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# Prognostic significance of epidermal growth factor receptor in surgically treated squamous cell lung cancer patients

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**Abstract:** Epidermal growth factor receptor (EGFR) is one of signalling pathways activated during premalignant proliferative changes in the airway epithelium. However there is no agreement about prognostic significance of EGFR expression in non-small cell lung cancer (NSCLC). Facts mentioned above prompted us to study EGFR expression in the group of 78 surgically treated squamous cell lung cancer (SqCLC) patients. The EGFR expression was visualized in formalin-fixed, paraffin-embedded sections, using immunohistochemistry. Three methods of assessment of EGFR expression were applied: percentage of cells with membranous EGFR expression - EGFR labellig index (EGFR LI), percentage of fields with membranous EGFR staining (PS%) and staining intensity (absent, weak or strong) in the whole specimen (SI). Mean EGFR LI and PS% values were 30.4 ± 3.5% and 51.6 ± 3.9%, respectively. Patients with higher EGFR expression (EGFR LI, PS%, SI) were significantly younger than those with low EGFR expression. EGFR LI was higher in pT3 tumours than in pT1+pT2 tumours, moreover, EGFR expression (EGFR LI, PS%, SI) was significantly higher in G1+G2 tumours than in G3 tumours. There were significant correlations between parameters used for assessment of EGFR expression. PS%≤50 indicated shorter disease-specific survival than PS%>50. However, patients with tumours with both very low and very high EGFR LI (13%≥EGFR LI>80%) showed significantly shorter survival than those with medium EGFR LI (13%<GFR LI≤80%). Additionally, pTNM and pN significantly influenced patients' survival. In multivariate analysis, EGFR LI and pTNM were independent prognostic parameters influencing disease-specific survival of patients.

**Key words:** Epidermal growth factor receptor - Squamous cell lung cancer - Surgery - Prognostic factors

## Introduction

Lung cancer (LC) is the most common cause of deaths among all malignant neoplasmas all over the world. The results of surgery are still not satisfactory. Patients after potentially curative surgery, with the same pathological stage of disease, display marked variability in recurrence and survival [29]. This prompts many authors to study the biology of LC. The following prognostic factors have been studied: DNA ploidy [21], proliferative activity of tumour cell [17], mutation and expression of proto-oncogens, loss of blood group antigens on tumour cells, loss of tumour suppressor gene function, angiogenesis and others [19].

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Epidermal growth factor receptor (EGFR) family is one of the best described signalling pathways activated during premalignant proliferative changes in the airway epithelium [14]. EGFR (ErbB-1) was found to be overexpressed in many types of squamous cell cancers, as well as in squamous cell lung cancer (SqCLC) [5, 9, 13, 31, 35]. Amplified EGFR signalling might induce cell growth and increase the metastatic potential of the tumour [37]. These facts suggest that EGFR expression might be a prognostic factor. In head and neck, ovarian, cervical, bladder and oesophageal cancers, the association between elevated EGFR level and poor patient outcome is particularly strong [28]. Not so strong relation has been noted in gastric, breast, endometrial and colorectal cancers [28]. In contrast, there is no agreement about prognostic significance of EGFR expression in NSCLC [10, 12, 28, 33, 36, 38]. This disagreement might be caused by lack of standard methods assessing EGFR expression and by histological heterogeneity of

**Table 1.** Correlations between clinicopathological parameters and parameters used for EGFR assessment in SqCLC patients

| Pathological parameter | N | EGFR LI (%) mean ± SE | PS (%) mean ± SE | SI | |
|---|---|---|---|---|---|
| | | | | absent or weak membranous staining - no. of cases (percentage) | strong membranous staining - no. of cases (percentage) |
| TNM stage | | | | | |
| I | 40 | 30.8 ± 5.2 | 53.4 ± 5.6 | 15 (19.2) | 25 (32.1) |
| II | 26 | 26.0 ± 5.2 | 50.6 ± 6.2 | 11 (14.1) | 15 (19.2) |
| III | 12 | 28.4 ± 9.5 | 48.3 ± 12.1 | 5 ( 6.4) | 7 ( 9.0) |
| pT | | | | | |
| pT1+pT2 | 1+62 | 27.8 ± 3.9 | 47.9 ± 4.5 | 28 (35.9) | 35 (44.9) |
| pT3 | 15 | 41.0 ± 7.5 a | 67.3 ± 6.9 | 3 ( 3.8) | 12 (15.4) |
| pTNM stage | | | | | |
| I | 32 | 33.6 ± 5.6 | 55.3 ± 6.1 | 12 (15.4) | 20 (25.6) |
| II | 31 | 23.8 ± 4.9 | 44.0 ± 6.3 | 13 (16.7) | 18 (23.1) |
| III | 15 | 37.1 ± 8.8 | 59.7 ± 9.1 | 6 ( 7.7) | 9 (11.5) |
| Grade* | | | | | |
| G1+ G2 | 14+25 | 41.0 ± 5.1 | 66.7 ± 4.8 | 9 (11.7) | 30 (38.9) |
| G3 | 38 | 20.2 ± 4.2 b | 37.1 ± 5.5 b | 21 (27.3) | 17 (22.1) b |

