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Molecular Factors and Criteria for Predicting the Response to Neoadjuvant Treatment in Patients with Esophageal Squamous Cell Carcinoma (ESCC) - responder / non-responder

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Rezumat

Criterii și factori moleculari de predicție responsivă la tratamentul neoadjuvant al pacienților cu carcinom esofagian (ESCC) – responder / non-responder

Introducere: Carcinomul esofagian (ESCC) ocupă locul 7 între cauzele de mortalitate prin boli neoplazice în Statele Unite și locul 6 în lume, cu o supraviețuire globală la 5 ani de 14%, mai mult decât modestă comparativ cu alte neoplazii digestive. În efortul de a îmbunătăți aceste valori, numeroase strategii au fost introduse, asociind gestului chirurgical variate terapii oncologice pre- și postoperatorii (radio şi/sau chimioterapie). Rezultatele la distanță au evidențiat ameliorări sensibile ale rezultatelor odată cu introducerea terapiei multimodale de tip neodjuvant.
Material şi metodă: Sunt evaluați 57 pacienți (2006-2010), preponderent de sex masculin (77%), cu vârsta medie 55 ani, toți cu diagnostic de ESCC. Protocolul terapeutic a presupus o abordare multimodală: timp I (RCT - radiochimiterapie), timp II (chirurgical) ± timp III (chimio- sau radioterapie). La 4 săptămâni post RCT bolnavii sunt evaluați pentru a se preciza caracterul responsiv la tratamentul neoadjuvant iar ulterior se intervine chirurgical cu executarea rezecției esofagiene. Pentru a identifica un predictor molecular al răspunsului sau rezistenței la CRT neoadjuvant, piesele de exereză sunt analizate HP şi IHC, markerii studiați fiind p53(Dako 1:50), Ki-67(Biogenex, 1:20), c-erbB-2(Dako, 1:250). Pe baza acestor rezultate, modelul de lucru utilizat pentru aprecierea răspunsului la terapia neoadjuvantă este gradul regresiei tumorale (TRG).
Rezultate: În urma analizării HP și IHC, pacienții au fost incluşi în două categorii: *responders* (celule tumorale $<10\%$) şi *non-responders* (celule tumorale $>10\%$). La 5 pacienți din grupul responders am obținut sterilizarea completă a neoplaziei. În concluzie, se poate afirma că identificarea markerilor cu potențial predictiv și, respectiv, corelarea cu rezultatele HP și IHC, reprezintă o alternativă de mare perspectivă în prognosticul terapiei ESCC. Depistarea agresivității tipului molecular al neoplaziei permite orientarea terapeutică, încadrarea sau îndrumarea către o anumită succesiune terapică sau chiar spre tratamente direcționate către ținte moleculare specifice.

Cuvinte cheie: cancer esofagian scuamo-celular, terapie multimodală, RCT neoadjuvantă + chirurgie, factori predictivi / prognostici la RCT neoadjuvantă

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Abstract

Regarding the mortality causes, esophageal squamous cell carcinoma (ESCC) is on the 7th place in the US and the 6th place in the world, with 5 year overall survival rate of 14%, which is still modest in comparison to other digestive neoplasia. Multiples strategies were involved to improve this percentage, associating surgical procedures and chemo- and radio- oncologic therapies. The distant results showed sensitive

improvement, after introduction of multimodality neoadjuvant therapies.
Material and method: Fifty-seven patients diagnosed with ESCC were evaluated between 2006 and 2010, male preponderance (77%), average age of 55. A multimodality therapeutic protocol was used: first – radio-chemotherapy (RCT), second - surgery and/or third – chemo- or radiotherapy. Four weeks post RCT all patients were evaluated to determine the response to neoadjuvant treatment followed by surgery - esophageal resection. Histopathological (HP) and immunohistochemical (IHC) analysis of the pathological specimens were performed in order to identify the molecular predictors with responsive or non responsive character; the studied markers were p53 (Dako 1:50), Ki-67 (Biogenex, 1:20), c-erbB-2 (Dako, 1:250). Based on these results, the working model used to determine the response to neoadjuvant therapy was tumor regression grade (TRG).
Results: After HP and IHC examination, the patients were included in two groups: responders (tumor cells < 10%) and non-responders (tumor cells > 10%). Complete neoplasia sterilization was achieved in 5 of the patients.
Conclusions: We are able to state that the identification of potential predictive markers along with HP and IHC results represents a great perspective alternative in the ESCC therapy outcome. The detection of molecular type aggressiveness of the neoplastic process allows therapeutic orientation or guidance to certain therapy sequences or even to specific molecular targeted treatments.

