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Epstein-Barr virus infection is strictly associated with the metastatic spread of sinonasal squamous-cell carcinomas

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Introduction

Sinonasal tumors represent 0.5% of all malignancies and 3% of head and neck carcinomas [1,2]. 40–50% of sinonasal cancers are squamous-cell carcinomas. Little is known regarding the clinical management of sinonasal squamous-cell carcinomas (SNSCC) because of their relatively low prevalence. A limitation of many studies of sinonasal carcinomas is that all entities are typically included and the recommendations for neck dissection are inconsistent [3,4]. The indications for neck dissection of squamous-cell carcinomas of the head and neck or the oral cavity, larynx and pharynx (HNSCC) are markedly more reliable because of the high prevalence of these tumors. In patients with suspicious lymph nodes or tumors in regions with a high rate of regional metastases, e.g., the supraglottis, prophylactic neck dissection or irradiation treatment of the neck is routinely recommended [5,6].

A number of genetic alterations contribute to the progression of HNSCC, as described by Califano et al. [7]. The TP53, EGFR, and $p16^{INK4}$ mutations are predominantly involved, as follows:

The TP53 mutation, found in 60–80% of HNSCC, is one of the most thoroughly investigated tumor suppressor genes. Additionally, these mutations are found in up to 73% of SNSCC [8,9]. Alterations in TP53 play a pivotal role in the carcinogenesis of HNSCC, and they are considered prognostic factors for survival. Additionally, a positive correlation was shown between TP53 mutations and the presence of lymph node metastases [10].

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The Epidermal Growth Factor Receptor (EGFR) pathway is another well-studied contributor to the carcinogenesis of HNSCC. Although only 1% of Caucasian and 7.3% of Asian patients have a mutated EGFR gene [11,12]. The receptor protein is frequently (95%) overexpressed in HNSCC, and it is associated with a poor outcome [9,13]. Hama et al. demonstrated an association of EGFR phosphorylation (pEGFR) with the degree of lymph node metastasis and suggested pEGFR as a poor prognosticator in HNSCC [14]. We formerly detected EGFR protein overexpression in 89% of SNSCC; however, we did not reveal an association with the disease outcome [15]. The data on the frequency of mutations in the downstream pathway of EGFR (KRAS, PIK3CA and BRAF) appear to be inconsistent [16]. As observed for TP53, there is no clinical or therapeutic relevance of the EGFR status and its downstream pathway [9]. There are only few data on mutations on SNSCC.

The $p16^{INK4A}$ protein belongs to the group of cyclin dependent kinase (CDK) inhibitors. The protein inhibits the activity of CDK 4 and CDK 6, which are responsible for the regulation of the G1 phase to the R-point of the cell cycle. High levels of $p16^{INK4A}$ lead to G1 arrest. Homozygous deletion, promoter methylation or rare point mutations of the gene involve disinhibition of the cell cycle. This inactivation of $p16^{INK4A}$ is found in 80% of HNSCC [17,18].

Extensive research in the field of viral carcinogenesis is in progress. Epstein-Barr-Virus (EBV) and human papillomavirus (HPV) could be found in some aerodigestive cancers. EBV infection associated with nasopharyngeal lymphoepithelial carcinoma (NPC) is suggested to play a key role in the metastatic process of NPCs [19]; however, diagnostic EBV detection in these tumors has no effect on decisions regarding individual therapy [20]. HPV has been found in nearly every location of the head and neck; however, it has been found in approximately 47% of oropharyngeal squamous cell cancers (OPSCC), and HPV DNA-positive OPSCC patients had a longer recurrence-free survival than did the HPV DNA-negative patients [21].

In this study, we explored potential genetic alterations in squamous-cell carcinomas of the sinus or nasal cavity and in those of the oral cavity, pharynx and larynx as a function of metastasis. We investigated whether SNSCC and HNSCC represent different tumor entities and assessed the role of HPV and EBV infection in the development of metastasis.