*in one case grade was not assessed;
a - pT1+pT2 vs. pT3; p=0.0432 (for EGFR LI);
b - G1+G2 vs. G3; p=0.0010 (for EGFR LI); p=0.0003 (for PS); p=0.0039 (for SI)

the analysed NSCLC groups. The latter cause is highly probable, as expression of EGFR is stronger in SqCLC than in adenocarcinomas and large cell carcinomas of lung [35, 13]. This might indicate different biology of different histological types of NSCLC. The facts mentioned above prompted us to analyse prognostic significance of three parameters applied for assessment of EGFR expression in a group of SqCLC patients. Recently, new EGFR-targeting anticancer agents are tested in clinical trials [1, 3, 6, 23, 24, 39]. Thus, development of simple and reliable methods measuring the level of EGFR expression could be helpful in selecting patients who might be qualified for the above treatment.

## Materials and methods

**Patients.** Between 1986-1999, seventy-eight patients with stage I-IIIA SqCLC were treated with radical surgery according to commonly accepted criteria [2, 25]. The mean age of patients was 58.9 ± 0.9 yrs (range 41-73 yrs). There were 71 men and 7 women, without difference between the mean age of males and females. None of the patients had been treated with radio- or chemotherapy before surgery. All the patients were operated on by the same surgeon. Thirty-nine patients underwent lobectomy, and 39 pneumonectomy, assessed as radical by the surgeon and pathologist [2, 25]. Mediastinal lymphadenectomy was performed only in suspected cases (enlargement of mediastinal lymph nodes or histologicaly confirmed metastases) - in the other patients only sampling was carried out [26, 27]. Nineteen patients were subjected to postoperative radiotherapy and two to chemotherapy. The clinical (TNM) and pathological (pTNM) stages were established according to TNM UICC 1997 criteria [34]. The stage and grading of the tumours are summarized in Table 1. Each patient was followed-up from surgery till death (for the purpose of this study follow-up was finished at 5th year). Thirty patients died: 7 patients (23.3%) of local cancer recurrence, 23 (76.7%) of distant metastases. Forty seven patients are still alive without progression of SqCLC, one is alive with metastases. The study has been approved by the Ethical Committee of the Centre of Oncology.

**Material.** Immediately after excision, fresh specimens (about 0.5 $cm^2$) from each patient were fixed in 10% neutral buffered formalin and embedded in paraffin for routine light microscopic analysis. The slides were examined by a pathologist in order to establish histology and grading.

**Immunohistochemistry.** Sections were cut at 4 μm, mounted on Super Frost® Plus (Menzel-Gläser, Germany) slides and then deparaffinized and hydrated through a series of xylenes and alcohols. Before staining, the sections were digested for 15 min at room temperature with DAKO® ready-to-use proteinase K (DAKO Ltd., proteolytic enzyme solution diluted in 0.05 M Tris-HCl, 15 mM sodium azide, pH 7.5). Then, the sections were rinsed in Tris-buffered saline (TBS), pH 7.4. Endogenous peroxidase activity was blocked by 0.03% hydrogen peroxide containing sodium azide (DAKO EnVision™+ system; DAKO Ltd.). Nonspecific binding of immunoglobulins was blocked by 20% normal swine serum (DAKO Ltd.). Next, slides were incubated for 40 min at room temperature with monoclonal mouse anti-human EGFR, clone H11 (DAKO Ltd.) which was diluted 1/200 in antibody diluent (DAKO Ltd.). DAKO EnVision™+ system was used to visualise the anti-EGFR antibody. Sections were counterstained with hematoxylin. For negative control, TBS was substituted for the primary antibody. Positive controls were performed on SqCLC sections known to exhibit membranous overexpression of EGFR.