Key words: esophageal squamous cell carcinoma, multimodal therapy, neoadjuvant RCT + surgery, predictive / prognostic factors for neoadjuvant RCT

Introduction

Regarding the mortality causes, esophageal squamous cell carcinoma (ESCC) is on the 7th place in the US and 6th place in the world (1,2). The 5 years overall survival rate of 14% is modest in comparison with other digestive neoplasia (3).

Reasons for such a small survival rate:

1. Inadequate screening methods and equivocal treatment guidelines;
2. Cancer diagnosed in an advanced stage of the disease; over 50% of patients are being diagnosed in an unresectable stage or with distant metastasis (4,5);
3. Increased risk of disease recurrence after esophagectomy and / or additional oncological therapy (RCT);
4. Insufficient non-invasive methods to assess RCT response;
5. Limited survival after palliative RCT for patients with metastatic or unresectable disease.

Summarizing, one can easily see that the high mortality rate reflects the rapid lymphatic spread and blood stream invasion and the lack of effective treatments, due to the impossibility of applying a treatment in early stage disease (6).

It is obvious the need of implementing additional strategies for detecting esophageal cancer in an early-stage and of improving the therapeutic possibilities, while diversifying multimodality therapy: surgery + oncology.

For many years the standard therapy for the esophageal squamous-cell carcinoma has been surgical resection. However, local control and survival were low even after radical resection with lymphadenectomy. Patients with locally advanced esophageal cancer showed an unfavorable trend when treated exclusively by surgery. In an effort to improve these values, esophageal cancer management has undergone a major evolution in the last 15 years. Many strategies that included various pre-and post-surgery oncological therapies (radio- and / or chemotherapy) were studied and applied, even removing completely the surgery from the therapy protocol. Remote results showed obvious improvements, so that neoadjuvant treatment became the current standard in this pathology (7-10). Neoadjuvant therapy is aiming to eradicate of lymphatic or hematogenous metastases and micrometastases, tumor volume reduction with improved resectability rate and also improving long-distance survival. However, it is already known that only patients with complete response to neoadjuvant therapy will show a significant survival. In addition, these therapies are expensive and associated with a high rate of complications. Nevertheless, a significant percentage of patients still show an insufficient response (11,12). These patients have no benefit in the neoadjuvant therapy and have to deal with toxic side effects; also, the appropriate surgical therapy is delayed by RCT (13). For these reasons, a predictive pre-therapeutic diagnostic test, that could be used to predict tumor response to neoadjuvant therapy, is crucial. Currently, there is no universally accepted method, reproducible and safe, for monitoring esophageal carcinoma response to RCT. The outcome after multimodality therapy of esophageal cancer is still modest, with long-term control rates of 25-35%. Modern studies (14-19) show that after RCT there is tumor "mitigation" in histological specimens from 11.4 to 51% on patients with microscopic tumor leftovers, but only in 54% of cases. Surprising was the identification of a subgroup of patients with excellent local disease control and an obvious improvement on long-term survival. Therefore, identification of responder to RCT patients has a prognostic significance that may change clinical management. Hence, the introduction of specific molecular markers indicating response or no response to neoadjuvant treatment would be extremely useful in selecting patients for future complex therapeutic protocols.

In these conditions, the clinician faces three major issues, leading to specific questions:

1. Can we identify the tumor response predictor factors to RCT and, if so, what would these be? A corollary of this dilemma is the identification of molecular markers that could predict the response to RCT.
2. Could we develop a post-RCT evaluation algorithm? Do we need a minimal standardization of clinical and paraclinical steps to follow?

3. What would be the criteria for patient assessment, so they can be included into one group or another, e.g. responder/non-responder?

1. Currently we are trying to identify a predictive factor of response or resistance to neoadjuvant RCT, based on demographic, imaging, HP, molecular or genetic information. The discovery of such factor would have an enormous applicability potential for getting better results and developing clinical protocols.

