HER2 EXPRESSION AND GENE AMPLIFICATION IS RARELY DETECTABLE IN PATIENTS WITH ORAL SQUAMOUS CELL CARCINOMAS

DISSERTATION

ZUR ERLANGUNG DES GRADES EINES DOKTORS DER MEDIZIN AN DER MEDIZINISCHEN FAKULTÄT DER UNIVERSITÄT HAMBURG

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AUS BERLIN

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„Wirkliches Neuland in einer Wissenschaft kann wohl nur gewonnen werden, wenn man an einer entscheidenden Stelle bereit ist, den Grund zu verlassen, auf dem die bisherige Wissenschaft ruht, und gewissermaßen ins Leere zu springen.“

Werner Heisenberg (1901-76)
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I. Publication

Her2 expression and gene amplification is rarely detectable in patients with oral squamous cell carcinomas

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PURPOSE: Her2 (ErbB2) transforms cells when overexpressed and is an important therapeutic target in breast cancer. Contrary to breast cancer, studies on Her2 overexpression and gene amplification in squamous cell carcinomas of the head and neck region described largely different results. This study was undertaken to learn more on the prevalence and clinical significance of HER2 amplification and overexpression in squamous cell carcinomas of the head and neck.

MATERIALS AND METHODS: Her2 expression and gene amplification was analyzed by immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) on two tissue microarrays composed of 427 squamous cell carcinomas of the head and neck region and 222 oral squamous cell carcinomas. Results were compared with clinicopathological features.

RESULTS: Her2 expression and gene amplification was rarely detectable in squamous cell carcinomas of the head and neck region and unrelated to tumor phenotype or survival of the patients with oral squamous carcinoma.

DISCUSSION: Our results demonstrate that Her2 protein and gene amplification was only detectable in a small subset of squamous cell carcinomas of the head and neck region as well as oral squamous cell carcinomas. However, it can be speculated that those few patients with Her2 overexpression and gene amplified tumors may possibly benefit from an anti-Her2 therapy.

Keywords: fluorescence in situ hybridization; head and neck squamous cell carcinoma; Her2, immunohistochemistry; oral squamous cell carcinomas

Introduction

Head and neck squamous cell carcinoma (HNSCC) is a common, morbid, and frequently lethal malignancy. HNSCC is the sixth most common non-skin cancer in the world, with an incidence of about 600,000 cases per year and mortality rate of about 50% (1). Despite recent advances in research, the survival rates for many types of HNSCC have improved little over the past 40 years (2). A crucial step to improve the prognosis is the identification of tumor-specific proteins that can be used as therapeutic targets.

The Her2 protein, a transmembrane tyrosine kinase growth factor receptor, is involved in controlling cell growth and differentiation (3). In normal cells, the activity of the Her receptors is strictly controlled (4), and in several other malignancies than breast cancer, abundant expression of Her2 has been reported and suggested as effective therapeutic target (4). Earlier studies on HNSCC reported a largely divergent Her2 expression and/or gene amplification rates, suggesting Her2 as promising target or not feasible for adjuvant treatment in these patients (5-19). These contrary results may be due to methodological differences and interpretations of the immunohistochemistry (IHC) assays and fluorescence in situ hybridization studies.

To learn more on the rate of HER2 gene amplification and overexpression of the receptor in HNSCC and the subanatomical entity of oral squamous cell carcinomas (OSCC), two tissue microarrays containing 427 HNSCC and 222 OSCC were analyzed by IHC using the Food and Drug Administration-approved test kit for measuring Her2 expression and fluorescence in situ hybridization (FISH) analysis. The aim of this study was to clarify the importance...
of HER2 gene amplification and expression in HNSCC and the subgroup of OSCC.

**Materials and methods**

**Patients and tissue samples**

Two tissue microarrays (TMA) were used for the IHC and FISH examinations. The first TMA contained a total of 427 carcinomas of the head and neck region HNSCC, including 92 larynx, 215 pharynx, and 120 oral carcinomas with data on age, gender, histologically grading, and tumor localization. The second TMA contained 222 OSCC from 157 male and 67 female patients. Among these oral carcinomas, 33 tumors were located at the rim of the tongue, 122 in the floor of the mouth, 35 in the alveolar ridge of the lower jaw, 10 were located at the buccal plane, and 22 in the upper jaw. The mean age of the patients was 56 years (range: 30-93 years). Clinical data on tumor size, grading, lymph node involvement, tumor relapse, distant metastasis, and survival were available for 216 patients with a median follow-up of 46 months (range: 1-306 months).

