SPANDIDOS PUBLICATIONS

Impact of EGFR immunoexpression on STAT3 activation and association with proinflammatory/regulatory cytokine pattern in laryngeal squamous cell carcinoma

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Abstract. Stimulation of epidermal growth factor receptor (EGFR) results in the activation of signal transducer and activator of transcription-3 (STAT3), a transcriptional factor associated with carcinogenesis. Proinflammatory cytokines are capable of activating a tumor cell death program by reducing EGFR tyrosine phosphorylation. This study aimed to identify EGFR expression in laryngeal carcinoma and determine the relationship with STAT3 and proinflammatory/regulatory cytokine secretion. An analysis of EGFR expression (membranous EGFR-m and cytoplasmic EGFR-c) was performed in tumor tissues by immunohistochemical (IHC) staining in 45 medical cases of laryngeal carcinoma. STAT3 expression in freshly isolated tumor and non-cancerous normal epithelial cells by RT-PCR was analyzed in 24 patients after total larynx resection. The concentrations of TNFα, IL-2, IL-6, IL-8 and IFNγ secreted by purified peripheral blood mononuclear cells (PBMCs) or contained in whole blood samples were measured by ELISA. The relationship between EGFR and mRNA STAT3 expression as well as the level of secreted cytokines was investigated. In our study, 93.3% tumors expressed EGFR-m and 37.8% EGFR-c. It also revealed a statistically significant dependence of the EGFR status on STAT3 expression in neoplastic tissues. Tumors with IHC EGFR-m positive staining >50% of the total number of cells, as well as with EGFR-c positive staining, were characterized by the most frequent presence of STAT3 expression. Our data demonstrate a significant negative relationship between EGFR-m expression and TNFα concentration, and a positive connection between membranous EGFR and IL-8 or IFNγ levels recorded in isolated PBMCs. Furthermore, this study revealed a significant relationship between EGFR-c immunoexpression and IL-8 or IFNγ concentration. Our findings have confirmed a key role of EGFR in determining the proliferative and malignant potential of laryngeal carcinoma.

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Introduction

Epidermal growth factor receptor (EGFR, ErbB1 and HER1) is a member of the ErbB receptor family and a type I tyrosine kinase receptor. It occurs as a transmembrane glycoprotein, which undergoes autophosphorylation and triggers signaling pathways involving the signal transducer and activator of transcription-3 (STAT3), phosphatydylinositol 3'-kinase/protein kinase B (PI_3K/Act), mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB), c-Myc, cyclin D1 and extracellular signal-related protein kinase 1/2 (Erk1/2) pathways (1-7). It is known as a key regulator of cellular proliferation and cell survival by cell cycle progression, inhibition of cell death, regulation of cell migration, tumor invasion and differentiation in a wide variety of human carcinoma types, such as head and neck carcinoma, cancer of the breast, colon, bladder, lung, kidney, esophagus, stomach and pancreas cancer (8-10).

A large number of malignancies of epithelial origin have been characterized by the overexpression of EGFR and/or its ligands, such as epidermal growth factor (EGF), transforming growth factor-α (TGFα) and amphiregulin (11,12). The amplification of genes encoding EGFR has also been noted in previous studies (13-15). The involvement of STAT3, as a latent transcriptional factor, in the activation of apoptosis and cell cycle progression, and its impact on carcinogenesis in various neoplasms were demonstrated (6,7,16-18). EGFR

stimulation, as with other mechanisms connected with IL-6R, VEGF and HGF signaling, results in the EGFR-dependent activation of the STAT3 controlling gene expression (19-22). A positive correlation between elevated EGFR activity, including kinase domain-activating mutant EGFR and STAT3 has been described for many human primary tumors (19,23-26). In squamous cell head and neck carcinomas (HNSCC), constitutively active STAT3 has been shown to be associated with EGFR signaling and deregulated cell growth (20,21,27, 28). Nevertheless, several studies have been reported, which demonstrate the interaction of proinflammatory cytokines with EGFR as well as the effects of antitumor cytokine connected with the activation of cell death program (29-33).

It is known that TNFα is capable of activating tumor cell apoptosis through receptor clustering and the stimulation of caspases (caspase-8) (31,32). Evidence for the stimulation of protein tyrosine phosphatase and the reduction of EGFR tyrosine phosphorylation by TNFα has been provided. Thus, decreased EGFR tyrosine phosphorylation leads to the blockade of the EGFR-mediated signal transduction (32). Another mechanism resulting in an increased EGFR expression and connected with the stimulation of TNFα receptor has also been noted (32,34-36). Evidence suggested that the over-expression of tyrosine kinase activity EGFR may suppress the antiproliferative or cytotoxic activity of TNFα (32,36). Interestingly, EGFR expression can be increased by TNFα via the p55 receptor (36). This effect of TNFα on EGF receptor tyrosine phosphorylation in tumor cells depended on the sensitivity or resistance to TNFα of cell variants selected for apoptotic and tyrosine kinase signaling processes (32). However, few studies introducing a new target of overexpressed EGFR by using the combination of monoclonal antibodies and TNFα to abrogate the host immune response resulting in enhanced tumor regression, have been presented in the literature (31,32).

Previous data suggested a new method of targeting EGFR signaling in epithelial tissue tumors. It has been demonstrated that the EGFR pathway was inhibited by blocking the activity of a key protease TNFα-converting enzyme (TACE, disintegrin and metalloproteinase 17), which regulates the bioavailability of EGFR ligands (3,37-39). Persistently activated mutant EGFR has been shown to be connected with the production of high IL-6 levels, leading to the increase in carcinogenesis via the IL-6/gp130/JAK pathway (19,20). The epigenetic regulation of gene expression resulting in IL-6 overexpression can escalate tumor progression by altering promoter methylation and gene expression of growth regulators such as EGFR (40). EGFR, TGFα and MAP kinase ERK1/2 are also involved in the mechanism of IL-8 production (3,41,42). Moreover, signaling through EGFR can lead to the PI_3K/Act pathway and may increase hypoxia-inducible factor (HIF) activity which is responsible for stimulating cytokines and growth factors (2).

