Neuroendocrine Carcinoma of the Head and Neck: HPV-Status and Co-Expression of Immunomodulating Receptors

Katharina Bahr1*, Stefanie Zimmer2, Erik Springer3, Christian Fottner4, Sven Becker5, Christoph Matthias5 and Julian Künel6

1Department of Otorhinolaryngology, Head and Neck Surgery, University Medical Center Mainz, Germany
2Institute of Pathology, University Medical Center Mainz, Germany
3Institute of Molecular Pathology, University Medical Center Mainz, Germany
4Department of Endocrine and Neuroendocrine Tumors, University Medical Center Mainz, Germany
5Department of ENT & Otorhinolaryngology, Head and Neck Surgery, University Medical Center Mainz, Germany

Abstract

Introduction: Lately, HPV-association of head and neck tumors has frequently been described in the literature, occurring not only in squamous cell carcinomas, but also in the less common neuroendocrine carcinomas (NEC) of the head and neck region.

The PD-1/PD-L1 checkpoint was found to be associated with a better outcome in patients with HPV-positive squamous cell carcinoma. It is source to antibody-therapies, such as Pembrolizumab.

Methods: The aim of this study was to determine the HPV-status of NEC of the head and neck region that were collected at our department during surgery in the course of the last 11 years. Additionally, the samples were analyzed in terms of their expression of PD-1, PD-L1 and PD-L2.

Results: Seven different NEC tumor samples of the head and neck region were analyzed; three of them (43%) showed HPV-association. All three HPV-positive samples showed HPV type 18 on molecular pathological analysis. Expression of PD-1 and PD-L1 differed widely between the samples and showed no correlation to the HPV-status. PD-L2 showed a stronger expression in HPV-positive samples.

Conclusion: HPV type 18 appears to be frequently associated with NEC. PD-L1 und PD-L2 expression varies widely in NEC. Its role in NEC to date remains unclear. Multicentric studies are required.

Keywords: Neuroendocrine carcinoma; HPV; PD-L1; Head and neck

Introduction

Neuroendocrine Tumors or Neoplasia (NEN) derive from neuroendocrine cells, which function is procession and secretion of neurohormones. To date, their precise role is not completely clarified. The head and neck region harbors a bewildering array of neuroendocrine tumor types. Common characteristics of NEN on pathological analysis are the expression of neuroendocrine markers, such as synaptophysin and chromogranin A and neuron-specific enolase. CD56/NCAM is an embryonic adhesion-molecule, which can be found in the cellular membranes of most NEN, but is also expressed in a few non-neuroendocrine neoplasms and therefore as a single marker, it is not ideal for the detection of NEN. [1] NEN can be of epithelial or neural/neuroectodermal origin. [1] NEN of neural/neuroectodermal origin in the head and neck region are represented by olfactoriusneuroblastoma and paraganglioma. NEN of epithelial origin are called neuroendocrine carcinoma (NEC) and derive from the so-called diffuse neuroendocrine system that expresses cytokeratines. They can either be skin- or mucosa associated. [1] Grading and differentiation is mostly equivalent to pulmonary NEC. They are mainly divided up into good- and moderate differentiated NEC (typical carcinoid, atypical carcinoid) and poorly-differentiated "high-grade"- carcinoma (small- and large-cell carcinomas) [2]. Some of these tumors are indolent and others highly aggressive. Besides, there are squamous cell- and adenocarcinomas that can show neuroendocrine differentiation by immunohistochemistry. They need to be differentiated from NEC, as well as basaloid squamous cell carcinoma, Sinunasal Undifferentiated Carcinoma (SNUC), malignant lymphoma and malignant melanoma. NEC make up to approximately 1% of mucosa-associated head-and-neck carcinomas.
Nicotine and alcohol abuse appear to be risk factors to their occurrence [4]. Recently, HPV in association with high-grade NEC has been mentioned in the literature (Table 1). The increase of HPV-associated tumors in the head and neck region has given HPV more and more importance and gives us reason to ask about its impact and clinical course of NEC [5,6]. PD-1 (programmed cell death protein 1) is a receptor expressed by B- and T-lymphocytes. Binding with its ligands PD-L1 or PD-L2, it is responsible for inhibiting T-cell-activation [7]. PD-L1 was found to be overexpressed by tumor cells while PD-L2 was found to be expressed by macrophages. PD-L2 expression was also found in various tumor types. The prevalence and distribution of PD-L2 seems to correlate with PD-L1-distribution, but was also found in squamous cell carcinoma in the absence of PD-L1 [8]. The PD-1/PD-L1 checkpoint was associated with a better outcome in patients with HPV-positive squamous cell carcinoma [9]. It is source to antibody-therapies, such as Nivolumab or Pembrolizumab [10,11]. Promising results have been found in phase 2 studies for small cell neuroendocrine lung cancer [12]. Published data concerning immunotherapy in NSCLC showed that patients with a strong expression of PD-L1 positive tumor cells showed a higher response rate and a longer survival than patients with a weaker PD-L1 expression and those with PD-L1 negative tumors [13-17]. But also two phase III studies showed that anti-PD-1 immunotherapy showed better responses than cytotoxic chemotherapy with docetaxel, regardless of the PD-L1-immunoassay results [11,14]. This implies that a negative PD-L1-status is not necessarily associated with a profitless immunotherapy. Besides, PD-1/PD-L1 pathway appears to be activated in the microenvironment of pulmonary high-grade-NEC and correlated with a higher mutation burden [18]. Currently, the anti-PD-L1 antibody avelumab is approved for treatment of the highly aggressive Merkel cell carcinoma.

