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Significance of transcriptionally-active high-risk human papillomavirus in sinonasal squamous cell carcinoma: Case series and a meta-analysis

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Sinonasal cancers represent a highly heterogeneous group of head and neck cancers, for which etiological and prognostic significance of high-risk human papillomavirus (HPV) infections has not yet been conclusively established. We investigated the presence of transcriptionally-active high-risk HPV in a series of 34 sinonasal squamous cell cancer (SNSCC) cases and evaluated the effect of transcriptionally-active HPV on the overall survival. In addition, we performed a meta-analysis of previously published studies, including this study, to summarize the prevalence of HPV positivity across histological subtypes of SNSCC. The presence of transcriptionally-active HPV was detected by HPV mRNA using the polymerase chain reaction (PCR) or *in situ* hybridization (ISH). p16 expression was evaluated as a surrogate marker for transcriptionally-active HPV infection by immunohistochemistry (IHC), the presence of high-risk HPV DNA was tested by PCR and the HPV genotypes were determined by sequencing of PCR amplicons. Transcriptionally-active HPV infections were found in ~25% of the SNSCC cases. The role of HPV infection in keratinizing SNSCC may be higher than previously reported (~32% in our study vs. ~0-6.3% in all other studies). Patients with transcriptionally-active HPV-positive SNSCCs were more likely to be diagnosed at earlier stages ($p<0.05$) and displayed better mean overall survival, although the difference between HPV-positive and HPV-negative groups was not statistically significant. In contrast to other non-oropharyngeal squamous cell carcinomas (non-OPSCCs) of the head and neck, in SNSCCs, p16/IHC and p16/IHC+HPV DNA displayed high specificity as surrogate markers of transcriptionally-active HPV infections. However, p16/IHC may have significantly lower sensitivity as a surrogate marker of transcriptionally-active HPV in SNSCCs compared to OPSCCs. Furthermore, in our group of SNSCCs, all cases positive for high-risk HPV DNA by PCR were also transcriptionally-active (causative) infections with positive HPV mRNA by ISH. Our results imply a possible different role of HPV-mediated carcinogenesis of squamous cell epithelium in oropharyngeal and sinonasal sites with the latter displaying a lower proportion of causative HPV infections; nevertheless, most cases positive for high-risk HPV DNA, p16/IHC or combination thereof were also found positive for transcriptionally-active HPV. The prognostic significance of HPV status in SNSCCs remains inconclusive and future studies should investigate the presence of transcriptionally-active HPV by direct HPV testing.

Key words: sinonasal, squamous cell carcinoma, human papillomavirus, survival, p16

High-risk human papillomavirus (HPV) is now recognized as the principal cause of the growing incidence of oropharyngeal squamous cell carcinoma (OPSCC) in some parts of the world. The HPV status is also recognized as an independent predictor of improved overall and disease-free survival (OS and DFS) in these patients [1]. While OPSCC represents the most common site and histological type of head and neck cancers, sinonasal squamous cell carcinomas (SNSCC) are among the least frequent tumors of the head and neck (~3–5%) [2].

The nasal cavity and paranasal sinuses represent small anatomical space with unmatched histological diversity of malignant tumors that could arise in these sites. Compared to the other head and neck subsites, the sinonasal tract shows

the lowest proportion of squamous cell carcinomas (SCC) relative to other carcinoma types (~65–70%) [2], but this proportion shows an increasing secular trend. This increasing proportion of SNSCC reflects a decreasing proportion of occupational risks-related sinonasal adenocarcinomas, at least in populations, where measures to prevent or diminish occupational exposures had been implemented [3].

In spite of the decreasing incidence of SNSCC over the last three decades, and a decreasing proportion of patients presenting with advanced disease, SNSCC remains a medical challenge due to its poor overall survival, which remained virtually unchanged over time [4, 5].

Development of SNSCC has been traditionally associated with exposure to wood dust, leather dust, some industrial chemicals, and smoking [2, 4, 6], and more recently, the sinonasal tract has been considered as another "hot-spot" for carcinomas with transcriptionally-active HPV infections [7]. Supporting the association with HPV, two large meta-analyses [8, 9] reported ~30% overall prevalence of HPV-positivity in sinonasal carcinomas. Nevertheless, studies included in these meta-analyses relied almost exclusively either on HPV DNA detection by the polymerase chain reaction (PCR), or HPV DNA by *in situ* hybridization (ISH), which do not distinguish between transcriptionally-active ("driving") and transcriptionally-inactive ("passenger" or "bystander") HPV infections. To date, only a limited number of studies evaluated the presence of transcriptionally-active HPV in SNSCCs either i) directly, through the presence of HPV mRNA by PCR or ISH, or ii) by inference, through the presence of diffuse (≥70%) nuclear and cytoplasmic p16 immunostaining in tissues positive for high-risk HPV DNA as a surrogate marker [10–16]. In addition, the prognostic significance of the HPV status in SNSCCs has not yet been conclusively established, as some investigators reported significantly better prognosis for HPV-positive cases [17], while others found no significant difference in survival between patients with HPV-positive and HPV-negative tumors [18]. Because of the lack of conclusive evidence for the association between the HPV status and treatment response or disease outcome, recently published guidelines of the College of American Pathologists do not recommend routine HPV testing in patients with sinonasal tumors [19].