Methods

Patients

Tumor samples from 44 SNSCC patients (nasal cavity and sinuses) and from 65 HNSCC patients (oral cavity, oropharynx, hypopharynx and larynx) were included in the study. The eligibility criteria were a pathologically confirmed squamous-cell carcinoma, patient age between 18 and 90 years and surgical or primary radio-chemical treatment. In the HNSCC cohort, we selected predominantly small tumor sizes (T1/T2 tumors: 73.4%), as in our SNSCC cohort (T1/T2 tumors: 75.0%). The specimens from patients with basaloid squamous-cell carcinomas or carcinomas that had formed an inverted papilloma were excluded. The HNSCC patients originated in a collective which had been formed for an earlier study [22]. The study was approved by the Medical Ethics Committee of the Technical University of Munich (project number 1420/05).

Laboratory studies

In situ hybridization

The presence of EBV-encoded RNA (EBER) and HPV DNA was detected by in situ hybridization (ISH). ISH was performed using an autostaining system (Leica Bond-Max). Probes specific for EBER and HPV subtypes 16, 18, 31, 33 and 51 were purchased from Leica Microsystems GmbH (Wetzlar, Germany). The samples with intense nuclear staining in at least 10% of the tumor cells were defined as positive. The antibody and probe specifications are listed in the supplemental information.

Immunohistochemistry

The $p16^{INK4a}$ expression was assessed using an autostaining system (Leica Bond-Max), the BOND Polymer Refine Detection Kit (both from Leica Microsystems GmbH), and a monoclonal mouse antibody (Roche Diagnostics GmbH, Mannheim, Germany). The stained tumor areas were dichotomized as follows: adopted from Schauer et al., we used an immunostaining score comprised of intensity and a stained tumor area that had values between 0 and 7 [23]. To perform the statistical analysis, we set a cut off at 4 and divided the samples into positive and negative.

The HPV activity in a cell could be shown by determining the level of the p16 expression [24]. Because p16 overexpression could occur independently from HPV infection, HPV patients with a positive HPV status and a high level of p16 expression were considered genuinely HPV positive. In this study, the cells that were both $p16^{INK4a}$ positive and HPV positive were considered genuinely HPV positive.

To measure EBV activity we stained the SNSCC sections with a monoclonal mouse antibody against LMP1 (Clone CS 1-4, DAKO®). Staining was performed on a manual base. Evaluation of stained areas was done accordingly to $p16^{INK4a}$. The cut off was set at 2. Samples with both, EBV ISH positivity and LMP1 expression were considered genuinely EBV positive.

Mutational analysis

The DNA-extracts were prepared from 3–5 deparaffinized specimen sections (10 μm) by Proteinase-K lysis. We performed a mutational analysis through a high-resolution melting curve analysis (HRMA) using a LightCycler 480 (LC480) and the High-Resolution Melting Master Mix (both from Roche Diagnostics GmbH), followed by Sanger sequencing of suspicious samples with MWG Eurofins. TP53 (exons 5, 6, 7, and 8), EGFR (exons 19 and 21), KRAS (exons 2 and 3), PIK3CA (exons 9 and 20) and BRAF (exon 15) were analyzed by HRMA.

Reverse transcription PCR (RT-PCR) and melting curve analysis

RNA was extracted from deparaffinized 10-μm sections. Lysis was performed using a mixture of 40 μl of Proteinase K, 100 μl of Tissue Lysis Buffer (both from Roche Diagnostics GmbH) and 16 μl of SDS (10%) at 55 °C overnight. For further washing and DNA digestion, the InviTrap Spin Tissue RNA Mini Kit (Stratec Molecular GmbH, Berlin, Germany) was used according to the manufacturer's protocol. The extracted RNA was stored at −80 °C. The concentration and purity of the RNA and DNA was determined using a NanoDrop 1000 system (Peqlab Biotechnologie GmbH, Erlangen, Germany).

The cDNA was synthesized from 250 ng of RNA using Maxima reverse transcriptase (Fermentas/Thermo Fisher Scientific, Inc., Waltham, MA, USA) and supplementary reagents following the provided protocol.

We combined a RT-PCR method developed by Yoshimoto et al. with melting-curve analysis using the CFX-96 cycler (Bio-Rad) [25]. To establish this method, we performed RT-PCR, initially using an EGFR vIII plasmid as a template, and then used a transfected cell line to determine the specific melting point of the PCR product.

In brief, the 25-μl reactions consisted of 2 μl of cDNA, 12.5 μl of SYBR mixture (2 x KAPA SYBR FAST Universal, Peqlab

Biotechnologie GmbH), 0.8 pmol/l of each primer, and 8.5 µl of water.

