Studying EGFR-receptor and claudin expression patterns in head and neck squamous cell carcinomas

PhD thesis

Balázs Szabó MD

Semmelweis University
Doctoral School of Pathological Sciences

Supervisor:
József Tóvári PhD, Head of Department

Official reviewers:
Margit Balázs PhD, DSc, university professor
Gálffy Gabriella MD, PhD, university associate professor

Head of the Final Examination Committee:
Janina Kulka MD, PhD, university professor

Members of the Final Examination Committee:
Judit Pápay MD, PhD, university assistant professor
László Tamás MD, PhD university associate professor

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LIST OF ABBREVIATIONS

Akt: protein kinase B (PKB)
CLDN: Claudin
EGF: Epidermal Growth Factor
EGFR: Epidermal Growth Factor Receptor
EGFRvIII: Epidermal Growth Factor Receptor variant III
FISH: fluorescent in situ hybridization
HNSCC: Head and Neck Squamous Cell Carcinoma
HPV: Human Papilloma Virus
kDa: kilodalton
KSH: Hungarian Central Statistical Office (Központi Statisztikai Hivatal)
MAPK: mitogen activated protein kinase
PI3K: phosphatidylinositide 3-kinase
TK: Tyrosine Kinase
PCR: Polymerase Chain Reaction
RAF: rat fibrosarcoma
RAS: rat sarcoma
RFLP: Restriction Fragment Length Polymorphism
RR: Relative Risk
TGF-α: Transforming Growth Factor-α
TJ: Tight Junction
TMA: Tissue Micro Array

1. INTRODUCTION

- ***Head and Neck Squamous Cell Carcinomas***

The majority (approx. 90%) of malignant head and neck tumours are squamous cell carcinomas (HNSCC). The majority of squamous cell carcinomas originating from the upper portion of the alimentary canal and upper respiratory epithelium derive from the oral cavity, pharynx and larynx; their histological structure is uniform and their etiology is similar. Head and neck squamous cell carcinoma was the sixth most common type of tumour in 2012 with some 600,000 new cases worldwide; the related mortality was 320,000. According to KSH data in 2011 approximately 4400 new patients were registered in Hungary, which represents the 3rd place after pulmonary and colorectal carcinomas among men regarding its incidence and mortality. Their prognosis is rather unfavourable; in the majority of the cases patients seek medical help with the malignant alteration only at a later stage. In addition to the two well-known risk factors, the most common chemical carcinogens, i.e. alcohol consumption and smoking, more and more data are suggestive of the potential role of viral etiology in the development of head and neck squamous cell

carcinomas. According to published studies integrated HPV could be detected in 19 to 35% of HNSCC-s. Histologically the HPV-positive group is usually highly differentiated, is often verrucous in nature, and according to several publications it is associated with a significantly better survival rate; this becomes evident with an increased radio- and chemotherapeutic sensitivity of the tumours.

Based on medical historical data of the Hungarian patient population HPV-positive cases are more characterized by concurrent chemical and viral carcinogenesis; confirmed viral origin is rare.

In the past decades surgical therapy, as well as combined chemo- and radiotherapy developed to a great extent, however, the survival rate of patients suffering from head- and neck tumours locoregionally progressed and/or giving remote metastases have not increased significantly in parallel. A promising possibility to increase treatment efficacy is completing available treatments with a target specific therapy. In addition, mapping and administration of new molecular biological prognostic and predictive factors is also very important. Since one major characteristics of HNSCC-s is EGFR-overexpression, medicinal products targeting the EGF-

receptor have been tried and introduced also in the treatment of head and neck carcinomas.

- ***EGFR and its abnormalities***

The Epidermal Growth Factor Receptor (EGFR) is a glycoprotein of 170 kDa weight; it consists of an extracellular ligand binding region, a hydrophobic transmembrane region and an intracellular domain with tyrosine-kinase activity. Several ligands can activate the receptor, among which the most significant ones are the epidermal growth factor (EGF) and the transforming growth factor-α (TGF-α). Activated receptors trigger several intracellular signalling cascades. In case of the EGF-receptor the most important pathway is the RAS-Raf-MAP-kinase and the phosphatidylinositide 3-kinase (PI3K) - Akt-kinase pathway. In normal cells these pathways are responsible for maintaining tissue homeostasis by facilitating proliferation and differentiation, however, their activation in tumorous cells increases angiogenesis, inhibits apoptosis, and results in cell adhesion, invasion and formation of metastases.