In this study, we investigated the presence of transcriptionally-active high-risk HPV in a series of SNSCC cases and evaluated the effect of transcriptionally-active HPV on the overall survival. In addition, we performed a meta-analysis of previously published studies, including this study, to summarize the prevalence of HPV positivity across histological subtypes of SNSCC. Our results indicate, that in SNSCCs, p16/IHC and p16/IHC+HPV DNA display appreciable specificity for the detection of transcriptionally-active HPV, which is remarkably different from other non-oropharyngeal SCCs of the head and neck. We also show that high-risk HPV may play a more significant role in keratinizing SNSCCs than previously considered. The HPV-positive status is associated with lower clinical stage at SNSCC diagnosis and improved overall survival, which however did not reach statistical significance.

Patients and methods

Patients and tissue specimens. The study was performed following the rules of the Faculty Hospital in Pilsen Ethics Committee. 34 patients with SNSCC diagnosed between the years of 2002 and 2014 were retrieved from the pathology files of two tertiary referral hospitals (Louis Pasteur University Hospital in Košice, Slovakia and Faculty Hospital in Pilsen, Czech Republic), and a large private pathology laboratory in Prešov, Slovakia. Hematoxylin-eosin and immunohistochemical stains were reviewed to confirm the diagnosis of SNSCC and to evaluate the histologic features. Demographic data, including occupational and smoking history, tumor localization, TNM stage, and the treatment modalities, including therapy at disease recurrence, were retrieved from medical records.

p16 immunohistochemical staining. For the immunohistochemistry (IHC), the most representative paraffin block with tumor tissue was selected in each case and 4 μm tissue sections were stained with the p16 antibody (CINtec® p16 Histology, Ventana) using the Ventana Benchmark automated stainer, according to the manufacturer's protocol with appropriate positive and negative control slides. The expression of p16 was evaluated as positive, if the nuclear and cytoplasmic staining were present in ≥70% of tumor cells because, at this cut-off level, the p16-immunostaining has been shown to correlate best with the presence of transcriptionally-active HPV in the HPV-related OPSCCs [20].

Polymerase chain reaction and *in situ* hybridization. Genomic DNA was isolated from paraffin-embedded tissue using the QIAsymphony SP instrument using special precautions to prevent contamination of DNA. The HPV DNA was detected using a set of PCRs with primer systems CPSGB, GP5+/GP6+, and type-specific primers for HPV 16, 18, 31, 33, 35, 45. Positive PCR samples were genotyped by sequencing and the sequences were analyzed by BLAST [21]. Expression of HPV16 E6 mRNA was examined through the detection of its most abundant splice variant E6*I [22].

HPV mRNA *in situ* hybridization was performed using the RNAscope HPV-test (Advanced Cell Diagnostics) with an HPV-HR18 probe on automated system Discovery Ultra by Ventana Medical systems. HPV-HR18 probe detects 18 HPV types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82). The result is considered positive if the RNAscope ISH signal is strong, with a pattern of clear punctate chromogenic dots in the cell nucleus and/or cytoplasm.

Meta-analysis search strategy and selection criteria. PubMed database was searched for all relevant peer-reviewed research reports written in English, using a combination of the following keywords: "squamous cell carcinoma",

"sinonasal", "paranasal", "nasal", "human papillomavirus", and "HPV". References listed in the retrieved literature, including those listed in two previously published meta-analyses [8, 9], were examined and included to the reference corpus if their titles contained the words "HPV" and any of the other keywords indicated above. The reference corpus was screened for the relevance using the following Population-Exposure-Comparator-Outcome (PECO) statement: Population: general population (non-occupational) groups of patients diagnosed with SNSCC; Exposure: N/A; Comparator: different SNSCC histological types; Outcome: prevalence of directly determined or inferred transcriptionally-active HPV status in at least 5 identified SNSCC cases. Reference corpus was screened for eligible studies using the title/abstract screening level and subsequently the full-text screening level. Eligible studies that met PECO criteria were assessed for methodological/reporting quality. Among studies reporting p16 status by IHC, only those that considered diffuse staining in ≥70% cells as a cut-off level were included to the meta-analysis. The selection of studies is depicted in PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) diagram (Figure 1).