The PCR cycling conditions included an initial denaturation step at 95 °C for 15 min, followed by 45 cycles of 95 °C denaturation for 30 s, 60 °C annealing for 30 s and a 72 °C extension for 15 s.

Melting was observed from 55 °C to 95 °C with a resolution of 1 acquisition/0.5 °C. The melting curves obtained were compared to that of the plasmid vector as a positive control sample and to that of a wild-type cell line (Cal 27) as a negative control sample.

Statistical analyses

To evaluate the correlation between the marker expression and the clinical data, contingency tables were produced using Pearson's chi-squared test for the larger groups and Fisher's exact test for the smaller groups. The analyzed markers were mutations of TP53, the expression of $p16^{INK4a}$ and the presence of HPV or EBV in the tumor cells. For SNSCC LMP1 was additionally evaluated to prove EBV activity. The clinical parameters, tumor and nodal stage, relapse rate and metastasis status were used.

The median follow up was calculated through a reverse Kaplan–Meier analysis, as published by Schemper et al. [26].

The disease-free (time to any form of relapse), overall and metastasis-free (time to relapse in lymph nodes or only distant metastasis) survival was analyzed using the Kaplan–Meier method.

The estimated 3-year survival probabilities and median survival times are presented, for the relevant subgroups. The follow up period in the SNSCC group was 5 years or longer in only 11% of the patients. The distributions of the survival times for the patients with different marker expressions were compared using the log rank test.

All the statistical tests were performed on a two-sided level of significance of 5%. For the relevant quantities, 95% confidence intervals are displayed. The statistical calculations were performed using SPSS, version 21 (IBM, Ehningen, Germany).

Results

Patient characteristics

The SNSCC patients were diagnosed between April 1994 and April 2013 and had an overall 5-year survival rate of 69.2%, with a median follow up period of 7.26 years (min–max, 0.03–14.83). The HNSCC patients ($n = 65$) had their first tumor diagnosis between 1993 and 1996 and had a similar 5-year survival rate of 70.3%, with a median follow up period of 11.14 years (min–max, 0.29–17.81). Surgical resection alone was performed in 30/44 (68.2%) of the SNSCC patients and 15/65 (23.1%) of the HNSCC patients. A subcohort of the patients underwent surgery, followed by adjuvant radiation, including 11/44 (25%) of the SNSCC and 50/65 (76.9%) of the HNSCC patients. Additionally, two of the SNSCC patients received radio-chemotherapy after surgery, and one of the SNSCC patients had primary radio-chemotherapy. Neck dissection was performed in 18 SNSCC cases (40.9%). The clinical characteristics, such as age, TNM status, tumor grading and location, as well as smoking and drinking behaviors are summarized in Table 1.

Table 1
The patient characteristics according to the patient groups. In the HNSCC group, we selected predominantly small tumors sizes (T1/T2 tumors: 73.4%), as in our SNSCC cohort (T1/T2 tumors: 75.0%). The patients with a basaloid squamous-cell carcinoma or carcinoma that had formed an inverted papilloma were excluded.