It raises the question if PD-1/PD-L1 and PD-L2 expression is found in NEC of the head and neck region, whether there is a correlation to their HPV-status and whether PD-1/PD-L1 expression rates correlate with clinical outcomes.

Materials and Methods

Reviewing the databases of the Department of Otorhinolaryngology and of the Institute of Pathology, eight cases of high-grade NEC of the head and neck region from 2006 to 2017 were found. Merkel cell carcinoma as a skin-based tumor and SCC or other tumor types with neuroendocrine differentiation was excluded from analysis. Tissue samples were provided by the tissue bank of the University Medical Center Mainz in accordance with the regulations of the tissue biobank and the approval of the ethics committee of University Medical Center Mainz. Clinical course and patient outcomes were retrieved from the medical records. For eight cases paraffin tumor samples were available for analysis. One of the cases, a cervical metastasis originating from a pulmonary primary, was excluded from analysis because of its origin. All collected samples had previously been fixed in formalin and routinely embedded in paraffin. A representative block of the carcinoma was selected for immunohistochemical and molecular analysis. Besides, the hematoxylin and eosin stained slides of the samples were also reviewed for correlation to immunohistochemistry. A retrospective analysis of HPV-status of the remaining seven NEC was performed including p16 staining as well as high-risk-multiplex PCR, followed by molecular pathological sequencing of the material. HPV-detection was performed by in situ hybridization. In order to quantify expression of the immunoregulating receptor PD-1 and its ligands PD-L1 and PD-L2, immunohistochemical analysis was realized with Anti-PD1-antibody (Abcam, ab52687), PD-L1 antibody (Dako 22C3) and PD-L2antibody (ab200377).

Immunohistochemistry

So far, there is no universally accepted consensus considering the evaluation of PD-L1 expression. Several different scores have been developed for different tumor entities, for example the tumor proportion score (TPS, used for non-small cell lung cancer) or the Cologne Score, while other publications simply differentiate between ‘PD-L1-negative’ and ‘-positive’ tumor cells with different cut-off rates [19,20]. Especially for NEC of the head and neck region, no scoring system has been established so far. The information that is relevant for the diagnosis and quantification of PD-L1 positivity is the rate of membraneously PD-L1 stained tumor cells. This rate can either be described as percentage or in a score. In this case evaluation of the PD-L1-expression was performed utilizing the Cologne Score [21]. The Cologne score is a categorization of the existing threshold values of PD-L1 positive cells and implies a score of six stages. Cologne Score 0 implies a PD-L1 positivity <1%. A positive staining in 1- <5% of the tumor cells corresponds to Cologne 1, whereas 5% - <10% positivity correlates with Cologne 2, 10% - <25% is equivalent to Cologne 3, 25% to <50% represents Cologne 4 and >50% meets with Cologne 5.

