ONCOLOGY

Squamous Cell Carcinoma of the Esophagus: Evaluation of the Status of Epidermal Growth Factor Receptors (EGFR and HER-2) by Immunohistochemistry and *in Situ* Hybridization

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We performed a parallel evaluation of the status of epidermal growth factor receptors EGFR and HER-2 in tumor samples from 31 patients with squamous cell carcinoma of the esophagus. Hyperexpression of proteins was detected by immunohistochemical methods and gene amplification and other chromosome abnormalities were studied using FISH reaction. Evaluation of EGFR status showed that amplification of EGFR gene was present in 25% cases and chromosome 7 polysomy was detected in 29.2% cases positive by protein expression (2+/3+). Immunohistochemically positive EGFR status was confirmed by the results of FISH reaction for gene amplification and chromosome 7 polysomy in 54.2% cases (p=0.002). During evaluation of HER-2 status in the tumor, hyperexpression of the protein detected histochemically was not confirmed by FISH reaction for detection of amplification of the corresponding gene in 16.1% cases. In 22.6% patients, chromosome 7 polysomy was detected; it was not accompanied by amplification of *HER-2* gene, but was related to immunohistochemically positive status of the tumor. Hyperexpression of EGFR protein significantly correlated with the presence of intravascular invasion (p=0.006) and increased depth of invasion (p=0.044), while amplification of *EGFR* gene (≥2.2) correlated with low differentiation degree of the tumor (p=0.006). The outcome of the disease was not associated with EGFR status at the gene and protein levels, whereas clinical course of the disease in patients with immunohistochemically negative expression of HER-2 protein was more favorable than in patients with positive expression (p=0.004). The results of this study suggest that hyperexpression/amplification of EGFR and hyperexpression of HER-2 are important clinical markers for evaluation of disease prognosis and development of new regimens of targeted therapy for patients with squamous cells carcinoma of the esophagus.

Key Words: *carcinoma of the esophagus; immunohistochemistry; fluorescent in situ hybridization; EGFR; HER-2*

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Biological peculiarities and molecular parameters predicting the risk of progressing growth of squamous cell carcinoma of the esophagus (SCCE) and efficiency of therapy are largely determined by abnormal expres-

sion of receptors EGFR and HER-2, representatives of the family of homologous transmembrane receptors of epidermal growth factor, in the tumor [5,9,11]. Stimulation of these receptors leads to activation of signal pathways and triggers transcription mechanisms accelerating proliferation of epithelial cells and the processes of invasion and neoangiogenesis in the tumor [9,13,15].

Genes *EGFR* and *HER-2* located in chromosomes 7 (7p12) and 17 (17q12-q21) encode the corresponding transmembrane proteins with pronounced tyrosine kinase activity [5,8,10]. Hyperexpression of EGFR and HER-2 on the membrane of tumor cells is often related to amplification of the corresponding genes and correlates with poor prognosis and aggressive course of some malignant neoplasms in humans [7,8,11,14].

In SCCE, EGFR abnormalities are detected in 50-80% cases [4,7,15], while the incidence of abnormal expression of HER-2 varies, according to different sources, from 0 to 56% [8,10-12]. In some studies, attempts were undertaken to reveal a correlation between amplification of *EGFR* and *HER-2* genes detected by fluorescent *in situ* hybridization (FISH) and hyperexpression of the corresponding proteins detected by the immunohistochemical method [5,7,10,12]. In some recent studies, a hypothesis was put forward that hyperexpression/amplification of these receptors in tumor cells correlates with unfavorable morphological parameters of SCCE [2,6], poor postoperation prognosis of the disease course, reduced total survival, and the risk of local relapses [3,4,14]. However, evaluation of the relationship between amplification of *EGFR* and *HER-2* genes or hyperexpression of the corresponding proteins and disease prognosis yields contradictory results. Moreover, there is a possibility that immunohistochemically-detected hyperexpression of these proteins is not related to amplification of *EGFR* and *HER-2* genes, but is determined by postreplication events or other chromosome abnormalities. According to published data, various malignant neoplasms in humans are often associated with chromosome 7 and 17 polysomy with preserved normal proportion between the gene and chromosome numbers [7,11,15]. The incidence and significance of detection of increased number of chromosomes 7 and 17 in tumor cell nuclei for evaluation of SCCE prognosis remain practically unstudied. Since FISH method allows us to evaluate not only the number of copies of the corresponding genes in the cell nucleus, but also the number of chromosomes containing these genes, comparison of the results of immunohistochemical and FISH studies is of particular interest for understanding of the mechanisms of EGFR and HER-2 hyperexpression in SCCE cells.