Characteristics	HNSCC	SNSCC	*P*-value[b]
	($N = 65$)	($N = 44$)	
Age – years			0.522
Median	73	61	
Range	56–90	37–84	
Sex – no. (%)			0.004
Male	61 (93.8%)	33 (75%)	
Female	4 (6.2%)	11 (25%)	
Tumor stage – no. (%)[a]			0.104
T1	21 (32.8%)	22 (50%)	
T2	26 (40.6%	11 (25%)	
T3	10 (15.6%)	6 (13.6%)	
T4	7 (10.9%)	5 (11.4%)	
Nodal stage – no. (%)[a]			<0.001
N0	34 (52.3%)	42 (95.5%)	
N1–3	30 (46.2%)	2 (4.5%)	
Metastasis stage – no. (%)			1.0
M0	65 (100%)	44 (100%)	
M1	0 (0%)	0 (0%)	
Mx	0 (0%)	0 (0%)	
Grading – no. (%)[a]			0.385
G1	4 (6.3%)	2 (4.5%)	
G2	34 (53.1%)	31 (70.5%)	
G3	26 (40.6%)	11 (25%)	
Primary site – no. (%)[a]			
Oral cavity	11 (16.9%)		
Oropharynx	21 (32.3%)		
Hypopharynx	13 (20%)		
Larynx	20 (30.8%)		
Nasal cavity (NC)		34 (77.3%)	
Paranasal sinuses (PS)		5 (11.3%)	
NC and PS		5 (11.3%)	
Alcohol consumption – no. (%)			0.006
Daily	36 (55.4%)	11 (25%)	
Occasional/never	10 (15.4%)	20 (45.5%)	
Unknown	19 (29.2%)	13 (29.5%)	
Tobacco exposure – no. (%)			0.025
Smoker	41 (63.1%)	14 (31.8%)	
Non-smoker	18 (27.7%)	17 (38.6%)	
Unknown	6 (9.2%)	13 (29.5%)	
Metastasis status – no. (%)[a]			0.003
Yes	32 (49.2%)	6 (13.6%)	
Distant metastasis	2 (3.1%)	1 (2.2%)	
Lymph node metastasis	30 (46.2%)	5 (11.4%)	
No	32 (49.2%)	38 (86.4%)	

[a] For 1 HNSCC patient, the single data (T, N, M, G) were missing.
[b] The *P*-values for differences between groups were calculated using Pearson's chi-squared test.

Mutational analysis and survival

In 9 of 44 SNSCC samples (24.3%), at least one mutation within the exons of the TP53 gene was detected. The SNSCC patients with TP53 mutations had a significantly ($p = 0.048$) shorter survival rate than those without the mutation; the median survival for the subgroup with mutations was 1.54 years (95% CI, 1.33–1.75), and the 3-year survival rate was 43.8% (95% CI, 0–88.1). The subgroup without mutations showed a median survival period of 7.26 years (95% CI, 4.81–9.72) and a 3-year survival rate of 84.1% (95% CI, 69.5–98.7). The Kaplan–Meier curves are displayed in Fig. 1. We could not find a significant correlation between the TP53 mutations and the clinico-pathological parameters or with the disease-free or metastasis-free survival.

In the HNSCC cohort, we found 10 of 65 specimens (15.2%) with one or more mutations for TP53. There was a tendency ($p = 0.084$) to shorter survival, with a 3-year survival rate of 86.6% (95% CI, 77.6–96.0) of the mutation-negative patients vs. 60% in the cohort that harbors mutations (95% CI, 29.6–90.0). The mutation analyses revealed no correlations with the clinical parameters.

The other gene analyses for exons with known hotspot mutations in different solid tumors showed only a small mutation rate, and an effect on the clinical outcome could not be revealed (Table 2).

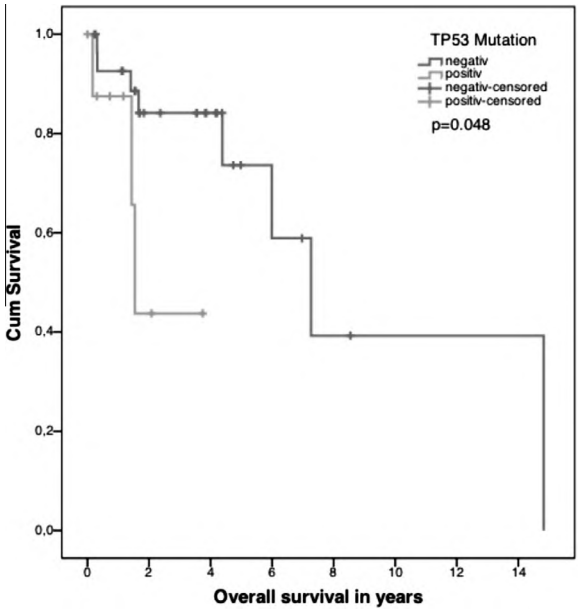

Fig. 1. For the correlation of TP53 mutations and the overall survival for SNSCC, a Kaplan–Meier analysis was performed, followed by a log-rank test. A significantly ($p = 0.048$) shorter survival was found in the patients with TP53 mutations compared with the patients without mutations.

Table 2
The mutational rates in the analyzed genes (in exons with known hotspot mutations) for SNSCC and HNSCC. Squamous-cell carcinoma of the sinus and the nasal cavity showed a similar genetic profile (with respect to the markers analyzed) to carcinoma of the pharynx/larynx.

