;**134**: 497-507.

6. Ndiaye C, Mena M, Alemany L, Arbyn M, Castellsague X, Laporte L, Bosch FX, de Sanjose S, Trottier H. HPV DNA, E6/E7 mRNA, and p16INK4a detection in head and neck cancers: a systematic review and meta-analysis. *The Lancet Oncology* 2014;**15**: 1319-31.

7. Arbyn M, de Sanjose S, Saraiya M, Sideri M, Palefsky J, Lacey C, Gillison M, Bruni L, Ronco G, Wentzensen N, Brotherton J, Qiao YL, et al. EUROGIN 2011 roadmap on prevention and treatment of HPV-related disease. *International journal of cancer Journal international du cancer* 2012;**131**: 1969-82.

8. Anantharaman D, Gheit T, Waterboer T, Abedi-Ardekani B, Carreira C, McKay-Chopin S, Gaborieau V, Marron M, Lagiou P, Ahrens W, Holcatova I, Merletti F, et al. Human papillomavirus infections and upper aero-digestive tract cancers: the ARCAGE study. *J Natl Cancer Inst* 2013;**105**: 536-45.

9. Attner P, Du J, Nasman A, Hammarstedt L, Ramqvist T, Lindholm J, Marklund L, Dalianis T, Munck-Wikland E. The role of human papillomavirus in the increased incidence of base of tongue cancer. *International journal of cancer Journal international du cancer* 2010;**126**: 2879-84.

10. 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, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2011;**29**: 4294-301.

11. Nasman A, Attner P, Hammarstedt L, Du J, Eriksson M, Giraud G, Ahrlund-Richter S, Marklund L, Romanitan M, Lindquist D, Ramqvist T, Lindholm J, et al. Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma? *International journal of cancer Journal international du cancer* 2009;**125**: 362-6.

12. Ryerson AB, Peters ES, Coughlin SS, Chen VW, Gillison ML, Reichman ME, Wu X, Chaturvedi AK, Kawaoka K. Burden of potentially human papillomavirus-associated cancers of the oropharynx and oral cavity in the US, 1998-2003. *Cancer* 2008;**113**: 2901-9.

13. Gajalakshmi V, Hung RJ, Mathew A, Varghese C, Brennan P, Boffetta P. Tobacco smoking and chewing, alcohol drinking and lung cancer risk among men in southern India. *International journal of cancer Journal international du cancer* 2003;**107**: 441-7.

14. Choudhury JH, Ghosh SK. Promoter Hypermethylation Profiling Identifies Subtypes of Head and Neck Cancer with Distinct Viral, Environmental, Genetic and Survival Characteristics. *PloS one* 2015;**10**: e0129808.

15. Patel KR, Vajaria BN, Begum R, Desai A, Patel JB, Shah FD, Shukla SN, Patel PS. Prevalence of high-risk human papillomavirus type 16 and 18 in oral and cervical cancers in population

from Gujarat, West India. *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology* 2014;**43**: 293-7.

16. Bahl A, Kumar P, Dar L, Mohanti BK, Sharma A, Thakar A, Karthikeyan V, Sikka K, Singh C, Poo K, Lodha J. Prevalence and trends of human papillomavirus in oropharyngeal cancer in a predominantly north Indian population. *Head & neck* 2014;**36**: 505-10.

17. Jamaly S, Khanehkenari MR, Rao R, Patil G, Thakur S, Ramaswamy P, Ajaikumar BS, Sahoo R. Relationship between p53 overexpression, human papillomavirus infection, and lifestyle in Indian patients with head and neck cancers. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* 2012;**33**: 543-50.

18. Barwad A, Sood S, Gupta N, Rajwanshi A, Panda N, Srinivasan R. Human papilloma virus associated head and neck cancer: A PCR based study. *Diagnostic cytopathology* 2012;**40**: 893-7.

19. Jalouli J, Ibrahim SO, Mehrotra R, Jalouli MM, Sapkota D, Larsson PA, Hirsch JM. Prevalence of viral (HPV, EBV, HSV) infections in oral submucous fibrosis and oral cancer from India. *Acta oto-laryngologica* 2010;**130**: 1306-11.

20. Gheit T, Vaccarella S, Schmitt M, Pawlita M, Franceschi S, Sankaranarayanan R, Sylla BS, Tommasino M, Gangane N. Prevalence of human papillomavirus types in cervical and oral cancers in central India. *Vaccine* 2009;**27**: 636-9.