In our previous studies, the peculiarities of the expression of EGFR and HER-2 in tumor cells were analyzed by immunohistochemical method and compared with the main clinical and morphological characteristics of SCCE [1].

The aim of the present study was to compare and to evaluate clinical significance of the status of epidermal growth factor receptors (EGFR and HER-2) in tumors of patients with SCCE at the protein and gene levels using immunohistochemical and FISH methods.

MATERIALS AND METHODS

We studied the peculiarities of the expression of EGFR and HER-2 in tumor specimens obtained during surgery from patients with stage II-III esophageal cancer (6 women and 25 men aging 44-72 years). At the moment of surgery, metastases in regional lymph nodes were detected in 19 (61.3%) cases. Retrospective analysis of treatment results showed that 16 (51.6%) patients developed local relapse and/or distant metastases within 1-3 years. In 15 (48.4%) cases, the patients survived more than 3 years without progression of the disease.

Histological analysis showed that all esophageal tumors were squamous cell carcinomas with differently expressed keratinization. Highly differentiated (G1), moderately differentiated (G2), and low differentiated (G3) carcinomas were diagnosed in 2 (6.5%), 20 (64.5%), and 9 (29%) patients, respectively.

Immunohistochemical staining was performed on paraffin sections of esophageal tumor tissue using antibodies against EGFR (Novocastra) and Super Sensitive Polymer-HRP detection systems (BioGenex). Expression of HER-2 was studied using HercepTest system (Dako). Deparaffinized sections were incubated in citrate buffer (pH 6.0) on a water bath at 95°C for 40 min. The results of the reaction were scored from 0 to 3+ using a HercepTest system.

FISH reactions were performed by double staining using HER-2FISH pharmDx™Kit (Dako), 17q12 and Vysis LSI EGFR SpectrumOrange/CEP7 SpectrumGreen, 7p12 kits on paraffin sections of the sample, which was analyzed by immunohistochemistry. Denaturation (82°C, 5 min) and hybridization (45°C, 20 h) were performed in an automatic hybridizer (Dako). Amplification was evaluated under a fluorescent microscope at ×100 by determining the ratio of red and green signals corresponding to *EGFR* and *HER-2* gene copies and centromere regions of chromosomes 7 and 17 in 100 tumor cell nuclei in each sample. Amplification was considered positive, when the ratio of the mean number of copies of the corresponding gene to the mean number of centromeres of chromosomes 7 and 17 (*EGFR*/*CEN7* and *HER-2*/*CEN17* genes) was ≥2.2. Fluorescent signals of cen-

tromeres of chromosomes 7 and 17 (*CEN7* и *CEN17*) in tumor cell nuclei in the presence of polysomy were also counted.

The results of immunohistochemistry were considered positive at 2+/3+ (3+ corresponded to hyperexpression and 2+ corresponded to ambiguous status of the marker) and negative at 0/1+. FISH reaction was considered positive in the presence of amplification of *EGFR* and *HER-2* genes or polysomy of chromosomes 7 and 17 and negative in the absence of amplification/polysomy.

RESULTS

Positive (2+/3+) immunoreactivity of EGFR and HER-2 in tumors of SCCE patients was presented by diffuse homogenous staining of cell membranes both at the periphery and in central parts of the tumors. In some cases, the reaction was heterogeneous and was primarily located in the basal parts of epithelial cords or looked like foci against the background of weakly stained cells. In esophageal mucosa adjacent to the tumor, weak reaction (1+) was detected in the basal epithelial layers.