**Immunohistochemistry**

Freshly cut TMA sections were analyzed on 1 day and in one experiment, HER2 expression was detected with the Food and Drug Administration-approved HercepTest kit (polyclonal rabbit antibody, undiluted; DAKO, Glostrup, Denmark). Bound antibody was then visualized using the EnVision Kit (DAKO). Slides were counterstained with hematoxylin and examined for the extent and intensity of nuclear and non-nuclear staining in tumor cells and for background staining by two pathologists in a blind manner. Divergent results were discussed and were decided by consent. Only spots with more than 20 tumor cells were evaluated. Tumors known to be positive for respective antibodies were used as positive controls, and tissue known to be negative for the used antibodies was used as negative controls. HER2 immunohistochemical expression was scored exactly as described for the Food and Drug Administration-approved HercepTest in four categories: 0, 1+, 2+, and 3+ (20).

**FISH**

For FISH analysis, TMA sections (4 μm) were treated with a commercial kit (Paraffin Pretreatment Reagent Kit; Vysis, Downers Grove, IL, USA) according to the guidelines of the manufacturer. A Spectrum-Orange-labeled HER2 probe was used together with a Spectrum-Green-labeled centromere 17 probe (Pathvision; Vysis). The sections were counterstained with DAPI/Antifade (Merck Millipore, Schwabach, Germany). A pathologist estimated the counts of the gene and centromere in a blind manner. HER2 amplification was defined if the signal ratio of HER2 to centromere is ≥2 in a tumor spot.

**Statistical analysis**

Statistical analyses was carried out with the JMP 5.0.1.2 (SAS Institute Inc., Böblingen, Germany). Contingency tables and chi-square test were performed to search for associations between molecular parameters and tumor phenotype. Survival curves were calculated according to Kaplan-Meier, whereas the raw survival times were set as an end point. The log-rank test was applied to detect significant survival differences between groups.

**Ethical committee approval**

The ethical committee of the Physicians Chamber of Hamburg approved the work, and the patients signed an informed consent for this work.

**Results**

**HER2 immunohistochemistry**

A total of 26 (11.7%) tissue spots of the 222 OSCC were non-informative for HER2 IHC due to the complete lack of tissue or absence of unequivocal cancer cells on the respective TMA spots. Representative images of HER2 IHC are given in Fig. 1. HER2 expression was detectable in 4 of 196 (2%) analyzable tumors including a score of 1+ in 0.5%, 2+ in 1%, and 3+ in 0.5% of cases (Table 1). HER2 expression was unrelated to gender (P = 0.4558), tumor stage (P = 0.4513), lymph node status (P = 0.4514) and tumor grading in OSCC (P = 0.9810; Table 1).

Within the head and neck cancer TMA containing squamous cell carcinoma of 92 larynx, 215 pharynx, and 120 oral carcinomas, HER2 expression was absent in the majority of HNSCC and detectable in 6 of 177 (3.2%) pharynx including a score of 1+ in 2.3% and 2+ in 1.1% and in 1 of 64 (1.6%) larynx carcinomas with a score of 2+ (Table 2).

![Image](A)

![Image](B)

**Figure 1** Representative pictures of (A) negative and (B) positive HER2 immunostaining in oral squamous cell carcinomas.
**Table 1** Association of Her2 immunohistochemistry with gender, tumor stage, lymph node status, and tumor grading in OSCC

<table>
<thead>
<tr>
<th>Variables</th>
<th>n TMA</th>
<th>0 (%)</th>
<th>1+ (%)</th>
<th>2+ (%)</th>
<th>3+ (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>196</td>
<td>192 (98)</td>
<td>1 (0.5)</td>
<td>2 (1.0)</td>
<td>1 (0.5)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>142</td>
<td>138 (97.2)</td>
<td>1 (0.7)</td>
<td>2 (1.4)</td>
<td>1 (0.7)</td>
<td>0.4558</td>
</tr>
<tr>
<td>Female</td>
<td>54</td>
<td>54 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Tumor spread</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT1</td>
<td>53</td>
<td>52 (98.1)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.4513</td>
</tr>
<tr>
<td>pT2</td>
<td>66</td>
<td>65 (98.5)</td>
<td>1 (1.5)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>pT3</td>
<td>24</td>
<td>23 (95.8)</td>
<td>0 (0)</td>
<td>1 (4.2)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>pT4</td>
<td>53</td>
<td>52 (98.1)</td>
<td>0 (0)</td>
<td>1 (1.9)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Lymph node</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nN0</td>
<td>102</td>
<td>100 (98)</td>
<td>1 (1)</td>
<td>0 (0)</td>
<td>1 (1)</td>
<td>0.4514</td>
</tr>
<tr>
<td>nN1</td>
<td>30</td>
<td>30 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>nN2</td>
<td>56</td>
<td>55 (98.2)</td>
<td>0 (0)</td>
<td>1 (1.8)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>nN3</td>
<td>8</td>
<td>7 (87.5)</td>
<td>0 (0)</td>
<td>1 (12.5)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Grading</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>15</td>
<td>15 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.9861</td>
</tr>
<tr>
<td>G2</td>
<td>144</td>
<td>140 (97.2)</td>
<td>1 (0.7)</td>
<td>2 (1.4)</td>
<td>1 (0.7)</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>35</td>
<td>35 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>2</td>
<td>2 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

