Epidermal growth factor receptor – its expression and copy numbers of EGFR gene in patients with head and neck squamous cell carcinomas*

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Received October 11, 2004

Signaling pathways activated by epidermal growth factor receptor (EGFR) are pathogenetically involved in the development of head and neck squamous cell carcinomas (HNSCC). A monoclonal antibody against the EGFR protein blocking the receptor activity (cetuximab – Erbitux – C225) is now available for therapeutic applications. The mechanisms of EGFR protein overexpression are poorly understood. Regulatory pathways, EGFR gene structural changes or its amplification may be involved. The aim of the study was to evaluate expression of the EGFR protein in patients with HNSCC, to identify EGFR gene copy numbers, and to find out whether the protein overexpression is associated with the EGFR gene amplification. In the case of a pathogenetical link of the EGFR gene amplification and the protein overexpression it would be useful to employ both diagnostic approaches to identify patients eligible for cetuximab therapy. We investigated 33 patients with HNSCC. The expression of EGFR protein was evaluated by immunohistochemistry, copy numbers of EGFR gene and the numbers of chromosome 7 centromeric signals were investigated by fluorescence *in situ* hybridization on interphasic nuclei (I-FISH). Histological sections from formalin fixed and paraffin embedded tissues were used. We observed three types of EGFR protein expression (homogeneous 3+ membrane positivity in 13 patients; membrane positivity varying from 1+ to 3+ in 12 patients; a strong membrane positivity at the periphery of the tumor cell clusters in 5 patients). In two cases the results were difficult to interpret. In one case single tumor cells only were positive. Numerical changes of chromosome 7 were present in 23 patients. We found the EGFR gene amplification in seven patients. The tumor cells with amplification of the EGFR gene were generally infrequent and were localized in small clusters, or they were randomly dispersed between the tumor cell population without the gene amplification. We did not find any correlation between the EGFR gene amplification and the EGFR protein overexpression. Thus, amplification of the EGFR gene is not pathogenetically involved in the EGFR protein overexpression. From the diagnostic aspect a standardized immunohistochemical assessment of the EGFR protein expression appears sufficient for detection of the EGFR status. Criteria for cetuximab treatment in patients with HNSCC may differ from those already used for patients with colorectal carcinomas and should take different patterns of the EGFR protein overexpression into consideration.

Key words: head and neck squamous cell carcinoma, epidermal growth factor receptor, protein expression, gene copy numbers, cetuximab

Head and neck squamous cell carcinomas (HNSCC) are relatively frequent neoplasms affecting middle aged and elderly patients. The prognosis of the disease is unfavorable. Tobacco smoking is one of the major risk factors and its deleterious effect may be enhanced by alcohol consumption. At a molecular level, oncogene activation, tumor suppressor gene inactivation, and increased expression of growth factor receptors and their ligands lead to the tumor development and progression. Out of growth factor receptors epidermal growth factor receptor (EGFR) appears pathogenically involved [13].

In treatment of patients with HNSCC new approaches are tested (alone or in a combination with a standard therapy – surgery, chemotherapy and radiotherapy). Drugs targeted

*This study was supported by the Grant of Faculty Hospital Motol 9730 and by Research Project of the Ministry of Health and Faculty Hospital Motol 000 000 64203/6073.

specifically to the molecular changes in tumor cells are applied – so called small molecules tyrosine kinase inhibitors (e.g. gefitinib – Iressa – ZD1839) or even more recently, monoclonal antibodies against key molecules in cancer development. Anti-EGFR monoclonal antibody (e.g. cetuximab – Erbitux – C225) already used in treatment of some patients with advanced colorectal carcinomas started to be tested in therapy of patients with advanced HNSCC. After binding of the antibody to the EGFR, a binding site for ligands at the EGFR is occupied, and activation of the receptor is blocked [16]. In cell lines derived from HNSCC, the antibody C225 increased effectiveness of radiotherapy [3]. In experimental conditions tumor-induced neovascularization and tumor cell migration is suppressed [4]. Bispecific monoclonal antibodies against the EGFR are now studied (one antigen-binding arm is specific for the EGFR, the second arm binds to CD3 and CD64 of immunologic effector cells) [8].

