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Original Article

Associations between clinicopathological prognostic factors and pAkt, pMAPK and topoisomerase II expression in breast cancer

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Abstract: This study aimed to examine the associations between mitogen activated protein kinase (MAPK), Akt, and topoisomerase II expression and other well established clinical and pathological prognostic factors in patients with breast cancer. A total of 42 women with breast cancer who underwent anthracycline based chemotherapy were included in this retrospective study. Immunohistochemical methods were utilized to examine the expression of phosphorylated MAPK (pMAPK), phosphorylated Akt (pAkt), HER-2/*neu* and topoisomerase IIα (topo IIα) in tissue blocks. Subsequently, the associations between pMAPK, pAkt, and topoisomerase IIα (topo IIα) expression characteristics and disease stage (T and N), tumor grade, estrogen/progesteron receptor status, and HER-2/*neu* expression were explored. Median age of the patients was 63 years (range, 37-82). There was a significant association between N stage and topoisomerase IIα expression ($P = 0.021$), with increasing rates of positivity in higher grades: N0, 22.7%; N1, 11.1%; N2, 42.9%; N3, 100%. In addition, topo IIα expression was higher in estrogen receptor-positive versus estrogen receptor-negative tumors (50% vs. 0%, $P = 0.0004$) and MAPK expression was more frequent among progesteron receptor-positive versus negative tumors (64.0 versus 20.0%, $P = 0.027$). Our results show that the tissue expression of topo IIα and MAPK, which play a role in the intracellular signal pathways, is associated with certain established prognostic factors in breast cancer. Further studies examining survival rates and involving larger sample populations are warranted to better define the importance of the observed associations.

Keywords: Breast cancer, mitogen activated protein kinase (MAPK), Akt, topoisomerase II, human epidermal growth factor receptor-2/*neu* (HER-2/*neu*)

Introduction

Breast cancer represents the most common cancer in women in the US and probably also among other nations, and is the second most common cause of cancer-related mortality among females worldwide [1]. Age-adjusted mortality rates for breast cancer has declined approximately 20% over the last 20-year period, with further expected decreases in mortality along with a better understanding regarding the biology and risk factors for breast cancer.

Principal prognostic factors that affect the course of the disease and guide the treatment include the Tumor-Nod-Metastasis (TNM) stage, axillary lymph node status, tumor size, tumor grade, hormone receptor expression, lymphatic and/or vascular invasion, histological type, human epidermal growth factor receptor-2/*neu* (HER-2/*neu*) expression and amplification. However, the current prognostic and predictive factors are far from being adequate in terms of their ability to provide accurate estimations at an individual level, warranting the discovery of novel histological/molecular and clinical determinants.

HER-2/*neu* is a cellular membrane receptor belonging to the family of epidermal growth factor receptors (EGFR) and its amplification or overexpression has been found to affect the

prognosis adversely in patients with breast cancer [2-4]. Activation of EGF receptors is associated with the phosphorylation of their intracellular tyrosine kinase components. Enzymes and proteins binding to these phosphorylated tyrosine kinases convey the specific signal to the nuclear transcription factors, playing a role in several intracellular processes including proliferation, angiogenesis, and apoptosis. The two main sub-pathways acted upon by the EGF receptors include Ras-Raf-MAPK (mitogen activated protein kinase) and PI3K (phosphatidylinositide 3-kinase)-Akt. The phosphorylated MAPK is transferred into the nucleus from cytoplasm, activating the nuclear transcription factors responsible for the transcription of target genes involved in cell proliferation through their phosphorylation. The PI3K enzyme family has been reported to be involved in many intracellular processes including increased proliferation/growth, motility, metastatic and invasive potential, and angiogenesis. The most important protein within the PI3K signal pathway is Akt, which is a serine-threonine kinase activated by a number of different stimulants. Normal Akt signaling pathways are disturbed in many different types of human cancer and this enzyme play a major role in the cancer progression and cell survival. Of the three Akt proteins, two, i.e. Akt2 and Akt3, have been detected in breast cancer cells [5, 6].

Akt, which is activated (i.e. phosphorylated) upon binding to phosphatidylinositols, regulates the functions of proteins involved in apoptosis (p21, p27, NFkB, caspases and BAD) and progression of cell cycle. In addition, it affects the functions of other proteins (BRCA 1 and estrogen receptor) closely related to the development of breast cancer.

The alpha-subtype of topoisomerase II is a key enzyme for DNA replication and also represents a target enzyme for several chemotherapeutic agents including anthracyclines and epipodophyllotoxins. Conflicting results have been obtained in many studies examining the association between amplification and/or deletion of topoisomerase IIα (topo IIα) and response to topo II inhibitors, probably due to methodological differences [7-10]. Some studies have found a significant association between overexpression of topo IIα and negative hormone receptor status, HER-2/*neu* expression, p53 mutation and poor histological differentiation [11, 12]. In a significant proportion of breast cancer patients with HER-2/*neu* amplification, concurrent occurrence of topo IIα amplification and deletion has also been observed [13, 14].