OSCC, oral squamous cell carcinoma; TMA, two tissue microarrays.

**HER2 FISH analysis**

A total of 15 (6.8%) tissue spots of the 222 OSCC were non-informative for HER2 FISH analysis due to the complete lack of tissue or absence of unequivocal cancer cells on the respective TMA spots. HER2 gene amplification with at least two copies per cell was found in 6 of 207 (3%) analyzable oral squamous cell carcinomas, including three tumors (1.5%) with of 4–6 HER2 gene signals and three tumors (1.5%) with more than 10 gene signals (Table 3). Representative examples of HER2 FISH analysis are given in Fig. 2. HER2 FISH results were unrelated to tumor phenotype (Table 3).

Within the head and neck cancer TMA, HER2 gene amplification was rarely detectable in the different tumor entities (Table 4). In detail, HER2 gene amplification was only found in 5 of 181 (4.4%) interpretable pharynx and in 3 of 59 (5.1%) analyzable larynx carcinomas (Table 4).

**Discussion**

Head and neck squamous cell cancer is a heterogenous and complex entity including diverse anatomical sites and a variety of tumor types displaying unique characteristics and different etiologies (21). Our TMA's enabled us to perform Her2 IHC and FISH analysis on a large number of oral squamous cell carcinomas as a subanatomical region of the HNSCC. Our study revealed that Her2 expression and gene amplification is rarely found in HNSCC and the subset of OSCC. However, it can be speculated that those few patients with Her2 overexpressing and gene amplified tumors may possibly benefit from a HER2-antibody therapy.

At the selected experimental conditions, detectable HER2 gene amplification and the expression of the receptor was found in a minority of HNSCC and the subset of OSCC. Earlier studies on Her2 in HNSCC and OSCC reported of largely divergent Her2 expression and/or gene amplification rates, suggesting Her2 as promising target or not feasible for adjuvant treatment in these patients (5–19). Most likely, these controversial data are due to divergent patient cohorts or differences in the methodical processes such as preparation of the tissue, antibodies, staining or evaluation.

**Table 2** Results of the Her2 immunohistochemistry in HNSCC, including 120 oral, 215 pharynx, and 92 larynx carcinomas

<table>
<thead>
<tr>
<th>Tumor entities</th>
<th>n TMA</th>
<th>Analyzable (n)</th>
<th>0 (%)</th>
<th>1+ (%)</th>
<th>2+ (%)</th>
<th>3+ (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral cavity</td>
<td>120</td>
<td>99</td>
<td>99 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0.002</td>
</tr>
<tr>
<td>Pharynx</td>
<td>215</td>
<td>177</td>
<td>171 (96.6)</td>
<td>4 (2.3)</td>
<td>2 (1.1)</td>
<td>0 (0)</td>
<td>0.4273</td>
</tr>
<tr>
<td>Larynx</td>
<td>92</td>
<td>64</td>
<td>63 (98.4)</td>
<td>0 (1)</td>
<td>1 (1.6)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

HNSCC, head and neck squamous cell carcinoma; TMA, two tissue microarrays.

