Combined EGFR/ALK Expression Analysis in Laryngeal Squamous Cell Carcinoma

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Abstract. Background/Aim: Epidermal growth factor receptor (EGFR) acts as an oncogene in malignancies. Our aim was to examine the role of combined EGFR/anaplastic lymphoma kinase (ALK) expression as molecular markers in laryngeal squamous cell carcinoma (LSCC) patients. Materials and Methods: Fifty (n=50) tissue sections derived from twenty-five (n=25) primary LSCCs were analyzed by immunohistochemistry (IHC). Results: EGFR overexpression was observed in 17/25 (68%) cases. Concerning ALK, 23/25 (92%) demonstrated low expression. EGFR expression was associated with grade (p=0.049), whereas ALK expression was correlated with stage (p=0.048). ALK overexpression was detected at advanced-stage EGFR-positive cases. A biphasic EGFR protein expression pattern was observed in five (n=5) LSCC cases, whereas ALK expression was stable in all cases. Conclusion: EGFR overexpression is frequently observed in LSCC combined with low ALK expression.

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LSCC patients with EGFR/ALK protein overexpression should be eligible for targeted therapeutic strategies.

Molecular analyses in solid malignancies – including laryngeal squamous cell carcinomas (LSCC) – have shown that up-regulation of specific growth factor receptors critically destabilize the cell micro-environment inducing signal transduction from the membrane to the nucleus (1). Among these molecules, epidermal growth factor receptor (EGFR) plays a crucial role in this process. The EGFR (other names include: ERBB, ERBB1, or HER1) gene is located on the short (p) arm of chromosome 7 at position 12 (cytogenetic chr band 7p12.1) (2). The protein encoded by the corresponding gene acts as a transmembrane glycoprotein. It is a member of the v-erb-b2 erythroblastic leukemia viral oncogene (ErbB)/human epidermal receptor (HER) family of receptor tyrosine kinases, that includes also three other cell membrane receptor tyrosine kinases: HER2/c-neu (ERBB2), HER3 (ERBB3) and HER4 (ERBB4). Three main EGFR depended pathways have been already identified including the PI3K-AKT-PTEN-mTOR, the RAS-(B) RAF-MEK-ERK/MAPK and also the IL6-JAK1/2-STAT3 (3). Concerning LSCC, a subset of patients exhibits EGFR activating mutations (approximately 10-30%) and also gene amplification (approximately 10-35%) leading to protein overexpression (4, 5). Similarly, deregulation of anaplastic lymphoma kinase (ALK) seems to be a novel marker in handling LSCC patients with EGFR aberrant expression. ALK gene is located at chromosome 2 (2p23 band) encoding a protein which acts as a transmembrane receptor tyrosine kinase.
to this, the examined cases were classified as follows: Score 0: no
levels were evaluated based on a staining score system. According
2 protein were considered as positive controls. Protein expression
and cytoplasmic/perinuclear staining for ALK. Microscopically
Ramon, CA, USA). Membranous and sub-membranous staining
patterns were considered acceptable for EGFR essential expression,
use of an automated staining system (I 6000 – Biogenex, San
primary antibody was omitted. IHC protocol was performed by the
dehydrated and cover-slipped. For negative control slides, the
antibodies at room temperature. Following incubation with the
in the following detection steps. Blocking solution was applied to
sections of the corresponding tissue blocks. The corresponding
between variables including protein expression levels, and
clinicopathological parameters such as gender, tumor grade and stage
were performed using Pearson Chi square test ($\chi^2$) estimated along
with its 99%CI and Spearman coefficient (SPSS v20 (SPSS Inc,
Chicago, IL, USA). Two-tailed $p$-values $\leq0.05$ were considered
statistically significant. Results and $p$-values are described in Table I.

Antibodies and immunohistochemistry assay (IHC). Ready-to-use
EGFR monoclonal mouse antibody (clone 31G7-Zymed/InVitrogen,
San Francisco, CA, USA) recognizing the extracellular domain of
EGFR protein, but not reacting with other erbB receptors was used.
Additionally, monoclonal mouse anti-ALK (CD246) antibody (clone
ALK-1, DAKO, Glostrup, Denmark; dilution at 1:100, 30 min at
25°C) was applied.