21. Mitra S, Banerjee S, Misra C, Singh RK, Roy A, Sengupta A, Panda CK, Roychoudhury S. Interplay between human papilloma virus infection and p53 gene alterations in head and neck squamous cell carcinoma of an Indian patient population. *Journal of clinical pathology* 2007;**60**: 1040-7.

22. Halec G, Holzinger D, Schmitt M, Flechtenmacher C, Dyckhoff G, Lloveras B, Hofler D, Bosch FX, Pawlita M. Biological evidence for a causal role of HPV16 in a small fraction of laryngeal squamous cell carcinoma. *British journal of cancer* 2013;**109**: 172-83.

23. Jung AC, Briolat J, Millon R, de Reynies A, Rickman D, Thomas E, Abecassis J, Clavel C, Wasylyk B. Biological and clinical relevance of transcriptionally active human papillomavirus (HPV) infection in oropharynx squamous cell carcinoma. *International journal of cancer Journal international du cancer* 2010;**126**: 1882-94.

24. Holzinger D, Schmitt M, Dyckhoff G, Benner A, Pawlita M, Bosch FX. Viral RNA patterns and high viral load reliably define oropharynx carcinomas with active HPV16 involvement. *Cancer Res* 2012;**72**: 4993-5003.

25. Chernock RD, Wang X, Gao G, Lewis JS, Jr., Zhang Q, Thorstad WL, El-Mofty SK. Detection and significance of human papillomavirus, CDKN2A(p16) and CDKN1A(p21) expression in squamous cell carcinoma of the larynx. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 2013;**26**: 223-31.

26. Jordan RC, Lingen MW, Perez-Ordonez B, He X, Pickard R, Koluder M, Jiang B, Wakely P, Xiao W, Gillison ML. Validation of methods for oropharyngeal cancer HPV status determination in US cooperative group trials. *The American journal of surgical pathology* 2012;**36**: 945-54.

27. Smeets SJ, Hesselink AT, Speel EJ, Haesevoets A, Snijders PJ, Pawlita M, Meijer CJ, Braakhuis BJ, Leemans CR, Brakenhoff RH. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. *International journal of cancer Journal international du cancer* 2007;**121**: 2465-72.

28. Rietbergen MM, Snijders PJ, Beekzada D, Braakhuis BJ, Brink A, Heideman DA, Hesselink AT, Witte BI, Bloemena E, Baatenburg-De Jong RJ, Leemans CR, Brakenhoff RH. Molecular characterization of p16-immunopositive but HPV DNA-negative oropharyngeal carcinomas. *International journal of cancer Journal international du cancer* 2014;**134**: 2366-72.

29. Shi W, Kato H, Perez-Ordonez B, Pintilie M, Huang S, Hui A, O'Sullivan B, Waldron J, Cummings B, Kim J, Ringash J, Dawson LA, et al. Comparative prognostic value of HPV16 E6 mRNA compared with in situ hybridization for human oropharyngeal squamous carcinoma. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2009;**27**: 6213-21.

30. Gheit T, Landi S, Gemignani F, Snijders PJ, Vaccarella S, Franceschi S, Canzian F, Tommasino M. Development of a sensitive and specific assay combining multiplex PCR and DNA

microarray primer extension to detect high-risk mucosal human papillomavirus types. *J Clin Microbiol* 2006;**44**: 2025-31.

31. Schmitt M, Dondog B, Waterboer T, Pawlita M, Tommasino M, Gheit T. Abundance of multiple high-risk human papillomavirus (HPV) infections found in cervical cells analyzed by use of an ultrasensitive HPV genotyping assay. *J Clin Microbiol* 2010;**48**: 143-9.

32. Halec G, Schmitt M, Dondog B, Sharkhuu E, Wentzensen N, Gheit T, Tommasino M, Kommoss F, Bosch FX, Franceschi S, Clifford G, Gissmann L, et al. Biological activity of probable/possible high-risk human papillomavirus types in cervical cancer. *International journal of cancer Journal international du cancer* 2013;**132**: 63-71.

33. Kumar RV, Kadkol SS, Daniel R, Shenoy AM, Shah KV. Human papillomavirus, p53 and cyclin D1 expression in oropharyngeal carcinoma. *International journal of oral and maxillofacial surgery* 2003;**32**: 539-43.

34. Kumar RV, Shenoy AM, Daniel R, Shah KV. Cyclin D1, p53, MIB1, intratumoral microvessel density, and human papillomavirus in advanced laryngeal carcinoma: association with nodal metastasis. *Otolaryngology--head and neck surgery : official journal of American Academy of Otolaryngology-Head and Neck Surgery* 2004;**131**: 509-13.