*chi-squared test vs. larynx.*

**Table 3** Association of HER2 FISH data with gender, tumor stage, lymph node status, and tumor grading in oral squamous cell carcinomas

<table>
<thead>
<tr>
<th>Variables</th>
<th>n TMA</th>
<th>Analyzable (n)</th>
<th>Normal (%)</th>
<th>Amplified (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>222</td>
<td>207</td>
<td>201 (97.1)</td>
<td>6 (2.9)</td>
<td>0.4925</td>
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<tr>
<td>Male</td>
<td>157</td>
<td>148</td>
<td>143 (96.6)</td>
<td>5 (3.4)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>65</td>
<td>59</td>
<td>58 (98.3)</td>
<td>1 (1.7)</td>
<td></td>
</tr>
<tr>
<td>Tumor spread</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT1</td>
<td>59</td>
<td>58</td>
<td>56 (96.6)</td>
<td>2 (3.5)</td>
<td>0.8624</td>
</tr>
<tr>
<td>pT2</td>
<td>75</td>
<td>66</td>
<td>65 (98.5)</td>
<td>1 (1.5)</td>
<td></td>
</tr>
<tr>
<td>pT3</td>
<td>28</td>
<td>27</td>
<td>26 (96.3)</td>
<td>1 (3.7)</td>
<td></td>
</tr>
<tr>
<td>pT4</td>
<td>60</td>
<td>56</td>
<td>54 (96.4)</td>
<td>2 (3.6)</td>
<td></td>
</tr>
<tr>
<td>Lymph node</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nN0</td>
<td>115</td>
<td>110</td>
<td>108 (98.2)</td>
<td>2 (1.8)</td>
<td>0.2315</td>
</tr>
<tr>
<td>nN1</td>
<td>34</td>
<td>30</td>
<td>29 (96.7)</td>
<td>1 (3.3)</td>
<td></td>
</tr>
<tr>
<td>nN2</td>
<td>65</td>
<td>60</td>
<td>60 (100)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>nN3</td>
<td>8</td>
<td>7</td>
<td>4 (57.1)</td>
<td>3 (42.9)</td>
<td></td>
</tr>
<tr>
<td>Grading</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>16</td>
<td>15</td>
<td>15 (100)</td>
<td>0 (0)</td>
<td>0.7762</td>
</tr>
<tr>
<td>G2</td>
<td>163</td>
<td>151</td>
<td>146 (96.7)</td>
<td>5 (3.3)</td>
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<tr>
<td>G3</td>
<td>41</td>
<td>39</td>
<td>38 (97.4)</td>
<td>1 (2.6)</td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>2</td>
<td>2</td>
<td>2 (100)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

FISH, fluorescence in situ hybridization; TMA, two tissue microarrays.
Table 4 Results of the HER2 FISH analysis in HNSCC, including 120 oral, 215 pharynx, and 92 larynx carcinoma.

<table>
<thead>
<tr>
<th>Tumor entities</th>
<th>n</th>
<th>Analyzable(%)</th>
<th>Normal (%)</th>
<th>Amplified (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral cavity</td>
<td>120</td>
<td>104 (83.3)</td>
<td>104 (100)</td>
<td>0 (0)</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Larynx</td>
<td>92</td>
<td>59 (75.3)</td>
<td>56 (94.9)</td>
<td>3 (5.1)</td>
<td></td>
</tr>
</tbody>
</table>

HNSCC: head and neck squamous cell carcinoma; TMA, two tissue microarrays.

*chi-squared test vs. larynx.

Therefore, standardized examination conditions should be a prerequisite for the clarification of Her2 expression and gene amplification in the squamous cell carcinomas of the head and neck region and the subgroup of oral squamous cell carcinomas. In this study, HER2 immunohistochemical expression was scored exactly as described for the Food and Drug Administration-approved HercepTest in four categories: 0, 1+, 2+, and 3+ (20). In literature, the accuracy and utility of this diagnostic test has been proven by demonstrating high concordance between the HER2 immunohistochemistry and HER2 fluorescence in situ hybridization (22). These data are in line with our results of high concordance between the two methods IHC and FISH of HER2 in OSCC, as tumors harboring a high HER2 gene amplification with more than 10 gene signals were also characterized by HER2 overexpression.

The results from our study virtually exclude Her2 protein as a clinically useful prognostic oral cavity carcinoma biomarker as Her2 IHC and FISH data were unrelated to clinicopathological features. However, it seems possible that despite the low HER2 overexpression/amplification rate of only 1–2%, those few patients may possibly benefit from anti-Her2 therapy. This suggestion is underlined by earlier studies, demonstrating an antitumor effect of combined gefitinib and trastuzumab or cetuximab and trastuzumab treatment on HNSCC in vitro (23, 24). Therefore, it might be interesting to include patients with HER2 overexpression/gene amplification HNSCC in clinical studies to further evaluate Her2 as therapeutic target in these tumors.