In the present study, the association between established clinical/histological prognostic factors and pMAPK, pAkt and topoisomerase IIα expression has been assessed in breast cancer tissues, in an attempt to disclose potential for them as novel prognostic factors.

Material and methods

Patients

Out of 108 patients with breast cancer who underwent adjuvant anthracycline-based chemotherapy in our institution (i.e. high-risk patients), tumor tissue samples were available in 42 for a retrospective assessment of pMAPK (phosphorylated MAPK), pAkt (phosphorylated Akt), HER-2/*neu*, and topo IIα expression through immunohistochemical methods.

Immunohistochemical analysis

HER-2/*neu* expression was immunohistochemically examined using CB-11 monoclonal antibody (1/40, Novocastra, Newcastle, UK). Membraneous staining was considered positive and the scoring was performed according to Herceptest (DAKO, Carpinteria, CA). In histological cross-sections with 2 + or 3 + HER-2/*neu* expression were checked using FISH. The expression of p-MAPK was assessed using monoclonal phospho-p44/42 MAPK (Thr202/Tyr204) antibody directed at the phosporylated pMAPKinase (Cell Signaling Technology, Beverly, MA). Nuclear staining was considered positive. The expression of p-AKT was assessed using phospho-AKT (Ser473) antibody directed at the phosphorylated Akt (1/20; Cell Signaling Technology, Beverly, MA). Membraneous, cytoplasmic and nuclear staining was considered as positive staining. Topoisomerase II Alpha expression was analyzed using Clone 3F6; MS-1755-S1 Neomarkers antibody. A nuclear staining equal to or more than 10% was considered positive. 6F11 (1/40 dilution) and 1A6 (1/20 dilution) monoclonal antibodies (Novocastra, Newcastle, UK) were used to assess the expression of estrogen and progesterone receptors. A percent staining below 10% in tumor cells was considered negative (i.e. ER and PR negative).

Table 1. Patient characteristics

Characteristic	n/n* (%)
T stage	
T1	17/42 (40.5%)
T2	20/42 (47.6%)
T3	4/42 (9.5%)
T4	1/42 (2.4%)
N stage	
N0	22/41 (53.7%)
N1	9/41 (21.9%)
N2	7/41 (17.1%)
N3	3/41 (7.3%)
Histological grade	
Grade 0	1/41 (2.4%)
Grade 1	1/41 (2.4%)
Grade 2	31/41 (75.6%)
Grade 3	8/41 (19.5%)
Topoisomerase IIα expression	29/42 (69.0%)
HER-2 expression (2+/3+)	23/36 (63.9%)
pAkt expression (cytoplasmic)	31/41 (75.6%)
pAkt expression (nuclear)	18/42 (42.9%)
MAPK expression	20/41 (48.8%)
Estrogen receptor positivity	24/41 (58.5%)
Progesteron receptor positivity	26/36 (72.2%)

*n/n denotes positive results/total number of patients with available data.

Statistical analysis

Data are presented as numbers and percentages. Between-group comparisons of categorical data were performed using Fisher's exact test or Chi-square test. A P value < 0.05 was considered as an indication of statistical significance.

Results

Patient characteristics are shown in **Table 1**. Of the 42 female patients included in the study, 41 were postmenauposal (97.7%). Median age of the patients was 63 years (range, 37-82).

Relation between prognostic parameters

There was a significant relation between N stage and topoisomerase IIα expression (P = 0.021), with increasing positivity in extensive nodal involvement: N0, 22.7%; N1, 11.1%; N2, 42.9%; N3, 100%. Furthermore, topo IIα expression was present in half of the estrogen receptor positive samples versus none in those without the estrogen receptor (50% vs. 0%, P = 0.0004). However, no significant relation could be found between topoisomerase IIα expression and T stage (P = 0.88), HER2/*neu* expression (P = 0.21), or progesteron receptor expression (P = 0.4).

pMAPK expression was more frequent among progesteron receptor positive samples as compared to those without progesteron receptor expression (64.0 versus 20.0%, P = 0.027). However, no significant associations could be observed between pMAPK (nuclear) expression and T stage, N stage, tumor grade, pAkt, topo IIα, estrogen receptor or HER2/*neu* expression (P > 0.05 for all).

In addition, the frequency of pAkt expression did not show any associations with any of the parameters tested in the study (P > 0.05 for all).

Discussion

Our results point out to the presence of a significant association between higher N stage, estrogen receptor positivity and topoisomerase IIα expression in addition to that between progesterone receptor positivity and pMAPK expression.