35. Bhosale PG, Pandey M, Desai RS, Patil A, Kane S, Prabhash K, Mahimkar MB. Low prevalence of transcriptionally active human papilloma virus in Indian patients with HNSCC and leukoplakia. *Oral surgery, oral medicine, oral pathology and oral radiology* 2016;**122**: 609-18.e7.

36. Bussu F, Sali M, Gallus R, Vellone VG, Zannoni GF, Autorino R, Dinapoli N, Santangelo R, Martucci R, Graziani C, Micciche F, Almadori G, et al. HPV infection in squamous cell carcinomas arising from different mucosal sites of the head and neck region. Is p16 immunohistochemistry a reliable surrogate marker? *British journal of cancer* 2013;**108**: 1157-62.

37. Castellsague X, Alemany L, Quer M, Halec G, Quiros B, Tous S, Clavero O, Alos L, Biegner T, Szafarowski T, Alejo M, Holzinger D, et al. HPV Involvement in Head and Neck Cancers: Comprehensive Assessment of Biomarkers in 3680 Patients. *J Natl Cancer Inst* 2016;**108**: djv403.

Table 1: Description of study group

Description	All HNC (N=364)		Oral Cavity (n=252)		Oropharynx (n=53)		Larynx* (n=59)		
	n	%	n	%	n	%	n	%	p-value
Age (in years)									
Median (Range)	53	(22-87)	50	(22-85)	60	(35-80)	58	(25-87)	
IQR	45-65		45-60		50-66		48-65		
Mean (SD)	53.6	(12.7)	52.2	(12.7)	58.2	(11.7)	55.6	(12.6)	0.01[&]
Gender									
Men	263	(72.3)	176	(69.8)	46	(86.8)	41	(69.5)	
Women	101	(27.7)	76	(30.2)	7	(13.2)	18	(30.5)	0.03[Ŧ]
Histological diagnosis									
NOS SCC	95	(26.1)	65	(25.8)	15	(28.3)	15	(25.4)	
Conventional NOS SCC	47	(12.9)	29	(11.5)	9	(17.0)	9	(15.3)	
Conventional Keratinizing SCC	117	(32.1)	94	(37.3)	10	(18.9)	13	(22.0)	
Conventional Non-keratinizing SCC	58	(15.9)	37	(14.7)	9	(17.0)	12	(20.3)	
Basaloid SCC	10	(2.7)	1	(0.4)	5	(9.4)	4	(6.8)	
Verrucous SCC	23	(6.3)	18	(7.1)	2	(3.8)	3	(5.1)	
Other SCC**	12	(3.3)	7	(2.8)	3	(5.7)	2	(3.4)	
Other non-SCC***	2	(0.5)	1	(0.4)	0	(0.0)	1	(1.7)	0.01[Ŧ]

Recruitment period: mid-2005-December 2011. "HNC": Head and neck cancers; "IQR": Interquartile range; "SD": Standard deviation; "SCC": Squamous cell carcinoma; NOS: Not otherwise specified; *: includes hypopharyngeal cancer (n=18), **: includes acantholytic (n=3), papillary (n=7) and sarcomatoid (n=2); ***: includes adenosquamous carcinoma (n=1) and undifferentiated carcinoma (n=1); [&]: ANOVA test p-value; [Ŧ]: Fisher's exact test.

Table 2: HPV DNA, RNA and p16^{INK4a} positivity in HNC subsites by HPV status

		All HNC (N=364)		Oral cavity (n=252)		Oropharynx (n=53)		Larynx* (n=59)		
HPV type	**Marker positivity**	Positive	Proportion (95%CI)	Positive	Proportion (95%CI)	Positive	Proportion (95%CI)	Positive	Proportion (95%CI)	Fisher's exact test p-value
Any HPV	DNA	50	13.7 (10.4-17.7)	30	11.9 (8.2-16.6)	10	18.9 (9.4-32.0)	10	16.9 (8.4-29.0)	0.266
	DNA & RNA‡	10	2.7 (1.3-5.0)	4	1.6 (0.4-4.0)	5	9.4 (3.1-20.7)	1	1.7 (0.0-9.1)	**0.015**
	DNA, RNA & p16^{INK4a}‡	4	1.1 (0.3-2.8)	0	0.0 (0.0-1.5)*	3	5.7 (1.2-15.7)	1	1.7 (0.0-9.1)	**0.003**
HPV16	DNA	36	9.9 (7.0-13.4)	23	9.1 (5.9-13.4)	8	15.1 (6.7-27.6)	5	8.5 (2.8-18.7)	0.385
	DNA & RNA	6	1.6 (0.6-3.6)	2	0.8 (0.1-2.8)	3	5.7 (1.2-15.7)	1	1.7 (0.0-9.1)	**0.033**
	DNA, RNA & p16^{INK4a}	3	0.8 (0.2-2.4)	0	0.0 (0.0-1.5)*	2	3.8 (0.5-13.0)	1	1.7 (0.0-9.1)	**0.017**
Non-HPV16 HR types	DNA	14	3.8 (2.1-6.4)	7	2.8 (1.1-5.6)	2	3.8 (0.5-13.0)	5	8.5 (2.8-18.7)	0.105
	DNA & RNA	4	1.1 (0.3-2.8)	2§	0.8 (0.1-2.8)	2ϕ	3.8 (0.5-13.0)	0	0.0 (0.0-6.1)**	0.149
	DNA, RNA & p16^{INK4a}	1	0.3 (0.0-1.5)	0	0.0 (0.0-1.5)*	1	1.9 (0.0-10.1)	0	0.0 (0.0-6.1)**	0.146