Positive EGFR expression in SCCE cells was detected in 24 of 31 samples (77.4%): 12 (38.7%) samples with moderate immunoreactivity (2+) and 12 (38.7%) samples with hyperexpression (3+). Negative reaction was noted in 22.6% cases (n=7): weak expression (1+) in 6 (19.4%) cases and the absence of reaction (0) in 1 (3.2%) case.

Positive HER-2 expression in SCCE cells was detected in 17 of 31 samples (54.8%), of them moderate immunoreactivity (2+) was observed in 12 (38.7%) samples and hyperexpression (3+) in 5 (16.1%) samples. Negative reaction was noted in 45.1% cases (n=14): weak expression (1+) in 9 (29%) cases and the absence of reaction (0) in 5 (16.1%) cases.

In 12.9% tumors (n=4), hyperexpression of HER-2 was associated with high EGFR expression in membranes of SCCE cells.

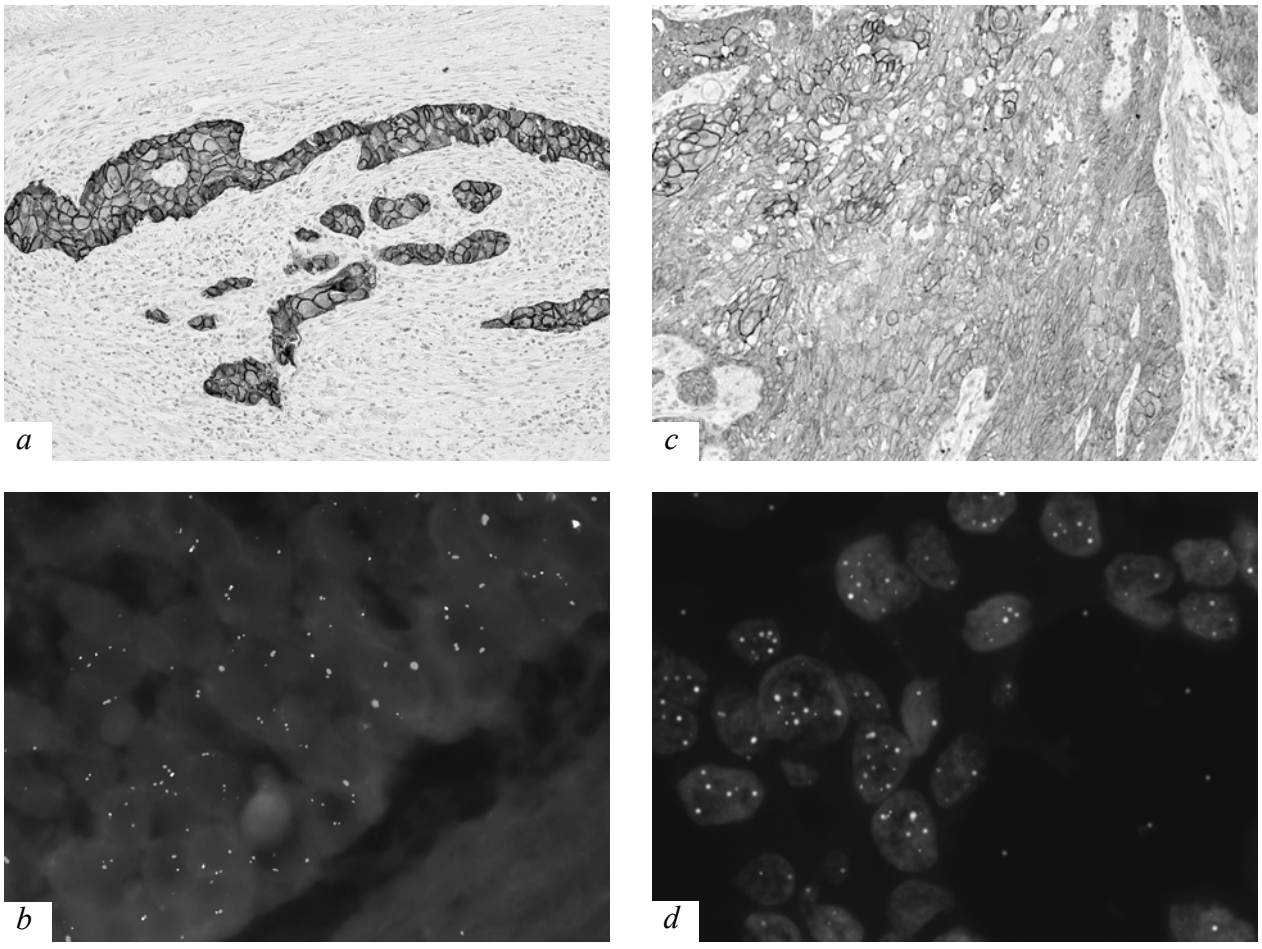

Fig. 1. Status of EGFR and HER-2 in tumors of patients with SCCE. Hyperexpression (*a*) of EGFR (3+) by tumor cells and amplification (*b*) of *EGFR* gene. HER-2-positive reaction of tumor cells (*c*) with ambiguous marker status (2+) and chromosome 17 polysomy (*d*) without amplification of *HER-2* gene. *a*, *c*) immunohistochemical reaction, cell nuclei are poststained with Mayer hematoxylin. *b, d*) FISH reaction. *a*) ×250; *b, d*) ×1000; *c*) ×400.

TABLE 1. Correlation between the Number of *EGFR* Gene Copies and Expression of EGFR Protein

Immunohistochemical reaction	FISH reaction			Total
	positive		negative	
	amplification ≥2.2	polysomy	amplification <2.2, no polysomy	
3+	4	4	4	12
2+	2	3	7	12
+/0	0	0	7	7
Total	6	7	18	31

Note. p=0.002 (exact Fisher test).

FISH analysis revealed amplification of *EGFR* gene in 6 of 24 (25%) EGFR-positive tumors (2+/3+ according to immunohistochemical analysis), which corresponded to 19.4% (n=6) of all cases. Amplification of *EGFR* gene was noted in 4 of 12 samples (33.3%) with hyperexpression of the protein (3+) and in 2 of 12 tumors (16.7%) with ambiguous immunological status (2+) (Fig. 1, *a*, *b*). Apart from