**Evaluation of EGFR expression.** Assessment of EGFR expression was based on EGFR protein staining in membranes of tumour cells. Three methods were used: SI - staining intensity within the whole specimen, PS% - percentage of fields with positive staining for EGFR, and EGFR LI (EGFR labelling index) - the percentage of cells with positive staining. The first method - SI was assessed after scanning of the whole specimen at × 200 magnification. Membran-

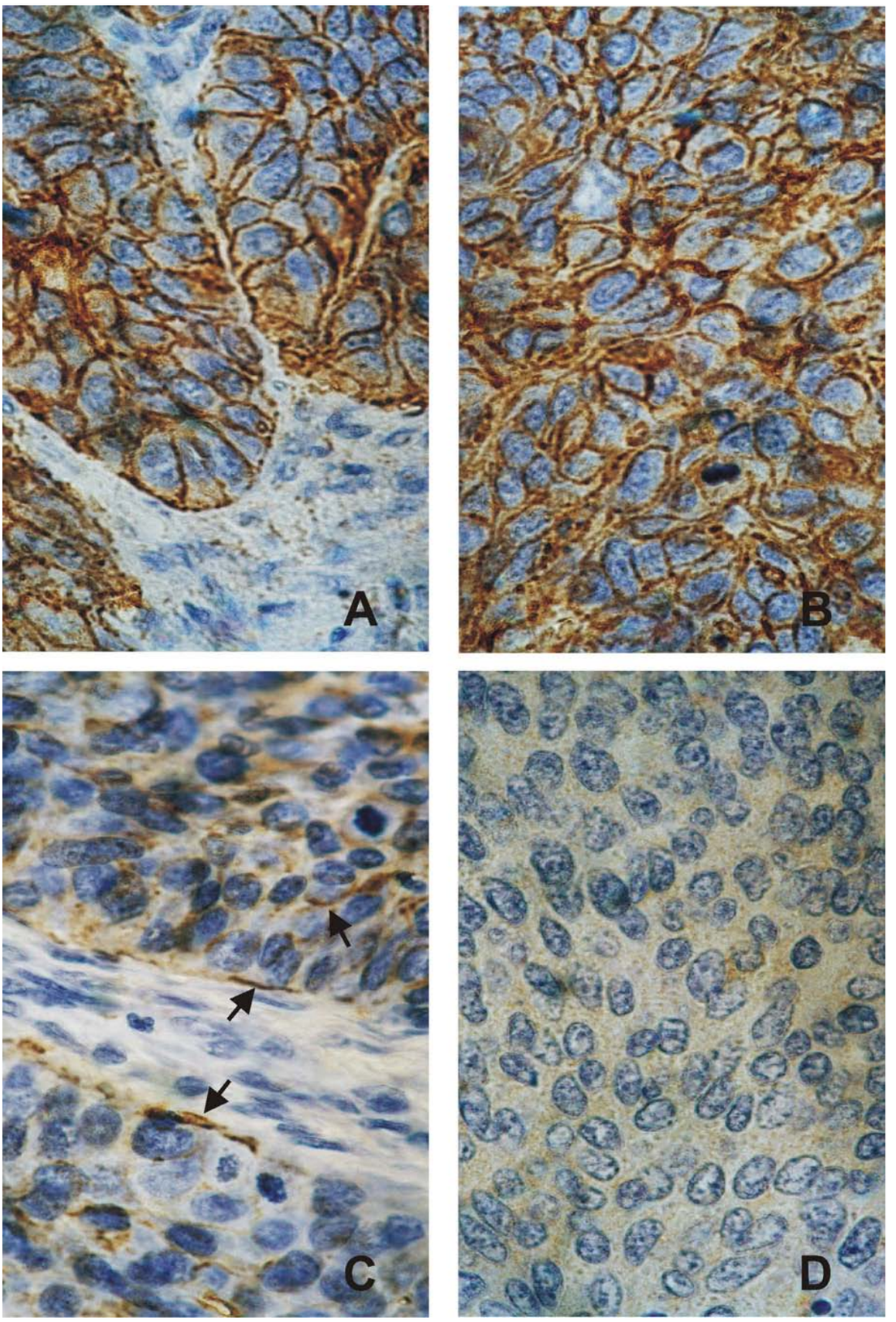


**Fig. 1.** Expression of epidermal growth factor receptor in squamous cell lung cancer. **A, B:** strong membranous EGFR expression in all tumour cells; **C:** weak membranous EGFR expression (arrows) in a few tumour cells; **D:** no EGFR expression in all tumour cells. × 630.