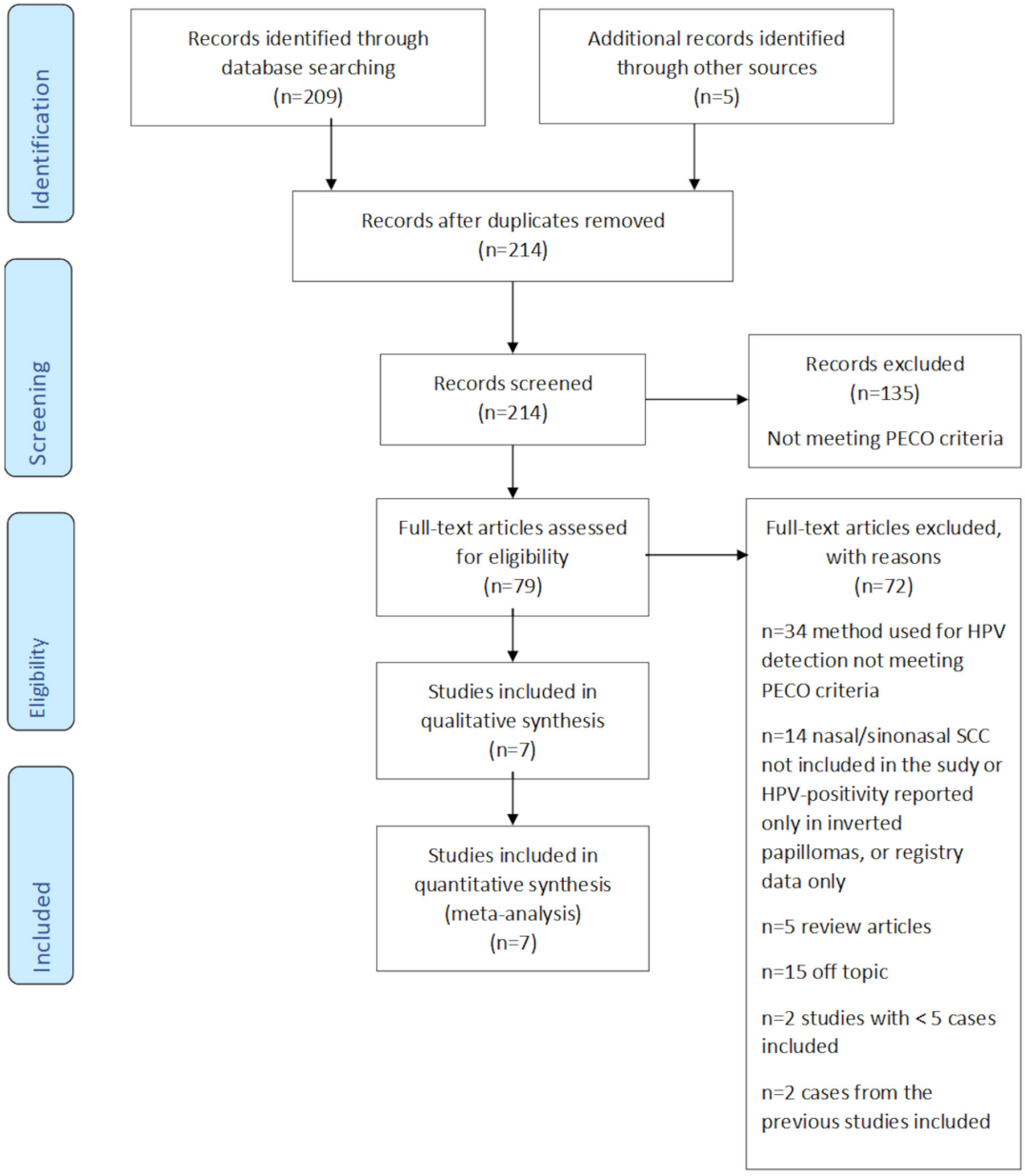

Figure 1. Flow diagram of selection of studies included in meta-analysis following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).

Statistical analysis. Statistical significance of differences between HPV-positive and HPV-negative groups was evaluated using the Mann-Whitney test and Fisher's exact test for continuous and dichotomous variables, respectively. Association between multilevel categorical variables and HPV-status was examined using the univariate logistic regression. Multivariate model was built using logistic regression modeling with forward selection of variables p16, stage, and age as predictors of HPV status and variables were entered if associated Wald test p-value <0.05 and removed if Wald test p-value >0.1. Median survival was determined by the Kaplan-Meir analysis for all patients and the significance of difference between HPV-negative and HPV-positive subgroups was tested by log rank test. The relative risk of dying for HPV-positive vs. HPV-negative patients (hazard ratio, HR) was determined by univariate Cox proportional hazards model. Meta-analysis for proportion of HPV-positive SNSCC cases was performed on studies meeting PECO eligibility and study quality criteria, including this study. Difference between proportion of HPV-positive and p16-positive cases was tested using McNemar's test.

Results

Demographic and clinical variables. Sinonasal carcinoma was diagnosed in 24 male and 10 female patients at a median age of 57 years (range 18–84 years). The men-to-women ratio in our sample is consistent with the reported two-fold higher occurrence of the disease in men relative to women [2].

Clinicopathological data for 34 SNSCC cases are summarized in Supplementary Table S1. The tumors were classified as keratinizing squamous cell carcinoma (K-SCC, n=19), non-keratinizing SCC (NK-SCC; including NK-SCC with maturation/hybrid SCC, n=14), and sarcomatous SCC (S-SCC, n=1). Other known SCC histological subtypes were not identified among cases included in this study. Only two SNSCCs developed from sinonasal inverted papilloma.