According to several studies, in head and neck tumours EGFR protein overexpression falls between 70 and 90%. EGFR overexpression and amplification is associated with a less

favourable survival also in patients suffering from head and neck tumours.

Currently there are two active substance groups available to inhibit the EGF-receptor: inhibition may be realized through monoclonal antibodies acting extracellularly, or through low-molecular weight tyrosine-kinase inhibitors that block tyrosine-kinase enzyme activity intracellularly. According to preclinical studies EGF-receptor seemed an ideal target in cancer-therapy, however, clinical results did not fully meet the expectations. Some publications even indicate that only 10 to 20% of tumorous patients respond satisfactorily to anti-EGFR treatment.

Several abnormalities of the EGF-receptor and signalling pathway are known in epithelial tumours, which affect anti-EGFR therapy efficacy. In addition to protein overexpression and gene amplification, mutation of the TK domain and deletion of the extracellular ligand binding domain (this results in the so called vIII variant) were described in various types of tumours. Being one of the most important proteins of EGFR-signalization and one of the most frequent oncogene in human tumours, KRAS may also carry mutations that influence the outcome of the anti-EGFR therapy.

- ***Claudins***

Claudins are proteins with molecular weight of 20-27 kDa playing a role in building up tight junction (TJ) type cell adhesion structures. They have an important role in the regulation of paracellular permeability and in the maintenance of cell polarity in epithelial and endothelial cells. They are built up of four transmembrane domains and two extracellular loops, and have cytoplasmic N-terminal and C-terminal endings in addition. During the development and progress of malignant tumours cell-cell relationships change, and during this process cell adhesion structures, like claudin molecules are altered. Such alterations can manifest in an increased or decreased expression of claudins as compared to normal tissues. This can be used later in histological differential diagnostics, and it also has some prognostic significance and may serve as basis for a target specific therapy.

2. AIMS AND OBJECTIVES

We studied the EGF-receptor epitope pattern on a Hungarian patient population using epitope-specific antibodies and studied potential abnormalities of the EGFR gene (amplification, vIII-, TK domain mutation) that are known to significantly influence the efficacy of targeted therapies. In addition, we aimed at identifying HPV infection and KRAS mutation in the tumours. As an additional objective we studied the claudin expression pattern of the same tumours and of the adjacent normal epithelium.

Questions addressed:

1. To what extent do head and neck tumours with various localizations express the EGFR protein?
2. What is the mutual relationship of the molecular properties mentioned above and examined by us; do they influence patient's survival and can they be accounted for any potential inefficacies of the anti-EGFR therapy?
3. Does the claudin pattern of normal and tumorous tissues in the head and neck region differ? Does it represent any localization-related difference? Are the potential

expressional differences related to the clinicopathological parameters tested by us?

4. Is the potentially different claudin pattern related to prognosis or not?

3. MATERIALS AND METHODS

Our samples are obtained from the surgical samples of 71 patients treated with head and neck squamous cell carcinoma at the Department of Ear-Nose and Laryngology and Head and Neck Surgery of Semmelweis University.

We mapped EGFR, vIII mutation and claudin expression using immunohistochemical methods (TMA). We used FISH technique to study EGFR gene amplification, PCR for determining any TK domain mutations (HRM), KRAS mutations (RFLP) and HPV 16, 18, and 33 positivity. We analyzed our results using adequate statistical methods.

4. RESULTS

- ***EGFR***

With regards to EGF-receptor gene numbers we found gene amplifications in 11.6% of the cases, and this was most frequently detected in the hypopharyngeal region; we found polysomia in 8.7% and trisomia in 24.6%. Altogether we found increased EGFR gene copies in 45% of the samples, and this was associated with a significantly worse survival rate.