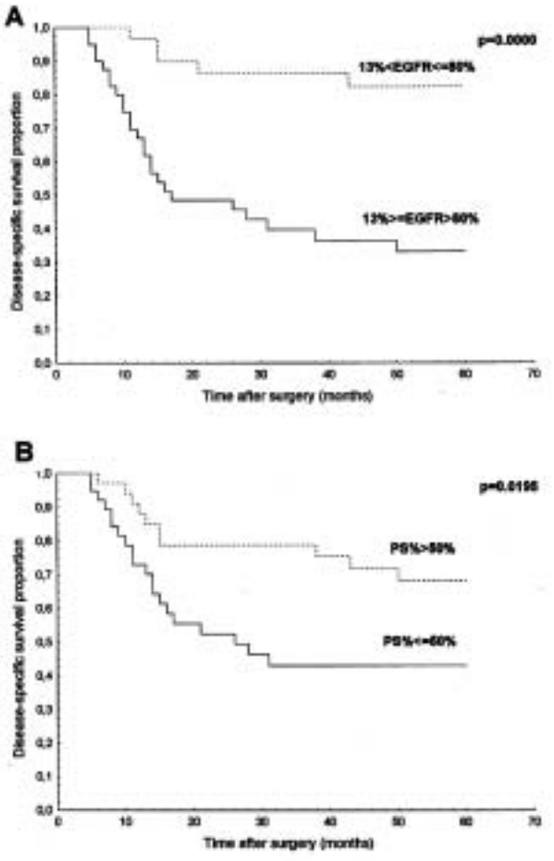


**Fig. 2.** Correlation between EGFR expression and patients' survival after surgery. **A:** patients survival according to EGFR labelling index; **B:** survival as a function of positive staining for EGFR (PS%), stratified using the PS%≤50% and PS%>50%.

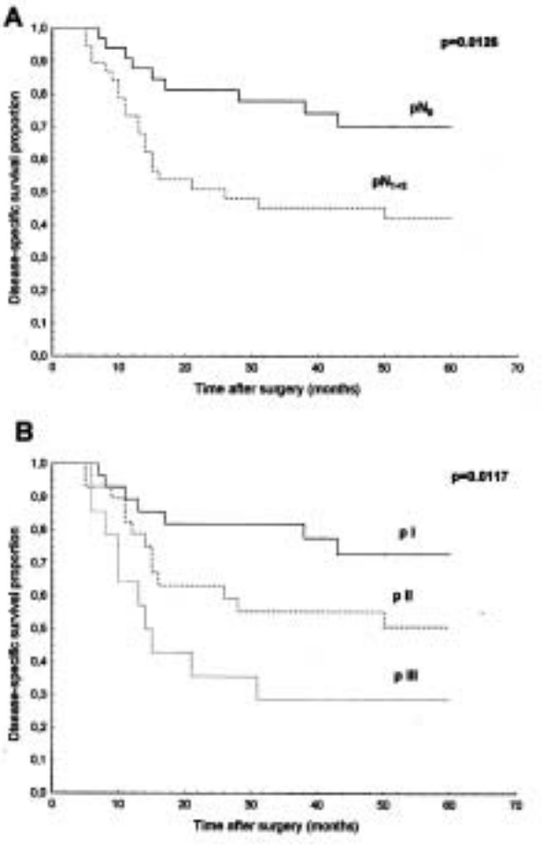


**Fig. 3.** Correlation between clinical parameters and patients' survival after surgery. **A:** correlation between pN and patients' survival; **B:** correlation between pTNM and patients' survival.

ous staining was considered strong (Fig. 1A, B), weak (Fig. 1C) or negative (Fig. 1D). In the mentioned above qualitative method, positive control slide showing strong membranous staining was used as a reference sample. In case of not uniform staining intensities in the specimen, the highest grade of intensity was considered. The second method - PS% was assessed in ten randomly selected fields (at × 400 magnification) and was computed as number of fields with positive membranous staining (weak or strong) divided by 10 (the number of examined fields). PS% was expressed as percentage. The third method - EGFR LI was assessed in ten randomly selected fields (at × 400 magnification), and computed as a number of cells with positive membranous staining for EGFR (weak or strong) divided by the number of all cells that were counted. EGFR LI was expressed as a percentage. The person involved in the EGFR expression assessment was not aware of the clinical outcome and follow-up status of patients.