Recently, using innovative techniques, there were identified molecular markers of response and prognosis in neoadjuvant therapy in esophageal cancer (20-27). Disadvantages of this method would be its semi quantitative type, the subjective nature of evaluation and its limitation due to sensitivity of monoclonal antibodies and tissue handling. There have been used more sophisticated techniques, such as gene expression evaluation of a certain factor, confirming a number of positive results. Micro-dissection techniques were developed to avoid contamination of normal tissue. Gene expression assessed by PCR was limited because of the need of fresh tissue; however, this technology has recently been improved, leading to histopathological data from tissues embedded in paraffin. Progress has been made in assessing genomic polymorphism, which requires only a blood sample from the patient; this seems to be the most advanced and accessible technology.

What is the difference (28) between prognostic and predictive factors?

Predictive factors (28) (see *Table 1*) allow assessing the patients by identifying the response to RCT treatment, selecting a specific treatment, even choosing one of the chemotherapeutic agents used in ESCC (esophageal squamous cell carcinoma). Thus, predictive factors will play a major role in determining whether the neoadjuvant treatment should be performed or not, allowing the choice of the oncologic therapy which would lead to a complete response.

By contrast, prognostic factors (28) are used for assessing

Table 1. *Main molecular predictive factors in ESCC24*
(after Vallböhmer and Lenz)

Type of cellular pathway/factor	Marker/type of change	Response to neo RCT
Growth factor receptors	HER2/neu protein/gene expression ↓	↑
Angiogenetic factors	Cox-2 protein expression ↑	↓
	VEGF protein expression ↓	↑
	TP protein expression ↓	↑
Tumor suppressor gene	p53 protein expression negative/positive (controversial)	↓↑
Cell cycle regulators	p21 protein expression ↑	↑
DNA repair system	ERCC1 gene expression ↓	↑
Apoptotic factors	Survivin gene expression ↓	↑

Table 2. *Main molecular prognostic factors in ESCC24*
(after Vallböhmer and Lenz)

Type of cellular pathway/factors	Marker/type of change	Correlation with clinicopathologic factors/survival
Growth factor receptors	EGFR protein expression ↑	Local recurrence ↑ Depth of tumor invasion ↑ Lymph node metastases ↑ survival ↓
Angiogenetic factors	Cox-2 protein expression ↑	Metastases ↓, tumor stage ↓
	bFGF protein expression ↑	Depth of tumor invasion ↑ Lymph-node metastasis ↑ TNM stage ↑
	TGF-α protein expression ↑	Cancer-specific death ↓ Survival ↑
	TGF-β receptor (type 1 and 2) protein expression ↓	Depth of tumor invasion ↑ Lymph node metastasis ↑ Survival ↓
	VEGF protein expression ↑ (in squamous cell cancer)	Metastases ↑, tumor stage ↑ Depth of tumor invasion ↑ Lymph-node metastasis ↑ Survival ↓
Tumor suppressor gene	p53 protein expression positive	Survival ↓
	p16 protein expression positive	Survival ↑
Cell cycle regulators	Cyclin D1 protein expression ↑	Survival ↓
	p21 protein expression ↑	Survival ↑
	p27 protein expression ↓	Survival ↑
DNA repair system	ERCC 3 protein expression ↓	Tumor progression ↑, tumor stage ↑ Survival ↓
	ERCC1 gene expression ↓	Risk of cancer recurrence ↑, survival ↓
Apoptotic factors	Bax protein expression ↑	Survival ↑
	Bcl-2 protein expression ↑	Histological gradesurvival ↑, T/N grade ↑
	Bcl-X protein expression ↑	Histological grade ↑, T/N grade ↑, Survival ↓
Matrix metalloproteinases	MMP-7 protein expression ↑	Nodal metastasis ↑
	MMP-9 protein expression ↑	Depth of tumor invasion ↑, nodal metastasis ↑, tumor stage ↑, survival ↓
	MMP-2 protein expression ↑	Depth of tumor invasion ↑, tumor stage ↑ Vascular/lymphatic vessel invasion ↑ Survival ↓
	MMP-11 protein expression ↑	Survival ↓