Our study aimed to analyze cytoplasmic and membranous EGFR expression in squamous cell laryngeal carcinoma, investigate the relationship between EGFR status and STAT3 expression in tumor cells, evaluate the secretion level of proinflammatory/regulatory cytokines (TNFα, IL-2, IL-6, IL-8 and IFNγ) and become familiar with their role in determining the proliferative and aggressive tumor potential.

Table I. Clinical characteristics of patients.

Feature	No. of patients	%
Total no.	45	
Gender		
Men	43	95.6
Women	2	4.4
Surgical treatment		
Complete laryngectomy	24	53.3
Partial laryngectomy	21	46.7
Lymphadectomy (+)	19	42.2
Lymphadectomy (-)	26	57.6
pT		
pT2	14	31.1
pT3	17	37.8
pT4	14	31.1
pN		
pN0	31	68.9
pN1-3	14	31.1
EGFR-m (%)		
<25	17	37.8
25÷50	9	20.0
>50	19	42.2
EGFR-c		
None	28	62.2
Weak to moderate	11	24.5
Strong	6	13.3

Materials and methods

Tissue samples, histological classification and morphological features. For this study, 45 cases (43 men and 2 women, aged 48-83 years, mean age 58±5 years) of archival tissue samples and paraffin-embedded tissues of surgically resected specimens from patients treated for squamous cell laryngeal carcinoma were utilized. Each patient underwent complete (53,3%; 24/45) or partial (46,7%; 21/45) surgical resection of the larynx and 42.2% (19/45) of the patients underwent dissection of the cervical lymph nodes with pathological confirmation of the metastases (pN1-3) in 31.1% (14/45) of cases. The lesions were assessed according to the criteria of the International Union Against Cancer (UICC-TNM 2002) (43). Criteria for patient participation in this study were: i) a histologically confirmed diagnosis of carcinoma planoepitheliale, ii) primary surgical resection without receiving prior immuno-, radio- or chemotherapy and iii) absence of distant metastases. Clinical characteristics of patients are shown in Table I.

Immunohistochemistry. Paraffin sections were mounted onto SuperFrost slides, deparaffinized, treated in a microwave

solution of citrate buffer, pH 6.0 for 30 min (2x5 min and 4x5 min 180W) and transferred to distilled water. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide in distilled water for 30 min, and sections were rinsed with Tris-buffered saline (TBS, DakoCytomation, Denmark) and incubated with monoclonal mouse anti-human GEFR antibody (Novocastra Laboratories Ltd. UK, clone: GGFR.113), dilution: 1:75. EnVision+Systems-HRP for mouse and rabbit (DakoCytomation) prepared according to the manufacturer's instructions were used. Visualisation was performed by incubating the sections in a solution of 3,3'-diaminobenzidine (DakoCytomation). After washing, the sections were counterstained with hematoxylin and coverslipped. For each antibody and sample a negative control was processed. Negative controls were carried out by incubation in the absence of the primary antibody and always yielded negative results. Within tumor cells, the EGFR protein was detected primarily in the cytoplasm and immunoreactive EGFR protein was detected primarily on the cell membrane. The intensity of immunohistochemical staining as a percentage of tumor cells with EGFR membranous (EGFR-m) expression was scored using a three-tier system: 1, <25%; 2, 25÷50% and 3, >50% positive cells. The cytoplasmic staining (EGFR-c) intensity was classified as 0, none; 1, weak to moderate and 2, strong. At least 10 high-power fields (magnification, x40) were assessed for each specimen. The slides were assessed in three independent sessions by two investigators.

Lymphocyte isolation and ELISA for TNFα, IL-2, IL-6, IL-8 and IFNγ measurement. For PBMC isolation the venous blood of each patient was obtained (10 ml) and transferred to test tubes containing heparin (10 U/ml). PBMCs were isolated by Ficoll-Hypaque (1077 density) and resuspended at a concentration of $1x10^6$ cells/ml in RPMI-1640 medium. An experiment in which samples were stimulated with 5 μg/ml of PHA was also performed. The recovered cells were checked and counted for viability with the trypan blue staining method. The isolated PBMC cultures and whole peripheral blood were incubated in 96-well plates at a final volume of 0.2 ml (per well). Culture supernatants were collected after 21, 42 and 72 h in 37˚C, 5% CO_2 (Cellstar Incubator) and the secretion pattern of cytokines TNFα, IL-2, IL-6, IL-8 and IFNγ were measured with specific enzyme-linked immunosorbent assays and an ELISA kit (EIA, Pharmingen, USA) according to the manufacturer's protocols. Absorbance was measured with an ELISA reader (Elx808, Bio-Tek Instruments, USA). Each sample was tested in at least three independent ELISA experiments and the data were calculated from three tests for each sample.

Non-cancerous/cancerous epithelium cell isolation. After radical laryngectomy, the surgical tissue specimens were excised aseptically immediately after the operation from at least four tumor sites: two central, two marginal and two sites of normal non-cancerous laryngeal epithelium of the same tumor patients. Tissue fragments were washed with PBS to remove contaminated blood and inserted in RPMI-1640 (Biomed, Lublin, Poland) supplemented with antibiotics streptomycin/penicillin/gentamycin 1% v/v (Sigma-Aldrich, Germany). The whole procedure was performed on an ice plateau.