Among the 34 SNSCC cases, transcriptionally-active HPV was detected by mRNA in 8 cases (4 positive by ISH and PCR, 3 positive by ISH, and 1 by PCR). Of them, 7 were positive and one tested negative for HR HPV DNA by PCR.

HPV DNA was found positive in 7 patients, all of whom were also positive for mRNA by at least one of the two assays employed for HPV mRNA detection. HPV 16 was detected in 5 patients, which makes it the most common genotype in our series of SNSCCs. HPV 18 and HPV 45 genotypes were each detected in one case.

HPV DNA was negative in 27 SNSCC cases, of which 26 were also negative for mRNA HPV. A single SNSCC case was negative for HPV DNA but positive for mRNA by ISH.

The proportion of tumors with transcriptionally-active HPV (detected directly by positive HPV mRNA) was higher in female than in male patients (40% vs. 16.7%), but the difference was not statistically significant (p=0.19, Supplementary Table S1). Similarly, HPV mRNA-positive and mRNA-negative groups did not differ significantly in mean ages, smoking status, or distribution of tumor histological subtypes (Supplementary Table S1). Nevertheless, HPV mRNA-positive sinonasal cancers displayed significantly higher proportion of immunoreactivity for p16 than HPV-negative cancers (62.5% vs. 7.7%; $DCI_{95=}$18.9–79.3%; p=0.004). p16-expression was also found to be a significant predictor of transcriptionally active-HPV status by a univariate logistic regression (OR=20.00; CI95:2.52–152.61; p=0.0039).

Sensitivity of detection of transcriptionally-active HPV status via p16 as a surrogate marker was 62.5% (CI95: 25.9–89.8%) and specificity 92.3% (73.4–98.7%) considering HPV mRNA ISH + HPV16 E6 mRNA as a "gold standard" test for transcriptionally-active HPV status. The differences between sensitivities and specificities of p16/IHC and the "gold standard" test were not statistically significant (McNemar's p=0.25 and p=0.5, respectively). The difference between proportions of p16/IHC-positive and transcriptionally-active HPV cases (detected by HPV mRNA) was not significant (20.6% vs 23.5%; McNemar's test p=1).

All SNSCC cases positive simultaneously for p16/IHC and HPV DNA (n=5) were also positive for HPV mRNA. Taken together, a surrogate marker requiring double-positivity of p16/IHC and HPV DNA/PCR would have an estimated sensitivity of 62.5% (CI95: 24.5–91.5%) and specificity of 100% (CI95: 86.8–100%).

SNSCC cases with transcriptionally-active HPV status tended to be diagnosed at lower clinical stages. In a univariate logistic regression (Supplementary Table S2), the odds ratios for HPV-positive status in stage I vs. stage IV (IVa + IVb + IVc) and stage III vs. stage IV were 48 (CI95:2.31–997.2) and 24 (CI95:1.62–356.65), respectively. Multivariate logistic regression modeling with the forward selection of variables p16 (negative, positive), stage (I–IV), and age as predictors of HPV status retained stage III variable ($OR_{stage\ III/IV=}$19.7; CI95:1.23–315.06) and p16-status (OR=59.27; CI95:2.98–1179.69) as statistically significant predictors of HPV status (Supplementary Table S3).

Analysis of survival. During the follow-up time of 2–148 months (median = 23.3 months), 16 patients died of disease and 18 patients survived or died of unrelated causes. The median overall survival determined by the Kaplan-Meir analysis was 46.6 months (CI95=19.4–90.4 months). Patients with transcriptionally-active HPV-positive tumors showed improved overall survival (Figure 2), but the difference was not statistically significant (Log-rank test p=0.60). The hazard ratio of dying for HPV-positive patients relative to HPV-negative patients was HR=0.71 (CI95:0.20–2.54) as determined by a univariate Cox proportional hazards model.

Occupational and lifestyle exposures. Occupational exposure that may be relevant for the risk of development of sinonasal carcinoma was identified in one case of SNSCC with transcriptionally-active HPV (firefighter by occupa-

tion) and one HPV-negative patient (occupational exposure to metallic dust). A small number of identified exposures, the incompleteness of exposure data, and potential co-exposures (both identified cases were current or past smokers) did not allow to assess associations between these exposures and SNSC.

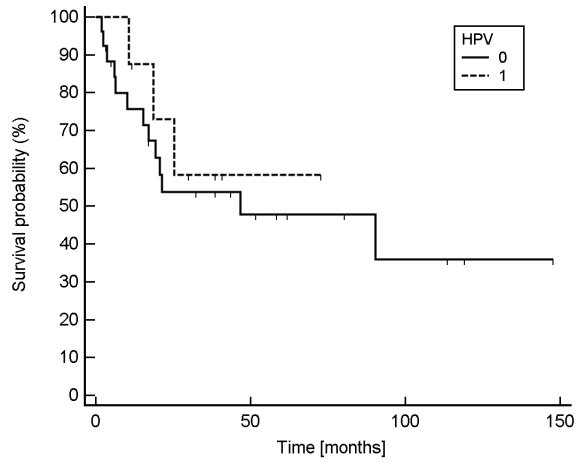

Figure 2. Kaplan-Meir analysis of overall survival of patients with sinonasal squamous cell cancers (SNSCC) with transcriptionally-active HPV status determined by HPV mRNA ISH: HPV 1-positive; HPV 0-negative.