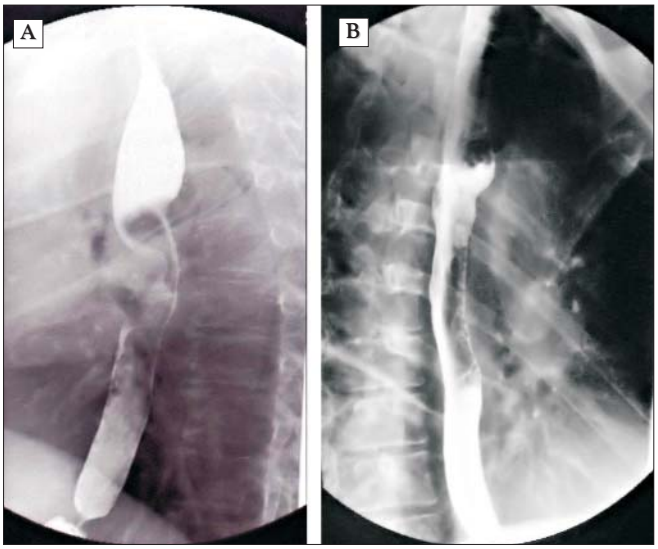


Figure 1. *(A, B) Oral barium meal showing mediothoracic esophageal tumor, before and after RCT*

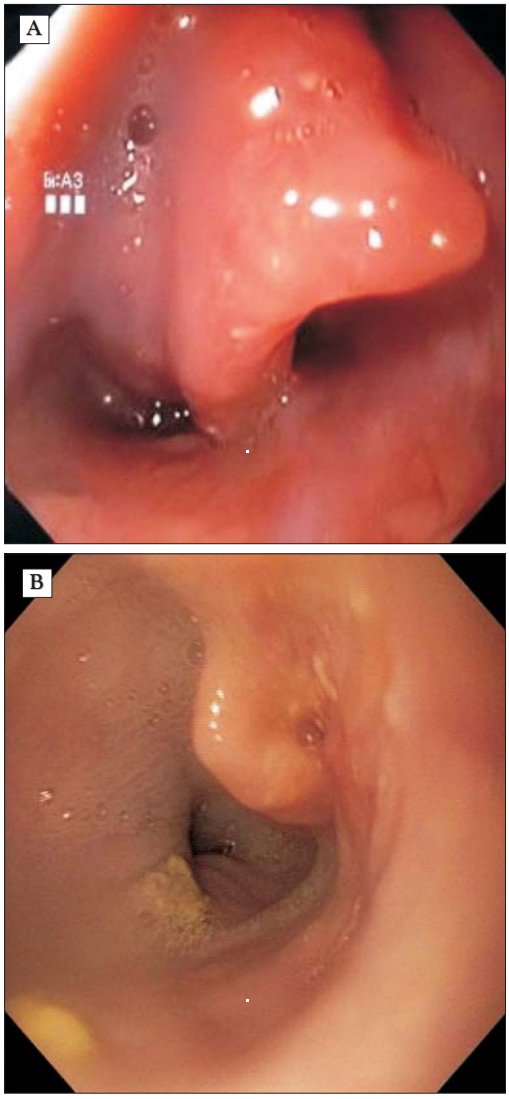


Figure 2. *(A, B) Endoscopic aspect of esophageal tumor - before and after RCT*

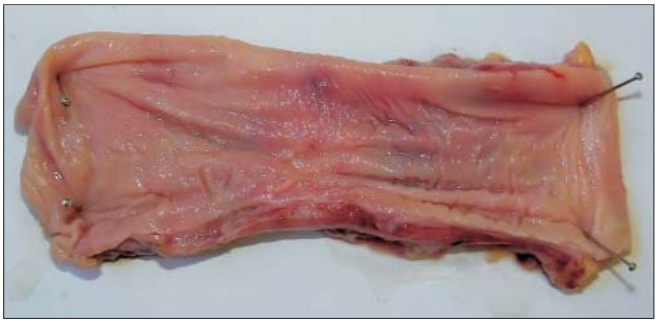


Figure 3. *Esophagectomy specimen with complete tumor sterilization, after neo-adjuvant RCT*

the overall results, i.e. the long-term cancer survival rate, disregarding the used treatment (see *Table 2*). Thus, a specific prognostic factor can be used in identifying the patients with high risk for tumor recurrence and, therefore, the patients whom would benefit of an adjuvant therapy.