Briefly, tissue specimens were cut with a surgical knife and minced with a scalpel. This was performed in RPMI-1640 (Biomed) supplemented with antibiotics streptomycin/penicillin/gentamycin 2% v/v (Sigma-Aldrich). Tissue fragments were washed 3 times with Hanks solution (Biomed). The tumor and normal epithelial samples were then digested overnight (for 18 h) in Nunc petri-dishes with 0.16 mg/ml hyaluronidase (Sigma-Aldrich), 0.55 mg/ml collagenase (Sigma-Aldrich) and antibiotics streptomycin/penicillin/gentamycin 1% v/v (Sigma, Aldrich) in 37˚C, 5% CO_2 (Cellstar Incubator).

The digested tissues were pressed gently through a 50-μm (mesh) sieve (Sigma-Aldrich) with RPMI-1640 (Biomed). Subsequently, the suspension was washed 3 times with PBS (without Mg^{2+} and Ca^{2+}) for 20 min in 8˚C by centrifugation in an MPW-350R centrifuge at 1800 rpm/500 rcf, poured over by a dyspase solution of 2.4 U/ml, incubated for 30 min in 37˚C and resuspended in 1 ml PBS. Cell concentration was estimated using a microscope and Bürker's chamber. To discard the apoptotic and necrotic cells the columns of magnetic cell sorting separator MACS (Miltenyi Biotec, Germany) and dead cell removal kit were used. Cells were resuspended at a concentration of $1x10^5$ cells/ml in RPMI-1640. The isolated laryngeal cancer (tumor marginal cells, TMC and tumor central cells, TCC) and non-cancerous cells (normal epithelial cells, NCC) were collected immediately after the procedure and frozen at -70˚C.

RNA extraction and reverse transcription PCR (RT-PCR) analysis. Total RNA was isolated using TRI reagent (Sigma-Aldrich Co., USA) and quantified by spectrophotometry at 260 nm. RNA with a 260/280 nm ratio in the range 1.8-2.0 was considered high quality. First-strand cDNA was synthesized from each RNA pool using RNA PCR Kit ver. 3.0 (Takara Shuzo Co., Ltd., Japan) according to the manufacturer's instructions. Briefly, 1 μg RNA was combined with 2.5 pmol of oligo dT-adaptor primer, 4 μl of 25 mM $MgCl_2$, 10X RNA PCR buffer, 2 μl of 10 mM dNTP mixture, 20 units of RNase inhibitor, 5 units of AMV reverse transcriptase XL and RNase-free water to a total volume of 20 μl. The STAT-3 and ß-actin genes, as a standard, were amplified using the specific primers: STAT3(f) 5'-TTG-CCA-GTT-GTG-GTG-ATC-3' and (r) 5'-AGA-ACC-CAG-AAG-GAG-AAG-3' [RefSeq: NM 003150] and ß-actin (f) 5'-CGT-GAC-ATT-AAG-GAG-AAG-CTG-TGC-3' and (f) 5'-CTC-AGG-AGG-AGC-AAT-GAT-CTT-GAT-3' [RefSeq: NM 001101]. The thermal cycling parameters were 30 rounds of 1 min at 94˚C, 1 min at 51˚C (STAT-3, ß-actin) and 3 min at 72˚C using a GeneAmp PCR system 9700 (Perkin-Elmer Co., Shelton, USA). The size of the amplified fragments was 313 and 374 bp for STAT-3 and ß-actin, respectively.

After amplification, 10 μl of PCR products were combined with 1 μl of gel-loading buffer and the mixture was electrophoresed on 8% polyacrylamide gel (acrylamide/bisacrylamide (30/2), 10% ammonium peroxydisulfate and 0.075% tetramethyl ethyl-enediamine, at 80 V for 3 h in Tris-borate, EDTA, pH 8.0 (TBE) buffer. The gel was silver-stained. To exclude genomic DNA contamination as a source for amplified

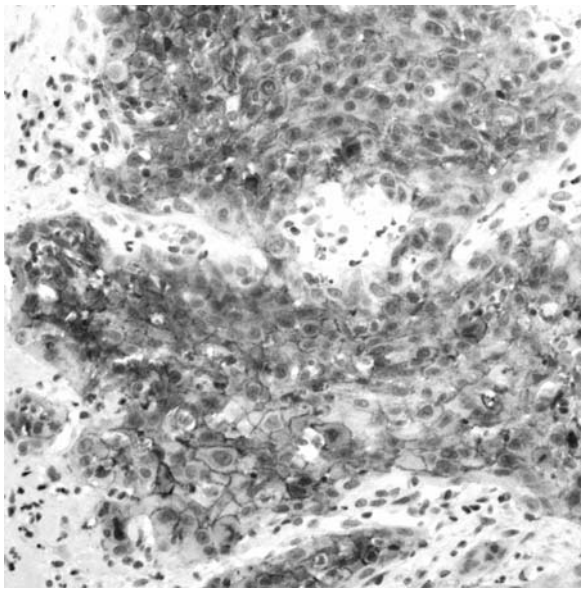

Figure 1. Representative immunohistochemical staining (IHC) for EGFR-m in tumor tissues with an index of <25%. Original magnification, x200.

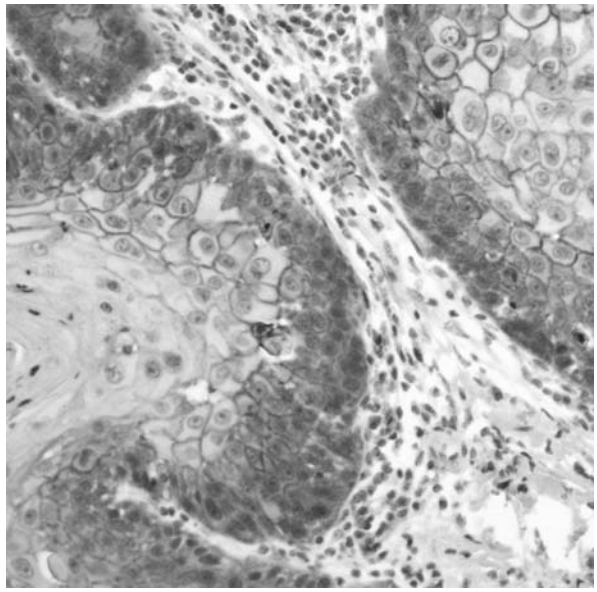

Figure 2. Representative immunohistochemical staining (IHC) for EGFR-m in tumor tissues with an index of 25÷50%. Original magnification, x300.