Out of the 42 participants, 69% had topoisomerase IIα expression. The reported topoisomerase IIα expression rates in the literature vary. In a study by Hellamans et al. [15] involving 63 patients with primary breast tumors, a strong association between topo IIα expression and higher grade, tumor size, nodal status, and presence of distant metastases was observed. In the same study, no such associations could be observed for the menopausal status, hormone receptor status, and the disease-free and overall survival. In another study, Koren et al. [16] examined the association between topo IIα expression and menopausal status, tumor type, tumor size, lymph node metastases, disease stage, and hormone receptor positivity in a total of 50 patients with breast cancer and found significantly higher expression of topo IIα in high grade tumors as compared to intermediate or low grade tumors. In addition, a significant association between ER positivity and topo IIα expression was observed. The authors concluded that topo IIα expression is an important prognostic factor in patients with breast

cancer [16]. Another study suggesting a link between topo IIα expression and other clinical/pathological parameters has reported an expression rate of 64% for topo IIα among 50 patients with breast cancer. In that study, Kalogeraki et al. [17] found a significant association between topo IIα expression and HER-2/*neu*, histological grade, and lymph node status. Another report has suggested a correlation between increased topo IIα expression and poor survival, higher disease stage, lymph node metastases, and HER-2/*neu* amplification, while no similar associations were observed for tumor size, tumor grade, hormonal receptor status, and relapses in the same study [18]. Recent studies with limited sample size have also examined the relationship between response to chemotherapy and topo IIα expression and reported some long-term results in patients with primary breast cancer patients. For example, in the study by Di Leo et al. [9] patients receiving CMF chemotherapy have been compared to those receiving an anthracycline-based chemotherapy regimen, and topo IIα amplification has not been found to adversely affect the survival as compared to non-amplification in subgroup analyses. In cell series treated with anthracycline, lesser efficacy of treatment has been reported with lower expression of topo IIα [13, 19]. In a retrospective subgroup analysis from a randomized trial, the prognostic effect of topo IIα gene amplification has been found to be limited to patients receiving non-anthracycline based regimens [9]. Again, in another study assessing the response to anthracycline, a correlation has been found between topo IIα gene amplification and the clinical response [10, 20, 21]. This finding contradicts with the retrospective report by Petit et al. [22] where no predictive value for HER-2/*neu* and topo IIα amplification was observed in terms of response to anthracycline. In our study, topo IIα expression has been found to show a significant association with lymph node metastasis and estrogen receptor expression, while no associations have been observed for tumor size, histological grade, pMAPK and pAKT. Despite the similarity to the study by Koren et al. with regard to the positive correlation between topo IIα expression and hormone receptor expression, our findings are at odds with certain other findings [15, 16]. Considering the fact that hormone receptor status is a well-established favorable prognostic factor in patients with breast cancer, the correlation observed in our study between topo IIα expression and receptor status is not well understood.

MAPK is an important signal protein that plays a key role in cell proliferation and apoptosis. Activated MAPK can be readily assessed both in fresh tissue samples and in paraffin blocks immunohistochemically [23]. Previous studies have reported a high percentage of cells with activated MAPK in breast tissue samples. In a study by Adeyinka et al. MAPK expression has been reported to occur in 48% of the primary breast cancer cells. In comparison with the normal breast tissue surrounding the tumor, a higher expression rate of MAPK has been observed in tumor tissues. In the same study, the presence of activated MAPK has been found to be associated with lymph node metastases and also a higher MAPK expression has been measured in metastatic lymph node samples as opposed to the primary breast cancer cells [24]. Also invasiveness, hormone receptor negativity, and advanced stage have been reported to be associated with the activation or overexpression of MAPK family [25, 26]. Decreased response to endocrine therapy has been reported in patients expressing activated MAPK [26]. In breast cancer patients with higher MAPK activity, significantly lower disease-free survival rate has also been observed [27, 28]. On the other hand, in another study MAPK has been found not to represent an independent prognostic factor in lymph-node positive patients undergoing adjuvant CAF chemotherapy [27]. Among our patients, MAPK expression was present in 48%, in line with previous reports. The significant positive association between MAPK and PR expression ($p < 0.005$) was most likely due to our limited sample size.

Akt is a serine-threonine kinase activated by a number of stimulants and with disturbed Akt signaling in many different types of human cancer. This enzyme plays important roles in cancer progression and in the maintenance of cell viability. Several recent studies have reported an association between akt activation and adverse prognosis [29, 30]. Patients undergoing tamoxifen and/or goserilen treatment and expressing Akt are more likely to relapse with distant metastases [29]. Also, in postmenopausal women Akt negativity has been found to be related with increased benefit from tamoxi-

fen treatment [30]. These findings suggest that Akt activation may have some predictive value in terms of treatment response for endocrine therapy. In our series, cytoplasmic pAkt positivity has been found in 76% of the sample, with 43% positivity for nuclear pAkt expression. Despite the similarity with the reported data in terms of staining percentage, no correlations have been observed in this study between pathological parameters and pAkt staining.

In conclusion, the results of this study indicate the presence of an association between several established prognostic factors in breast cancer and expression of topo IIα and MAPK. A better understanding of the potential role of the molecules involved in intracellular signaling pathways on disease prognosis and response to treatment may help discover novel molecular targets. However, this requires further studies with larger sample size and longer follow-up that also evaluate the effect on survival.

Disclosure of conflict of interest

None.

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