2. An involution or tumor regression assessment protocol involves a full assessment (29) imagistic and endoscopic (barium, endoscopy + echoendoscopy, CT or MRI) associated with the HP examination of sample tissues (*Fig. 1, 2, 3*).

To identify molecular markers, the method would require associated specific IHC.

Recently, PET appears to improve sensitivity, specificity and accuracy compared with CT in the staging of ESCC (9,30-34). This improvement has led to avoid unnecessary resections in patients with occult metastases missed by CT, MRI or EUS. Also, CT and EUS proved to be weak predictors for response to RCT, with a low accuracy in evaluation after RCT (35). In comparative studies, PET has proven a superior method of assessment and evaluation of response to RCT, much better that CT or Echo-endoscopy. Some preliminary studies, using PET for evaluation after RCT, are now available, showing strong associations between PET and histological response and improving overall survival rate (33,34,36,37). To better define the usefulness of PET, compared with CT, in detecting RCT response in esophageal cancer, the same prospective studies have attempted to correlate CT evaluation before and after RCT and the tumor pathologic response in esophagectomy specimens. In addition, PET test characteristics were evaluated for determining re-staging after RCT. Conclusion was simple: CT assessment of tumor response after RCT is imprecise and, therefore, not recommended. Echoendoscopy and FDG-PET appears to have similar accuracy. Echoendoscopy can be used to identify patients who achieved a response, but is not feasible during or immediately after RCT and, therefore, not practical for therapeutic response assessment. If we also consider the method's impossibility to detect secondary metastasis, 3-4 cm beyond the esophageal adventitia, the technique's limitations are obvious. FDG-PET, used to measure alterations in tissue metabolism, seem to be a promising non-invasive method for assessing response to neoadjuvant RCT in patients with

esophageal cancer. Evaluation of FDG uptake values, measuring metabolic response, may be a marker of cell death and requires, of course, further investigation.

3. The classification of patients into one group or another (responder/non-responder) has proven to be difficult (19), due to extremely hard result quantification or to evaluation deficiencies. Finally, an easy but prone to biases way was adopted:

Responder = patients with < 10% residual tumor cells on histopathological specimen.

Non-responder = patients with > 10% residual tumor cells on histopathological specimen.

Another working model used (38-40) to assess response to neoadjuvant therapy is tumor regression grade (TRG), identifying a range of response rates. There are 5 types of TRG:

- I. Pathological complete regression pCR;
- II. Subtotal regression pSR (< 10%);
- III. Partial regression pPR (10-50%);
- IV. Minimum regression pMR (>50%);
- V. Non-regression.

Response to neoadjuvant therapy (i.e. surgical specimen free of residual cancer) is the best predictor factor for overall survival rate. The absence of residual disease occurs in approximately 15-30% of the cases; these have a 3 year survival rate around 60% which is, apparently, independent of the applied protocol, histological type and tumor stage (38-40).

Material and Method

Fifty-seven patients were evaluated (2006-2010), most of them male (77%), with the average age of 55, all diagnosed with ESCC. The overweight patients, the patients with non-surgical prognosis and the ones with distant metastasis were excluded from the study. We did not have any T1 patients. The therapeutic protocol entailed a multimodal approach: time I (RCT), time II (surgical) ± time III (chemo- or radiotherapy). The neoadjuvant protocol (see *Table 3*) entailed the use of Cisplatin 100 mg/m^2 iv, administered in the days of 1st, 21st, 42nd, together with 5-FU 1000 mg/m^2 iv in the days of 1st to 4th, 21st to 25th, 42nd to 45th, as well as external irradiation of 40-45 Gy for 4 weeks. Four weeks after RCT the esophageal resection was made, the excision parts being analyzed, HP and IHC. Only 51 patients entered the study, as 6 patients proved to be non-surgical cases. From the multitude of markers, we selected only 3 for the study (*Table 4*): p53 (Dako 1:50), Ki-67 (Biogenex, 1:20), c-erbB-2 (Dako, 1:250) via the indirect Hsu three stage Avidin-Biotin-Peroxidase (ABC) method, modified by Bussolati and Gugliotta (41,42) (*Fig. 4,5,6*). The correlation of the tumor molecular titer with the response to the neo adjuvant treatment was made on the excision parts.