**Statistical analysis.** Descriptive statistics were used to determine mean values of EGFR LI and standard error of mean (SE). The normality of EGFR LI distribution was assessed using Shapiro-Wilk W test. The statistical significance of differences between the means was assessed by Mann-Witney U test, (in case of variables with not normal distribution) and with the Student t-test (in case of variables with normal distribution). The Pearson $\chi^2$ test was used to analyse the associations between categorical variables. In all statistical procedures, p values lower than 0.05 were considered significant. Disease-specific survival (the patients whose cause of death was not malignancy were treated as alive) was analysed. The probability of survival was calculated using the Kaplan-Meyer method [20]. The first step in the analysis of prognostic factors was univariate analysis done using the log-rank test. Because mean or median values for EGFR LI were not important, the optimal cut-off points, ('minimal' p values) were chosen by log-rank test. Covariates that in univariate analysis were significantly related to outcome variable, were entered into multivariate analysis. The joint effect of remaining covariates, were analysed using Cox proportional hazard model and stepwise regression procedure [7]

## Results

In the analysed group of 78 SqCLC, strong membranous staining for EGFR was found in 47 cases (60.3%) and weak staining in 21 cases (26.9%). In 10 cases (12.8%),

**Table 2.** Univariate analysis for SqCLC patients treated with surgery. Data for 5-year disease-specific survival

| Parameter | N | The Kaplan-Meier estimated 5-yr survival (%) | Median survival (months) | (log-rank test) p value |
|---|---|---|---|---|
| EGFR LI | | | | |
| 13%<GFR LI≤80% | 36 | 82 | - | |
| 13%≥EGFR LI>80% | 42 | 33 | 16 | 0.0000 |
| PS% | | | | |
| >50% | 38 | 4368 | - | |
| ≤50% | 40 | | 25 | 0.0195 |
| TNM | | | | |
| I | 40 | 58 | - | |
| II | 26 | 48 | 42 | |
| III | 12 | 55 | - | 0.9 |
| pN | | | | |
| pN0 | 38 | 70 | - | |
| pN1 + pN2 | 33+7 | 42 | 23 | 0.0126 |
| pTNM stage | | | | |
| I | 32 | 73 | - | |
| II | 31 | 51 | - | |
| III | 15 | 29 | 14 | 0.0117 |

**Table 3.** Final results of Cox multivariate analysis. Disease-specific survival for 78 SqCLC patients treated with radical surgery

| Final results | | |
|---|---|---|
| Parameter | RR* | (Cox proportional hazards) p value |
| EGFR LI | | |
| 13%<GFR LI≤80% | 1 | |
| 13%≥EGFR LI>80% | 6.7 | 0.0001 |
| pTNM | | |
| I + II | 1 | |
| III | 3.3 | 0.0030 |

*RR - relative risk

membranous staining was absent. The EGFR LI ranged from 0 to 100% with a mean value of 30.4% ± 3.5. Mean EGFR LI for 68 tumours (87.2%) with positive (weak or strong) membranous staining for EGFR was 34.8 ± 3.7. PS% ranged from 0 to 100%, with a mean value of 51.6% ± 3.9. The variables, EGFR LI and PS%, did not show normal distribution (p<0.0000). As expected, tumours with strong membranous staining had significantly higher values of EGFR LI and PS% (47.5 ± 4.1, 70.2 ± 3.9 respectively) than tumours with weak staining (6.5 ± 1.5, 34.8 ± 5,1 respectively), (p=0.0000). Moreover, correlation between PS% and EGFR LI has been found (p=0.000).

There was no association between EGFR expression and gender of patients. Significant correlation between age and EGFR LI or PS% was found (p=0.009, 0.019) - older patients had lower EGFR LI and PS%. Moreover, patients with tumours having strong membranous EGFR staining were significantly younger (p=0.0217), (mean age 57.3 ± 1.1 years) than those with tumours having weak or absent membranous staining for EGFR (62.2 ± 1.3).

There was no association between T, N, TNM, pN, pTNM and EGFR expression. However, significantly higher EGFR LI was found for pT3 than for pT1+pT2 tumours (Tab. 1). All parameters: EGFR LI, PS% and SI showed higher values in well and moderately differentiated tumours (G1+G2) than in poorly differentiated tumours (G3), (Tab. 1).

