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Protein expression, gene amplification, epidermal growth factor receptor mutations and lung carcinoma

Proteinska ekspresija, genska amplifikacija, mutacije receptora za faktor rasta epiderma i karcinom pluća

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Ključne reči:
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Introduction

Despite significant advances in the detection and treatment of lung cancer it causes the highest number of cancer-related mortality.

Recent advances in the detection of genetic alterations facilitated the development of potent and specific target therapies.

Epidermal growth factor receptor (EGFR)

EGFR belongs to the family of structurally and functionally similar ErbB or HER receptors on cell surfaces, which have intracellular tyrosine kinase activity. What all of these receptors have in common is that they consist of extracellular and membrane components to which ligand and intracellular components are bound *via* the tyrosine kinase activity [1]. Under normal conditions the activity of ErbB receptors is controled by ligands which belong to the group of EGF related growth factors [2].

Protein expression of EGFR and lung carcinoma

EGFR expression was found in normal tissue and lung carcinoma. In normal lung tissue, according to some authors, EGFR expression was localized to the pole with ciliated bronchial cells, whereas EGFR expression was not detected in the bronchial glands [3]. According to others, however, increased protein expression of EGFR in the bronchial epithelium was localized in the basal layer of epithelial cells (Figure 1). Increased EGFR expression is present in 40–60% of primary non-small cell lung carcinomas (NSCLC) and in all histological types, although with squamous cell carcinoma the levels were higher [4]. According to some studies the level of expression is associated with the disease prognosis, as a higher level of expression is found in cancer patients who are in stage III of the disease compared to patients in stages I and II. Moreover, some studies have shown that tumors with high EGFR expression have larger metastatic potential, poorer histological differentiation and higher proliferative potential [5, 6]. EGFR expression is determined by immunohistochemical method, and evaluated by semiquantitative method (Figure 2).

EGFR gene amplification and lung carcinoma

The main mechanism of increased protein expression of EGFR is gene amplification.

Six basic types of EGFR gene amplification in NSCLC have been defined. According to some authors and analyzed by fluorescent *in situ* hybridization (FISH), most often, in about 60% of cases, the classic form occurs in the shape of large clusters with more than 20 gene copies. This type of characteristics is the amplicon with the „homogenously staining region“. In about 10–15% of tumors with amplification, there is another form, created by forming small clusters containing 4–10 EGFR gene copies. The third form of gene amplification (15–20% of tumors), EGFR amplicon includes the CEP 7 sequence so that a cluster of EGFR and CEP 7 signals occurs. In about 5% of the tumors, a fourth form of EGFR gene amplification occurs, with atypically large and

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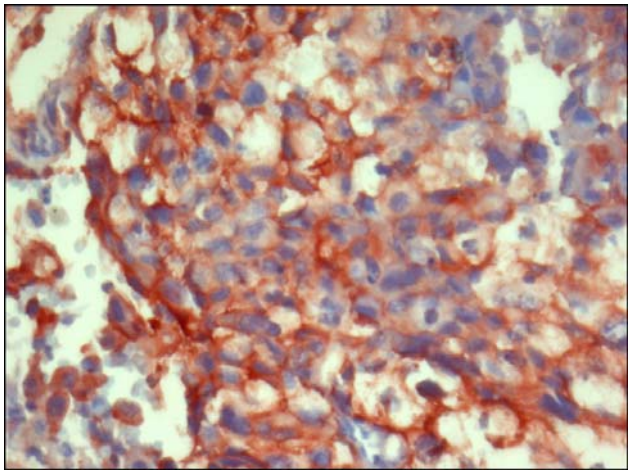

Fig. 1 – Strong, complete membranous staining of tumor cells within lung adenocarcinoma
(anti EGFR antibody, × 200).

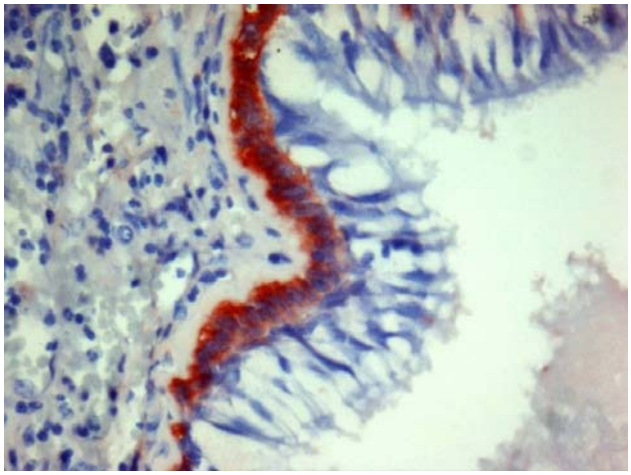

Fig. 2 – EGFR protein expression in the basal cell layer of the bronchial epithelium
(anti EGFR antibody, × 200).

bright signals. In 1% of the tumors, a fifth form of EGFR gene amplification can be found, in the form of fine volatile signals that represent extrachromosomal „double minute". The last, sixth form of EGFR gene amplification occurs in approximately 5% of the tumors as a large number of copies in cells with chromosomal aneusomy [7]. The incidence of the increased number of gene copies, i.e. gene amplification of EGFR in NSCLC, ranges, in published work, between 8% and 10% [8, 9].