In summary, the results of this study show that Her2 expression and gene amplification is rarely found in HNSCC and the subset of OSCC. In line with most other Her2-positive tumors, Her2 overexpression occurs in the presence of gene amplification in OSCC, which argues for a possible anti-Her2 therapy in these tumors.

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neu expression in epithelial dysplasia and oral squamous cell
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and neck squamous cell carcinoma. *Otolaryngol Head Neck
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45.
concordance between HercepTest immunohistochemistry and
ERBB2 fluorescence in situ hybridization before and after
implementation of American Society of Clinical Oncology/
College of American Pathology 2007 guidelines. *Mod Pathol*
molecular targeted drug therapy for EGFR and HER-2 in
head and neck squamous cell carcinoma cell lines. *Int J Oncol*
gefitinib on head and neck squamous cell carcinoma enhanced
Her2 expression and gene amplification is rarely detectable in patients with oral squamous cell carcinomas

II. Introduction

The Her2 oncogene seems to be important for the growth of many different tumours. It is most strongly associated with the important therapeutic target in breast cancer. This gene product which shows a significant association to cell proliferation and cell cycle control, is of high scientific interest not only for breast cancer but also for other cancers like in our case for squamous cell carcinomas of the head and neck region, because it could be a possible target for a specific therapeutic approach and eventually be a prognostic marker. Since there is inconsistent data to this topic our objectives were to clarify the significance of HER-2 expression and HER-2 gene amplification for the squamous cell carcinoma of the head and neck region.

III. History and state of knowledge of Her2

Her2 (human epidermal growth factor receptor 2, also known as erbB2 (standing for its origin in the Erb-b gene which is responsible for avian erythroblastosis virus)), is an oncogene which has been localised to chromosome 17q21 and encodes for one of the epithelial growth factor receptors on the cell. It has a molecular weight of 185,000 dalton (Stern et al. 1986) and belongs to the HER family of four transmembrane receptor tyrosine kinases involved in signal transduction pathways that regulate cell growth and differentiation. It was discovered at the Massachusetts Institute of Technology by the Weinberg scientists Group in 1982-1984. From then on it was in the focus of science and research. It was shown that it was amplified in a range of tumor types including breast, ovarian, bladder, salivary gland, endometrial, pancreatic and non-small-cell lung cancer (NSCLC)(Scholl et al. 2001). Her2 is involved in disease initiation and progression, associated with poor prognosis, and may also predict the response to chemotherapy and hormonal therapy(Scholl et al. 2001). Based on this data anti-Her2 monoclonal antibodies have been designed to specifically antagonise the function of the HER2 receptor in Her2-positive tumours (Scholl et al. 2001). The therapy with these agents such as Trastuzumab „targeted therapy of HER-2“ is well established for the metastasized HER2 positive
breast cancer (Tripathy et al. 2004). Although there is a positivity of HER2 reported for many human tumor types the data of positivity varies. This applies to immunohistochemical examination procedures (IHC), which result in a wide span of positive HER2 test results through the use of several different reagents and records of the examination criteria. It is reported for example that there is a HER2 overexpression of 5.7-88.8% of the non-small cell lung cancer cases (Ugocsai et al. 2005) and in 3-54% of the colon cancer cases (Ooi et al. 2004). On the other hand there is a variability of HER2 positivity in the clinical analysis of amplification. Different detection methods for example Southern blot or fluorescent in situ hybridization (FISH) and different evaluation criteria seem to be responsible for the different HER-2 amplification results given in the literature. Reports on tumour examinations at the head and neck region state also a different extent of HER2 overexpression in 11%-60% of the cases and a HER2 amplification in 18%-46% of the cases (Scheer 2003).

IV. Head and neck squamous cell carcinoma/Her2

Head and neck cancer is a broad term used to describe malignancies that arise in the nasal and oral cavities, pharynx and larynx, as well as the paranasal sinuses. Most of these epithelial cancers are squamous cell carcinomas. Head and neck squamous cell carcinoma (HNSCC) is a common, morbid, and frequently lethal malignancy (Hanken* et al. 2014). HNSCC is the sixth most common non-skin cancer in the world, with an incidence of about 600,000 cases per year and mortality rate of about 50% (Ferlay et al. 2010). Besides alcohol and tobacco abuse, it is becoming evident that human papilloma virus (HPV) infection is a more and more important risk factor for the development of HNSCC (Jain et al. 2013). Despite recent advances in research, the survival rates for many types of HNSCC have improved little over the past forty years (Gupta et al. 2009). A crucial step to improve the prognosis is the identification of tumour specific proteins like Her2 that could be used as therapeutic targets. As mentioned above, reports on tumour examinations at the head and neck region state a different extent of HER2 overexpression in 11%-60% of the cases and a Her2 amplification in 18%-46% of the cases (Scheer 2003). Her2 could be a possible target for a specific therapeutic approach and eventually be a prognostic marker. Therefore it is important to clarify the significance of HER-2 expression and HER-2 gene amplification for the squamous cell carcinoma of the oral cavity.
V. Aims of our study

