Expression and activation of P38 MAP kinase in invasive ductal breast cancers: Correlation with expression of the estrogen receptor, HER2 and downstream signaling phosphorylated proteins

JEAN-LOUIS MERLIN[1,2,3], ALEXANDRE HARLÉ[1,2,3], MAËVA LION[1,2,3], CAROLE RAMACCI[1] and AGNÈS LEROUX[1,3]

[1]Department of Biopathology, Institut de Cancérologie de Lorraine, 54519 Vandoeuvre les Nancy;
[2]Université de Lorraine, Faculty of Pharmacy, 54001 Nancy;
[3]CNRS UMR 7039 CRAN, 54506 Vandoeuvre les Nancy, France

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Abstract. MAP kinase signaling proteins have major implications in the molecular oncogenesis of breast cancers and have been extensively investigated as putative targets for therapy. This study reports the investigation of the expression of P38 MAPK and its phosphorylated form (p-P38 MAPK) in clinical specimens of invasive breast carcinomas and their correlation with estrogen receptor (ER) and HER2 expression, as well as MAPK and PI3 kinase-AKT pathway signaling phosphorylated proteins. Expression levels of P38 MAPK and p-P38 MAPK as well as p-AKT, p-GSK3β, p-S6 kinase, p-MEK1 and p-ERK1/2 were quantitatively assessed using multiplex bead immunoassay in frozen specimens from 45 invasive ductal breast cancers. Twenty-nine specimens were ER^+, 15 were $HER2^+$ and 10 were triple-negative breast cancers (TNBCs). P38 MAPK was found to be expressed in all tumor specimens and was significantly (P=0.002) overexpressed in ER^+ tumors. P38 MAPK expression was lower in TNBCs than in all of the other tumors. The median expression of p-P38 MAPK was also higher in ER^+ tumors while lower in the TNBCs. HER2 status had no effect on P38 MAPK and p-P38 MAPK expression. No variation in the phosphorylation rate of P38 MAPK was observed in relation with ER, HER2 or TNBC status. Significantly higher (P=0.0048) expression of p-AKT was observed in $HER2^+$ tumors. No significant difference in p-MEK1, p-GSK3β and p-S6K expression was found in any other comparisons based on ER and HER2 expression subtypes. Investigation of the expression of multiple phosphorylated signaling proteins can be used for personalized targeted therapy. In invasive breast cancer, the overexpression of P38 MAPK may serve as a biomarker for the evaluation of P38 MAPK inhibitors.

Correspondence to: Professor Jean-Louis Merlin, Institut de Cancérologie de Lorraine, 6 Avenue de Bourgogne, CS30519, 54519 Vandoeuvre les Nancy, France
E-mail: jl.merlin@nancy.unicancer.fr

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Introduction

Breast cancer is the most common cancer in women with more than one million new cases. Breast cancer is one of the leading causes of cancer-related mortality in women (1). Apart from surgery, adjuvant radiation therapy and chemotherapy, treatment of breast cancer is based on the identification of molecular targets, mainly estrogen receptor (ER) expression, HER2 overexpression and amplification. In ER-expressing tumors, endocrine therapy consisting of mainly tamoxifen and anti-aromatase drugs is prescribed. In human epidermal growth factor receptor 2 (HER2)-positive ($HER2^+$) tumors, anti-HER2-targeted therapies such as the anti-HER2 monoclonal antibody trastuzumab and the tyrosine kinase inhibitor lapatinib have been proposed. Moreover, in $HER2^+$ tumors also expressing ER, the combination of both endocrine and anti-HER2 therapies have been proven to improve the outcome of patients through the blockade of signaling crosstalk leading to resistance to ER-targeted therapy. In triple-negative breast cancers (TNBCs) i.e. ER^-, PR^- and $HER2^-$ tumors, no targeted therapy has been proven effective to date, and the molecular characteristics of TNBCs have been extensively studied in order to identify putative molecular targets for drug development. Since several different growth factor pathways can stimulate breast cell growth, targeting a unique pathway may have limited effect on the inhibition of breast cancer proliferation, and the inhibition of signal transduction at a deeper point in the cascade has been envisaged.

In this context, mammalian target of rapamycin (mTOR) has been identified as the point of convergence of many mitogenic signals, which has led to the recent registration of the mTOR inhibitor everolimus in association with anti-

aromatase therapy for ER^+ breast cancers (2). Another point of convergence of intracellular downstream growth factor receptor signaling is the P38 mitogen-activated protein kinase family (P38 MAPK). P38 MAPK is a member of the MAPK family which includes the extracellular signal-regulated kinase (ERK), the c-Jun N-terminal kinase (JNK), and P38 MAPK (3). P38 MAPK is comprised of four isoforms (α, β, γ and δ) that can be activated by various growth factors, inflammatory cytokines, and chemical or physical stress. The α isoform is the most abundant and is subject to a larger inter-individual variability than the other three isoforms (12). P38 MAPK plays a complex role in the regulation of cell growth, differentiation, apoptosis, and responses to inflammation or stress (4,5). P38 MAPK activity was found to be upregulated in breast, head and neck carcinomas, lymphomas, gliomas and squamous cell carcinomas (6).