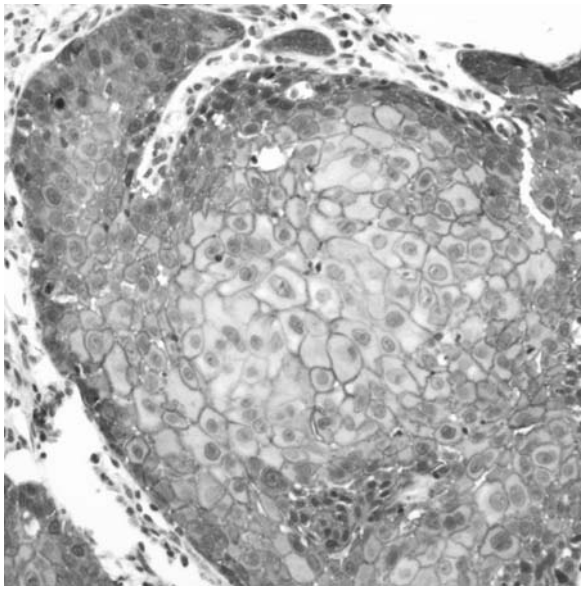

Figure 3. Representative immunohistochemical staining (IHC) for EGFR-m in tumor tissues with an index of >50%. Original magnification, x300.

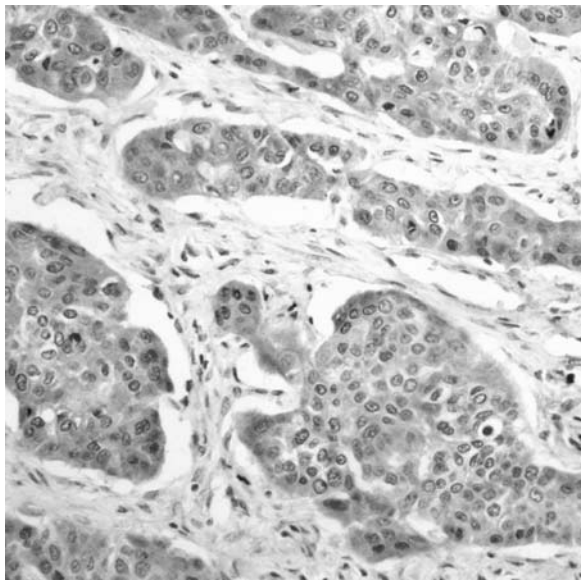

Figure 4. Representative immunohistochemical positive expression for EGFR-c. Original magnification, x200.

products, each reaction was also carried out without reverse transcriptase. In addition, no template control was run for the RT and PCR stages for each of the primer sets and none showed any visible PCR products. For the qualitative and quantitative analysis of silver nitrate-stained gels a video densitometer (Biotec-Fischer, Reiskirchen, Germany) with the software program Gel-Pro® analyzer 3.0 (Media Cybernetics, USA) was used. RT-PCR reactions were repeated 3 times for each sample. The integrated optical density (IOD) of the bands in a digitized picture was measured. STAT-3 gene expression was determined as the ratio of STAT-3 to ß-actin. In the PCR reactions, ratios were normalized to the appropriate control ratio. In normal non-cancerous laryngeal samples STAT-3/ß-actin ratios were 0.263±0.01 (mean ± SD), respectively. The cut-off value for the STAT-3 positive results (mean ± 3SD) was calculated as 0.293, respectively.

Statistical analysis of data. The statistical calculations were made by the StatPlus 2007 software (AnalystSoft, Vancouver, Canada). None of the parameters recorded in the tumor material passed tests for being normally distributed (Kolmogorov-Smirnow test). Therefore, non-parametric statistical tests for the association between the EGFR IHC index and mRNA STAT3 expression were used to analyze the results (Mann-Whitney U test and Spearman's rank analysis). The non-parametric Kruskal-Wallis and one-way ANOVA tests were

Distribution of EGFR membranous and cytoplasmic coexpression.

EGFR-m	1 (<25%)	2 (25÷50%)	3 (>50%)	Total
EGFR-c				
0 (None)	11	6	11	28/45 (62.2%)
1 (Weak or intermediate)	6	2	3	11/45 (24.5%)
2 (Strong)	0	1	5	6/45 (13.3%)
Total	17/45 (37.8%)	9/45 (20%)	19/45 (42.2%)	45

used to estimate the association between the EGFR index and median concentration ratio of each cytokine. Dunnett correction was applied to adjust for multiple comparisons. $P<0.05$ was considered to be significant.

Results

Our experiments confirm the membranous EGFR (EGFR-m) positive expression in 93.3% of tumor samples and the cytoplasmic EGFR (EGFR-c) positive expression in 37.8% cases of squamous cell laryngeal carcinoma. An EGFR membrane staining index of <25% manifested itself in 17 out of 45 (37.8%) samples and the index of 25÷50% in 9 out of 45 (20.0%) cases and >50% was observed in 19 out of 45 (42.2%) cases. Cytoplasmic staining intensity, classified as negative, was observed in 62.2% (28/45) of tumor samples. The remaining 37.8% (17/45) of cases revealed positive (weak to moderate or strong) cytoplasmic staining. Representative IHC stainings for EGFR-m and EGFR-c are shown in Figs. 1-4. Immunohistochemical scores of EGFR-m and EGFR-c coexpression of epidermal growth factor receptor are shown in Table II. Non-cancerous normal epithelial cells used as a control and laryngeal neoplastic cells used as a studied sample isolated from the tumors of 24 patients after total larynx resection were evaluated for the expression of STAT3 mRNA by RT-PCR.