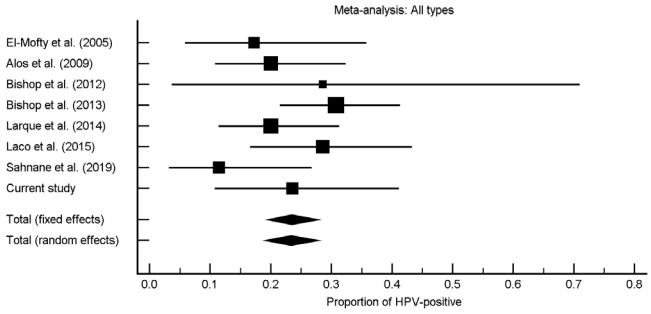

Figure 3. Forrest plot of the results of the meta-analysis for the proportion of transcriptionally-active HPV-positivity (detected by mRNA or inferred from p16/IHC+HPV DNA) among patients diagnosed with sinonasal squamous cell cancers (SNSCC).

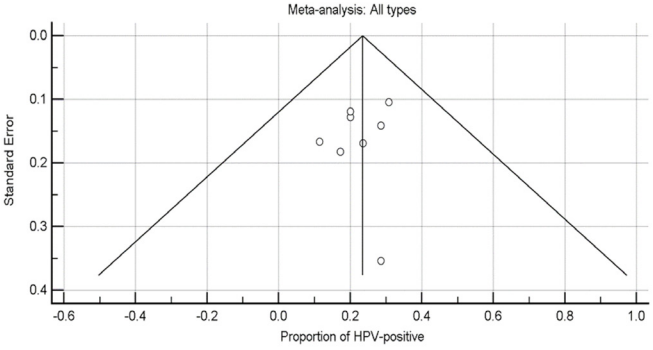

Figure 4. A funnel plot for meta-analysis of the proportion of HPV-positivity among SNSCC cases.

Meta-analysis. This meta-analysis was performed to compare and aggregate the results of the studies that reported the presence of transcriptionally-active HPV in SNSCC. Since only a few studies employed mRNA-based methods for direct detection of transcriptionally-active HPV, our meta-analysis also included those studies that inferred transcriptionally-active HPV status based on simultaneously positive high-risk HPV DNA and p16/IHC statuses.

The search and evaluation of references identified seven studies that met the criteria for inclusion (Supplementary Table S4) [10–16]. A meta-analysis of the proportion of cases with transcriptionally-active HPV in these studies (by mRNA or by inference from p16 and DNA), as well as the current study, estimated mean proportion of positive cases to be 23.5% (CI95:19.3–28.0%) for the fixed effects model, and 23.3% (CI95:19.9–28.0%) for the random effects model (Figure 3). The Cochran`s Q test (Q=7.88; p=0.344) and I^2 (I^2=11.14%) support consistency of the results across all included studies. Furthermore, the distribution of studies among the summary line is not indicative of publication bias (Figure 4).

Further, to determine whether keratinizing (K-SCC) and non-keratinizing (NK-SCC) subtypes differ in proportions of transcriptionally active HPV-positivity, we performed meta-analysis separately for each of these histological subtypes of SNSCC (Figures 5 and 6).

For K-SCC, meta-analysis identified significant statistical heterogeneity (Q=12.7; DF=5; p=0.0265; I^2=60.6%). The heterogeneity was introduced by our study, which found the proportion of HPV-positivity as 31.6% (CI95:12.6–56.6%), while in the remaining studies, this proportion ranged from 0% to 6.3%. Meta-analysis limited to these remaining studies found the total proportion of HPV-positivity in K-SCC as 4.8% (CI95:2.0–8.7%) with no statistically significant heterogeneity (Q=2.17, DF=4, p=0.70, I^2=0.00%).

Meta-analysis for NK-SCC determined mean proportion of HPV-positive cases as 39.10% (CI95:30.5–48.3%) for the fixed-effects model and 40.0% (CI95:29.0–51.6%) for the random-effects model, with no statistically significant heterogeneity (Q=7.8; DF=5; p=0.17; I^2=36.2%).