EGFR LI was significantly higher in tumours from 6 patients, who later suffered from loco regional recurrence than in 25 patients who suffered from metastases (56.9 ± 15.7 vs. 24.8 ± 6.8 respectively), (p=0.0404).

The Kaplan-Meier estimated 5-year disease-specific survival was 55%. In univariate analysis, 36 patients with EGFR LI ranging between 13% and 80% survived significantly longer than 34 patients with EGFR LI lower than 13% or 8 patients with EGFR LI higher than 80% (p=0.0003, p=0.0001 respectively). There was no difference in length of survival between patients with EGFR LI≤13% and those with EGFR LI>80% (p=0.3). This prompted us to join the two groups into one: 13≥EGFR LI>80% (Fig. 2A, Tab. 2). Patients with PS%≤50%, survived significantly shorter than those with PS%>50% (p=0.0195), (Fig. 2B, Tab. 2). Also patients with pN1+pN2 tumours survived significantly shorter than those with pN0 tumours (p=0.0126), (Fig. 3A, Tab. 2). Pathological stage was the next factor that significantly influenced patients' survival (p=0.0117), (Fig. 3B, Tab. 2). Other factors: SI, clinical stage, pathological grade, radiotherapy, chemotherapy were not significant in univariate analysis.

In Cox multivariate analysis, only EGFR LI and pTNM were significant for disease-specific survival (Tab. 3).

## Discussion

The main goal of this study was the assessment of EGF receptor expression using three methods (based on number of cells positively stained for EGFR and on staining intensity) and estimation of their prognostic significance. Only SqCLC were analysed. In our study, positive EGFR staining was found in 87.2% of cases. In SqCLC, Cerney *et al*. [5] found EGFR expression in 85.7% of cases, Veale *et al*. [35] in 87.5%, Pfeiffer *et al*. [31] in 94.1%, Pastorino *et al*. [30] in 59% and Cox *et al*. [9] in 38.1% of analysed cases. In our study, the mean value of EGFR LI was 30.4% ± 3.5 while in the study of Fontanini *et al.* [13] it was 42.1 ± 26.2. Other authors did not show EGFR expression as an absolute percentage, instead they classified tumours into groups with

**Table 4.** Relation between EGFR expression and survival of NSCLC patients after surgery (literature data)

| Authors | Year | No. of patients | Method of EGFR visualization | Results of analysis | |
|---|---|---|---|---|---|
| | | | | U | M |
| Dazzi *et al.* [10] | 1989 | 152 | IHC | NS | NS |
| Veale *et al.* [36] | 1993 | 19 | PCR | S * | S |
| Volm *et al.* [38] | 1993 | 121 (SqCLC) | IHC | S * | S |
| Giatromanolaki *et al.* [18] | 1996 | 107 | IHC | NS | NS |
| Pfeiffer *et al.* [31] | 1996 | 186 | IHC | NS | NS |
| Pastorino *et al.* [30] | 1997 | 137 (pT1N0) | IHC | S * | S |
| Rusch *et al.* [33] | 1997 | 96 | IHC | S ** | NS |
| Fontanini *et al.* [12] | 1998 | 195 | IHC | NS | NS |
| Pfeiffer *et al.* [32] | 1998 | 190 | IHC | NS | NS |
| Fu *et al.* [15] | 1999 | 158 | IHC | NS | NS |
| Cox *et al.* [9] | 2000 | 169 | IHC | NS | NS |
| Brabender *et al.* [4] | 2001 | 83 | PCR | NS | NS |
| Cox *et al.* [8] | 2001 | 167 | IHC | NS | NS |

U - univariate, M - multivariate, S - significant, NS - not significant, IHC - immunohistochemistry, PCR polymerase chain reaction;
* high level of EGFR expression indicates shorter overall patients' survival; ** low level of EGFR expression indicates shorter overall patients' survival

different EGFR protein expression [8, 9, 10, 30, 31, 33] or analysed staining intensity only [18].

We found significantly higher EGFR LI values for well and moderately differentiated than for poorly differentiated tumours. Our results are in agreement with those of Dazzi *et al.* [10] who found strong EGFR expression more often in well-differentiated tumours than in less differentiated and undifferentiated ones. Other authors found inverted correlation [9] or did not find any correlation between tumour grade and EGFR expression [4, 13, 31, 32, 35].