amplification of *EGFR* gene, balanced polysomy of chromosome 7 in tumor cell nuclei was detected in 7 of 24 (29.2%) EGFR-positive (2+/3+) tumor samples, which corresponded to 22.6% (n=7) of all cases. In positive cases, polysomy (mean number of chromosomes 7 was 3-6 per tumor cells) was observed. Thus, the positive FISH reaction including gene amplification and chromosome 7 polysomy was detected in 41.9% cases (13 of 31 analyzed samples); a statistically significant correspondence of FISH-positive observations, which constituted 54.2% (13 of 24), to the results of immunohistochemical analysis was revealed (p=0.002; Table 1). In 45% cases (11 of 24 samples), FISH reaction demonstrated the absence of *EGFR* gene amplification and chromosome 7 polysomy in cell nuclei of EGFR-positive tumors (2+/3+ by the results of immunohistochemical analysis).

TABLE 2. Correlation between *EGFR* Gene Amplification and Tumor Differentiation Degree

Parameter	FISH reaction		Total
	amplification ≥2.2	amplification <2.2	
Differentiation degree, %	G1 (high)	0	2
	G2 (moderate)	1	19
	G3 (low)	5	4
Total		6	25

Note. p=0.006 (exact Fisher test).

Amplification of *EGFR* gene (≥2.2) in tumor cell nuclei significantly correlated with low histological differentiation of the tumor (p=0.006; Table 2).

Hyperexpression of EGFR protein was more frequently detected than amplification of the corresponding gene and significantly correlated with the depth of invasion (penetration of the tumor through all layers of the esophageal wall, adventitia, and fatty tissue) and invasion into blood vessels (p=0.044 and p=0.006, respectively). The positive FISH status of *EGFR* (gene amplification and chromosome 7 polysomy) was more often observed in low differentiated tumors and in the presence of invasion into blood vessels (differences approximated the level of significance, p=0.084 and p=0.058, respectively). The outcome of the disease was not associated with EGFR status at both the gene and protein levels (Table 3).