‡HPV RNA and p16 expression were examined in all HPV DNA-positive cases (n=50) and a random of HPV DNA negatives (n=38; 12%). All HPV DNA negative were RNA-negative. Three cases were p16^{INK4a}-positive.

*includes hypopharyngeal cancer (n=18)

CI: Confidence interval; **: 97.5%CI

§HPV18 (n=1) and HPV35 (n=1)

ϕHPV35 (n=1) and HPV56 (n=1)

Table 3: p16^{INK4a} IHC data stratified per HPV DNA and HPV RNA status

HPV type		**Any HPV DNA-positive** (n=50)				**Any HPV DNA-negative*** (n=38)	
		Any RNA+ (n=10)		Any RNA- (n=40)		Any RNA- (n=38)	
		p16+	p16-	p16+	p16-	p16+	p16-
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
	HNC	4 (40.0)	6 (60.0)	11 (27.5)	29 (72.5)	3 (7.9)	35 (92.1)
Subsite	Oral cavity	0 (0)	4 (40.0)	8 (20.0)	18 (45.0)	2 (5.3)	27 (71.1)
	Oropharynx	3 (30.0)	2 (20.0)	0 (0)	5 (12.5)	1 (2.6)	3 (7.9)
	Hypopharynx/Larynx	1 (10.0)	0 (0)	3 (7.5)	6 (15.0)	0 (0)	5 (13.2)
HPV type		**HPV16 DNA-positive** (n=36)				**HPV16 DNA-negative*** (n=52)	
		HPV16 RNA+ (n=6)		HPV16 RNA- (n=30)		HPV16 RNA- (n=52)	
		p16+	p16-	p16+	p16-	p16+	p16-
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
	HNC	3 (50.0)	3 (50.0)	9 (30.0)	21 (70.0)	6 (11.5)	46 (88.5)
Subsite	Oral cavity	0 (0)	2 (33.3)	7 (23.3)	14 (46.7)	3 (5.8)	33 (63.5)
	Oropharynx	2 (33.3)	1 (16.7)	0 (0)	5 (16.7)	2 (3.8)	4 (7.7)
	Hypopharynx/Larynx	1 (16.7)	0 (0)	2 (6.7)	2 (6.7)	1 (1.9)	9 (17.3)
HPV type		**Other HR-HPV DNA-positive** (n=14)				**Other HR-HPV DNA-negative*** (n=74)	
		Other HR-HPV RNA+ (n=4)		Other HR-HPV RNA- (n=10)		Other HR-HPV RNA- (n=74)	
		p16+	p16-	p16+	p16-	p16+	p16-
		n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
	HNC	1 (25.0)	3 (75.0)	2 (20.0)	8 (80.0)	15 (20.3)	59 (79.7)
Subsite	Oral cavity	0 (0)	2 (50.0)	1 (10.0)	4 (40.0)	9 (12.2)	43 (58.1)
	Oropharynx	1 (25.0)	1 (25.0)	0 (0)	0 (0)	3 (4.1)	9 (12.2)
	Hypopharynx/Larynx	0 (0)	0 (0)	1 (10.0)	4 (40.0)	3 (4.1)	7 (9.5)

*None of the HPV DNA-negative cases were RNA-positive; Represents column percentages.

p-value for the comparison of p16^{INK4a} result in HPV DNA- cases between subsites=0.62; p-value for the comparison of p16^{INK4a} result in HPV DNA+ cases between HPV types=0.51

p-value for the comparison of p16^{INK4a} result in HPV DNA+/RNA+ cases between subsites=0.10; p-value for the comparison of p16^{INK4a} result in HPV DNA+/RNA+ cases between HPV types=0.57