Gene	HNSCC	SNSCC
BRAF – no. mutated (%)	2 (3.25)	0 (0)
PIK3CA – no. mutated (%)	0 (0)	2 (4.5)
EGFR – no. mutated (%)	0 (0)	0 (0)
KRAS – no. mutated (%)	0 (0)	0 (0)
TP53 – no. mutated (%)	10 (15.2)	9 (24.3)
EGFR vIII – no. mutated (%)	6 (9.2)	4 (10.5)

Effect of viral infection on the metastatic spread in SNSCC patients

A total of 7.1% (3/42) of the patients in the SNSCC and 21.7% (5/18) of the patients in the HNSCC collective (25% of oropharyngeal and oral cavity cancer, 0% of hypopharynx cancer, and 37.5% of larynx cancer) were diagnosed as HPV positive (Table 3). For both HNSCC and SNSCC, we could not find a significant correlation between the HPV status and the metastasis status or survival (SNSCC: $p = 0.3$; HNSCC: $p = 0.3$). In the HNSCC collective, the HPV-positive patients showed a significantly higher tumor stage at diagnosis ($p = 0.039$) than did the HPV-negative patients.

The EBV ISH test was positive in 21/44 SNSCC specimens (47.7%) and in 7/48 HNSCC specimens (14.6%) (Table 3) (Fig. 2A). There was a strong correlation between the EBV status and the rate of lymph node or distant metastases in the SNSCC patients. A positive metastasis status was defined for the patients with lymph node metastases at tumor diagnosis or at relapse. The contingency tables illustrate that all the SNSCC patients with lymph node or distant metastases were positive for EBV (Fisher's exact test, $p = 0.008$) (Fig. 2B). This becomes even more evident when taking EBV activity through expression of LMP1 into account. 14/44 SNSCC patients were EBV and LMP1 positive and every patient with a positive metastasis status was LMP1 positive, yielding a p-value of 0.001 by Fisher's exact test (Fig. 2C). Further statistical analyses could not be performed because all patients with

Table 3
The expression patterns for EBV, HPV and p16. Here, the HPV patients with a positive HPV status and a high p16 expression level were considered genuinely HPV positive. A total of 7.1% of the SNSCC patients and 21.7% of the HNSCC patients were diagnosed as HPV positive. Using in situ hybridization, we found a high percentage of EBV positivity in the SNSCC samples in contrast to the percentage in the HNSCC samples. EBV activity in SNSCC was confirmed through LMP1 staining.

Marker	HNSCC	SNSCC
EBV – no. (%, valid cases)	7 (14.6, 48)	21 (47.7, 44)
LMP1 – no. (%, valid cases)	–	28 (63.3%, 44)
EBV/LMP1 matching – no. (%, valid cases)	–	14 (32.8%, 44)
HPV – no. (%, valid cases)	8 (21.6, 37)	9 (20.5, 44)
p16 – no. (%, valid cases)	21 (75, 28)	13 (29.5, 44)
HPV/p16 matching – no. (%, valid cases)	5 (21.7, 23)	3 (7.1%, 42)

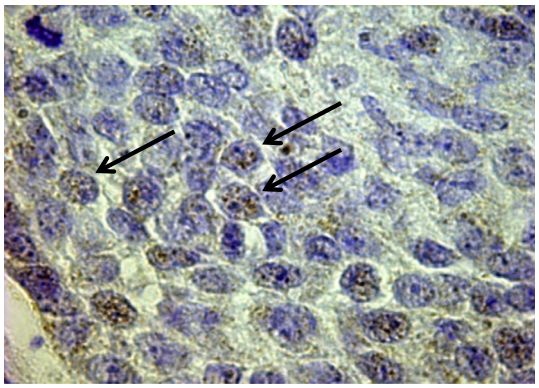

Fig. 2A. A 63× magnification of a histologically confirmed squamous cell carcinoma. The arrows indicate positive nuclear staining of EBER in the in situ hybridization. We found 21 positive cases in SNSCC (47.7%, 44 valid cases).

