Changes in gene expression of EGFR and LAD1 in patients with metastatic squamous cell laryngeal carcinoma

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I. Introduction

Numerous genetic mechanisms are known to be closely related to oncogenesis, disease progression and survival prognosis of patients. It has been proven that gene transformation responsible for normal cell proliferation has a key role in tomour growth. A classical example are genes encoding tyrosine kinase cell membrane receptors, which is part of cellular signalisation and transduction of growth signals. Molecular defects pertaining such receptors (point mutaions, deletions or overexpression) could lead to neoplasia. The epidermal growth factor receptor (EGFR) is a member of the ErbB/HER family of receptor tyrosine kinases (RTKs) and is essential for several cellular survival processes [1,2]. EGFR is one of the most implicated genes in carcinogenesis due to its frequent overexpression and mutations in multiple cancers [3,4]. Up to 80–90% of HNSCCs overexpress or harbour mutations in EGFR, and these alterations directly impact overall and progression-free survival [5]. Targeting EGFR therapeutically using anti-EGFR monoclonal antibodies or kinase domain inhibitors with concomitant radiation remains one therapeutic option for patients with HNSCC. EGFR status is increasingly being recognized as a predictor of survival as well as chemoradiation response in HNSCC.

EGFR structure: EGFR is a transmembrane protein comprised of an extracellular ligand-binding domain (ECD), a transmembrane domain (TD), a juxtamembrane (JM) segment, a tyrosine kinase domain (TKD), and a C-terminal regulatory tail. The conformational change caused by ligand binding leads to auto- and trans-phosphorylation of the TKD with subsequent recruitment of adaptor proteins such as Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains. These domains then activate downstream pathways essential for cell survival and proliferation [6]. Mutations in various domains of the tyrosine kinase recetor (RTK) could lead to a constitutive activation in the absence of a ligand as some of these mutations could be related to EGFR up regulation and it`s phosphorilation as it has been observed in missence mutations in the extracelullar domain (P596L, G598V, A289V) in glioblastoma [7]. Overexpression of RTK, often through gene amplification, leads to increased receptor concentration. Such overexpression even in a structurally normal receptor (with or without the presense of a ligand) bolsters neoplastic transformation. Increased receptor expresiion makes the cell more sensitive to EGF family signalisation as the speed with which formation of heterodimers occurs is increased as well. That results in cell proliferation stimulus at an unusually low EGF concentra-

tion. Since EGFR plays a critical role in regulating various cellular processes, oncogenic mutations in any of these domains result in aberrant expression and/or dysregulated signaling while also altering responses to EGFR targeting agents

EGFR has been shown to be of prognostic significance in Head and Neck squamous cell carcinoma (HNSCC). Multiple studies have shown that the overexpression of EGFR in HNSCC directly correlates with worse outcomes. [8,9] Meta-analyses have shown that EGFR overexpression is associated with reduced overall survival (OS), and progression-free survival (PFS), and disease-free survival (DFS) with the magnitude of these effects varying from study to study. [10] A phase III EXTREME study that evaluated EGFR copy number as a predictive marker in patients treated with a combination of Cetuximab and platinum/5-fluorouracil (5-FU) as a first-line treatment of recurrent and/or metastatic squamous cell carcinoma of the head and neck which lines the importance of further discernment of EGFR expression and therapy responce. [11]

LAD1 is a gene encoding an anchoring filament that is a component of basement membranes. It may contribute to the stability of the association of the epithelial layers with the underlying mesenchyme and communication between cytoskeleton and extracellular matrix. It has been proven that there is a connection between mutations in LAD1 and worse outcome in laryngeal, thyroid and lung cancer. Byul Moon et.al. Investigate expression levels of LAD1 and their relation to increased metastatic potential in colorectal cancer. In animal test subjects high levels of gene expression have been established in metastatic tissues. The results show a relationship between LAD1 overexpression and increased metastatic potential, and overall worse survival prognosis and disease progression of colorectal cancer in which migration and invasion of cancer cells is promoted. [12] Ianping Li et al. Examine the role of LAD1 expression in therapy response to Docetaxel in prostatic cancer. The studies show increased expression in cancer cells which leads to the question if it could be implemented as a prognostic marker if further investigation in that line is conducted.[13]