EGFR (transmembrane 170 kD molecule) is a member of ERB protein family. EGFR gene coding for the receptor is localized at chromosome 7 (7p12). Synonyms used for the EGFR protein are ERBB-1 and HER1. After ligand binding (e.g. epidermal growth factor, transforming growth factor alpha) homodimerization or, after interaction with other members of the ERB family, heterodimerization occurs. Tyrosin kinase of the receptor's intracytoplasmic domain is activated and several signal pathways are triggered off contributing to the cell proliferation, invasion and increased metastatic potential of the tumor cells [2, 10]. EGFR protein is expressed on the cell membrane of all squamous cells at a low level. Increased expression of the EGFR is found in many tumors of epithelial origin (e.g. carcinomas of the mammary gland, ovary, prostate, urinary bladder, lungs, head and neck, uterine cervix, endometrium, colon) [2]. Functional or structural gene changes may contribute to the increased protein expression. One of such changes common in the ERB family is gene amplification [14].

There are limited data in the literature concerning mutual relationships between the EGFR protein expression and copy numbers of the EGFR gene in patients with HNSCC. The aim of this study was to find out if such a relation exists and to determine a potential value of detecting the EGFR protein expression by immunohistochemistry alone or in a combination with establishing the EGFR gene copy numbers to make patients with HNSCC eligible for the cetuximab therapy.

Material and methods

Patients and investigated material. We investigated a group of 33 patients with HNSCC who had initially undergone surgery without any prior treatment (27 men and 6 women; the age of the patients varied from 33 to 79 years at diagnosis; median 56 years, average 57 years). The primary tumors were localized in the palatinal tonsils (13 patients), larynx (12 patients), base of the tongue (4 patients), floor of the oral cavity (2 patients), hypopharynx (1 patients) and tongue (1 patient). The International Union Against Cancer (UICC 2002) system of TNM classification was used to categorize the patients. Tumor sizes were classified as follows: T1 (2 cases), T2 (12 cases), T3 (11 cases) and T4 (8 cases). Regional metastases were found in 27 patients with nodal status N1 (10 cases), N2 (13 cases) and N3 (4 cases). Six patients presented without lymph node involvement. Accordingly, there was one patient stage I, 3 patients were stage II, 9 patients were stage III and there were 20 patients stage IV.

For immunohistochemistry and for fluorescence *in situ* hybridization histological sections from formalin fixed and paraffin embedded tissue were used.

Immunohistochemical (IHC) detection of the EGFR protein expression. Expression of the EGFR protein was evaluated using diagnostic system "EGFR pharmDx" from DakoCytomation (the U.S. FDA for identifying colorectal cancer patients eligible for treatment with cetuximab). The kit includes mouse monoclonal antibody (clone 2-18C9) against the extracellular domain of the EGFR. The antibody does not cross-react with other members of the ERB protein family. The appropriate tissue for the IHC investigation was selected using hematoxylin and eosin staining. The instructions of DakoCytomation data sheet were strictly followed. Negative controls to each patient tissue, cell lines supplied with the kit (CAMA-1; HT-29), and control tissues with a non-neoplastic squamous epithelium (tongue, tonsil, vocal chord, nasal vestibule, uterine cervix) were included in each experimental run. The EGFR protein expression intensity (0, 1+, 2+, 3+) was evaluated as recommended by DakoCytomation in "EGFR pharmDx Image Gallery".