We conducted these studies in order to learn more on the rate of HER2 gene amplification and overexpression of the receptor in head and neck squamous cell carcinoma (HNSCC) and to find out more about the subanatomical entity of oral squamous cell carcinomas (OSCC) and the significance of HER2 gene amplification and expression in HNSCC and the subgroup of OSCC.

VI. Brief summary of methods and materials

Two tissue microarrays (TMA) were used for the immunohistochemical (IHC) and fluorescence in situ hybridization (FISH) examinations to reach maximum standardization of investigation for the tumour collective. This technique for the assessment of Her2 status is well established. Several authors have validated the use of TMA technology in this setting in comparative studies using archived material. Over the years of use it has been shown that the TMA technology is a useful tool for the validation of different HER-2 FISH protocols and for assessment of interlaboratory reproducibility (Graham et al. 2008). In the presented studies, in order to clarify the significance of HER-2 expression and HER-2 gene amplification for the squamous cell carcinoma of the oral cavity, the method authorised by the FDA (US Food and Drug Administration) has been used together with the associated evaluation scoring of the immunohistochemical examination (Hercep Test™, DAKO, Glostrup, Denmark) and the fluorescent in situ hybridization (PathVysion™; Vysis). The studies were conducted at a Tissue-Micro-Array (TMA) in order to reach maximum standardisation of investigation for the tumour collective.

a. Tissue-Micro Array (TMA)

Tissue Micro Arrays consist of paraffin blocks in which up to 1000 separate tissue cores are brought into an array format and can be analysed simultaneously. Based on paraffin-embedded tissue blocks, core needle biopsies are taken from specific locations and are re-embedded into one arrayed block from which the analysis is taking place. The TMA technology is a fast, cost-effective, and statistically powerful method that will substantially facilitate translational research (Simon & Sauter 2004). The first TMA in this study contained 222 oral squamous cell carcinomas (OSCC) from 157 male and 67 female patients treated in the department for oral- and maxillofacial surgery of the University Medical Center Hamburg-Eppendorf, Germany, between 1988 and 2007. Among these oral carcinomas, 33 were at the tongue’s margin, 122 at the floor of the mouth, 35 in the
alveolar process, 10 located oropharyngeal and 22 in the upper jaw. The second well-established TMA from Basel was a comparative analysis consisted of 427 carcinomas of the head- and neck region (HNSCC) including 92 laryngeal carcinomas, 215 pharyngeal carcinomas and 120 OSCC’s.

b. Immunohistochemistry (IHC)

Immunohistochemistry refers to the process of detecting antigens (e.g. proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues. The IHC technique is a combination of immunologic and chemical reactions visualised with a photonic microscope. It starts with deparaffination of tissue sections, then includes preincubation steps (e.g. antigen retrieval, blocking of nonspecific activities), incubation with the primary antibody, and labeling of the antigen-antibody reaction and ends with slide counterstaining and coverslipping (Ramos-Vara & Miller 2014). In our TMA sections we used the Food and Drug Administration–approved HercepTest kit (polyclonal rabbit antibody, undiluted, DAKO, Glostrup, Denmark) to detect the Her2 expression. For the visualisation of the bound antibody we used the EnVision Kit (DAKO, Glostrup, Denmark). A hematoxylin counterstain was done. Two pathologist examined all spots of the TMA in a blind manner and rated the extent and intensity of background staining as well as nuclear and non-nuclear staining of tumour cells. Only spots with more than 20 tumour cells were evaluated. HER2 immunohistochemical expression was scored exactly as described for the Food and Drug Administration–approved HercepTest in four categories: 0, 1+, 2+, and 3+ (Wolff et al. 2013) by the producer (DAKO (Hercep-Test®, Hamburg DAKO; table 1) (M. Blessmann 2008).
### Staining pattern