Discussion

High-risk human papillomavirus (HPV) has been established both as an etiological agent and a positive prognostic marker in oropharyngeal carcinoma [23]; however, the role of persistent infections by high-risk HPV in the etiology and the disease outcome of sinonasal cancers remains unclear at this time. Based on the cancer registry data, which do not distinguish between transcriptionally-active and "passenger" HPV infections, HPV status in SNSCC has been reported as a favorable prognostic factor [17] or a variable not associated with survival [18]. However, only a few studies reported the HPV-positivity of sinonasal carcinomas in the context of transcriptionally-active high-risk HPV [10–16]. Two

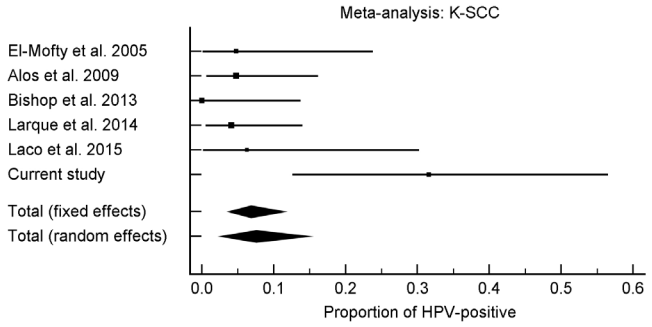

Figure 5. Forrest plot of the results of the meta-analysis for the proportion of transcriptionally-active HPV-positivity (detected by mRNA or inferred from p16/IHC+HPV DNA) among patients diagnosed with keratinizing sinonasal squamous cell cancers (K-SCC).

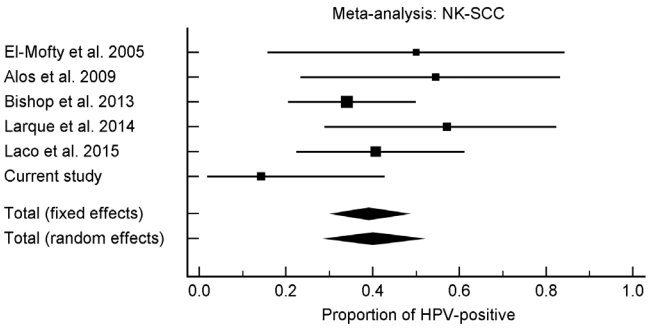

Figure 6. Forrest plot of the results of the meta-analysis for the proportion of transcriptionally-active HPV-positivity (detected by mRNA or inferred from p16/IHC+HPV DNA) among patients diagnosed with non-keratinizing sinonasal squamous cell cancers (NK-SCC).

of these studies found significantly improved OS and DFS in HPV-positive groups [11, 13], and two studies showed a trend towards better prognosis without statistical significance [14, 15].

Our data also suggest a trend towards better survival of patients with transcriptionally-active HR-HPV status, albeit not a statistically significant difference between the HPV mRNA-positive and mRNA-negative groups. In addition, our results, as well as the results of other investigators, imply that HPV-positive SNSCCs tend to be diagnosed at lower clinical stages than HPV-negative cancers [23], and this finding may be the underlying cause behind the improved survival reported in some studies, rather than biological and clinical differences between these disease entities. Nevertheless, small sample sizes together with the lack of control for potential confounders in all these studies, including ours, imply the need for further investigations in this matter.

Our results demonstrated transcriptionally-active high-risk HPV in 23.5% (CI95:10.7–41.2%) cases of SNSCC. This finding is consistent with the results of 7 other studies that found proportions of transcriptionally-active HPV (by mRNA or inference from DNA and p16/ISH) in SNSCCs ranging from 11.4–31.1% [10–16]. Consequently, our results support the etiological role of high-risk HPV in some squamous cell carcinomas arising in the sinonasal tract.

Previous studies of SNSCC reported HPV-positive status most commonly in non-keratinizing squamous cell carcinomas [13], papillary and basaloid carcinomas [17], adenosquamous carcinomas [2], and carcinoma with adenoid cystic-like features [24]. In contrast, keratinizing SNSCC reportedly displayed a much lower proportion of transcriptionally-active high-risk HPV cases [2]. It is therefore intriguing that our study found a considerably higher proportion of HPV mRNA-positivity in K-SCC than six other studies that also considered transcriptionally-active HPV through mRNA or p16/IHC+DNA statuses (~32% in our study vs. ~0–6.3% in all other studies) [10–15]. This difference may reflect differences in populations included in the analysis. For instance, the study by Laco et al. [15] included patients diagnosed in the same narrow geographic region as our study; however, SNSCC patients differed between the two studies at least in age distributions, with the median age at cancer diagnosis of 61 years vs. 57 years for K-SCC, and 67 years vs. 56.5 years for NK-SCC. Our dataset included younger patients to the meta-analysis (median 57 years; range: 18–84 years), than other studies such as Laco et al. (median: 62 years; range 23–85 years) [15] or Larque et al. (median: 63.6 years; range 40–93) [14]. Since our HPV-positive cases with K-SCC histology tended to be diagnosed at a younger age than HPV-negative K-SCC cases (median: 48 years vs. 57 years), we hypothesize that other studies included fewer of these young patients, for whom the K-SCC tumor histology may be associated with HPV-positive status. This could have projected into the lower proportion of HPV-positivity for K-SCC histology groups in studies that tended to include older patients. Nevertheless, we cannot rule out other reasons for the difference, including intricacies that may arise in the morphological diagnosis of K-SCC.

p16 expression with the cut-off set at ≥70% strongly correlates with the HPV infection in OPSCC [20]. Since HPV-positive OPSCCs display more favorable prognosis than HPV-negative OPSCCs, pathologists are currently recommended to test primary OPSCCs by p16/IHC, which serves as a surrogate marker for transcriptionally-active HPV, including additional HPV-specific tests at the discretion of the pathologist, treating clinician, or in the context of a clinical trial [19]. In contrast, the survival benefit of HPV-positive status has not yet been conclusively established for SNSCC.