We have demonstrated that laryngeal cancerous epithelial (marginal and central tumor) and non-cancerous normal epithelial cells expressed mRNA for STAT3. Additionally, we have found that the mean expression of the signal transducer and activator of transcription-3 factor in tumor cells was 0.23±0.11 $IOD_{STAT3/ß\text{-}actin}$. Our study has revealed that central cancer and marginal tumor cells demonstrated 0.25±0.13 and 0.22±0.09 $IOD_{STAT3/ß\text{-}actin}$ expression of STAT3, respectively. The level of STAT3 in the non-cancerous normal epithelial cells was 0.19±0.12 $IOD_{STAT3/ß\text{-}actin}$, which is the lowest recorded STAT3 level in comparison with the expression in cancerous laryngeal cells. In the present experiment, the supernatants of purified PBMC cultures and whole peripheral blood samples, with and without PHA stimulation, were collected after 21, 42 and 72 h in the 45 cases. The secretion patterns of proinflammatory/regulatory cytokines (TNFα, IL-2, IL-6, IL-8 and IFNγ) were measured using an ELISA test. Among these five cytokines, either secreted by freshly isolated PBMC cultures or measured in whole peripheral blood samples, IL-6 and -8 have been detected in the highest concentrations. The cytokine concentrations either produced by purified PBMC cultures or measured in whole blood are shown in Table III.

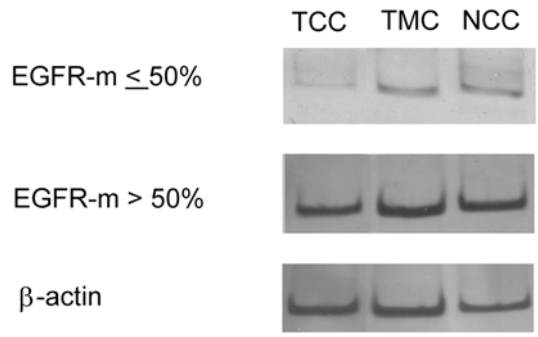

Figure 5. STAT3 mRNA expression by RT-PCR in neoplasm cells isolated from tumors with an EGFR-m index of ≤50% and >50% positive cells.

Relationship between EGFR-m status and STAT3 expression in neoplastic cells. To verify whether EGFR-m determines STAT3 expression in tissues of squamous cell laryngeal carcinoma or not, we juxtaposed the mRNA amplification level obtained by PCR for STAT3 with EGFR immuno-expression. Our study has revealed a statistically significant dependence of EGFR status on STAT3 expression in neoplastic tissues. We have found that the mean expression in tumor central and marginal cells, isolated from tumors with an EGFR-m index of ≤50% positive and non-cancerous normal epithelial cells were: TCC, 0.16±0.9 $IOD_{STAT3/ß\text{-}actin}$; TMC, 0.2±0.04 $IOD_{STAT3/ß\text{-}actin}$ and NCC, 0.19±0.06 $IOD_{STAT3/ß\text{-}actin}$, respectively. Our study has revealed that the mean expression of STAT3 in tumor central and marginal cells, isolated from neoplasms with an EGFR-m index of >50% positive and in non-cancerous normal epithelial cells were: TCC, 0.32±0.15 $IOD_{STAT3/ß\text{-}actin}$; TMC, 0.31±0.09 $IOD_{STAT3/ß\text{-}actin}$ and NCC, 0.3±0.05 $IOD_{STAT3/ß\text{-}actin}$, respectively. Moreover, we have observed that tumors, in which >50% of cells were sensitive to IHC EGFR-m staining were characterized by the most frequent presence of STAT3 expression (Spearman's test: $r=0.85$, $p=0.01$). The expression of STAT3 mRNA by RT-PCR in neoplasm cells isolated from tumors with an EGFR-m index of ≤50% and >50% is shown in Fig. 5.

Relationship between EGFR-c status and STAT3 expression in neoplastic cells. To verify whether EGFR-c determines

Table III. Mean concentrations of cytokines secreted by purified PBMC cultures or measured in whole blood.

	TNFα [ng/ml]	IL-2 [ng/ml]	IL-6 [ng/ml]	IL-8 [ng/ml]	INFγ [ng/ml]
Without PHA stimulation					
Purified PBMCs					
21 h	0.37±0.07	0	66.01±11.49	126.54±28.06	0.004±0.002
42 h	0.37±0.06	0.0001±0.0001	104.25±18.93	267.98±43.33	0.012±0.009
72 h	0.27±0.06	0.0014±0.0015	143.72±24.73	246.66±38.64	0.033±0.018
Whole blood					
21 h	0.33±0.04	0.0002±0.0001	63.46±7.67	109.00±12.70	0.003±0.001
42 h	0.21±0.03	0.0006±0.0004	88.64±12.58	345.32±48.94	0.016±0.009
72 h	0.12±0.03	0.0016±0.0001	141.14±34.89	291.13±47.68	0.026±0.022
With PHA stimulation					
Purified PBMCs					
21 h	0.88±0.12	0.41±0.11	96.97±13.32	234.74±33.15	1.32±0.41
42 h	0.64±0.01	0.38±0.11	150.74±23.41	711.58±298.60	2.50±0.52
72 h	0.38±0.07	0.17±0.04	191.57±28.68	601.27±63.73	2.25±0.33
Whole blood					
21 h	0.53±0.14	0.67±0.02	94.43±17.22	157.57±25.76	0.49±0.11
42 h	0.31±0.05	0.13±0.03	128.28±22.30	423.96±61.33	1.28±0.32
s72 h	0.17±0.05	0.15±0.03	164.25±36.23	379.21±81.98	1.59±0.54

