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

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SUMMARY

Background: Sinonasal squamous-cell carcinomas (SNSCC) are relatively rare. Thus, data regarding the rate of lymph node metastases are inconsistent in contrast with well-known high metastasis rates in squamous-cell carcinomas of the head and neck (HNSCC) (oral cavity, pharynx and larynx). Hence, the indication for elective neck dissection is difficult in SNSCC. The aim of this study was to assess common genetic alterations and EBV and HPV status as a function of metastasis in SNSCC and HNSCC.
Methods: We retrospectively analyzed 44 SNSCC and 65 HNSCC for TP53, EGFR, KRAS, PIK3CA and BRAF mutations using a high-resolution melting analysis followed by Sanger sequencing. EBV and HPV detection was performed using in situ hybridization for virus encoded RNA. Tumor-associated $p16^{INK4a}$ expression was visualized by immunohistochemistry and correlated with HPV infection. The mutation data, EBV and HPV status were statistically compared with the clinical data in SNSCC and HNSCC.
Results: TP53 mutations were exclusively associated with shorter survival in SNSCC ($p = 0.048$). All the other markers had no effect on the metastasis rate and survival. In total, 20 of 44 SNSCC were EBV-positive. Only these EBV positive tumors developed lymph node or distant metastases ($p = 0.008$). LMP1 was positive in 14/44 patients. When combining both methods significance for a correlation between EBV/LMP1 positive patients and metastases was even higher ($p = 0.001$).
Conclusion: In SNSCC, the presence of EBV is strictly associated with metastasis. We recommend an elective neck dissection in patients with EBV-positive SNSCC.

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