Even though FISH is the standard technique for the detection of gene amplification, a disadvantage of this method is that the fluorescence signals are not stable and may fade or disappear within weeks. In addition, it is more difficult to observe the morphology of cells. Some of these issues can be overcome by applying chromogen *in situ* hybridization (CISH). CISH signals do not disappear over time, and it is possible to distinguish stromal and tumor cells [10] (Figures 3 and 4). Gallegos et al. [11] were the first to, in order to analyze the CISH method for the detection of EGFR gene copies in NSCLC, compare the results obtained by FISH and CISH method in the same group of patients and there was concordance of results using these two techniques. It has been confirmed earlier that CISH provides similar results to FISH regarding the detection of HER2/neu gene in breast cancer [10, 12, 13].

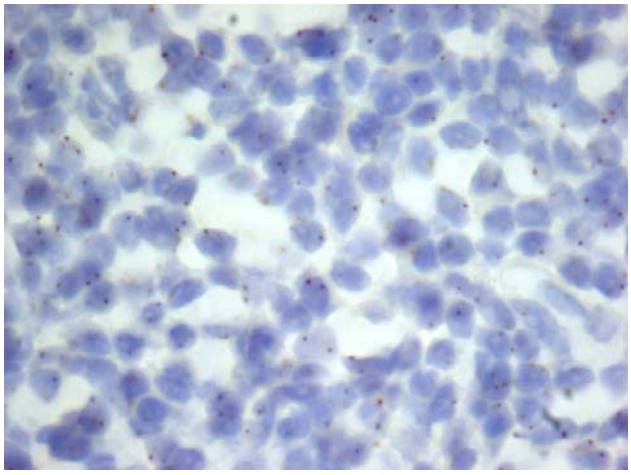

Fig. 3 – Tumor cells of lung adenocarcinoma with normal EGFR gene copy number
(anti EGFR antibody, × 200).

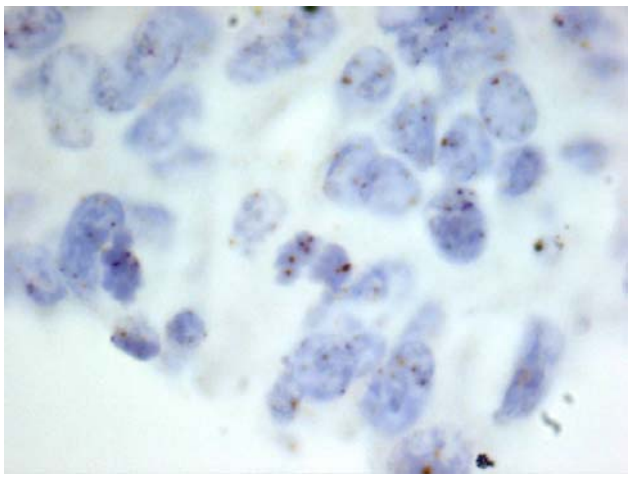

Fig. 4 – EGFR gene amplification in tumor cells of lung adenocarcinoma
(anti EGFR antibody, × 200).

Mutation of the gene for EFGR in lung carcinoma

The EGFR gene is located on the 7p12 chromosome.

Even though a great number of EGFR gene mutations has been documented so far, out of the seven exons encoding the TK domain (exons 18–24), mutations usually occur in the first four exons. A limited number of mutations has been identified in lung adenocarcinomas. These mutations lead to an extension in receptor activation once the ligand is bound. In addition, a consequence of mutations is a change in the TK domain, especially in places near the ATP binding site and the signal activation of pathways that lead to apoptosis resistance [14].

The most commonly used methodology for this purpose has been, and will probably continue to be, direct sequencing of PCR products. The main drawbacks of this method are its

low sensitivity (20–50%) and the significant risk of contamination involved in handling post-PCR products [15–17]. Furthermore, recent advances in molecular techniques have enabled the development of more sensitive methods for detecting mutations with real-time quantitative PCR, using specific probes or amplified refractory mutation system (ARMS™) technology [18]. Most recently, the development of EGFR mutant-specific antibodies for immunohistochemistry (IHC) has presented a new method for consideration [19, 20].