STAT3 expression in tissues of squamous cell laryngeal carcinoma or not, we juxtaposed the mRNA level for STAT3 with EGFR immunoexpression. Our study has revealed that the mean expression of STAT3 in tumor central and marginal cells, isolated from neoplasms with a negative EGFR-c index and in non-cancerous normal epithelial cells were TCC, 0.2±0.15 $IOD_{STAT3/ß\text{-}actin}$; TMC, 0.17±0.07 $IOD_{STAT3/ß\text{-}actin}$ and NCC, 0.16±0.12 $IOD_{STAT3/ß\text{-}actin}$, respectively. The mean expression of STAT3 in tumor central and marginal cells, isolated from neoplasms with a positive EGFR-c index was TCC, 0.32 ± 0.08 $IOD_{STAT3/ß\text{-}actin}$; TMC, 0.31±0.14 $IOD_{STAT3/ß\text{-}actin}$ and NCC, 0.3±0.12 $IOD_{STAT3/ß\text{-}actin}$. Thus, we found the relationship between EGFR-c status and STAT3 expression in laryngeal carcinoma. In our experiment, neoplasm tissues with positive EGFR staining in cell cytoplasm have demonstrated a higher level of STAT3 expression (Spearman's test: r=0.74, p=0.02). Interestingly, no relationships of statistical significance were found between EGFR and STAT3 expression after isolated tumor cells had been divided into two subgroups (central and marginal). The expression of STAT3 mRNA by RT-PCR in neoplasm cells isolated from tumors with EGFR-c positive and negative expression is shown in Fig. 6.

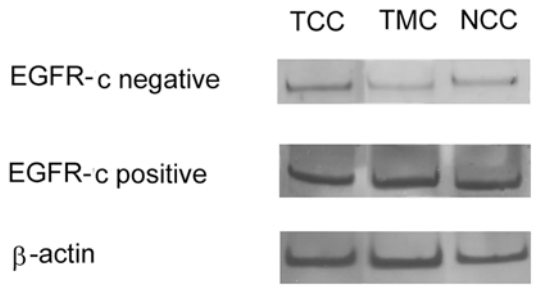

Figure 6. STAT3 mRNA expression by RT-PCR in neoplasm cells isolated from tumors with an EGFR-c positive and negative expression.

Relationship between EGFR-m status and proinflammatory/regulatory cytokine pattern. To confirm whether proinflammatory/regulatory cytokines, expressed by purified PBMCs and measured in whole blood, determine EGFR expression, we examined the concentrations of selected cytokines and juxtaposed them with immunohistochemical results. Our data demonstrate significant differences between EGFR-m expression and TNFα concentration as recorded in isolated PBMC supernatants in a 72-h culture with and without PHA stimulation (Kruskal-Wallis and one-way ANOVA tests: p=0.03 and p=0.04). The highest mean TNFα concentrations in the supernatants of purified PBMCs were 0.94±0.15 and 0.68±0.02 ng/ml in 21- and 42-h cultures, with PHA stimulation for tumors with EGFR-m >50% and 0.54±0.02 ng/ml in a 72-h culture, with PHA stimulation for tumors with an EGFR-m index of 25÷50%. In addition, the highest mean TNFα concentrations in these samples were 0.61±0.13, 0.55±0.01 and 0.41±0.03 ng/ml in 21-, 42- and 72-h cultures,

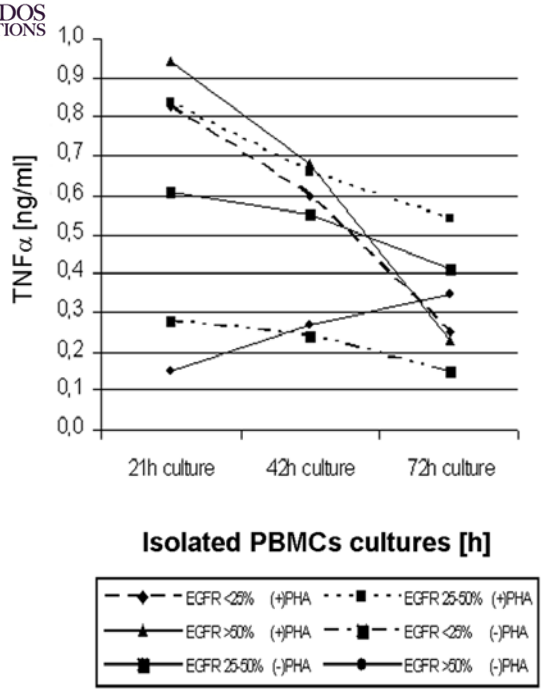

Figure 7. Mean TNFα concentrations secreted by purified PBMC cultures, for tumors scored using a three-tier system of EGFR-m immunoexpression.

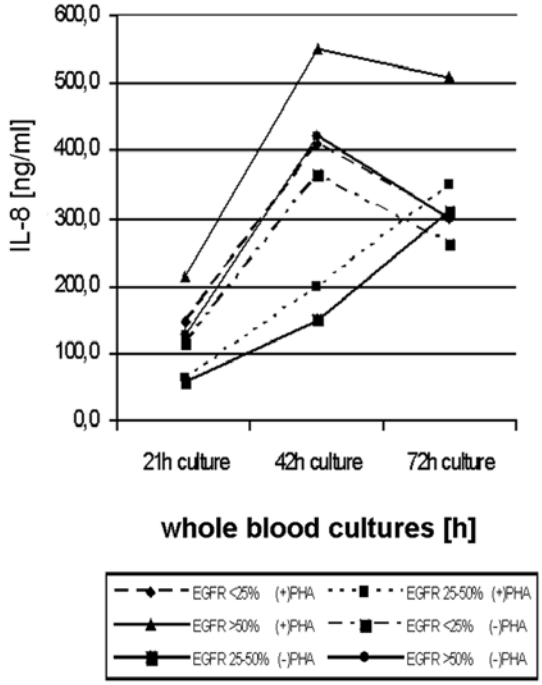

Figure 8. Mean IL-8 concentrations measured in whole blood, for tumors scored using a three-tier system of EGFR-m immunoexpression.

without PHA stimulation, for tumors with an EGFR-m index of 25÷50%, respectively. The mean TNFα concentrations secreted by purified PBMC cultures, for tumors with EGFR-m immunoexpression are shown in Fig. 7.

