Immunohistochemical expression and significance of epidermal growth factor receptor (EGFR) in breast cancer

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Abstract
Breast cancer is a heterogeneous disease that includes several molecular types, characterized by the expression profile of sex hormone receptors, HER2 protein, cytokeratin 5, p53, and Bcl-2. EGFR is an additional marker predominantly expressed by basal-like carcinoma, but its significance in the other types is not completely understood. The aim of this study was to analyze the immunohistochemical expression of EGFR and its relationships with other factors of prognosis. There were investigated benign lesions and 84 cases with invasive breast carcinoma that were submitted first to the molecular classification. Next, we performed the staining for EGFR and two patterns of the final product of reaction were described. EGFR expression was found in 41.66% of the cases with basal-like carcinoma, in 50% of the cases with luminal B carcinoma, and in 21.42% of the cases with HER2 overexpression. A significant correlation was found between EGFR expression and degree of differentiation and distant metastasis. No significant correlation was found with the lymph node status, excepting for the basal-like carcinoma in which an inverse correlation was noticed. Our results suggest that EGFR expression by tumor cells of the breast cancer defines a specific subset of tumors with poor prognosis and potential resistance to the adjuvant therapy.

Keywords: breast cancer, molecular classification, epidermal growth factor receptor (EGFR), immunohistochemistry, prognosis.

Introduction
Breast cancer is nowadays the most frequent malignant tumor in females. Moreover, despite progresses made in the field of early detection and therapy, morbidity and specific mortality continue to increase. An important event in the therapy of breast cancer was the introduction of targeted therapy, based on the hormone receptors and HER2 protein expression. Unexpectedly, it was shown that hormone therapy does not influence the prognosis in long-term follow-up studies. On the other hand, resistance to the therapy with trastuzumab was already reported, and it is thought to be the consequence of PTEN gene loss. Therefore, there is a strong need to identify new therapeutic targets [1, 2].

A significant improvement in this field was the recent introduction of the new molecular classification that recognizes five different types of breast carcinoma: basal-like, HER2, luminal A and B, and unclassified [3, 4]. The main goal of this classification was to clarify which are the molecular markers of prognosis and to show the potential response to adjuvant therapy in individual cases. The five molecular types of breast cancer were demonstrated by gene profile analysis and immunohistochemistry was shown to be a good and accurate surrogate to define a particular type [5, 6].

In the panel of markers used to perform a molecular classification of breast cancers are included estrogen receptors (ER), progesterone receptors (PR), HER2, cytokeratin 5/6, epidermal growth factor receptor (EGFR), p53, and Bcl-2. From these markers, EGFR plays an important role in defining basal-like carcinoma, and previous studies suggested that this receptor could be a target for specific inhibitors [7].

EGFR is a member of the ErbB family of receptors, and its stimulation by endogenous ligands (epidermal growth factor or transforming growth factor-alpha) results in activation of intracellular tyrosine kinase, therefore, leads to the inhibition of apoptosis, activation of cell proliferation, and increases the metastatic potential [8]. Based on these properties, EGFR was investigated in many human malignant tumors and it is now regarded as a potential target for cancer therapy [9–11]. In breast cancer EGFR expression was found mainly in basal-like carcinoma, but many reports already signaled out positive cases associated with HER2 or luminal types [12, 13]. It was shown that EGFR plays a crucial role not only in the molecular diagnosis of breast cancer, but also induces resistance to chemotherapy and radiation treatment, and therefore, is a marker of poor prognosis and survival [14, 15].

The immunohistochemical expression of EGFR is not restricted to the basal-like carcinoma. Its expression was also found with low rates in luminal and HER2 types, but its significance in these conditions is still elusive, and even not taken into account by definition of these types.

The purpose of this study was to evaluate the expression of EGFR by the five molecular subclasses of breast cancer, and to demonstrate the correlation with clinical and pathological conventional factor of prognosis.
Material and Methods

Specimens and primary processing

There were investigated specimens of mammary gland taken by open surgery from normal tissue at distance from the tumor (n = 4), fibroadenoma (n = 5), atypical ductal hyperplasia (n = 4), ductal carcinoma in situ (n = 9) and invasive carcinoma (n = 84).

In patients with invasive carcinoma, in situ lesions were found in 15 cases. Lymph node metastases were found in 49 from 84 cases, and distant metastases in three cases.

All specimens were formalin-fixed paraffin embedded, and step section 5 µm thick were performed from each block. Initial sections were stained with the routine Hematoxylin–Eosin method for the pathological diagnosis and grade. Information about tumor stage, lymph node status and metastasis were available in all cases with carcinoma.

Immunohistochemistry

Additional slides were prepared for immunohistochemistry and the first purpose was to classify cases with carcinoma according to the molecular classification, based on the expression of ER, PR, HER2, cytokeratin 5, p53, and Bcl-2. Next, we addressed to the expression of EGFR in the molecular types in order to evaluate its diagnostic and prognostic value. All reagents used in the present study were from DakoCytomation (Glostrup, Denmark). Details on the immunohistochemical technique, regarding antigen retrieval, antibodies, dilution and working system are shown in Table 1. For all methods, the chromogen was 3,3’-diamino-benzidine and nuclei were stained with Lillie’s modified Hematoxylin.