It is widely accepted by numerous authors that loss of cell cohesion, extracellular tissue damage and epithelial mesenchyme transition lead to increased metastatic potential. [14] EMT is a process encompassing phenotypic changes where epithelal cells acquire mesenchymal features: lose cell-cell junctions, apical-basal polarity, epithelial markers, and acquire cell motility, a spindle-cell shape, and mesenchymal markers. Metastasing is a complex process encomassing various cancer cell interactions and especialy cell membrane proteins and the surrounding extracellular matrix (ECM). EMT can be induced in various oncogenic pathways – including Src, integrin and Wnt/−β-catenin [14]. The tumor-promoting role of LAD1, respectively LAD1 mutations, in laryngeal cancer could be associated with the property of LAD1 to regulate epithelial-mesenchyme relations. That highlights the improtance of LAD1 in cancer genesis. Processes similar to EMT occur in tumor progression and malignant transormation. EMT facilitates the mobility and invasiveness of cancer cells, which is further proven by Klobučar, MarkoSedić and co by investigating the overexpression of anchoring filaments in metastatic tissues, respectively LAD1 overexpression. It has been also established through a Western blot analysis that non-metastatic tissues have no indication of such overexpression. Which is further proof of the relevance of anchor filament overexpression and disease progression and metastasaing potential in laryngeal squamous cell carcinoma.

II. Matherials and Methods

Collecting tissue samples

Collection of targeted surgical specimens, kept in buffer solution and cryoconserved in liquid soluton. Pathohistological verification of the collected tissue samples.

RNA isolation

In terms of conducting a genetic analysis total mRNA was used, isolated from cryostated tissue. mRNA isolation was performed with a ready kit for extraction of purified RNA in terms of shortening the extraction prosedure, obtaing high molecular high quality RNA without the use of unwanted purifing agents following the manufacturing protocols (Rneasy Mini Kit, Qiagen).
Quantitative analysis and spectrophotometric analysis were performed on all RNA (NanoDrop ND 1000, Thermo Fisher Scientific Inc.). mRNA was kept at a -80°C temperature.

Reversed transcription and DNA synthesis

The following kit was used – QuantiTect Reverse Transcription Kit (Qiagen), which synthesizes DNA based on total mRNA isolated from non-fixed tissues with the help of enzyme reverse transcritase following manufacturing protocols.
DNA samples were kept at -20°C temperature.

Real time PCR

The following kits were used: QuantiTect Primer Assay (Qiagen) for the following genes – *ACTB* (control gene) and *LAD1*, and QuantiTect SYBR Green PCR Kit (Qiagen).
Real – time PCR mix was prepared according manufacturing protocols.
In the RT-PCR reactions the DNA concentration was unified – 100ng for each one.
The real time PCR was performed on 7900HT Fast Real Time PCR System (Applied Biosystems) as follows:

Table 1. Real-time PCR running program:

Steps	**Polymerase activation**	**PCR**		
	Primary denaturation	45 cycles		
		denaturation	Aniling	Elongation
Temperature	95ºC	94ºC	55ºC	72 ºC
Time (min	15:00	0:15	0:30	0:30