<table>
<thead>
<tr>
<th>Staining pattern</th>
<th>HER-2 Overexpression</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absence of fluorescence or membrane coloring in less than 10% of tumour cells.</td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>In more than 10% of tumour cells weakly/hardly visible membrane coloring. Only part of membrane.</td>
<td>Negative</td>
<td>1+</td>
</tr>
<tr>
<td>In more than 10% of tumour cells weakly to moderately visible coloring of entire cell membrane.</td>
<td>weakly positive</td>
<td>2+</td>
</tr>
<tr>
<td>In more than 10% of tumour cells strongly visible coloring of entire cell membrane.</td>
<td>strongly positive</td>
<td>3+</td>
</tr>
</tbody>
</table>

**Tab.1:** Evaluation criteria for Hercep-Score (HER-2).

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**Figure 1:** Representative pictures of (A) negative and (B) positive Her2 immunostaining in oral squamous cell carcinomas (Hanken* et al. 2014).
c. Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridisation is a technique that is used to detect and localise the presence or absence of specific DNA sequences on chromosomes. FISH uses fluorescent probes that bind to only those parts of the chromosome with which they show a high degree of sequence complementarity. Fluorescence microscopy can be used to find out where the fluorescent probe is bound to the chromosomes (Amann & Fuchs 2008). We treated our TMA sections with a commercial kit (Paraffin Pretreatment Reagent Kit, Vysis, Downers Grove, IL, USA) corresponding to the guidelines of the manufacturer. We used a Spectrum-Green–labeled centromere 17 probe with a Spectrum-Orange–labeled HER2 probe (PathVysion; Vysis), counterstaining the sections with DAPI (4′,6-Diamidin-2-phenylindol)/Antifade (Merck Millipore, Schwalbach, Germany). Two pathologists estimated the counts of the gene and centromere in a blind manner. Only if there was a signal ratio of Her2 to centromere >2 in a tumour spot the HER2 amplification was defined.

![Representative examples of (A) negative and (B) positive HER2 gene amplification in oral squamous cell carcinomas. Green: centromere region; Orange: HER2 gene. HER2 amplification was defined in the signal ratio of HER2 to centromere is >2 in a tumour spot (Hanken* et al. 2014).](image_url)
d. Statistical analysis

We analysed the clinical data on tumour size, grading, lymph node involvement, tumour relapse, distant metastasis, and survival for a total of 216 patients. For statistical analysis JMP 5.0.1.2 (SAS Institute Inc., Böblingen, Germany) was used. To search for associations between molecular parameters and tumor phenotype, contingency tables and chi²-test were performed. Survival curves were calculated according to Kaplan-Meier, setting raw survival times as an end point. In order to detect survival differences that are significant between the groups a Log-Rank test was applied.