Significantly higher EGFR expression in pT3 tumours than in pT1+pT2 tumours found in our study, was not observed by other investigators [4, 11, 13, 16]. We noted lower EGFR expression in older patients, whereas Cox *et al.* [9] found inverted relation, and other authors did not find any association between age and EGFR expression [16, 31, 32].

In our and other authors' results there was no significant relationship between EGFR level and gender, TNM, pTNM [4, 9, 11, 31, 32]. However, Fujino *et al.* [16] and Veale *et al.* [35] found significantly lower EGFR expression in early stage cancers (pI+pII, I+II respectively) than in advanced stage cancers (pIII+pIV, III).

In our study, patients with tumours with EGFR LI between 13% and 80% survived significantly longer than those with EGFR LI less than 13% and more than 80%. This fact might indicate that during progression of SqCLC, a cancer cell may overexpress or not express EGFR protein on cell membrane. Patients with tumours with PS%≤50% survived (or survived without evidence of disease) significantly shorter than patients with PS%>50%. It is possible that this method is not sensitive enough to distinguish the group of patients with very high EGFR expression and poor prognosis. The least sensitive (qualitative) method, SI, based on intensity of EGFR staining, was not correlated with disease-specific survival of patients.

In the literature, there is still a disagreement about prognostic significance of EGFR expression in NSCLC patients (Tab. 4). In most studies, EGFR expression was not correlated with patients survival [4, 8, 9, 12, 15, 18, 31, 32]. However, some authors found EGFR overexpression as a positive [33] and some as a negative [30, 36, 38] factor influencing patients' survival. Findings mentioned above may confirm, to some extent, our results, as we found that both very high (EGFR LI>80%) and low (EGFR LI≤13%) EGFR expressions had negative impact on patients' survival. In our opinion, methods applied by all quoted above authors, based on classification of tumours into groups with different EGFR expression (for example: no staining, P%, <80% ≥80%of stained cells [31]), might cause oversight of groups with very low or very high EGFR expression. In our study, oversight of the group of tumours with very high EGFR expression and poor prognosis occurred, when we applied a method based on percentage of positively stained fields (PS%). Using the least sensitive method, SI, it was impossible to show correlation between EGFR expression and patients' survival.

One should not be surprised that high expression of EGFR is correlated with progression of malignant disease. It is well known that lung cancer is a multistep mutational process in which overexpression of EGFR is a common alteration. It may be observed in preinvasive bronchial epithelium, in the thickened basal cell zone of basal cell hyperplasia and in carcinoma *in situ* [14]. Additionally, activation of EGFR may lead to cell proliferation, metastases, angiogenesis and reduction of apoptosis [14]. In this context, it is difficult to explain why low EGFR expression predicts shorter survival. However, we have to remember that the family of EGFR genes includes 4 oncogenes: EGFR/HER1/Erb B-1, HER2/Erb B-2, HER3/Erb B-3 and HER4/Erb B-4, and not only EGFR level but also other EGFR family members are overexpressed in lung cancer and premalignacy [14]. Therefore, in the group of tumours with low EGFR expression and poor prognosis, overexpression of other EGFR family members might be responsible for conversion of cancer cell into a more aggressive phenotype.

We have found higher EGFR LI in tumours from patients who later suffered from loco regional recurrence than in tumours from patients who later developed metastases. Similarly, in NSCLC, Lai *et al*. [22], found relationship between the expression of ErbB-1 and ErbB-3 and higher risk of loco regional recurrence, and between the expression of ErbB-3 and higher risk of metastases. Those results might suggest that in lung cancers, EGFR (ErbB-1) is involved in local tumour growth processes, while ErbB-3 (not ErbB-1) in the processes of metastasis formation. These observations might indicate that different members of EGFR family are involved in different processes of tumour progression.

We conclude that the problem of prognostic significance of EGFR expression in lung cancer is far from being resolved. However, as shown for head and neck tumours, overexpressed EGFR might be a valid target for cancer therapy. The two most extensively tested strategies involve monoclonal antibodies against EGFR (for example IMC-C225) and inhibitors of the receptor tyrosine kinase (ZD1839). They can be applied alone or in combination with chemotherapy [23, 24]. There is a need for further studies considering not only the level of EGFR expression but also other parameters that may be connected with signalling *via* EGF receptor.

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