The Cologne scoring system was selected because it gives a more detailed differentiation of the staining compared to the division into only three categories of the TPS (<1% = no PD-L1 expression, 1-50% = expression, >50% = high expression). Besides, Cologne Score

### Table 1: Published data of NEC cases with tumor sites and results of HPV-sequencing.

<table>
<thead>
<tr>
<th>Author</th>
<th>Cases</th>
<th>Site</th>
<th>HPV-Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bishop et al. (2011) [29]</td>
<td>4</td>
<td>Oropharynx</td>
<td>16</td>
</tr>
<tr>
<td>Kraft et al. (2012) [4]</td>
<td>6</td>
<td>Oropharynx</td>
<td>16, 18, 33</td>
</tr>
<tr>
<td>Bates et al. (2014) [31]</td>
<td>2</td>
<td>Oropharynx</td>
<td>16, 18</td>
</tr>
<tr>
<td>Misawa et al. (2016) [25]</td>
<td>2</td>
<td>Oropharynx</td>
<td>16, 18</td>
</tr>
</tbody>
</table>

Figure 1: A: Membranous Staining of PD-1 with Anti-PD1 (Abcam, ab52687), the arrow marks tumor cells that show membranous staining. B: Expression of PD-L1 with Dako 22C3, the arrow marks tumor cells that show membranous staining. C: Staining of PD-L2 (ab200377), membranous and cellular in tumor cells D: strong p16-expression.
has been used before in other studies concerning neuroendocrine carcinomas [22].

The Dako 22C3 PD-L1 antibody may also stain tumor associated immune cells. This needs to be considered on evaluation. For this analysis, only PD-L1-positive tumor cells and not PD-L1-positive immune cells were counted.

The intensity of the staining does not give additional information on the positivity and is irrelevant for analysis [13] hence, all tumor cells must be taken into account for proper quantification, which required a strong microscopic enlargement of the tumor samples [21].

Results

Seven samples of NEC were available for analysis (n=4 cervical CUP-syndrome; n=2 Larynx; n=1 Hypopharynx). Five of the tumors were of male patients, two female, age ranged between 44 and 94 years (median age 66.71 years). Four tumors were surgically removed; three of them received adjuvant therapy with either radiation, chemotherapy or a combination of both. The clinical parameters (tumor site, therapy and follow-up) of the patients are summarized in Table 2. Table 2 additionally shows the results of HPV-analysis and PD-1, PD-L1/PD-L2-expression. Figure 1 shows examples of the immunohistochemical staining. Six NEC cases showed positive staining of p16, whereas only three of them were proven to be HPV-positive in molecular pathological analysis. In a consensus PCR using common MY09/11-Primers no PCR-products could be amplified in any of the samples. A parallel multiplex-PCR covering 14 high-risk strains showed for the indicated samples specific fragments sized 121 nucleotides which were then sequenced and identified as HPV18, PD-1 and PD-L1 expression was sparse, PD-1 expression in the samples varied between 0% and 20%, whereas PD-L1-expression showed Cologne Scores between 0 and 3, PD-L2 expression varied between 20% and >50%. Staining intensities in the samples were inhomogeneous from mild to strong. Evaluation of the patient’s outcome showed that patients, who had HPV-positive NEC, suffered from almost the same rather aggressive clinical course than HPV-negative cases. In both groups one patient appears free of tumor, one suffered from relapse of the tumor that required further treatment, and one showed distant metastases either at first diagnosis or shortly afterwards and has died by now. A particular case was one of a 44-year old female, who presented with left cervical swelling, noticed four weeks ago. A lymph node biopsy, taken in an outpatient clinic from the cervical region, had revealed a poor-differentiated carcinoma. ENT examination and CT-staging revealed no further lesions leading to a primary tumor. A bilateral tonsillectomy, bilateral neck dissections were performed and biopsies were taken from the base of the tongue and the nasopharynx. Histological finding of the neck dissection on the right revealed 16 lymph nodes, two of them with metastases of poorly differentiated NEC. The neck dissection on the left included 33 lymph nodes, one of them also with poorly differentiated NEC, exceeding the capsule. Molecular pathological examination revealed HPV-positive DNA-sections in the PCR. Sequencing of the PCR products enabled the identification of the HPV-type 18 (high risk). To the best of our knowledge this was the first case to be discovered and described as a neuroendocrine CUP-Syndrome with a positive HPV-status in the literature to date. In the course of her treatment an FDG-PET-Scan was performed to exclude further tumor sites or residual tumor masses. Despite the fact that there was no literature evidence for adjuvant chemoradiotherapy for poorly-differentiated NEC of the head and neck, interdisciplinary tumor board consensus recommended chemoradiotherapy, mainly due to capsule-exceeding growth of the tumor and the high risk of recurrence. Following common guidelines that recommend adjuvant therapy of cisplatin/carboplatin and etoposide in treating gastroenteropancreatic neuroendocrine neoplasms, [22] the patient received four cycles of Cisplatin/Etoposid, following radiotherapy of nasopharynx, fossa tonsillaris and base of the tongue as well as lymph node levels I-V bilaterally with cumulative dose of 60 Gy. Despite the rather aggressive therapy, the patient suffered from two more tumor relapses in the upcoming two years, which were surgically removed and further treated with chemotherapy.