Our results suggest that p16/IHC may have lower sensitivity as a surrogate marker of transcriptionally-active HPV in SNSCCs compared to OPSCCs. Lewis et al. [25] reported that 158 of 163 HPV-positive OPSCC cases were also positive for p16/IHC, while our study found 5 of 8 HPV mRNA-positive SNSCC cases to be positive for p16/IHC.

The difference in the prevalence of p16-expression between HPV-positive OPSCC and HPV-positive SNSCC (96.9% vs. 62.5%) is statistically significant (D=34.4%; CI95=10.3–66.4%; p<0.0001) and suggests a more frequent occurrence of SNSCC cases, which are positive for transcriptionally-active HPV, but also p16-negative, compared to OPSCCs. Nevertheless, our results provide only an imprecise estimate for the sensitivity of p16/IHC as a surrogate marker of transcriptionally-active HPV (CI95:24.5–91.5%).

Conversely, our results suggest higher specificity of p16/IHC as a surrogate marker for transcriptionally-active HPV in SNSCCs relative to OPSCCs. The study by Lewis et al. [25] reported p16/IHC-positivity in 26 of 73 HPV-negative OPSCC cases, while our study found only two cases among 26 HPV-negative SNSCCs, which were also positive for p16/IHC. As a result, HPV-negative OPSCC cases were more likely p16/IHC-positive (35.6%) than HPV-negative SNSCC in our study (7.7%), and this difference is statistically significant (D=27.9%; CI95:8.6–40.6%; p=0.007). Thus, the estimated specificity of p16/IHC as a surrogate marker for the detection of transcriptionally-active HPV seems to be higher in SNSCCs (92.3%; CI95:74.9–99.1%) than in OPSCCs (64.4%; 52.3–75.2%). Based on our results, this specificity in SNSCCs may be further increased, if the positivity of both p16/IHC and HPV DNA/PCR is required for positive inference of transcriptionally-active HPV status (100%; CI95: 86.8–100%). Our finding of higher specificity of p16/IHC and/or p16IHC+HPV DNA as surrogate markers of transcriptionally-active HPV in SNSCCs is consistent with results of the study reported by Laco et al. [15], that indicates 100% specificity (CI95:89.4–100%) for p16/IHC as a surrogate marker for HPV positivity detected by E6/E7 mRNA ISH. These findings suggest that specificity of p16/IHC for the detection of causative HPV is substantially higher in SNSCCs than in other non-oropharyngeal head and neck squamous cell carcinomas [26, 27]. This finding also substantiated our decision to include in our meta-analysis also those studies that inferred transcriptionally-active HPV status based on p16/IHC and HPV DNA, in addition to the studies that detected this status directly by mRNA HPV.

In conclusion, transcriptionally-active HPV infection plays an etiological role in ~25% of SNSCC and the role of HPV infection in keratinizing SNSCC may be higher than previously reported. Overall survival of patients with transcriptionally-active HPV status was found better in this study, in comparison with patients with HPV-negative status, but the difference between groups did not reach statistical significance. p16/IHC and p16/IHC+HPV DNA display high specificity but may have lower sensitivity as surrogate markers for transcriptionally-active HPV in SNSCCs compared to OPSCCs.

Supplementary information is available in the online version of the paper.

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Significance of transcriptionally-active high-risk human papillomavirus in sinonasal squamous cell carcinoma: Case series and a meta-analysis

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Supplementary Information

Supplementary Table S1. Clinicopathological data of the 33 patients with SNSCC.

	HPV mRNA-negative n=26	HPV mRNA-positive n=8	p-value (Test)
Sex			0.194
Male	20	4	(Fisher's exact test)
Female	6	4	
Median age (range)	58 (18-84)	51 (43-81)	0.255 (Mann-Whitney test)
Smoking history			1.00
Never smokers	7	4	(Fisher's exact test)†
Current or past smokers	8	3	
Unknown	11	1	
Occupational risks			NA
Yes	1	1	
Unknown	25	7	
Tumor type			0.416
K-SCC	13	6	(Fisher's exact test)‡
NK-SCC	12	2	
S-SCC	1	0	
p16 status by IHC			0.004
positive	2	5	(Fisher's exact test)
negative	24	3	
Tumor site			NA
Nasal cavity	10	4	
Maxillary sinus	7	1	
Multiple subsites	3	0	
Other/Unknown	6	3	
Clinical stage, AJCC 7th ed.			NA
I	1	3	
II	3	0	
III	2	3	
IVa	8	0	
IVb	6	1	
IVc	2	0	
Unknown	4	1	
Grade			NA
1	4	1	
2	6	1	
3	15	6	
4	1	0	
Primary therapy			NA
Biopsy only	3	1	
Radical surgery	4	0	
Surgery+RAT	3	3	
Surgery+CHT	1	0	
Surrgery+RAT+CHT	2	1	
CHT only	1	0	
CHT+RAT	8	1	
RAT only	3	2	
Unknown	1	0	