Fluorescence in situ hybridization on interphasic nuclei (I-FISH) for evaluation of the EGFR gene copy number and number of chromosome 7. For enumeration of copy numbers of the EGFR gene and chromosome 7 we used LSI EGFR Dual Color Probe-Hyb Set (Abbott Laboratories/Vysis, Inc.). The set is a mixture of two directly labeled probes – locus specific probe against EGFR (7p12) labeled with "Spectrum Orange" and alpha satellite centromere probe (7p11.1-q11.1) for chromosome 7 (CEP7) labeled with "Spectrum Green". Vysis instructions were used ("Paraffin Pretreatment"; http://www.vysis.com/ParaffinPretreatment_32958.asp) and followed by "LSI protocol" (http://www.vysis.com /LSI_22729.asp) with one modification of post-hybridization washing (0.4x SSC/0.3% NP-40 was substituted by 2x SSC/0.3% NP-40). To achieve a balanced proportion between preserved tissue morphology and a sufficient intensity of the fluorescence we increased or decreased the time of enzymatic proteolytic digestion. The region suitable for applying the probe was chosen after checking the tumor tissue morphology using hematoxylin and eosin staining, and the results of IHC.

Evaluation of I-FISH results. The I-FISH results were evaluated under fluorescence microscope (Provis AX70, Olympus) with a dual-band filter. In each case we counted the signals in at least one hundred of tumor cell nuclei.

If more than 10% of tumor cells had more than 10 signals for EGFR gene per a nucleus, the tumor was evaluated as having a strong EGFR gene amplification. A moderate amplification was reported if more than 10% of tumor cells had 10 or a lesser number of signals of the EGFR gene per a nucleus. The evaluation of the gene amplification was performed in relation to the CEP7 signal count. It is necessary to take an increased number of locus specific signals caused by chromosome duplication during the cell cycle into account – a typical appearance of metaphase chromosome after DNA replication is represented by two sister chromatids linked together at the centromere, resulting in two CEP7 signals and four EGFR signals per interphasic nucleus. Similarly, PAULETTI et al [11] and LEBEAU et al [7] evaluate tumors (breast carcinomas) containing up to four ERBB2 gene signals per nucleus as not amplified. The presence of such a signal pattern (two signals for centromere of chromosome 7 and three or four signals for EGFR gene per nucleus) in investigated HNSCC, occurred in about 1 to 10% of the tumor cells, depending on the proliferative activity of the tumors. Thus, the moderate gene amplification was described, for example, if the tumor had cells with two CEP7 signals and 5–10 EGFR signals, or the cells had three CEP7 signals and 7–10 EGFR signals. Cases without an increased number of the EGFR gene signals or with an increased number of signals in up to 10% of the tumor cells were reported as non-amplified. Similar models using the 10% limit, were used for ERBB2 gene amplification [7, 11, 12] and for evaluation of ERBB-2 protein positivity by FDA approved HercepTest.

Cases with more than two CEP7 signals per a tumor cell nucleus in more that 10% of tumor cell population were reported as having severe numerical changes of chromosome 7.

Results

EGFR protein expression. In general, we found three main patterns of the EGFR protein expression. In the first type there was a strong homogeneous membrane positivity (3+) of nearly all tumor cells (13 patients) (Fig. 1). The second type was characterized by a variable positivity (from 1+ to 3+) of all tumor cells (12 patients) (Fig. 2). The third type, found in 5 patients, presented as a strong membrane positivity of the tumor cells at the periphery of the tumor nests, the intensity of the expression decreased towards the central parts, and the centers were negative (Fig. 3). In samples of two patients the only tumor cells positive were at the periphery of the sample; repeated IHC procedure yielded an identical result. In one case, we found 3+ membrane positivity in single tumor cells only, the remaining tumor tissue was negative.

In keratinizing tumor cells, the intensity of the EGFR protein expression decreased significantly, most of these cells were negative (Fig. 1). In basal layers of the non-neoplastic squamous cell epithelium (the epithelium was present in samples of 28 investigated patients) the EGFR protein was 3+ positive; the intensity of the EGFR protein expression decreased toward the upper layers of the epithelium, the top layers were negative (Fig. 3). In two cases the intensity of the EGFR protein expression in basal layers of the epithelium varied between 1+ and 2+; the tumor cells of this case expressed the EGFR protein strongly (3+).

