SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 12: 2127-2132, 2016

Evaluation of EGFR as a prognostic and diagnostic marker for head and neck squamous cell carcinoma patients

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Received October 23, 2015; Accepted May 26, 2016

DOI: 10.3892/ol.2016.4896

Abstract. Approximately 90% of all head and neck tumors are squamous cell carcinomas. The overall survival of patients with head and neck squamous cell carcinoma (HNSCC) is low (≤50%). A non-invasive marker of disease progression is sorely required. The present study focused on the plasmatic levels of epidermal growth factor receptor (EGFR) in HNSCC patients (N=92) compared with healthy (N=29) and diabetic [type 2 diabetes mellitus (T2DM); N=26] controls. Enzyme-linked immunosorbent assay using antibodies against the extracellular region of EGFR (L25-S645) was performed. No significant changes were observed between diabetic and healthy controls. However, there were significantly higher EGFR plasma levels in HNSCC patients compared with both control groups (P=0.001 and 0.005, respectively). Receiver operating characteristic curve analysis identified a sensitivity of 76.09%, a specificity of 67.27% and an area under curve of 0.727 for this comparison. No significant association was observed between EGFR plasma levels and tumor stage, tumor grade, lymph node or distant metastasis occurrence, smoking habit or hypertension. However, the presence of human papillomavirus infection and T2DM in HNSCC patients had borderline effect on the plasma EGFR levels. Survival analysis revealed no significant influence of plasmatic EGFR levels on the overall and disease-specific survival of HNSCC patients. In conclusion, EGFR plasma levels appear to be a relatively promising diagnostic, but poor prognostic, HNSCC marker.

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Key words: spinocellular cancer, biomarker, EGFR, head and neck tumors, plasma, diagnosis

Introduction

Epidermal growth factor receptor (EGFR), also known as ErbB1, is a 170-kDa transmembrane glycoprotein belonging to the ErbB/human epidermal growth factor receptor family of receptor tyrosine kinases (1-3). EGFR is composed of an extracellular highly glycosylated ligand-binding domain (ECD) comprising amino acids 1-621, a hydrophobic transmembrane domain (amino acids 622-644) and an intracellular domain with tyrosine kinase activity for signal transduction (amino acids 645-1,186) (Fig. 1) (1-3). Upon binding of a ligand-like amphiregulin, EGF or transforming growth factor α (TGFα) undergoes a conformational change by homo-dimerization or hetero-dimerization with another member of the erbB family, followed by auto-phosphorylation (4,5). This results in tyrosine kinase activation and triggering of signaling cascades. Activation of EGFR leads to the activation of intracellular signaling pathways that regulate cell proliferation, invasion, angiogenesis and metastasis (4,5). EGFR has been selected as a target of anticancer treatments due to its critical roles in cell survival and proliferation (6). EGFR is a strong prognostic marker in head and neck, ovarian and cervical cancer (7-9). EGFR expression has been associated with a higher proliferative index, advanced tumor stage and increased tumor angiogenesis in HNSCC (9). Overexpression of EGFR and TGFα significantly predicted a shorter disease-free and overall survival (9). EGFR activation also resulted into increased cell invasiveness and motility (10) via the induction of epithelial-to-mesenchymal transition (11,12). Furthermore, EGFR can interact with the receptor cluster of differentiation 44, resulting in a migratory cell phenotype (13). In addition to membrane-bound EGFR, tumor

cells express soluble EGFR proteins that can be produced by alternative messenger (m)RNA splicing events, aberrant translocation or disintegration of circulating tumour cells (14,15). Another 110-kDa soluble EGFR isoform, termed proteolytic isoform-soluble (PI-s)EGFR, is disengaged by proteolytic cleavage partially caused by metalloproteases (16,17). Sanderson *et al* (18) have also reported two soluble isoforms of EGFR (150 and 100-kDa) within exosomes.