Abbreviations: K-SCC-keratinizing squamous cell carcinoma (SCC); NK-SCC-non-keratinizing SCC, S-SCC-sarcomatoid SCC; IHC-immunohistochemistry; RAT-radiotherapy; CHT-chemotherapy; †test for difference between never smokers vs. past/current smokers groups; ‡test for difference between K-SCC vs. NK-SCC groups

Supplementary Table S2. Logistic regression model

	b	SE	Wald statistics	p-value	Odds Ratios (CI95)
St I	3.87	1.55	6.26	0.0124	48.00 ($2.31–9.97\times10^{2}$)
St II	-17.21	7.63×10^{3}	5.08×10^{-6}	0.9982	0.00
St III	3.18	1.38	5.33	0.0210	24.00 $1.62–3.57\times10^{2}$
St IV	Baseline				
Constant	-2.77	1.03	7.24	0.0071	
Hosmer-Lemeshow test	$\chi^2=6.31\times10^{-9}$; p=1.00				
Pseudo R^2 (Nagelkerke)	0.5473				
n	29				

Abbreviations: mRNA HPV Status ~st. (st-clinical stage; levels I-IV; baseline-stage IV)

Supplementary Table S3. Logistic regression model with forward selection of variables.

	B	SE	Wald statistics	p-value	Odds Ratios (CI95)
St III	2.98	1.42	4.43	0.0354	19.65 ($1.23–3.15\times10^{2}$)
St IV	Baseline				
p16_1	4.08	1.53	7.16	0.0075	59.28 ($2.99–1.18\times10^{3}$)
p16_0	Baseline				
Constant	–2.96	1.03	8.34	0.0039	–––
Overall model fit	$\chi^2=14.03$;p=0.0009				
Pseudo R^2 (Nagelkerke)	0.5736				
n	29				

Abbreviations: Final model: mRNA HPV Status ~ st+p16 (st = clinical stage; levels I-IV; baseline = stage IV; p16-status of p16; levels 1 = positive; 0 = negative)

Supplementary Table S4. Studies reporting transcriptionally-active HPV infections in sinonasal squamous cell carcinoma directly (through HPV mRNA) or by inference (diffuse positivity or ≥70% neoplastic cells positive for p16/IHC and HPV DNA-positivity).

		HPV+ / SCC subtype								
Study [Reference number]	**HPV detection methods**	**HPV-positive cases**	**K-SCC**	**NK-SCC**	**B-SCC**	**P-SCC**	**Ad-SCC**	**V-SCC**	**S-SCC**	**Comment on prognosis**
El-Mofty et al., 2005 [10]	DNA PCR + p16	**5/29 (17.2%)**	1/21	4/8						
Alos et al., 2009 [11]	DNA PCR + p16	**12/60 (20.0%)**	2/42	6/11	2/5	2/2				Improved OS and PFS in HPV-positive group
Bishhop et al., 2012 [12]	DNA and mRNA ISH	**2/7 (29.0%)**								
Bishop et al., 2013 [13]	DNA ISH + p16	**28/91 (31.1%)**	0/25	15/44	4/8	4/5	5/6		0/3	A trend toward improved survival in HPV-positive group
Larque et al., 2014 [14]	DNA PCR + p16, DNA ISH, mRNA PCR	**14/70 (20%)**	2/49	8/14	2/51	2/2				Improved OS and PFS in HPV-positive group
Laco et al., 2015 [15]	DNA and mRNA PCR, DNA and RNA ISH	**14/49 (28.6%)**	1/16	11/27	2/3	0/1	0/1	0/1		A trend towards improved survival in HPV-positive group
Sahnane et al., 2019 [16]	DNA ISH + p16, DNA PCR	**4/35 (11.4%)**								
Current study	DNA PCR + p16, mRNA PCR and ISH	**8/34 (23.5%)**	6/19	2/14 (incl. 1 hybrid SCC)					0/1	A trend towards improved survival in HPV-positive group
Total		87/374 (23.3%)	12/172 (6.97%)	46/118 (38.98%)	10/21 (47.61%)	8/10 (80%)	5/7 (71.42%)		0/4 (0%)	

Abbreviations: PCR-polymerase chain reaction; ISH-in situ hybridization; K-SCC-keratinizing squamous cell carcinoma (SCC); NK-SCC-non-keratinizing SCC; B-SCC-basaloid SCC; P-SCC-papillary SCC; Ad-SCC-adenosquamous carcinoma; V-SCC-verrucous SCC; S-SCC-sarcomatoid SCC; OS-overall survival; PFS-progression free survival