If the primary antibody against the EGFR protein was substituted by monoclonal IgG1 antibody (negative control – part of the detection kit), we did not observe non-specific staining in any of the cases. Control cell lines reacted as expected – CAMA-1 was negative (score 0), HT-29 was positive (2+). In control tissues the EGFR protein was overexpressed in the basal layer of the squamous epithelium. We found the EGFR protein positivity also in other types of cells than keratinocytes: tongue – myoepithelial cells of small salivary glands, fibroblasts, Schwann's cells, epineural cells (striated muscle cells were negative); tonsil – perifollicular cells; vocal cord – cells around nerve branches; uterine cervix – smooth muscle cells (the cells of the columnar endocervical epithelium were negative).

Copy number of the EGFR gene. We found amplification of the EGFR gene in seven of 33 investigated patients. In five of them, the number of EGFR signals was increased to a moderate level of amplification (Fig. 4). In two patients, a strong gene amplification was present (Fig. 5). The tumor cells with an increased copy number of the EGFR gene, irrespective whether they showed a moderate or strong gene amplification, formed small clusters, or they were singly scattered among non-amplified tumor cell populations. In 19 of 26 patients classified according to the criteria used in this study as non-amplified we found, after a careful review of the whole tumor sample, two to four tumor cells with an increased copy number of the EGFR gene.

In patients with HNSCC, the cells of all layers of the non-neoplastic squamous cell epithelium as well as other non-neoplastic tissues (muscle cells, inflammatory cells) did not show any increase in numbers of the EGFR gene signals. In the control cell lines CAMA-1 and HT-29 the EGFR gene was not amplified.

The number of chromosome 7 centromeric signals (CEP7). In 23 patients we found numerical changes of chromosome 7 (Fig. 6). In a majority of the cases the tumor cells with an increased number of CEP7 signals did not exceed 50% of the tumor cell population. The number of CEP7 signals varied up to 5 signals per a nucleus. In one case the tumor cells with seven CEP7 signals were present. The number of CEP7 signals in cells of squamous cell epithelium and in other non-neoplastic cells was not increased. In addition to cells with two CEP7 signals we found also cells with one CEP7 signal per a nucleus, a finding attributed most probably to the effect of tissue cutting. In the control cell lines CAMA-1 and HT-29 numerical changes of CEP7 signals were present.

We found no correlation between the individual types of EGFR protein expression and EGFR gene amplification and/or numerical changes of chromosome 7 in this study but

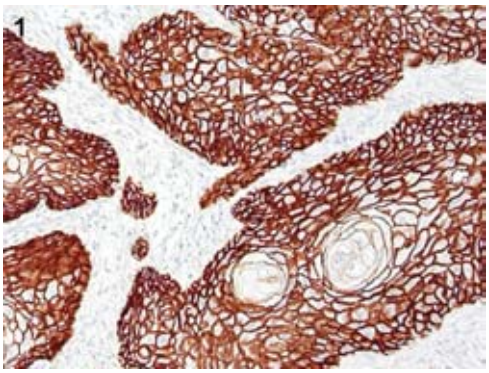

Figure 1. A strong homogeneous positivity (3+) of EGFR protein on membranes of the tumor cells in HNSCC. In keratinizing tumor cells the intensity of the EGFR protein expression decreased. Stromal cells are negative. IHC (EGFR pharmDx – DakoCytomation), formalin fixed and paraffin embedded tissue section.