Results

The patients were included in two categories: 15 (29%) responders (tumor cells < 10%) and 36 (71%) non-responders (tumor cells > 10%). We managed to obtain complete sterilization of the neoplasia in 5 patients from the 15 responders. The statistical integration (SPSS) showed that the increased expression of c-erbB-2 is suggestive for the weak response to RCT. As proof, 13 out of 15 patients with response to RCT had a minimum or even absent c-erbB-2 expression (absent in 1 case only), while in all the non-responders the expression level was high. Absence of the p53 with Ki-67 > 10% lead to excellent results of the RCT and the reverse is also valid: increased titers of the p53 with Ki-67 < 10% has been associated with modest results of the RCT. In *Table 5*, the expression levels of the three studied markers at tumor cell level, with effect over the neoplastic aggressiveness, and, as consequence, over its response to the alternative oncologic therapies, are very well emphasized. The distinct positioning of

Table 3. *Neoadjuvant protocol type in patients with ESCC*

Neoadjuvant RCT		
Chemotherapy Drugs Radiotherapy	**Dose**	**Therapy day**
Cisplatin	100 mg/m^2 iv	1,21,42
5-FU	1000mg/m^2 iv	1-4, 21-25, 42-45
External radiation	40-45Gy	4 weeks

Table 4. *Molecular markers in studies for assessing responsiveness to neoadjuvant RCT in patients with ESCC*

Antibody	Producer	Dilution	Clone	Specificity
CerbB2	Dako	1:250	Polyclonal	Her2-neu/ErbB2 protein gene
Ki67	Dako	1:50	MB1	Proliferation cell nuclear antigen
p53	Dako	1:50	DO-7	p53 protein gene