In breast cancer, a high level of expression of P38 MAPK has been found to correlate with poor prognosis and to be involved in invasiveness and metastasis in relation with the urokinase plasminogen activator system (7). Activation of P38 MAPK has been observed in Scarff-Bloom-Richardson (SBR) grade 2 or 3 ductal tumors (8). Expression of phosphorylated-P38 MAPK (p-P38MAPK) has been reported in ~20% of primary breast carcinomas (9). P38 MAPK overexpression has been correlated with HER2 amplification and tamoxifen resistance (10) and has been proposed as a potential prognostic marker in breast cancer (11). Specifically, phosphorylation of P38 MAPK was found to be a negative prognostic indicator in HER2-negative, lymph node-positive breast cancers (9).

The role of P38 MAPK in the regulation of breast cancer cell proliferation remains to be elucidated and has been suggested to have dual activities that include regulation of survival and proliferation depending on the expression of mutant TP53 (12) as observed in most ER^- breast tumors therefore justifying the development of P38 MAPK inhibitors for the treatment of TP53-mutated, ER^- breast cancers or TNBCs. Furthermore, the activation of P38 MAPK has recently been reported to regulate signaling by EGFR/c-Src crosstalk in breast cancer (13).

In addition, P38 MAPK has been recently reported to play a role in the resistance of ER^+ breast tumors to endocrine therapy (14). Although the cellular mechanisms underlying the development of tamoxifen resistance in breast cancer cells are not totally understood, recent research has found that alteration of the signaling pathways (15-17) can decrease the cell sensitivity to tamoxifen. More precisely, the development of crosstalk between ER and growth factor-mediated activation of the MAPK cascade, through the activation of HER2 has been reported to increase both genomic and non-genomic ER actions in breast cancer leading to tamoxifen resistance. This justifies the combination of endocrine therapy together with aromatase inhibitors and anti-HER2 therapies with trastuzumab-based or lapatinib-based therapies for breast cancer (18). Recent studies have noted a positive correlation between activated P38 MAPK levels and tamoxifen resistance (19). P38 MAPK has been reported to potentiate ER agonist activity through increased phosphorylation of ER and enhanced ER signaling through coactivator regulation (20). P38 MAPK has been shown to play a role in breast cancer progression and invasion (21) in association with other signaling proteins such as integrins and urokinase plasminogen activator (22) as well as H-RAS (23).

P38 MAPK isoform γ has been recently shown to be selectively activated by exposure to tamoxifen, consequently recruiting nonclassical ER signaling and increased estrogen cell sensitivity (24). Therefore, increased P38 MAPK activation could define a more malignant, resistant and metastatic breast cancer phenotype and justify the evaluation of P38 MAPK inhibitors in the treatment of invasive and tamoxifen-resistant breast carcinomas (14). A number of P38 MAPK inhibitors are currently being investigated in clinical trials (25).

In the present study, we investigated the expression of P38 MAPK and p-P38 MAPK in clinical specimens of invasive breast carcinomas. We first investigated the correlation of their expression with ER and HER2 expression, and subsequently evaluated the correlation with expression levels of MAPK and PI3K signaling phosphorylated proteins such as p-AKT, p-GSK3β, p-S6 kinase, p-MEK1 and p-ERK1/2 quantitatively determined using multiplex bead immunoassay as previously described and validated in breast cancer (26).

Materials and methods

Patients and tumor characteristics. Frozen tumor samples of breast cancer from 45 patients with infiltrative ductal carcinoma were obtained from the tumor bank of our Institute (agreement with French National Cancer Institute and Ministry of Health). All patients were informed of the tumor banking procedure and no opposition was expressed. The median age at diagnosis was 56.3 years (range, 28-91).

Breast cancer tissues macroscopically selected by the pathologists were obtained immediately after surgery and were shock frozen in liquid nitrogen then cryopreserved at -80˚C. The mean tumor (SD) specimen weight was 15.2 (4.2) mg. None of the patients received any preoperative adjuvant endocrine therapy or chemotherapy. Thirty-four tumors were SBR grade 3 (Scarff-Bloom-Richardson) and 11 tumors were grade 2.