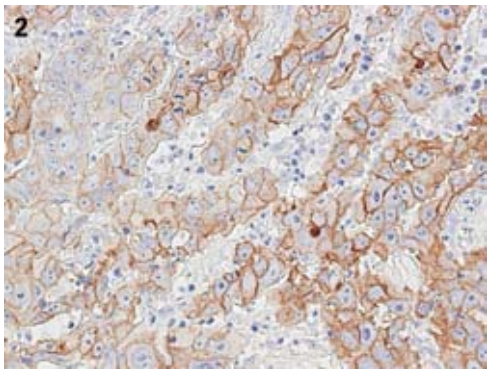

Figure 2. A variable positivity of EGFR protein on membranes of the tumor cells in HNSCC. The intensity of the protein expression vary from 1+ to 3+. Stromal cells are negative. IHC (EGFR pharmDx – DakoCytomation), formalin fixed and paraffin embedded tissue section.

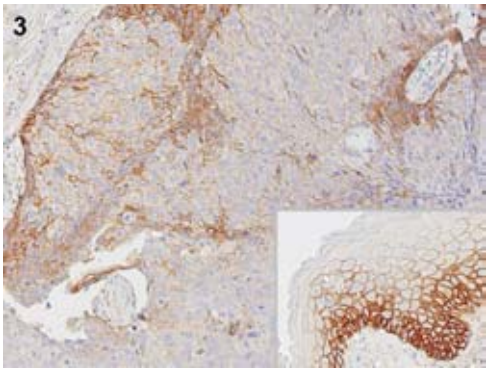

Figure 3. A strong EGFR membrane positivity of the HNSCC tumor cells at periphery of the tumor cell nests. The intensity of expression decreases towards the central parts. Inserted picture – a strong EGFR protein expression in basal layers of the non-neoplastic squamous cell epithelium. IHC (EGFR pharmDx – DakoCytomation), formalin fixed and paraffin embedded tissue section.

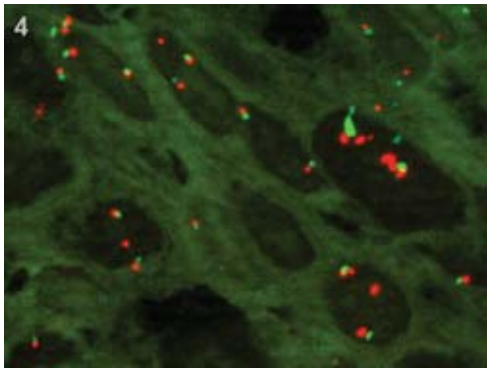

Figure 4. A moderate level of the EGFR gene amplification in HNSCC. One tumor cell with a moderate EGFR gene amplification in the tumor cell population with the EGFR gene non-amplified. I-FISH (EGFR locus – Spectrum Orange, CEP7 – Spectrum Green; Vysis), formalin fixed and paraffin embedded tissue section.

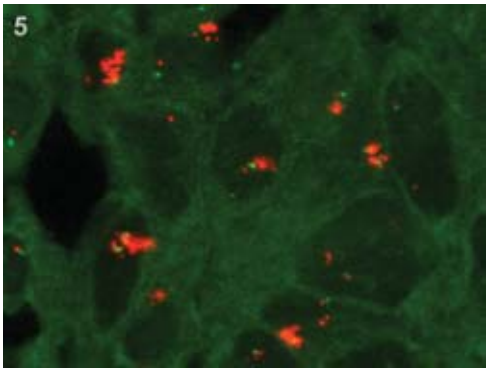

Figure 5. A strong EGFR gene amplification in HNSCC. A cluster of tumor cells with a strong gene amplification. I-FISH (EGFR locus – Spectrum Orange, CEP7 – Spectrum Green; Vysis), formalin fixed and paraffin embedded tissue section.