EGFR protein expression and activity demonstrated differences in tumours of various localizations, and various epitopes of the receptor stained with different intensity, as well. In addition, we also observed a great extent of heterogeneity within the same single tumour. Using the antibody that recognizes the intracellular domain, 3+ protein expression intensity was associated with a significantly worse survival rate. Patients with an elevated EGF-receptor activity in the tumour (when activity was studied with phospho-EGFR specific antibodies) showed a better survival rate. This result, however, did not reach the level of statistical significance.

In our samples vIII mutation frequency was 21%, and the majority of such mutations occurred in the laryngeal region.

We found no TK domain mutations in our tumour samples, whereas KRAS mutations frequency was 2.8%.

Integrated, high risk HPV (HPV 16, 18 and 33) was present in 19.7% of the samples, predominantly in tongue base and tonsillar localization. As opposed to previous literature data, HPV positivity was not associated with a better survival rate in our tested population.

We performed a statistical co-evaluation of EGFR abnormalities, histological characteristics and HPV infection. We determined that in all of the vIII variants, the gene copy number was elevated, while in tumours expressing the wild EGFR increased number of gene copies were only observed in 30%. The extent of EGFR activation and HPV infection do not correlate with the presence of the vIII mutant receptor. We could not demonstrate statistical differences either between HPV infection and vIII mutation, or between HPV infection and EGFR activity. Gene amplifications were not seen in any HPV positive tumour samples. We demonstrated that in case of the studied head and neck tumours inflammatory infiltration and vessel invasion have prognostic significance from among the classical clinicopathological factors.

- ***Claudins***

Our samples from normal and tumorous epithelium were both negative for CLDN3 -8 and -10.

CLDN1, -2, and -7 expression showed significant differences between normal and tumorous tissues, which were predominantly independent of localization, except for CLDN1 expression that was significantly higher in oropharyngeal tumours than in the other two localizations. As compared to normal epithelium, we found elevated CLDN1 and -7, and decreased CLDN2 expression in our tumorous samples. The extent of CLDN1 expression in the tumorous samples was higher as compared to CLDN7 expression. CLDN1, -2, and -7 expression proved to be independent of most of the known clinical and pathological parameters, for example of age, gender, HPV status, alcohol consumption, staging and tissue differentiation.

CLDN1 and CLDN7 expression do not modify overall survival, however, a worse survival is associated with the lower expression of CLDN2 expression.

5. CONCLUSIONS

1. Based on our results the Hungarian HNSCC patient population is not different from the European average with regards to biological characteristics of the EGFR. Traditional pathological parameters (extent of tumour differentiation, vessel invasion, inflammatory infiltration) show strong correlation with survival. EGFR overexpression could be detected in the majority of our tumorous samples. EGFR protein expression and activity, as well as staining of different epitopes showed significant heterogeneity both in tumours of various localizations and within the same tumour, as well. As a result, we do not consider this to have any diagnostic or prognostic significance.
2. The elevated copy number and overexpression of EGFR was associated with a significantly worse survival. As opposed to available literature data, HPV positivity of our own tumorous samples (19.7%) was not associated with a better survival rate. This can be attributed to the fact that based on the medical history data in our patients chemical and viral carcinogenesis concurred. We had no TK domain mutations in our tumorous samples at all, and KRAS mutation was

also rare (2.8%). As opposed to this, the 21% frequency of vIII mutation indicates that the vIII variant of the EGFR could explain the inefficacy of anti-EGFR therapy. It would be therefore desirable to detect the presence of the vIII variant in addition to testing the extent of EGFR expression before any therapeutic antibodies are administered.

3. Our samples from normal and tumorous epithelium were negative for CLDN3, -8, and -10. CLDN1, -2 and -7 expression showed significant differences between normal and tumorous tissues, which were predominantly independent of localization, except for CLDN1 expression that was significantly higher in oropharyngeal tumours than in the other two localizations. As compared to normal epithelium, we found elevated CLDN1 and -7 expression in our tumorous samples; however it was not significantly correlated to survival. In the studied tumours CLDN1 expression was the most expressed, thus we predict this to be potentially the most efficacious therapeutic target in HNSCC.
4. In our tumorous samples decreased CLDN2 expression was associated with a significantly worse prognosis, thus we consider its presence or absence a promising prognostic factor.

6. LIST OF THE AUTHOR'S OWN PUBLICATIONS

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