The present study focused on plasmatic EGFR levels of HNSCC patients, which were analyzed by enzyme-linked immunosorbent assay (ELISA) using anti-EGFR antibodies raised against the L25-S645 region of full-length EGFR. Notably, information about binding sites of ELISA antibodies are often not provided in the literature, despite that it could be very important for interpretation of the results obtained. Blood markers are less invasive than tissue biopsies, and sample collection can be repeated, which enables real-time monitoring of disease progression and treatment response in patients. As a control group, a gender- and age-matched healthy cohort, and a gender- and age-matched cohort of patients with type 2 diabetes mellitus (T2DM), were used. The T2DM group was included because a proportion of the present HNSCC patients also exhibited T2DM, and certain studies have shown that diabetes suppresses the expression of EGFR (19). Since EGFR is affected by both female estrogen receptors (20,21) and male androgen receptors (22), EGFR may be a potential mediator of gender-related differences in HNSCC. Based on these facts, female HNSCC patients were excluded from the current study.

Materials and methods

Samples preparation. The present study was approved by the ethical committee of St. Anne's Faculty Hospital (Brno, Czech Republic). All surgical tissue samples were obtained from male HNSCC patients treated at St. Anne's Faculty Hospital between April 2013 and June 2015 upon providing informed consent. Histologically verified primary HNSCC carcinoma tissues were collected (92 samples). The tissue material harvested at surgery was placed into RNAlater® solution for RNA stabilization and storage (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The material was maintained cold, and RNA was isolated within 24 h. Additional information about the patients and controls is presented in Table I.

Blood samples from HNSCC patients and healthy (N=29) and T2DM (N=26) controls were obtained by venipuncture, and 5 ml was placed into an S-Monovette® 4.9 ml, K3EDTA test-tube (Sarstedt AG & Co., Nümbrecht, Germany) for plasma preparation. The blood samples were centrifuged at 1,200 x g at 4˚C for 10 min within 60 min of collection. Plasma was aliquoted and stored at -80˚C until analysis.

ELISA analysis. Plasma levels of EGFR were determined with a commercial ELISA kit (RayBiotech, Inc., Norcross, GA, USA) according to the manufacturer's protocol. The ELISA was designed to detect human EGFR in plasma or serum with a detection limit of 4 pg/ml, a 10% intra-assay varaibility and a 12% inter-assay variability, as described in the manufacturer's instructions. For the assay, plasma samples were diluted 100-fold, and evaluated with anti-EGFR antibodies raised against the L25-S645 region of EGFR.

Table I. Characterization of patients and controls.

Group	Factor	Number of cases	Age, years (range)
HNSCC patients		92	62.90 (44-89)
	TNM T1-2	39	62.42 (44-89)
	TNM T3-4	52	63.17 (47-87)
	TNM N0	42	65.04 (44-89)
	TNM N1	49	61.26 (44-77)
	TNM M0	86	62.86 (44-89)
	TNM M1	5	62.12 (55-71)
	Grade 1	6	63.46 (53-79)
	Grade 2	50	63.26 (44-89)
	Grade 3	32	61.49 (47-75)
	Non-smoker	28	66.99 (46-89)
	Smoker	59	62.01 (44-78)
Healthy controls		29	64.38 (54-69)
Diabetic controls		26	56.73 (50-83)

TNM, tumor-node-metastasis; HNSCC, head and neck squamous cell carcinoma.

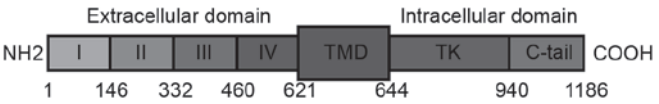

Figure 1. Schematic representation of EGFR protein (170-kDa). EGFR is composed of an extracellular highly glycosylated ligand-binding domain (comprising amino acids 1-621; exons 1-15), a hydrophobic transmembrane domain (amino acids 622-644; exons 15-17) and an intracellular domain with tyrosine kinase activity for signal transduction (amino acids 645-1,186; exons 18-28). I-IV denote subdomains of the extracellular domain. The figure was adapted from Albitar *et al* (1). TMD, transmembrane domain; TK, tyrosine kinase; EGFR, epidermal growth factor receptor.