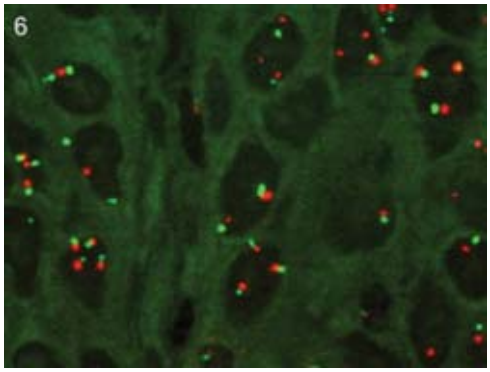

Figure 6. HNSCC with numerical changes of chromosome 7. Tumor cell nuclei with three or four signals of EGFR gene and CEP7 are present. A disproportion between the number of red and green signals in some nuclei (for example: two green and one red signal) is caused by tissue sectioning. I-FISH (EGFR locus – Spectrum Orange, CEP7 – Spectrum Green; Vysis), formalin fixed and paraffin embedded tissue section.

the number of patients investigated is small for a statistical evaluation.

Discussion

The availability of a new therapeutic agent (cetuximab) – a monoclonal antibody against the EGFR protein raised the importance of EGFR assessment in patients with HNSCC. Because protein expression may be enhanced by amplification of its gene we made an attempt to evaluate relationship between the expression of the EGFR protein and the number of copies of the EGFR gene in patients with HNSCC. This issue has not been documented well in the literature.

Until now, there is no consensus in choosing a type of the primary antibody and IHC detection system for investigation of EGFR protein expression and the criteria for evaluation of the results are not well established [1]. Similarly as with HercepTest (ERBB-2 protein expression/carcinomas of the mammary gland), DakoCytomation developed a diagnostic kit "EGFR pharmDx" for evaluation of the EGFR protein expression in patients with metastatic colorectal carcinomas who may benefit from cetuximab therapy. We used "EGFR pharmDx" kit in this study. At present, DakoCytomation recommends to report the results as "EGFR positive" or "EGFR negative". As "EGFR positive" they evaluate any positivity over the background (1+, 2+, 3+) in at least 1% of the tumor cells. In the group of patients with HNSCC, we found the EGFR protein expression quite intensive but heterogeneous. There were three major patterns of IHC positivity: a) a homogeneous 3+ membrane expression in nearly all tumor cells, b) a variable 1+ to 3+ expression in the tumor cells, c) a strong positivity at the periphery of the tumor nests with a decreased intensity to a negative result in the central parts. Based on this result we consider the evaluation recommended by DakoCytomation inadequate for HNSCC. We believe that response to cetuximab therapy in patients with 3+ expression in all tumor cells might be different than that in patients with the second or third type of the expression. In this context it is noteworthy that there is a molecular heterogeneity in HNSCC as mapped by cDNA microarray techniques – according to CHUNG et al patients with HNSCC may be stratified according to their gene expression profile to several subgroups [5]. It is possible, that subtypes of EGFR protein expression we found in our group of patients may result from different regulatory pathways and that the different patterns of IHC positivity may at least to some extent reflect groups of HNSCC pooled on the basis of expression profiles. To find out whether the different subtype of the EGFR protein expression has an impact on prognosis and/or on a prediction of the response to cetuximab therapy in patients with HNSCC, it is necessary to perform clinical studies with a long term follow up, a goal beyond the scope of this study.

In our group of patients with HNSCC, we found an increased expression of the EGFR protein in 30 of 33 cases. In two, it was possible to interpret the IHC result as positive, but the finding was ambiguous – the EGFR protein was expressed only in tumor cells at the periphery of the samples. Because of sensitivity of the EGFR protein to formalin fixation, we cannot rule out a partial damage of the antigen epitope during the tissue fixation. In one patient we found only scattered individual tumor cells 3+, the majority of the tumor was negative, which suggests a downregulation of the EGFR protein expression.