In addition, we discovered significant differences between membranous EGFR immunoexpression and IL-8 concentrations in whole peripheral blood in 21- and 42-h observations, with (ANOVA test: p=0.007 and p=0.02) and without (ANOVA test: p=0.01 and p=0.008) PHA stimulation. Dunnett correction disclosed significant differences between tumors with an EGFR-m index of 25÷50% and >50% in IL-8 concentrations produced by mononuclear cells in whole blood (p=0.02 and p=0.04 for a 21-h culture with and without PHA stimulation, respectively, and p=0.03 for a 42-h culture with PHA). The highest mean IL-8 concentrations in whole peripheral blood samples were 216.2±49.7, 550.3±110.5 and 510.4±236.3 ng/ml in 21-, 42- and 72-h cultures, with PHA stimulation for tumors with EGFR-m of >50%, respectively. Moreover, the highest mean IL-8 concentrations in introduced cultures were 127.1±22.4 and 424.5±77.5 ng/ml in 21- and 42-h cultures, without PHA stimulation, for an EGFR-m index of >50% as well as 312.4±93.3 ng/ml in a 72-h culture, without PHA stimulation, for tumors with an EGFR-m index of 25÷50%. Mean IL-8 concentrations measured in whole blood, for tumors with EGFR-m immunoexpression are shown in Fig. 8.

Our results showed a significant difference between EGFR-m expression and IFNγ level in whole blood in a 42-h culture with PHA (ANOVA test: p=0.02). The highest mean IFNγ concentration was 0.68±0.19 ng/ml in whole blood in a 21-h culture with PHA stimulation for an EGFR-m index of 25÷50%. Moreover, the highest mean IFNγ concentrations in

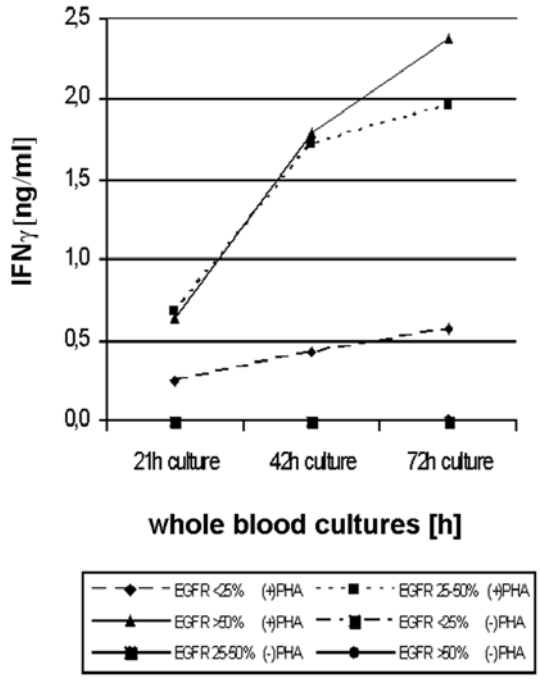

Figure 9. Mean INFγ concentrations measured in whole blood, for tumors scored using a three-tier system of EGFR-m immunoexpression.

whole blood samples were 1.79±0.66 and 2.38±1.76 ng/ml in 42- and 72-h cultures, with PHA stimulation, for an EGFR-m index of >50%. Mean IFNγ concentrations measured in whole blood, for tumors with EGFR-m expression are shown in Fig. 9. However, no statistical difference between the EGFR-m index and IL-2, IL-6 concentration has been recorded.

Relationship between EGFR-c status and proinflammatory/regulatory cytokine pattern. To verify whether proinflammatory and regulatory cytokines determine EGFR expression in the cytoplasm of laryngeal carcinoma cells, we juxtaposed the results of our analyses. We observed significant differences between EGFR-c expression and IL-2, IL-8 or IFNγ concentrations recorded in isolated PBMC supernatants in a 21-h culture stimulated with PHA (ANOVA test: p=0.005, p=0.04 and p=0.03 for IL-2, IL-8 and IFNγ, respectively). The highest mean concentrations of the selected cytokines were detected in tumors lacking EGFR staining. The mean concentrations of analyzed cytokines were 0.5±0.05 pg/ml for IL-2, 247.3±19.54 pg/ml for IL-8 and 1.57±0.19 pg/ml for IFNγ. In addition, after Dunnett correction, further significant differences were disclosed in IL-2 levels produced by mononuclear cells in 21-h cultures stimulated with PHA for tumors with EGFR-c expression, and scored with the use of a three-tier system, (p=0.04 and p=0.04 for EGFR classified as 0 vs. 1 and 0 vs. 2, respectively). A significant relationship between EGFR-c immunoexpression and IFNγ concentration in whole peripheral blood for a 21-h culture with PHA stimulation (ANOVA test: p=0.02) has also been revealed in our study, with the mean concentration of IFNγ being 0.63±0.16 pg/ml. Dunnett correction disclosed significant differences in secreted IFNγ levels for tumors with EGFR cytoplasmic stages classified as 0 or 1 (p=0.04). No statistical relationship between EGFR-c staining and TNFα or IL-6 concentration has been found.