RNA isolation and reverse transcription (RT). TriPure Isolation reagent (Roche Diagnostics, Basel, Switzerland) was used for RNA isolation. The isolated RNA was used for complementary (c)DNA synthesis. RNA (1,000 ng) was reverse transcribed using Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics) according to manufacturer's protocol. The cDNA (20 μl) prepared from total RNA was diluted with RNase-free water to 100 μl, and 5 μl cDNA was directly analyzed using the LightCycler® 480 II System (Roche Diagnostics).

RT-quantitative polymerase chain reaction (qPCR). RT-qPCR was performed using TaqMan® Gene Expression Assays (Life Technologies; Thermo Fisher Scientific, Inc.) with the LightCycler® 480 II System, and the amplified DNA was analyzed by the comparative $\Delta\Delta$Cq calculation (23) using β-actin as an endogenous control. The primer and probe sets for β-actin (Hs99999903_m1), metallothionein (MT)2 (Hs02379661_g1), MT1 (Hs00831826_s1), tumor protein p53 (TP53) (Hs01034249_m1), B-cell lymphoma (BCL)-2 associated X protein (BAX) (Hs00180269_m1), BCL-2 (Hs00608023_m1), vascular endothelial growth factor A

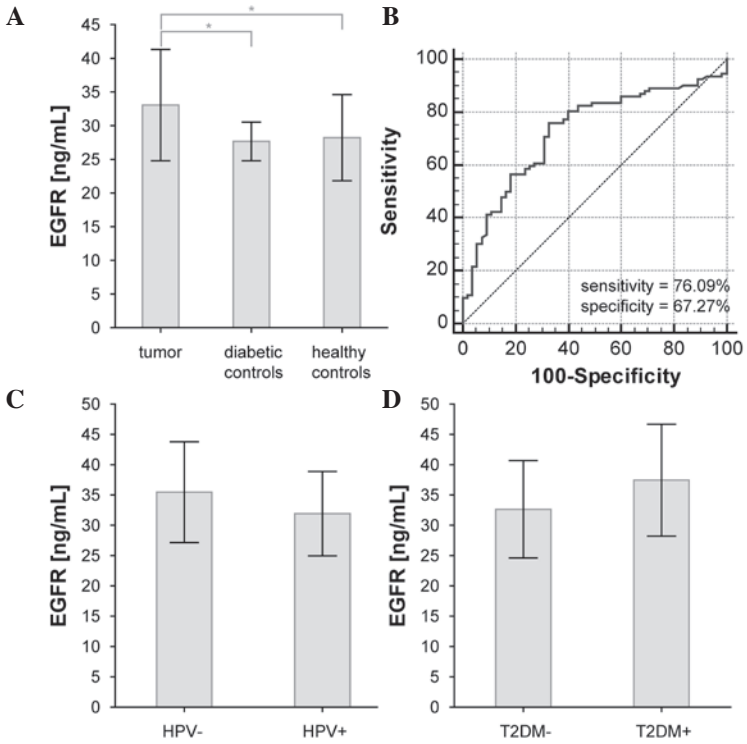

Figure 2. Plasmatic EGFR levels. (A) Plasma EGFR levels in head and neck squamous cell carcinoma patients and in two control groups (*P<0.05 vs. controls). (B) Receiver operating characteristic curve analysis indicating the sensitivity and specificity of plasma EGFR detection. (C) Plasma EGFR levels in human papillomavirus-positive and -negative patients. (D) Plasma EGFR levels and type 2 diabetes mellitus status in patients. EGFR, epidermal growth factor receptor; T2DM, type 2 diabetes mellitus; HPV, human papillomavirus.