The limit of detection (LOD) of the real-time PCR method is lower than that of direct sequencing while the mutation specific IHC produces excellent specificity. It is necessary to have methods available that give us access to results rapidly and accurately [21].

EGFR gene mutation is more common in nonsmokers than in smokers (51% *vs* 10%), with adenocarcinoma than with other types of cancer (40% *vs* 3%), in patients of Asian descent than those of different descent (30% *vs* 8%) and in women compared with men (42% *vs* 14%). According to some authors EGFR mutation status is not associated with the patient's age, clinical stage of the disease, histologic characteristics and overall survival rate, and was not found in normal lung tissue [22, 23]. According to others, mutations in the tyrosine kinase domain of the epidermal growth factor receptor have prognostic significance, since patients with EGFR-mutant NSCLC have prolonged disease-free survival, compared with those with wild-type disease, regardless of the treatment received [24, 25].

Gene amplification is known to increase the expression of oncoproteins, as is the case with ErbB2 and breast cancer. The combination of gene amplification and mutation in lung carcinoma is relatively rare. However, studies show that EGFR amplification occurs more frequently in tumors with mutations [22, 23]. EGFR gene amplification is detected in some EGFR mutation–positive patients, and it is reported to be associated with disease progression [26]. A subset of lung adenocarcinomas shows activation of EGFR by mutations and/or amplification but the interaction between them is complex and unclear. Amplification can also occur independently, without the mutation, and may explain the increased expression of EGFR in squamous cell carcinomas and other epithelial tumors of the lung [22, 23].

EGFR inhibition in carcinomas

Significant progress has been made in recent decades in the study of specific cellular, molecular and genetic mechanisms that contribute to tumor growth and progression. This progress has instigated development and clinical evaluation of various tumor-specific anticarcinoma therapeutic approaches. A promising antitumor strategy is the inhibition of EGFR signaling. The best studied mode of EGFR inhibition includes monoclonal antibodies (mAbs), which are aimed directly against the extracellular part of EGFR and a small molecule of TK inhibitors (TKI) is directed against the very TK. Monoclonal antibodies bind to the ligand-binding site in the extracellular domain, while the TK inhibitors bind to the cytoplasmic TK receptor domains [27].

Great efforts have been invested into the strategy of choosing the patients for TK inhibitor therapy in those with NSCLC. A history of smoking, performance status, drug-induced itching and molecular biomarkers such as EGFR mutations, EGFR protein expression (immunohistochemically analyzed) and EGFR gene amplification (analyzed by fluorescent *in situ* hybridization) are factors that point to a better response to treatment with TK inhibitors.

The 2 TKI agents currently approved for use in lung cancer, which target lung cancer with EGFR mutations, are gefitinib (2002) and erlotinib (2003). EGFR mutation is a specific target for therapy by TKIs and is a validated biomarker of a treatment response. The clinical utility of this biomarker is supported by prospective clinical trials that have demonstrated a progression-free survival benefit of TKI as first-line therapy in EGFR-mutant patients. Based on current data, predictive biomarker tests for EGFR should involve mutational analysis. EGFR FISH testing is less predictive of TKI response rate than mutation testing in clinical studies, and currently should not be used as a method for EGFR TKI treatment selection [28].

Susceptibility and resistance to EGFR inhibitors

Most frequently detected mutations are those that determine the TK region, i.e. deletion mutations with or without insertion in exon 19 and missense mutation in exon 21. These mutations are more common in patients of Asian descent than in patients of different descent (40% *vs* 19%) and are more common in patients with adenocarcinoma, nonsmokers and women, although they are found in other groups of patients. The response to erlotinib and gefitinib treatment is stronger in patients with mutations than in patients with wild-type EGFR. All EGFR mutations are heterozygous, i.e. affect only one allele and have a dominant oncogenic effect, regardless of the presence the wild-type allele. Mutations are usually located near the ATP binding sites, but also on places where gefitinib binds. It can be assumed that mutations lead to achieving a more stable binding of ATP but also of its competitive inhibitor gefitinib. This could explain the increased activity after receptor ligand binding, as well as the greater susceptibility to the TK inhibitor therapy. Susceptibility to gefitinib has also been demonstrated in patients without EGFR mutations, suggesting that other mechanisms, such as gene amplification can sensitise tumor cells [29–35].