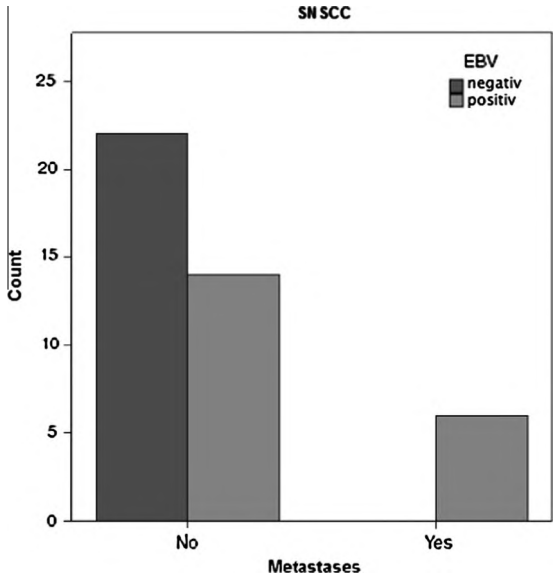

Fig. 2B. There was a strong correlation between the EBV status and the metastasis status in the SNSCC patients. A positive metastasis status was defined for patients having lymph node metastases at tumor diagnosis or at relapse.

metastases were EBV/LMP1 positive. Four nodal metastases from SNSCC tumors, that could be analyzed after removal by neck dissection, were positive for EBV infection. A total of 7/22 EBV negative and 10/21 EBV positive patients underwent neck dissection at the time of tumor diagnosis and surgical tumor resection. One patient of those who received a neck dissection had a lymph node recurrence, which was diagnosed within 3 months after the

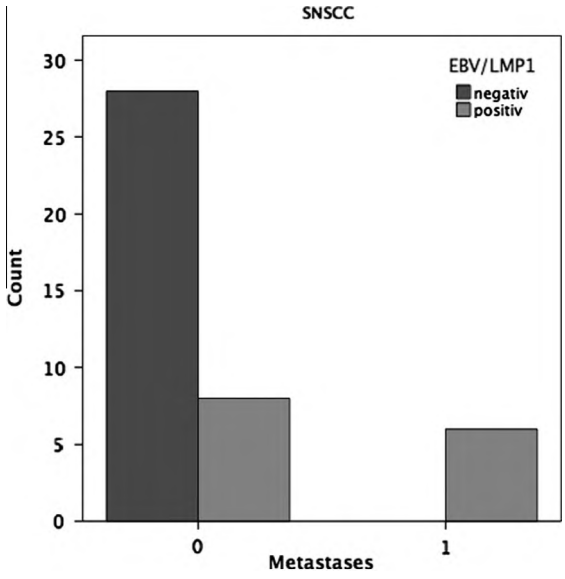

Fig. 2C. Taking LMP1 as parameter of EBV activity in a cell into account the correlation between EBV and the metastasis status is even more evident.

primary treatment. The other patients who developed recurrence of lymph node ($n = 2$) or distant metastases ($n = 1$) did not undergo neck dissection. The overall ($p = 0.54$) and disease-free survival ($p = 0.07$) of the HNSCC patients were not affected by the presence of EBV. In sinonasal malignancies and in HNSCC, no correlation of a co-infection of HPV and EBV with the course and outcome of disease could be found.

Discussion

Only the TNM classification enables the prediction of the course and outcome of SNSCC and HNSCC disease. Whereas an indication for neck dissection could be derived from the high metastasis rate of HNSCC, little is known regarding the clinical management of SNSCC because of its relative low prevalence. An additional problem is that most studies on sinonasal carcinomas include all the entities, wherefore the data regarding the metastasis rates appear inconsistent [3,4]. In the largest single center SNSCC cohort in Germany to date, we analyzed the frequency of the genetic alterations in SNSCCS, which are well known to (frequently) occur in HNSCC. Additionally, we investigated a potential effect of HPV and EBV infection on SNSCC, with respect to lymph node or distant metastases, and evaluated the risk profile that could indicate neck dissection.