(VEGFA) (Hs00900055_m1), fms-related tyrosine kinase 1 (FLT1) (Hs01052961_m1), matrix metalloproteinase 2 (MMP2) (Hs01548727_m1), MMP9 (Hs00234579_m1), proto-oncogene c-Fos (FOS) (Hs00170630_m1), c-Jun (JUN) (Hs00277190_s1), marker of proliferation Ki-67 (MKI67) (Hs00606991_m1), EGF (Hs01099999_m1) and EGFR (Hs01076078_m1) were selected from TaqMan® Gene Expression Assays. RT-qPCR was performed under the following amplification conditions in a total volume of 20 μl (5 μl cDNA, 10 μl TaqMan® Gene Expression Master Mix, 4 μl molecular-grade water and 1 μl TaqMan Gene Expression Assay): Initial incubation, 50˚C for 2 min, followed by denaturation at 95˚C for 10 min and then 45 cycles of 95˚C for 15 sec and 60˚C for 1 min.

Human papillomavirus (HPV) detection. The 142 bp-long sequence of the conservative major capsid protein L1 gene were amplified using general primers, GP5 and GP6, for non-specific identification of HPV-positive subjects. The PCR mixture from New England BioLabs, Inc. (Ipswich, MA, USA) contained PCR buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl and 2.5 mM $MgCl_2$), 0.05 mM of each deoxynucleotide, and 0.05 mM of GP5 (5'-TTTGTTACTGTGGTAGATAC-3') and GP6 (5'-GAAAAATAAACTGTAAATCA-3') primers. The DNA amplification was performed during 40 cycles that included a denaturation step at 94˚C for 30 sec, annealing at 45˚C for 30 sec and extension at 72˚C for 30 sec.

As internal quality control of the isolated DNA, the β-actin gene (600 bp) was amplified (forward primer 5'-CCTGAA CCCTAAGGCCAACC-3' and reverse primer 5'-GCAATG CCTGGGTACATGGT-3'). Each PCR product was analyzed using electrophoresis on 1% agarose gels stained with ethidium bromide.

Data analysis. Differences between the two groups were calculated using the *t*-test. Survival analysis was conducted using Cox proportional hazard regression analysis with plasma EGFR levels as covariates. Receiver operating characteristic (ROC) curves were calculated using the DeLong methodology. Subsequently, Kaplan-Meier analysis was used with continuous data being divided into two groups as follows: Low expression (<mean values) and high expression (≥mean values) groups. The associations between the continuous variables were analyzed using Pearson's correlations. Unless noted otherwise, P<0.05 was considered to indicate a statistically significant difference. Software STATISTICA 12 (StatSoft, Inc., Tulsa, OK, USA) and MedCalc 15.8 (MedCalc Software bvba, Ostend, Belgium) were used for analysis.

Results

Association between plasma levels of EGFR and HNSCC occurrence. No significant changes in EGFR plasma levels were observed between diabetic and healthy controls (P=0.690). However, there was a significant difference between EGFR plasma levels in HNSCC patients and in both control groups (P=0.001 and 0.005, respectively) (Fig. 2A and Table II). If both control groups were assessed together, the statistical significance was P=0.0001. ROC curve analysis identified a sensitivity of 76.09%, a specificity of 67.27% and an area

Table II. Plasma levels of EGFR and clinical characteristics in HNSCC patients.

Factor	Status (number of cases)	EGFR levels, ng/ml mean ± standard deviation	P-value
Cases vs. controls	HNSCC patients (92)	33.1±8.3	-
	Healthy controls (29)	28.2±6.4	0.001
	Diabetic controls (26)	27.7±2.9	0.005
Smoking	Yes (59)	31.8±7.7	0.150
	No (28)	34.5±8.5	-
Hypertension	Yes (28)	34.4±8.8	0.380
	No (58)	32.7±8.0	-
Diabetes mellitus	Yes (10)	37.4±9.2	0.085
	No (76)	32.6±8.0	-
TNM T-staging	T1-2 (39)	32.5±8.1	0.580
	T3-4 (52)	33.4±8.5	-
TNM N-staging	N+ (49)	33.7±8.2	0.430
	N- (42)	32.3±8.5	-
TNM M-staging	M+ (5)	31.3±8.1	0.640
	M- (86)	33.1±8.3	-
Tumor grade	High (82)	32.8±8.1	0.270
	Low (6)	36.8±12.3	-
HPV status	HPV+ (49)	32.0±7.0	0.084
	HPV- (18)	35.5±8.3	-

EGFR, epidermal growth factor receptor; HNSCC, head and neck squamous cell carcinoma; TNM, tumor-node-metastasis; HPV, human papillomavirus.