<table>
<thead>
<tr>
<th>No.</th>
<th>Antibody</th>
<th>Clone</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
<th>Incubation time</th>
<th>Working system/Chromogen</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Estrogen receptor</td>
<td>1D5</td>
<td>RTU</td>
<td>MW, 30’ citrate buffer pH 6</td>
<td>30’, RT</td>
<td>LSAB+/HRP, DAB</td>
<td>Nuclear</td>
</tr>
<tr>
<td>2</td>
<td>Progesterone receptor</td>
<td>PgR636</td>
<td>RTU</td>
<td>MW, 30’ citrate buffer pH 6</td>
<td>30’, RT</td>
<td>LSAB+/HRP, DAB</td>
<td>Nuclear</td>
</tr>
<tr>
<td>3</td>
<td>Ki67</td>
<td>MI81</td>
<td>RTU</td>
<td>MW, 30’ citrate buffer pH 6</td>
<td>30’, RT</td>
<td>LSAB+/HRP, DAB</td>
<td>Nuclear</td>
</tr>
<tr>
<td>4</td>
<td>HER2/neu</td>
<td>Rabbit polyclonal</td>
<td>RTU</td>
<td>MW, 30’ antigen retrieval solution HercepTest</td>
<td>30’, RT</td>
<td>HercepTest Visualization reagent, DAB</td>
<td>Membrane pattern</td>
</tr>
<tr>
<td>5</td>
<td>p53</td>
<td>DO7</td>
<td>RTU</td>
<td>MW, 30’ citrate buffer pH 6</td>
<td>30’, RT</td>
<td>LSAB+/HRP, DAB</td>
<td>Nuclear</td>
</tr>
<tr>
<td>6</td>
<td>Bcl-2</td>
<td>124</td>
<td>RTU</td>
<td>MW, 30’ citrate buffer pH 6</td>
<td>30’, RT</td>
<td>LSAB+/HRP, DAB</td>
<td>Nuclear, cytoplasmic</td>
</tr>
<tr>
<td>8</td>
<td>Cytokeratin 5/6</td>
<td>D5/16 B4</td>
<td>1:80</td>
<td>MW, 30’ citrate buffer pH 6</td>
<td>30’, RT</td>
<td>LSAB+/HRP, DAB</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>10</td>
<td>Cytokeratin 18</td>
<td>DC10</td>
<td>1:25</td>
<td>MW, 30’ citrate buffer pH 6</td>
<td>30’, RT</td>
<td>LSAB+/HRP, DAB</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>12</td>
<td>EGFR</td>
<td>Polyclonal</td>
<td>RTU</td>
<td>MW, 30’ citrate buffer pH 6</td>
<td>30’, RT</td>
<td>EGFR Pharnix visualization reagent, DAB</td>
<td>Membrane, cytoplasmic</td>
</tr>
</tbody>
</table>


Evaluation of immunohistochemical reactions

Examination was performed with the microscope Eclipse 80i Nikon and images captured as JPEG format were evaluated with Lucia G microscopic analysis soft.

The expression of ER and PR was evaluated on the base of Allred score, and overexpression of HER2 protein was estimated on the base of the conventional system indicated by the manufacturer and applied as HercepTest.

Staining for cytokeratin 5, cytokeratin 18, Bcl-2 and EGFR were considered positive only if a minimum of 15% of definite tumor cells show positive reaction. EGFR scoring was based on the pattern of the final reaction product, intensity and percentage of positive tumor cells, and graded from 0 (negative) to +3 (strong positive reaction).

External control slides

Slides provided by the manufacturer were tested together with human specimens and the intensity of the reaction was interpreted according the control.

Statistical analysis

Statistical analysis was performed with SPSS13.0 soft, and included Chi square and Student tests, p<0.05 being considered as significant.

Results

The reaction for EGFR was negative in all cases of normal breast tissue, atypical hyperplasia and fibroadenoma. DCIS as isolated lesion was found in nine cases, and from these, three showed weak positive reaction in tumor cells, restricted to the cell membrane. In DCIS, the reaction was heterogeneous and the average showed between 15 and 25% of positive tumor cells.

In the control slides (Figure 1) the positive reaction was restricted to the membrane of tumor cell line isolated from an EGFR-positive human breast carcinoma.

We next investigated 84 cases with invasive carcinoma, and from them, there were 78 ductal invasive carcinomas, two lobular invasive carcinomas, one papillary carcinoma and three mucinous carcinomas. According to the grading system accepted by WHO, there were 21 cases with G1, 45 cases with G2, and 18 cases with G3. In all these cases it was applied the panel of antibodies necessary to perform the molecular classification.