Gene-expression analysis

Gene-expression analysis of the tumor tissue was performed using quantitative real-time Polymerase Chain Reaction – RT-PCR of the gene LAD1 in 12 tumor tissue samples, where the results were compared to the LAD1 expression in normal laryngeal tissue sample as well as to the *ACTB (control gene)* expression. Levels of gene expression in normal and cancerous laryngeal tissue were compared.
The methodology is based on RT-PCR which allows precise quantitative DNA analysis during the amplification reaction by taking note on the amount of fluorescent dye – SYBR Green I. It allows the identification of all double stranded DNA products of the reaction by the increase of the SYBR Green fluorescentic signal.
The quantitative analysis is based on measuring the amount of a surtain nucleic acid during the cycles of amplification with the use of the software SDS v.2.2.2 adapted to 7900HT Fast Real Time PCR System (Applied Biosystems). This software uses $2^{-\Delta\Delta Ct}$ method for relative quantification of the expression. At the end of the analysis the number of times the expression was changed is calculated (fold change or Relative Quantification-RQ) of both the experimental and control groups. The formula **fold change = $2^{-\Delta\Delta Ct}$** was used.
If the end value is >2,000 it is considered a positive expression change (fold upregulation) of the gene. If the end value is <0,500 it is condidered a negative expression change (fold downregulation) and is measured through the formula – $1/2^{-\Delta\Delta Ct}$.

III. Results

We have evaluated 10 patients with pathohistological diagnosed neck metastasis (Table 2) 1-doesn't have family history of cancer 2-does have family history of cancer.
100 % of the patents are male. Mean age of the patent group-67,5 years,100% of the evaluated patients are in a T4 stage of the disease.100 % of the participants are heavy tabaco and alcohol users. 100% of the evaluated patents have pathohistological diagnosed metastatic lymph nodes.100% of the evaluated patents have pathohistological diagnosed squamous cell laryngeal carcinoma.60 % of the patients have form of toxic effect in their work environment (for example heavy metals, noxious fumes). 80% of the patients have poor oral hygiene, and significant tooth decay. 66,7% of evaluated patients have G2 grade. 55,56 %of evaluated patients have family history of cancer.
Most of the patients with upregulation in LAD1 and EGFR and higher Gleason score (lower differentiation), and family history of cancer have a worse prognoses and faster progression compared to other patients.
There is association with the N stage of the patient and the levels of LAD1 and EGFR upregulation in the tissues.

IV. Conclusion

In conclusion we want to discuss the following findings.

1. In patients with downregulation, the patient theoretically will have good response to target therapy with EGFR inhibitors.
2. Levels of expression of EGFR can be used for predictors of the patients s response towards tar-

Table 2

stage	grade	LAD1 tc	LAD1 tp	LAD1m	EGFRtc	EGFRtp	EGFRm	family
T4N1Mx	G2	Normal expression	Normal expression	Downregulation	Normal expression	Normal expression	Downregulation	1
T4N2Mx	G2	Downregulation	Upregulation	Downregulation	Downregulation	Normal expression	Downregulation	2
T4N2Mx	G2	Upregulation	Upregulation	Downregulation	Upregulation	Upregulation	Downregulation	2
T4N3Mx	G1	Downregulation	Downregulation	Downregulation	Normal expression	Normal expression	Downregulation	1
T4N2Mx	G2	Upregulation	Normal expression	Normal expression	Normal expression	Normal expression	Normal expression	1
T4n1Mx	G1	Normal expression	Normal expression	Downregulation	Normal expression	Normal expression	Downregulation	2
T4N2mx	G2	Normal expression	Normal expression	Normal expression	Normal expression	Normal expression	Upregulation	1
T4N3Mx	G2	Downregulation	Normal expression	Upregulation	Upregulation	Normal expression	Normal expression	2
T4N3Mx	G3	Upregulation	Upregulation	Upregulation	Upregulation	Upregulation	Upregulation	2

get therapy. Practical result- predictor for EGFR-inhibitor resistance and overall patient prognosis.

3. There is an developed antibody against ladinin – the protein product of LAD1 gene, and expression levels can be evaluated via immunohistochemistry evaluation of a routine histological biopsy. That can lead to more accurate prognosis for the patients.
4. In all evaluated patients differences in the levels of gene expression are diagnosed in the tumour centre, periphery and metastatic lymph nodes.

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