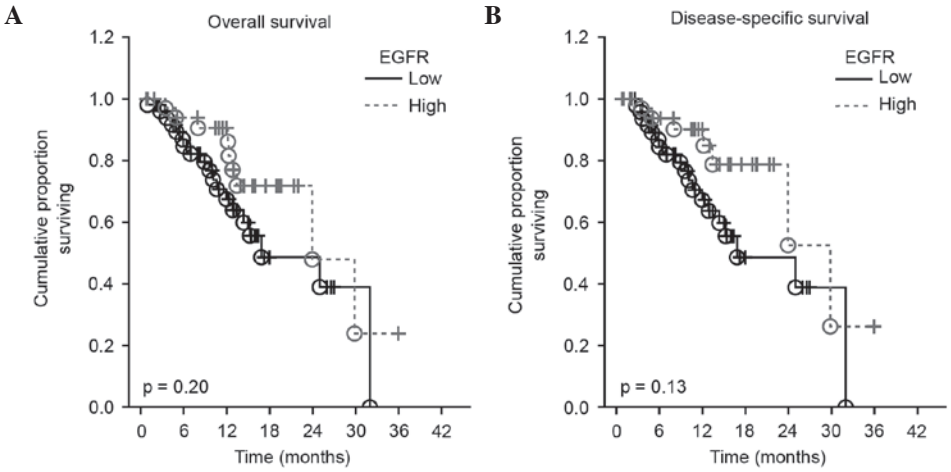

Figure 3. Survival analysis. (A) Kaplan-Meier overall survival analysis. (B) Disease-specific survival analysis. High/low EGFR indicate EGFR values above/below the mean EGFR values. P-value was calculated by Cox proportional hazard regression analysis. EGFR, epidermal growth factor receptor.

under the curve (AUC) of 0.727 for this comparison (Fig. 2B). Additional information about the patients and controls is contained in Table I.

Correlation between tumor gene expression and EGFR plasma levels. Correlations between plasma EGFR levels and expression of genes in tumor tissues of HNSCC patients were examined. There was no significant correlation between plasma EGFR levels and tumor tissue EGFR mRNA expression, and only a weak negative correlation with MMP9 mRNA was observed (r=-0.21, P=0.040). Gene expression analyses of EGF, EGFR, MKI67, BCL-2, BAX, FOS, JUN, TP53, VEGFA, FLT1, MMP2, MMP9, MT1A and MT2A genes in HNSCC tumor tissue compared with tumor adjacent tissue and tonsillectomies have been published elsewhere (24).

Association between plasma levels of EGFR and clinico-pathological characteristics. By examining the associations

between plasma EGFR levels and clinicopathological characteristics of HNSCC patients, no significant association was identified for smoking habit, T2DM, hypertension, HPV infection, tumor stage, tumor grade, or lymph node or distant metastasis occurrence. However, the presence of HPV infection and T2DM in HNSCC patients had a borderline effect on the plasma EGFR levels (Table II and Fig. 2C and D).

Association between plasma levels of EGFR and disease-free and overall survival. The prognostic value of EGFR plasma levels on overall and disease-free survival was studied by Cox proportional hazard regression analysis and Kaplan-Meier curves. Survival analysis revealed no significant influence of plasmatic EGFR levels on overall or disease-specific survival in the present cohort of HNSCC patients [hazard ratio (HR)=0.97; 95% confidence interval (CI)=0.92-1.01; P=0.200, and HR=0.96; 95 CI=0.91-1.01; P=0.130 for overall and disease-free survival, respectively] (Fig. 3).