Based on the tandem expression of ER, PR, HER2, cytokeratin 5, cytokeratin 18, p53 and Bcl-2, we found a specific molecular profile for basal-like carcinoma in 12 (14.29%) cases, luminal A in 44 (52.38%), luminal B in four (4.76%), HER2 type in 14 (16.66%) (Figure 2), and unclassified in 10 (11.9%) cases.
The immunohistochemical evaluation of EGFR expression showed two patterns (Figure 3, a and b). The first, found in the majority of positive cases, is characterized by a membrane-restricted pattern of the final product of reaction. The second that we called 'aberrant expression', was found only in two cases, and showed diffuse cytoplasmic pattern without membrane enhancement.

The distribution of EGFR positive reaction in the five molecular types of breast cancer was the following: basal-cell carcinoma was positive in five (41.66%) from 12 cases, luminal A in none from 22, luminal B in two (50%) from four cases, HER2 in three (21.42%) from 14 cases, and unclassified in none. Our findings clearly demonstrate that EGFR expression is not found in type luminal A and unclassified breast cancer.
No correlation was found between the expression of EGFR, tumor stage, pathological form and degree of differentiation. From the overall 11 positive cases, nine were G3 and two were moderately differentiated. A significant correlation was found between the expression of EGFR with distant metastases (all three cases were positive in the primary) \((p<0.000032)\). Regarding the correlation between EGFR expression and lymph node metastases, a significant inverse correlation was found only in cases with basal-like carcinoma \((p<0.001)\).

**Discussion**

EGFR expression was demonstrated by many authors to be a useful marker in the molecular classification and a good predictor of prognosis in breast cancer \([16–18]\). Moreover, detection of EGFR on the surface on circulating tumor cells derived from individuals with metastatic breast cancer seems to be useful to stratify patients for anti-EGFR therapy \([19]\). We found two model of distribution of the final product of reaction in breast cancer. One is the typical described in the literature, with membrane-restricted pattern. To the best of our knowledge, this paper describes for the first time the aberrant expression, with diffuse cytoplasmic pattern. The significance of this model of distribution is not known and the number of cases from this subgroup was too low \((n=2)\) for statistical analysis.

Our results strongly suggest that EGFR-positive reaction is restricted to the basal-like, HER2 and luminal B-types of breast cancer. This finding confirms previous data that showed the expression of EGFR in HER2-type and in a reduced number of luminal types. We found that EGFR expression in the later is restricted to the luminal B-type, and this could support the existence of a carcinoma arising from luminal cells that cluster close to the basal-like carcinoma. A baso-luminal type was already purposed but this entity is not yet fully characterized and accepted. It can be speculated that EGFR-positive luminal carcinoma is more aggressive and is likely to believe that this cases have a higher risk for metastases.

The expression of EGFR by a subset of HER2-type breast cancer clearly defines a subtype that is also ER-negative, and consequently, associated with worst prognosis.

In terms of prognosis, it was shown that basal-like carcinoma gives rise less frequently to lymph node metastases, and more frequently to systemic metastases. In our study, the expression of EGFR strongly correlates with the presence of systemic metastases and an inverse correlation was found with lymph node status.

Tyrosine kinase inhibitors are effective anti-cancer therapies but resistance to these agents was already reported and includes EGFR inhibitors \([20]\). Phosphorylation of EGFR was detected in triple-negative tumors and was blocked by gefitinib treatment in vitro \([21]\). These results are promising in terms of clinical trials with EGFR inhibitors and this may be extended to a subset of metastatic HER2-positive cases that show poor response to trastuzumab therapy \([22]\). Recently, in a phase II trial with an EGFR inhibitor in metastatic breast cancer it was shown that the combination between erlotinib and bevacizumab (anti-vascular endothelial growth factor antibody) had only a limited efficiency \([23, 24]\). This is not a surprising result, because our findings demonstrated that only a limited number of cases with breast carcinoma \((11 \text{ from } 84)\) do express EGFR. An anti-EGFR antibody, cetuximab, alone or in combination with cisplatin, was effective in vitro on breast carcinoma cells \([25]\), but its clinical application needs further validation. A high-rate of local recurrence was observed in EGFR-positive cases \([26]\). This is in accord with our findings regarding the significant correlation found between EGFR expression, grade of the tumor and metastatic potential. Based on these data, the immunohistochemical evaluation of EGFR expression becomes a useful tool to perform characterization of breast cancer and to define new therapeutic targets.

**Conclusions**

Our study demonstrated the immunohistochemical expression of EGFR in 13.09% of the cases with invasive breast carcinoma. No expression was found in the normal mammary tissue, fibroadenoma and atypical hyperplasia. EGFR is an important marker to stratify cases with breast cancer according the molecular classification. The expression of EGFR correlated with the degree of differentiation, inversely with the lymph
node status only in basal-like carcinoma, and with distant metastases. In a subset of patients with breast cancer, EGFR could be considered an effective target for specific therapy.

Acknowledgements

This work was supported by Grant 41054/2007/PNII and Grant 96/2007/PNII of the Romanian Ministry of Education and Research. The authors are grateful to Diana Tătucu for her excellent technical assistance.

References


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Received: February 15th, 2009

Accepted: April 10th, 2009

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