IHC for the markers’ expression was carried out on 4-μm serial
sections of the corresponding tissue blocks. The corresponding
slides were deparaffinized, rehydrated and enzyme digested for 10
min at 37°C. The EnVisionTM+ (Dako) detection system was used in
the following detection steps. Blocking solution was applied to
the slides for 10 min, followed by incubation for 1 h with the
antibodies at room temperature. Following incubation with the
secondary antibody for 10 min, diamobenzidine-tetrachloroide-
DAB (0.03%) containing 0.1% hydrogen peroxide was applied as a
chromogen and incubated for 5 min. Sections were counterstained,
dehydrated and cover-slipped. For negative control slides, the
primary antibody was omitted. IHC protocol was performed by the
use of an automated staining system (I 6000 – Biogenex, San
Ramon, CA, USA). Membranous and sub-membranous staining
patterns were considered acceptable for EGFR essential expression,
and cytoplasmic/perinuclear staining for ALK. Microscopically
normal appearing laryngeal epithelia tissue sections expressing Bcl-
2 protein were considered as positive controls. Protein expression
levels were evaluated based on a staining score system. According
to this, the examined cases were classified as follows: Score 0: no
staining or membrane/cytoplasmic staining in <10% of tumor cells;
Score 1+: faint membrane/cytoplasmic staining in >10% of tumor
cells; Score 2+: weak or moderate complete membrane/ cytoplasmic
staining in >10% of tumor cells and Score 3+: strong, complete
membrane/ cytoplasmic staining in >10% of tumor cells. Scores of
0 and 1+ were considered as negative for EGFR/ALK expression
while Scores 2+/3+ as positive (overexpression), respectively.

Results

According to IHC evaluation guidelines, the examined cases
demonstrated different EGFR/ALK expression levels. EGFR
overexpression (Score 2+/3+, moderate to high expression
levels) was observed in 17/25 (68%) LSCC tissue sections,
whereas the rest of them demonstrated low expression (Score
1+). EGFR overall expression was correlated to the gender
of the examined patients ($p=0.007$). Concerning ALK
expression, the majority of the examined tissue sections
(23/25 - 92%) demonstrated low expression levels. EGFR
expression was associated with the grade ($p=0.049$), whereas
ALK expression was correlated with the stage of the
examined malignancies ($p=0.048$). No statistical significance
was observed correlating EGFR with ALK overall expression
($p=0.133$). Interestingly, ALK overexpression was detected
mainly in EGFR positive cases with advanced stage cancer.
Biphasic (score 1+/2+) EGFR protein expression pattern was
observed in five (n=5) LSCC cases. Score 2+ was considered
prominent for categorizing these cases in protein analysis. In
contrast to EGFR partial biphasic expression, ALK
expression pattern was stable (homogenous) in all examined
cases (Figure 1).

Discussion

Identification of specific gene deregulation mechanisms leads
to optimal management of patients suffered by solid
malignancies. Concerning LSCC, anti-EGFR targeted
therapeutic strategies based on monoclonal antibodies (mAbs)
or tyrosine-kinase inhibitors (TKIs) represent very promising
approaches in modern oncology (10, 11). EGFR
overexpression – due to gene amplification or point
mutations – is frequently observed in LSCC cases analyzed by
IHC (12, 13). In our protein analysis, overexpression of the
marker was correlated with the grade of the examined cases.
Another study has shown similar association combined, also,
with overall advanced disease characteristics, including stage
and metastasizing potential leading to poor prognosis (14).
Interestingly, another study has concluded that the synergetic over activation of EGFR and hepatocyte growth factor tyrosine kinase receptor MET proto-oncogene, (c MET-gene locus: 7q31) is associated with poor survival especially in patients with glottis LSCC (15). Similarly, another LSSC study has evaluated the efficacy of ME22S agent, a novel EGFR/Met bi-specific antibody using an in vitro and also a xenograft model (16). The study has indicated that dual inhibition of EGFR and Met suppressed the invasion and growth potential of the corresponding LSCC cell cultures. They suggested that this dual action should be a modern approach in therapeutic strategies for the treatment of LSCC subsets of patients with specific molecular signatures. Concerning the EGFR protein expression in LSCC, a biphasic (membranous to cytoplasmic) expression pattern (DA chromogen, original magnification 100×).

Figure 1. Histogram showing the EGFR and ALK expression patterns (Score 1+/2+/3). Biphasic expression (Score 2+/1+) was reported only for EGFR in some cases. Inside plates: overexpression of EGFR and ALK assayed by IHC (Score 2+/3+). Note the membranous/cytoplasmic staining pattern (DAB chromogen, original magnification 100×).