Discussion

Numerous studies have shown that EGFR is overexpressed in HNSCC tumor tissue, but only few studies focused on soluble EGFR levels (24-26). There are contradictory studies on soluble EGFR levels, which could be either decreased or elevated in cancer patients compared with a healthy cohort. For example, Partanen *et al* reported that patients with asbestosis-induced lung cancer have elevated serum soluble EGFR ECD levels (27). Increased soluble EGFR ECD levels were also reported in the urine of patients with squamous cell carcinomas of the lung, head and neck (28), whereas patients with ovarian cancer had decreased levels of serum p110 EGFR compared with the normal population (29). In the present study, ELISA using antibodies against the L25-S645 region of EGFR was used to measure the levels of EGFR in plasma samples of HNSCC patients. Female patients were excluded from the present study due to possible gender-specific EGFR interactions with estrogen or androgen receptors (20-22). Significantly higher EGFR plasma levels were detected in HNSCC patients compared with the healthy cohort and the diabetic control group (P=0.001 and 0.005, respectively). This finding is in accordance with that of Perez-Torres *et al* (17), who suggested that the mechanism of proteolytic cleavage of EGFR and shedding of PI-sEGFR into the plasma may be activated in malignant cells that overexpress the full-length receptor. The cleavage of EGFR probably occurs in the transmembrane domain between G625 and M626 (17). In HNSCC patients, EGFR expression is supposed to be higher in tumor tissues compared with tonsillectomy samples and tumor-adjacent tissues (24). Furthermore, the release of two soluble EGFR isoforms within the exosomes is activated by EGF (17), which is highly produced by HNSCC tumor-adjacent tissues (24).

No significant changes in EGFR plasma levels were observed between diabetic and healthy controls, which is not in accordance with the Vairaktaris *et al* hypothesis that diabetes suppresses the expression of EGFR (19). However, a slight change on the borderline of statistical significance was observed between HNSCC patients with or without diabetes (P=0.085), while HNSCC patients with diabetes tended to have higher EGFR plasma levels. Borderline changes in EGFR plasma levels were also noticed between the HPV-positive and HPV-negative groups of HNSCC patients (slightly higher levels of plasmatic EGFR were detected in the HPV-negative cohort), although these changes were not significant.

Survival analysis revealed no significant influence of the plasmatic EGFR levels on overall and disease-specific survival in the present cohort of HNSCC patients. By contrast, Ye *et al* demonstrated that non-small-cell lung cancer patients with lower plasma EGFR concentrations (<27.24 ng/ml) had a significantly shorter overall survival compared with patients who had higher plasma EGFR concentrations (≥27.24 ng/ml) (18.2 vs. 33.4 months, P=0.021) (30).

In conclusion, EGFR plasma levels appear to be a relatively promising diagnostic, but poor prognostic, HNSCC marker. However, further studies are required to determine the clinical value of plasmatic EGFR levels in HNSCC patients. The next important step in soluble EGFR research should be a precise distinction between 110-kDa PI-sEGFR originating from full-length EGFR protein cleavage and EGFR isoforms originating from alternative splicing of EGFR gene transcripts. These EGFR isoforms could readily have slightly different functions. For example, 110-kDa PI-sEGFR originating from full-length EGFR protein cleavage could reflect the presence of malignant cells that overexpress the full-length receptor (17) or a necrotic disintegration of tumor cells. Such form of soluble EGFR was probably originally involved in a proliferative signaling pathway, and could be marker of poor prognosis, while the soluble EGFR isoform originating from alternative splicing was probably not an activator of these proliferative signaling pathways due to the missing intracellular domain, and could exhibit a high affinity binding for EGF, which should result in decreased proliferative signaling and better prognosis.

Acknowledgements

The present study was supported by the Ministry of Health of the Czech Republic (Prague, Czech Republic; grant no. IGA MZ NT 14337-3/2013) and by Specific University Research Grants (grant nos. MUNI/A/1365/2015 and MUNI/A/1426/2015) provided by the Ministry of Education, Youth and Sports of the Czech Republic (Prague, Czech Republic) in 2016 and by Czech Science Foundation (GACR GA16-12454S).

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