VII. Summary of results with discussion

As mentioned above, we conducted these studies in order to learn more on the rate of HER2 gene amplification and overexpression of the receptor in head and neck squamous cell carcinoma (HNSCC) and to clarify the subanatomical entity of oral squamous cell carcinomas (OSCC) as well as to clarify the importance of HER2 gene amplification and expression in HNSCC and the subgroup of OSCC. The clinical data on tumor size, grading, lymph node involvement, tumour relapse, distant metastasis, and survival were available for 216 patients with a median follow-up of 46 months (range: 1–306 months) (Hanken* et al. 2014). Our study revealed that the Her2 expression in the immunohistochemical stains was rarely detectable. Furthermore, gene amplification in the TMA of Her2 was absent in the majority and unrelated to tumour phenotype or survival of the patients suffering from oral squamous cell carcinomas as a sub anatomical region of the HNSCC. In the immunohistochemical stains 26 (11.7%) tissue spots of the 222 OSCC had to be excluded due to the complete lack of tissue or absence of unequivocal cancer cells on the respective TMA spots. The Her2 expression could be detected in 4 of 196 (2%) analysable tumours including a score of 1+ in 0.5%, 2+ in 1%, and 3+ in 0.5% of cases and was unrelated to gender (p=0.4558), tumour stage (p=0.4513), lymph node status (p=0.4514) and tumour grading in OSCC (p=0.9810) as shown in table 1 in the attached paper above. Within the TMA containing squamous cell carcinomas of 92 larynx, 120 oral and 215 pharynx carcinomas, Her2 expression was absent in the majority of HNSCC and detectable in 6 of 177 (3.2%) pharynx including a score of 1+ in 2.3% and 2+ in 1.1% and in 1 of 64 (1.6%) larynx carcinomas with a score of 2+ as shown in table 2 in the attached paper (Hanken* et al. 2014). We could demonstrate a high concordance between the Her2 immunohistochemistry and HER2 fluorescence in-situ hybridisation. Also in the HER2 FISH analysis a total of 15 (6.8%) tissue spots of the 222 OSCC were excluded due to the complete lack of tissue or absence of
unequivocal cancer cells on the respective TMA spots. HER2 gene amplification with at least 2 copies per cell was found in 6 of 207 (3%) analysable oral squamous cell carcinomas including 3 tumours (1.5%) with 4-6 HER2 gene signals and 3 tumours (1.5%) with more than 10 gene signals. These findings were unrelated to tumour phenotype shown in table 3 of the attached paper. As shown in table four of the attached paper, HER2 gene amplification was only found in 8 of 181 (4.4%) interpretable pharynx and in 3 of 59 (5.1%) larynx carcinomas within the head and neck cancer TMA (Hanken* et al. 2014). To summarise the results with the selected experimental conditions, detectable HER2 gene amplification and the expression of the receptor was found in a minority of HNSCC and the subset of OSCC. In earlier studies it was either reported that the Her2 expression and gene amplification in HNSCC and OSCC was high or not feasible. Making it on the one hand a promising target for adjuvant treatment or on the other hand not relevant. By analysing these studies it sticks out that there was not a uniform methodically approach such as preparation of antibodies, the tissue, staining or evaluation. We suggest using standardised examination condition would make the study of Her2 expression and gene amplification in the squamous cell carcinomas of the head and neck region with the subgroup of oral squamous cell carcinomas more transparent and more meaningful. Looking at our data of IHC and FISH three tumours harboring a high gene amplification with more than 10 gene signals were also characterised by Her2 protein overexpression. Therefore we can conclude that there is a meaningful linkage between expression and amplification. Looking at the data from our study one is likely to exclude Her2 protein as a clinically useful prognostic oral cavity carcinoma biomarker as Her2 IHC and FISH data were unrelated to clinic-pathological features. But it is underlined in the literature that the minority of 1-2% patients of our study possibly benefits from anti-Her2 therapy demonstrating an antitumour effect of combined gefitinib and trastuzumab or cetuximab and trastuzumab treatment on HNSCC in vitro. For that reason, it makes sense to clarify in further studies the best treatment for HER2 highly amplified and simultaneously HER2 positive oral cavity carcinomas. This study should also give an impulse for further studies for the importance of oncogenes as tumour markers in Head and neck squamous cell carcinomas (HNSCCs).
VIII. References


IX. Erklärung des Eigenanteils an der Publikation


X. Danksagung

Mein besonderer Dank gilt Herrn Prof. Dr. Dr. Max Heiland, Direktor der Klinik und Poliklinik für Mund-, Kiefer- und Gesichtschirurgie am Universitätsklinikum Hamburg-Eppendorf, für die Bereitstellung der Doktorarbeit.

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10.2013 – 01.2014 Datenanalyse und –akquise zur Auswertung und Erstellung des Manuscripts
"Her2 expression and gene amplification is rarely detectable in patients with oral squamous cell
carcinomas" bei PD Dr. Dr. Marco Blessmann in der MKG am UKE
Forschung bei Professor Gieri Cathomas, Leiter der Pathologie, Kantonspital Liestal, Schweiz, Bearbeitung und Auswertung einer Datenbank zu HPV-Tumoren im Oropharynx

- HER2 in head and neck Squamous cell carcinoma (Doktorarbeit, MKG UKE PD Dr. Dr. Blessmann), Veröffentlicht 2014 im: Journal of Oral Pathology and Medicine

- Case Report: Henoch- Schönlein Purpura in adults (Institut Innere Medizin, Universitätsspital Liestal, Schweiz, Professor Leuppi), Veröffentlicht 2014 im: Die PRAXIS Journal Schweiz
  -> Posterpräsentation auf dem European and Swiss Congress of Internal Medicine in Genf am 15.05.2014

- Ausstehend: Paper zu HPV orophanryngeal cancer (Harvard Medical School, Professor Rocco)

- Ausstehend: Paper: “Bilateral tonsilar manifestation of Her2 positive oropharyngeal cancers” Professor Gieri Cathomas, Pathologie, Kantonspitaal Liestal, Schweiz

- Ausstehend: Evaluation einer neuartigen mikrochirurgischen Anastomose - Technik mit einem resorbierbaren ST-Stent im Tiermodell (MKG UKE Professor Heiland)
XII. **Eidesstattliche Versicherung**

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: ..........................................................