Mutation analysis

The presence of missense or non-sense mutations in the TP53 gene is a strong and independent prognostic factor for the survival of HNSCC patients [27]. Here we show that 24.3% of the SNSCC patients had one or more protein-changing mutation(s), which is significantly correlated to a reduced median survival ($p = 0.048$). Other studies reported higher mutation rates of between 29% and 73% [8,28]. We hypothesized that this finding is due to the selection of only squamous-cell carcinomas. Adeno-carcinomas of the nose show a higher frequency of TP53 mutations. Another possible explanation is that we analyzed only the four exons harboring hotspots, in contrast to other studies that included uncommon exons [8]. Similarly to the effect on the HNSCC patients, TP53 mutations adversely affect the prognosis of SNSCC patients. That the HNSCC patients had a relatively low mutational frequency and those with TP53 mutations show only a tendency toward shorter survival is most likely because of the selection of predominantly low-risk cases (T1/T2 tumors: 73.4%) [22], as in the SNSCC cohort (T1/T2 tumors: 75.0%). Only patients with surgically resectable tumors were included, which might explain the 5-year survival of 70%, which is much higher than the expected rate of between 10% and 40% [29]. Additionally, this selection criterion could explain the lack of PIK3CA and EGFR mutations in the collective we investigated here. PIK3CA mutation rates between 11% and 16% [30,31] and EGFR mutation rates in the range of 1% and 7.3% have been published elsewhere [11,12]. Overall, squamous cell carcinoma of the sinus and the nasal cavity show similar genetic alterations to those of carcinomas of the pharynx/larynx. The metastatic spread of the entities is very different, and the effect of HPV and EBV on the development of metastases was examined.

EBV/HPV-status

A recent meta-analysis of HPV infection in sinonasal carcinomas revealed that 27% of the samples were positive for HPV, regardless of the detection method employed (dot blot hybridization, in situ hybridization, or PCR) [32]. With respect to HNSCC, Kreimer et al. described an overall HPV prevalence of 25.9% in HNSCC, with a significantly higher prevalence (35.6%) in oropharyngeal SCC [33]. In this study, we found positive HPV status in 9.7% of the SNSCC and in 21.7% of the HNSCC. Layer and Buchwald recommended combining at least two recognized methods to determine the presence of clinically relevant HPV [34]. Applying this recommendation to our study, we found HPV-positive tumors without p16 expression, which might explain that the rates we found are lower than those reported elsewhere. Alos et al. found an improved 5-year survival rate of 80% for HPV-positive SNSCC patients vs. 31% for HPV-negative patients [35]. We observed a slightly, not significantly, favorable overall survival of HPV-positive SNSCC and HNSCC patients over those patients without HPV infection. We identified a significant association of HPV infection with an advanced tumor stage at diagnosis ($p = 0.039$), which has been described [34].

We demonstrate here for the first time a strong association of EBV infection and the prevalence of SNSCC (47.7%), and we revealed a significant correlation between the presence of EBV and metastatic spread. More specifically, all the patients who developed lymph node or distant metastases were EBV and LMP1 positive. In 14.6% of the HNSCC specimens in which EBV infection was found, EBV was not correlated to metastases or survival. The development of metastasis in SNSCC is a relative rare phenomenon and occurs most frequently within the first year upon diagnosis of the disease [36]. In all the histological subtypes of sinonasal malignancies, lymph node involvement has been reported in 14.3% of the specimens, and distant metastases have been found in 1.6% of the tumors [37,38]. By comparison, we recently found 4% of SNSCC patients with initial lymph node metastases and none with distant metastases at diagnosis [36]. Within the follow-up period, 1 of the 44 (2.3%) patients developed a distant metastasis, and 3 of the 44 patients (6.8%) developed regional lymph node metastases. Although these rates are low, some clinicians recommend prophylactic neck dissection or sentinel lymph node biopsy [6,39]. There is an obvious connection between the metastasis rate and the presence and activity of EBV in SNSCC. Patients with an EBV/LMP1 positive tumor appear to be a high risk subcohort. They require a close-meshed follow up for local and distant metastases, and a neck dissection should be recommended to the patients in this group.

Conclusion

It appears highly advisable to include EBV/LMP1 detection in the first staging process of every newly diagnosed SNSCC. An early EBV diagnosis could be indicative for the need of very close-meshed follow-up or could demonstrate the eligibility of the patient for neck dissection. We recommend a neck dissection based on EBV positivity. Our results should be verified in prospective studies.

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Conflict of interest statement

None declared.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.oraloncology.2015.07.008.

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