Discussion

EGFR is a member of the ErbB family receptors, involved in cellular proliferation in many various types of carcinomas and may be an important therapeutic target (8-10, 29-31,35). EGFR is a key transmembrane glycoprotein triggering a network of downstream signaling pathways, including not only cell proliferation, cell-cell adhesion and migration, but also the invasion of the tumor front, apoptosis and DNA-damage repair in neoplasm cells (8-10). Over-expression of EGFR and its ligands, such as EGF, TGFα, amphiregulin and the presence of tyrosine kinase domain mutations have been noted in many human cancer types, such as HNSCC (11-15).

Membranous and cytoplasmic EGFR immunoexpression was presented in this study. We observed that 93.3% of tumor samples possessed membranous EGFR expression. Our study shows that 37.8% of samples of the carcinoma expressed EGFR cytoplasmic staining. Based on a review of the literature overexpression of the EGFR protein is found in 34-93% of various types of human carcinomas (4,5,44,45). A dispersion of results report EGFR expression or its ligands which stems from different antibodies and methods that have been used. For instance, Cohen *et al* (45) evaluated IHC staining for the patterns of EGFR overexpression, including membranous, cytoplasmic and total expression score. These authors demonstrated that the prognostic value of EGFR expression differs significantly when various methods are used to evaluate EGFR overexpression and that the immunohistochemical analysis is qualitative rather than quantitative. Thus, the results obtained are not consistent (44).

Investigators have confirmed the relationship between EGFR status and STAT3, and between the EGFR status and cytokine expression, such as IL-6, IL-8 or TNFα (19,20,29-33,40-42). Our results show the relationship between EGFR-m and EGFR-c immunoexpression, and the mRNA amplification level, obtained by PCR for STAT3 as well as the concentrations of proinflammatory/regulatory cytokines secreted by PBMCs, especially IL-8, IFNγ and TNFα. Kuwahara *et al* (3) claim that IL-8 gene transcription and protein release are regulated through p38/NF-κB activation via EGFR transactivation in a lung epithelial cell line. Nakanaga *et al* (41) demonstrated that EGF receptor and MAP kinase ERK1/2 are involved in IL-8 expression by these stimuli and that TGFα mediates IL-8 production. Gao *et al* (19) reported that cell lines expressing persistently activated mutant EGFR also produce high IL-6 levels. Similarly, Wehbe *et al* (40) pointed out that IL-6 over-expression resulted in an altered expression and promoter methylation of several genes, including EGFR. These mechanisms contribute to carcinogenesis and enhance tumor growth. Moreover, they indicated that an increased IL-6 expression is connected with the sensitivity of tumor cells to therapeutic treatment using methylation inhibitors (40).

In contrast, our study has failed to demonstrate an association of EGFR with the IL-6 level. Discrepancies in conclusions may be due to a high IFNγ concentration assessed in our experiment. Croker *et al* (46) suggested that the SOCS (suppressor of cytokine signaling) family is involved in the negative regulation of IL-6 *in vivo*. They indicated an enhanced STAT phosphorylation in response to IFNγ. However, an alternative mechanism of EGFR activation with an indirect impact on the STAT3 activity pathway has also been described (21). Yang *et al* (29) demonstrated that IFNγ promotes an antiproliferative effect of HER1/EGFR inhibitors on colon cancer cell lines. It has been shown that TNFα can reduce EGFR tyrosine phosphorylation, which results in the blockade of EGFR-mediated signal transduction. The signal transduction, in turn, can switch TNFα-induced apoptosis by stimulating caspases or by inducing ligand (TRAIL) action as an anticancer agent (30-32). Many investigators have demonstrated that the presence of TNFα, a proinflammatory cytokine, is characteristic of antitumor effects and manifests itself as an increasing vascular permeability of tumor microvasculature, which leads to an improved infiltration of monoclonal antibodies in the tumor (32). Hambek *et al* (31) found synergistic effects of the interaction between TNFα and EGFR after blocking it with monoclonal antibodies, which resulted in tumor size reduction in squamous cell carcinoma xenografts of the head and neck, uterine cervix cancer and adenocarcinomas with a high level of EGFR expression. They also noted that the combination treatment caused the increase in tumor cell apoptosis for carcinomas with a low EGFR expression (31). In several other studies, investigators demonstrated that the EGFR pathway is inhibited by blocking the activity of TNFα-converting enzyme, which induces metastatic properties, including the adhesion to fibronectin, mRNA expression of α-integrin and the production of matrix metalloproteinase-9 (38,39). Notably, Ando *et al* (35) indicated that therapy with TNFα induces autophosphorylation of EGFR in cross-talk signaling in lung

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ses with acquired resistance to the EGFR tyrosine hibitor. Many investigators have suggested that a constitutive STAT3 activation, in response to cytokine signals, such as proinflammatory interleukins (IL-6 and TNFα) or chemokines (IL-8), results in apoptosis inhibition under many human primary malignant conditions, including head and neck squamous carcinomas (HNSCC) (47-49). It is well known that proinflammatory cytokines influence the inflammatory response to tumor antigens and are implicated in the regulation of STAT3 signaling (47,50). An important alternative mechanism of STAT3 activation with an impact on the epidermal growth factor receptor (EGFR) pathway has been noted in recent studies (19,24). Tumor cells expressing persistently activated mutant EGFRs activate the gp130/JAK/STAT3 pathway by IL-6 up-regulation (19,51). Interestingly, in STAT3 activation the IL-6R cascade is more often reported on than the EGFR pathway (50,52). Of note is that Garcia (2) showed that signaling through EGFR leads to the activation of multiple effectors, including hypoxia-inducible factors (HIFs), which stimulate transcriptional activators, cytokines, growth factors and their receptors.

In conclusion, this investigation confirms a key role of EGFR in determining the proliferative and aggressive potential of the tumors studied. It also demonstrates that pro-inflammatory and regulatory cytokines can be committed to the activation of downstream molecules participating in neoplasm growth.

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