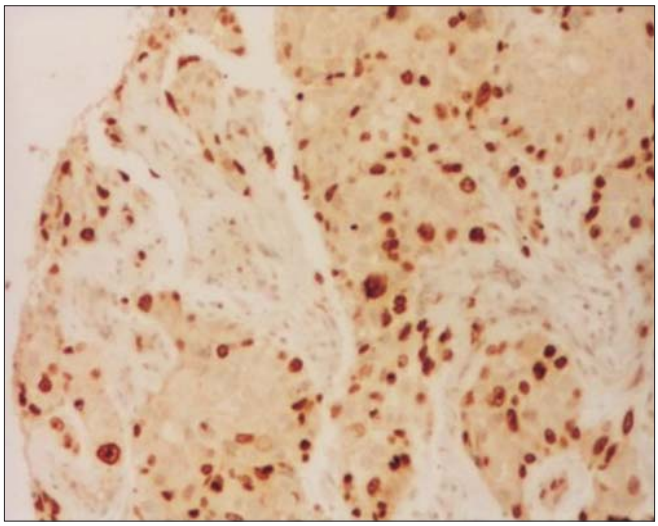


Figure 4. *IHC test image - Ki-67 (+) 20% expression {p53 (-)}*

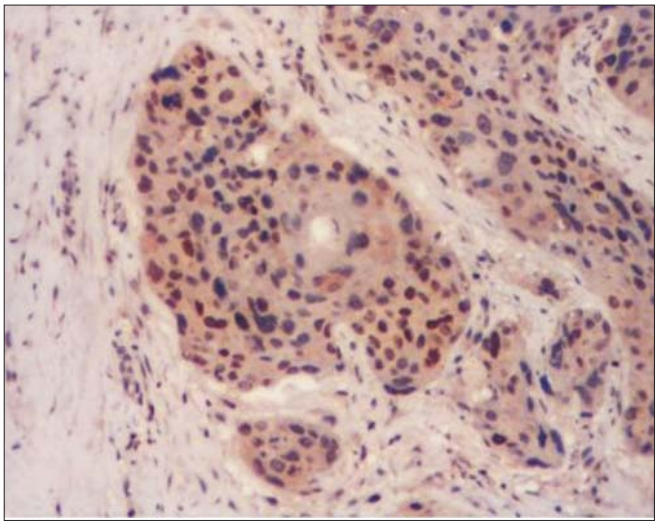


Figure 5. *IHC test image with p53 expression (+) 30%, 10x*

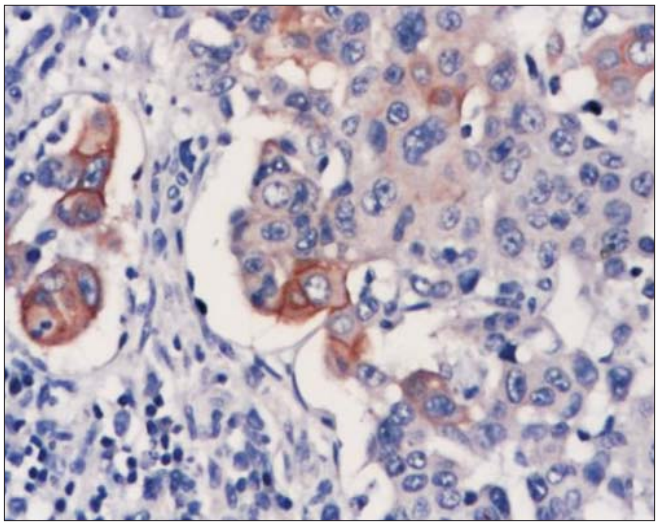


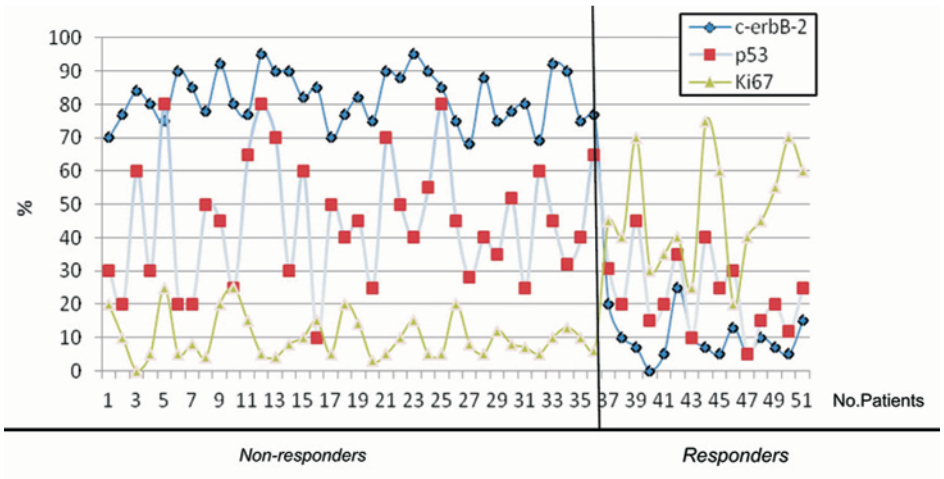
Figure 6. *IHC test image with c-erB-2 expression (+), 20x*

the two groups responder – non-responder makes this difference easy to notice.

We may conclude that identifying the markers with predictive and respectively prognostic potential in the ESCC represents a viable alternative, with great perspective. The undoubted advantages it offers, especially for determining how aggressive the molecular type of the neoplasia is and also for the possibilities of therapeutic orientation, turn it into an important weapon in the medical anti-cancer arsenal. The method becomes truly valuable with an important increase for both the sensitivity and the specificity, especially under the circumstances of associations for identification of multiple tumor markers.

Data in the specialized literature has shown a constant improvement in defining the tumor biological behavior, especially through multiple screenings of specific receptors at tumor cell membrane level. As a consequence, over the past few years, various treatments appeared in the medical

Table 5. *Tumor cells expression levels of the three studied markers and patient correlation in responder / non-responder groups*



practice, directed straight to specific molecular targets (43,44), various trials suggesting that this is a method with great future. An example of such treatment is the angiogenesis inhibitors (eg. Avastin® - bevacizumab) or the monoclonal antibodies for the tumors with an increased receptor expression to the epidermal growth factor (Erbitux® - cetuximab). Adding the association of the chemotherapy treatment to it seems to improve the results even more (45).

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