Resistance to TK-targeted therapies rises as a problem. This resistance was first identified in patients with advanced chronic lymphocytic leukemia and an association with point mutation was found or, less commonly, with gene amplification. In gastrointestinal stromal tumor and NSCLC, resistance to TK inhibitor therapy is associated with some types of TK mutations. Research showed that EGFR mutation is connected to resistance to therapy in NSCLC, T790M, and that it causes a blockage in TK inhibitor binding to the ATP site (secondary resistance). In addition, activating mutations GTPase and K-ras also lead to primary resistance [36, 37].

EGFR status and the clinical-pathological parameters of lung cancer with specific reference to adenocarcinoma

According to the current WHO classification, the histological types of adenocarcinomas are: papillary, acinar, solid and the bronchoalveolar. The majority of tumors was built from a combination of these histologic patterns and has thus been classified as a mixed form of adenocarcinoma. However, certain types and subtypes of tumors, i.e. adenocarcinoma of the lung, are associated with the corresponding molecular alterations [38]. Moreover, the subject of numerous studies is the connection between the smoking status, disease stage, tumor differentiation, as well as the patient's gender with the molecular status and especially with the EGFR profile of the tumor.

In their study, Hirsch et al. [39] found no difference when it comes to the respondents' gender, smoking status and histological type of tumor between the groups of patients with and without EGFR gene amplification, demonstraded *via* FISH analysis. In their work they analyzed patients with bronchoalveolar carcinoma in an advanced stage and came to the conclusion that the presence of EGFR gene amplification may be a predictor of better prognosis and better therapy response in patients treated with gefitinib.

Đačić et al. [9] found no correlation between the clinical-pathological parameters and the protein expression of EGFR in the tumor, but have found a greater occurence in the presence of gene amplification in poorly differentiated squamous cell carcinoma.

In patients with lung adenocarcinoma with bronchoalveolar characteristics who were treated with gefitinib and erlotinib the response to the treatment was better [40, 41].

Many studies analyzed K-ras and EGFR alterations in adenocarcinomas of the lung. The frequency rate of both mutations ranges between 10% and 30%. EGFR mutations are more common in the Asian population, non-smokers and nonmucinous tumors whereas K-ras mutation is more common in the non-Asian population, smokers and invasive mucinous adenocarcinoma, and these mutations are mutually exclusive [42].

Under the new proposed classification of lung adenocarcinoma (International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society International Multidisciplinary Classification of Lung Adenocarcinoma) [43] certain types of lung adenocarcinomas differ based on molecular alterations, too, and so it is possible to, based on histological appearance of the tumor, predict its molecular profile, with greater or lesser certainty. EGFR mutations are more frequently found in adenocarcinoma *in situ* (AIS), adenocarcinoma with predominantly lepidic growth (LPA), papillary and micropapillary adenocarcinoma, although they may be found in other histologic types. In a large study on 806 NSCLC, EGFR mutations were found more frequently in adenocarcinoma previously classified as BAC or adenocarcinoma with lepidic growth (under new classification AIS, minimally invasive adenocarcinoma – MIA, and LPA). Predominantly solid type of adenocarcinoma is more frequently associated with the presence of K-ras mutations [44].

EGFR gene amplification is more frequently found in primary tumors than in metastases and is more often found in invasive tumors and more poorly differentiated types than in precursor lesions such as atypical adenomatous hyperplasia (AAH) and earlier bronchioalveolar cancer, indicating greater aggressiveness of these tumors in the presence of EGFR gene amplification [45–47].

In our study, performed on 90 patients with lung adenocarcinoma, the presence of EGFR protein expression in tumors was found in 37.78%, while the EGFR gene amplification in tumors, analysed using the CISH method, was present in 11.11% of respondents. Multivariate analyses were conducted on the group of patients with present EGFR protein expression in tumors, and certain characteristics were distinguished: mixed adenocarcinoma with broncholoalveolar characteristics, solid and papillary type, moderately and poorly differentiated tumors, smokers and women; while the analyses conducted on the group of patients with the presence of EGFR gene amplification in tumors distinguished the following characteristics: mixed adenocarcinoma with bronchioalveolar characteristics, papillary and other variants of adenocarcinoma, moderately differentiated tumors, non-smokers and women. There was no difference regarding the presence of protein expression and gene amplification of EGFR in the tumor in relation to T and N status of the patient, recurrence of disease, nor between stages I, II and IIIA of the disease [48].

Conclusion

The classification algorithm, based on histological and molecular characteristics of tumors, is useful in the selection of therapy that targets molecular alterations. In the era of genomics and proteomics, progress is expected in early diagnosis of lung cancer, its classification and selection of therapeutic agents. The profile of the disease can be induvidualised, and with it the choice of therapy for each patient, which will in the future certainly lead to more successful treatment and longer survival rates of patients with lung cancer as well as those with malignant tumors in general.

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