Table I. Clinicopathological parameters and total EGFR/ALK protein expression results.

| Clinicopathological parameters | EGFR | p-Value | ALK | p-Value |
|--------------------------------|------|---------|-----|---------|
| OE | LE | OE | LE |
| LSCC (n=25) | 17/25 (68%) | 2/25 (8%) | 8/25 (32%) | 23/25 (92%) |
| Gender | | | | |
| Male (n=21) | 16/25 (64%) | 2/25 (8%) | 5/25 (20%) | 19/25 (76%) |
| Female (n=4) | 1/25 (4%) | 0/25 (0%) | 3/25 (12%) | 4/25 (16%) |
| Grade | | | | |
| 1 (n=7) | 6/25 (24%) | 2/25 (8%) | 1/25 (4%) | 5/25 (20%) |
| 2 (n=17) | 9/25 (36%) | 8/25 (32%) | 0/25 (0%) | 17/25 (68%) |
| 3 (n=1) | 1/25 (4%) | 0/25 (0%) | 0/25 (0%) | 1/25 (4%) |
| Stage | | | | |
| I (n=7) | 6/25 (24%) | 0/25 (0%) | 1/25 (4%) | 7/25 (28%) |
| II (n=15) | 10/25 (40%) | 2/25 (8%) | 5/25 (20%) | 13/25 (52%) |
| III (n=3) | 2/25 (8%) | 0/25 (0%) | 1/25 (4%) | 3/25 (12%) |

OE: Over expression (2+/3+); LE: low expression (0/1+).
pattern was observed in some cases. Intratumoral heterogeneity in EGFR protein expression – due to different genetic substrates - in LSCCs has been similarly detected by IHC (17). Another study co-analyzing EGFR at the DNA and protein levels has identified a correlation between cytoplasmic predominantly expression and gene amplification, especially in LSCCs derived from glottis (18). In contrast to this association, E-cadherin and not EGFR aberrant expression seems to be correlated with advance stage (distant high metastatic potential, poor prognosis) in LSCC (19).

ALK aberrant expression – due to its gene rearrangements (fusion or mutations) – is, in contrast to NSCLC, not a frequent event in LSCC (20, 21). Low expression is common in these malignancies. In our protein analysis, only a small subset of specimens demonstrated overexpression of the marker. Concerning its inhibition strategies, ceritinib, crizotinib, alectinib and brigatinib have been developed and have been FDA approved as targeted agents (22, 23). Interestingly, a study analyzing in vitro (cell cultures, xenografts) the potential interaction between EGFR and ALK aberrant expression has shown that the induction of ALK acts as a novel mechanism of EGFR inhibitor resistance in these carcinomas (24). In fact, application of gefitinib in combination with ceritinib and brigatinib, induced ALK expression. In contrast to LSCC, specific HNSCC pathological entities demonstrate differences regarding ALK gene expression profiles. Controversial data have been published for ALK alterations in sarcomatoid HNSCC referring to its translocation (25, 26).

Conclusion

Assessment of ALK and EGFR aberrant protein expression due to gene deregulation (fusions/translocations, mutations, amplification) seem to be critical for applying targeted therapeutic regimens in subsets of LSCC patients with specific molecular aspects. Although ALK expression is mainly low in these malignancies, interaction between the two proteins composes a mechanism that modifies resistance to anti-EGFR targeted therapies. This observation influences also other HNSCCs, such as oral SCC. ALK inhibitors seem to induce the anti-tumor activity of EGFR inhibitors in these carcinomas by increasing ALK expression, especially through abolition of intermediate signalling transduction molecules, such as AKT activation (27).

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors’ Contributions

Anastasia Politi: researcher; Evangela Tsiambas researcher, paper writing; Nicholas S Mastronikolis: academic advisor; Dimitrios Peschos: academic advisor; Ioannis Aspridis: academic advisor; Efthymios Kyrodimos: academic advisor, paper writing; Ilianna Armati: statistical analysis; Asimakis Asimakopoulos: academic advisor; Anna Batistatou: academic advisor; Vasileios Rogos: academic advisor; Aristeidis Chrysovergis: clinical advisor; Vasileios S Papanikolaou: clinical advisor.

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