In contrast to the fact that most patients overexpressed the EGFR protein, we found the EGFR gene amplification in only seven patients. In general, the amplification was of a moderate degree, and it was found in a low percentage of the tumor cells. Tumor cells with the increased EGFR gene copy number were present in small clusters or such cells were distributed singly and haphazardly in the tumor tissue. In a view of a low percentage of the EGFR gene amplification in relation to the EGFR protein expression, I-FISH evaluation of the EGFR gene copy number in patients with HNSCC has a limited value. Data on the EGFR gene amplification in the literature are scarce. Tissue homogenization was used for evaluation of the EGFR gene amplification in the past. ISHIZUKA et al applied comparative genomic hybridization and Southern blotting and found EGFR gene amplification in only 1 of 33 patients (3%) with esophageal squamous cell carcinoma [6]. NAGATSUKA et al detected the EGFR gene amplification by competitive PCR in 4 of 20 oral squamous cell carcinomas (20%) [9]. In contrast to these findings, the EGFR gene amplification is more frequent in patients with glioblastomas – SHINOJIMA et al described the amplification in 40 of 87 patients (46%) [15].

Using I-FISH on histological sections in HNSCC has an advantage over homogenization techniques in that the tumor cells can be identified in a context with the tissue morphology and the tumor cell heterogeneity can be thus revealed. Individual tumors differ in differentiation, degree of keratinization, and in the presence and composition of the fibrous stroma and inflammatory reaction. Because of such a variability of the tissue used for I-FISH examination, it is necessary to individualize the laboratory procedure preceding the application of the fluorescence probe. The best effect to achieve an optimal FISH signal intensity was reached by a modification of the time of proteolytic digestion.

Generally, one of the frequent and known causes of a protein overexpression in carcinomas is gene amplification. However, based on this study we conclude, that the increased expression of the EGFR protein is not caused by such a mechanism in patients with HNSCC. Because we investigated the presence of the gene amplification in a view of tissue morphology, we were able to compare results of I-FISH and IHC in the same tumor tissue region. We did not observe any difference of the EGFR protein expression between the tumors with and without the EGFR gene amplification. Because EGFR protein expression in keratinizing tumor cells is decreased (similarly as in upper layers of non-neoplastic squamous cell epithelium) it can be deduced, that the expres-

sion of the EGFR protein is a controlled process regulated by the tumor cells. Stimulation of transcription, activated translation or a prolonged life span of the protein may participate in the regulatory pathways. Activation mutations of the EGFR gene are also described. The most frequent mutation is known as EGFRvIII. This mutation leads to a deletion of the extracellular domain of the receptor and to its constitutive activation [1]. Because the primary antibody in diagnostic EGFR pharmDx kit detects the wild type of the EGFR protein (170 kD) and also its mutant form – EGFRvIII (145 kD), the effect of therapy with monoclonal antibodies against deleted epitopes in a subgroup of patients with this type of mutation is questionable.

In patients with HNSCC numerical changes of chromosome 7 are frequent [17]. In our group of patients we found numerical changes of CEP7 signals in 70% of HNSCC. An increased number of changes of chromosome 7 leads implicitly to an increased number of the EGFR gene. If the mixture of probes against the EGFR gene and chromosome 7 is used for I-FISH simultaneously as in this study, there is no risk to misinterpret numerical changes of the chromosome as EGFR gene amplification.

There was no correlation between numerical changes of CEP7/EGFR gene amplification and the expression patterns of the EGFR protein found in this study.

We may conclude that in the group of 33 patients with HNSCC, the overexpression of the EGFR protein was not caused by the EGFR gene amplification and it was not related to the increased numbers of CEP7 signals. The impact of cetuximab treatment in patients with different patterns of the EGFR protein expression is not clear at present, and the question should be addressed by studies with a long-term follow up of larger cohorts of patients. Until the problem is solved, we would prefer to investigate the EGFR protein expression and its patterns (subtypes) by immunohistochemistry before starting cetuximab therapy in all patients with advanced HNSCC who might become eligible for this type of treatment.

The authors thank Mrs. M. NERADOVÁ for technical assistance.

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