Discussion

Apart from HPV-association of NEC that are sporadically mentioned in other studies, this study additionally examined a junction to PD-1-PD-L1/PD-L2 checkpoint and gives us reason to further investigate the linkage between NEC and the expression of immunomodulating cells.

Whereas squamous cell carcinoma shows a better prognosis, when associated with HPV, NEC appears to be extremely aggressive in their clinical course regardless of HPV-status. The results of p16
staining differed from molecular pathological HPV-analysis. This reinforces, as indicated before by Alos et al. [23], that p16 is not a reliable marker of HPV-positivity in NEC. In this study, HPV 18 was interestingly the only HPV-type found in the samples. This differs from other tumor types and other studies concerning NEC, in which HPV 16 and 18 was consistently found (Table 1). Furthermore, among the seven samples found in our pathological database, there is not a single one of the oropharynx, where HPV-associated cancers like squamous cell carcinoma typically occur. Even though compared to (Table 1) oropharyngeal NEC seem to be common. Most of the studies mentioned in table 1 refer to real NEC, which is to say pure small cell carcinoma or carcinoids. Only Kraft [4] and Misawa [24] either mention squamous cell carcinomas with just a neuroendocrine differentiation or leave the subtype unclear in one of their cases, which could partly be an explanation for the high oropharyngeal incidence of NEC cases. Further investigations are needed to determine the incidence of oropharyngeal NEC as well as the role of high risk HPV types. It also needs to be investigated, whether the incidence of HPV-infection in NEC of the head and neck region is due to their localization or whether the tumor characteristics of NEC favor a HPV-association. In our series of neuroendocrine carcinoma we could confirm HPV infection in some cases as almost reported in the literature. Compared to PD-1 and PD-L1-expression, PD-L2-expression appeared to correlate with HPV-positivity. PD-L2 had a stronger expression in the HPV-positive tumor samples with expression rates over 50%. According to other studies, PD-L2-expression is usually much more restricted than PD-L1 expression [25]. Since PD-L2 is mainly expressed by macrophages this could be explained by an inflammatory response to the tumor. But still, our results differ from other studies dealing with SCC or NEC, that mostly showed an equal or higher PD-L1 than PD-L2 expression in the tumor samples [26]. The affinity of PD-L2 for PD-1 is three times higher than the affinity of PD-L1. On the other hand it is believed that PD-L2 appeared to be slightly less potent than PD-L1 at inhibiting T cell activation [27].

Especially our lymph node samples, that represented metastasis of unknown primary, were highly positive for PD-L2. Stated that some tumor entities expressing PD-L2 were associated with a poorer prognosis. But still, little is known about the significance of this ligand Ohaegbulam et al. [28]. The results of immunohistochemistry expression analysis presented here, including a rather small cohort of NEC, does not allow conclusions whether there is a correlation to the tumor’s HPV-status. Besides, any conclusion from these data in terms of prognosis and outcome needs to be drawn very carefully, since the study population is rather small and patients were treated with a variety of regimens from curative to palliative. Due to the rarity of the disease, a multicentric analysis of NEC of the head and neck region is needed to further evaluate the role of HPV as well as immunohistochemistry with regard to inflammatory immune response in generation and clinical course of this tumor entity.

References