In none SCCE samples with positive expression of HER-2 (2+/3+ by the results of immunohistochemical analysis) amplification of *HER-2* gene was detected by FISH method. Hyperexpression of HER-2 (3+ by the results of immunohistochemical analysis) was not confirmed by the results of FISH analysis for detection of amplification of the corresponding gene in 16.1% (5 of 31) patients with SCCE. Amplification of *HER-2* gene was detected in only one SCCE sample demonstrating HER-2-negative immunoreactivity (0). At the same time, FISH reaction for detection of *HER-2* gene amplification in tumor cells revealed chromosome 17 polysomy. In 22.6% (7 of 31) cases, the disease was associated with polysomy (mean number of chromosomes 17 was 3-5 per tumor cells). Polysomy was accompanied by enhanced expression of HER-2 protein (2+/3+ by the results of immunohistochemical analysis, Fig. 1, *c*, *d*).

TABLE 3. Correlation of Immunohistochemical and FISH Status of EGFR with Clinical and Morphological Parameters of SCCE (Analysis of Subgroups)

Parameter		Immunohistochemical status		*p*	FISH status		*p*
		+ (+2/+3), *n*=24	— (0/+1), *n*=7		+ (amplification ≥2.2/ polysomy), *n*=13	— (amplification <2.2/ no polysomy), *n*=18	
Differentiation degree	G1/G2	17	5	0.681	7	15	0.084
	G3	7	2		6	3	
Depth of invasion	P1/P2	10	0	0.044*	4	6	0.597
	P3/P4	14	7		9	12	
Invasion into blood vessels	no	3	5	0.006*	1	7	0.058
	yes	21	2		12	11	
Metastases into lymph nodes	0	8	4	0.241	5	7	0.638
	1	16	3		8	11	
Progression	no	8	4	0.241	7	5	0.137
	yes	16	3		6	13	

Note. *Significant differences ($p<0.05$).

No correlations between hyperexpression of HER-2 in the tumor and clinical and morphological parameters of the disease were found. Changes in chromosome 17 were more incident in SCCE patients with metastases in regional lymph nodes and early progression, but the differences did not attain the level of statistical significance. The outcome of the disease was not associated with *HER-2* status at the gene level, whereas clinical course of the disease in patients with immunohistochemically negative expression of HER-2 protein was more favorable (survived more than 3 years without distant metastases and local relapses) than in patients with positive expression (died because of disease progress at early terms after surgery; p=0.004).

Synchronic chromosome 7 and chromosome 17 polysomy in tumor cell nuclei was observed in 12.9% (4 of 31) patients with SCCE. The combination of chromosome 7 polysomy and amplification of *HER-2* gene was found in one patient.

Thus, immunohistochemical and FISH status of EGFR was not associated with the outcome of the disease; at the same time, increased number of *EGFR* gene copies in SCCE cell nuclei (amplification ≥2.2) significantly correlated with low differentiation degree of the tumor (p=0.006), and hyperexpression of the protein significantly correlated with intravascular tumor invasion (p=0.006) and depth of invasion into the esophageal wall (p=0.044).

The results of our study suggest that the increase in the number of *EGFR* gene copies can lead to enhanced expression of EGFR protein associated with unfavorable morphological parameters of the disease and sufficient for targeted therapy with monoclonal antibodies, which enables the use of this molecule in the development of new regimens of targeted therapy in SCCE [6,7,14].

Hyperexpression of HER-2 in the tumor significantly correlated with the development of local relapses or distant metastases at early terms after surgery (p=0.004). It should be noted that increased production of the protein was not associated with considerable increase in the number of *HER-2* gene copies, as it was in case of its amplification. These data suggest that hyperexpression of HER-2 protein in SCCE cells is not always associated with amplification of the corresponding gene, the increased production of the corresponding protein can be determined by other mechanisms, in particular, chromosome 17 polysomy.

Thus, hyperexpression of HER-2 in the tumor caused by different chromosome abnormalities without amplification of the corresponding gene is important, from clinical point of view, for evaluation of individual peculiarities of the disease course.

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