Immunohistochemistry (BenchMark Ventana) was used to detect estrogen and progesterone receptor expression and HER-2 overexpression as part of the routine clinical diagnostics using polyclonal antibody A485 (Dako, Trappes, France) immunostaining of HER2 and monoclonal antibodies 6F11 and Pgr312 (both from Novocastra, Leica Microsystèmes, Nanterre, France) for determination of estrogen and progesterone expression, respectively.

Protein extraction. The tumor specimens were first disrupted using steel bead TissueLyser (Qiagen, Courtaboeuf, France) for 15 min, and then exposed to the lysis solution (Cell Lysis kit, Bio-Rad, Marnes-la-Coquette, France) containing PMSF anti-protease for 10 sec. After centrifugation (4,500 x g for 20 min at 4˚C), the protein-containing supernatants were collected and stored frozen at -80˚C until analysis. Before being analyzed, the protein concentration was determined in each extract using 690 nm colorimetric DC protein assay kit (Bio-Rad) based on Lowry technique and adjusted to 250 μg/ml.

Multiplex bead immunoassay. The expression of the signaling phosphoproteins was analyzed using multiplex bead immunoassay as described and validated previously (26). Briefly, protein extracts were transferred into 96-well dishes and diluted with 25 μl buffered solution. Fluorescence capturing beads coupled to antibodies directed against P38 MAPK, p-P38 MAPK, p-AKT, p-GSK3β, p-P70S6K, p-MEK1, p-ERK1/2 phosphoproteins were mixed. The beads were added into each well and incubated overnight at 37˚C. Biotinylated antibodies and then streptavidin-phycoerythrin solution were then added. The positive control consisting of standard protein extract from cell lines was added to each series. The multiwell plates were then analyzed according to the manufacturer's instructions (Bio-Plex; Bio-Rad, Hercules, CA, USA). Frozen protein extracts from an EGFR-overexpressing human breast cancer cell line exposed to EGF were used as positive controls as reported previously (26). The results were recorded as mean fluorescence intensities expressed as arbitrary units and considered as significant when exceeding a signal/noise ratio of 3.

Statistics. All analyses were performed as triplicate and are presented as mean fluorescence intensities (SD). All statistical analyses were performed using the Wilcoxon test using R software (v.2.15.1.; the R Foundation for Statistical Computing) and the level of significance was set at $P<0.05$.

Results

Immunohistochemistry. The breat cancer tumor characteristics are summarized in Table I. The ER status as determined by immunohistochemistry was positive in 29 patients (64%) and negative in 16 patients (36%). Progesterone receptor (PR) status was positive in 18 patients (40%) and negative in 27 patients (60%). Fifteen tumors (33%) were $HER2^+$ and 30 tumors (67%) were $HER2^-$. Ten (22%) tumors were triple-negative breast cancers (TNBCs), i.e. ER^-, PR^- and $HER2^-$.

P38 MAPK and phosphorylated-P38 MAPK expression. P38 MAPK and p-P38 MAPK were found to be expressed in nearly all tumor specimens (44/45, 98%) and were significantly ($P=0.0016$) overexpressed in ER^+ tumors (Fig. 1A). The median expression of p-P38 MAPK was also higher in ER^+ when compared with that in ER^- tumors. HER2 status had no influence on P38 MAPK and p-P38 MAPK expression (Fig. 1B). P38 MAPK expression was lower in TNBCs (Fig. 1C) when compared with the expression level in all other tumor types as was p-P38MAPK expression but without reaching statistical significance. No significant variation in the phosphorylation rate of P38 MAPK, as calculated from the phosphorylated/unphosphorylated P38 MAPK expression ratios, was observed in association with ER, HER2 and TNBC status, or SBR grade.

Phosphorylated-AKT and phosphorylated-ERK1/2 expression. Significant expression of p-AKT and p-ERK1/2 was detected in 33/45 (73%) and 17/45 (38%) of the tumor extracts, respectively. p-AKT expression was found to be significantly higher ($P=0.0048$) in $HER2^+$ tumors (Fig. 2) than in $HER2^-$ tumors. No other significant difference was observed regarding either ER and TNBC status or SBR grade. No difference in p-ERK1/2 expression was observed regarding ER, HER2 and TNBC status. Expression of p-ERK1/2 was found to be significantly higher ($P=0.0235$) in SBR grade 3 than in SBR grade 2 tumors (Fig. 3).

Table I. Patient demographics and tumor characteristics.

Characteristics	Patients, n (%)
No. of patients	45 (100.0)
Age (years)	
≤50	13 (28.9)
51-69	24 (53.3)
≥70	8 (17.8)
Tumor size (mm)	
T1 (10-20)	16 (35.6)
T2 (21-50)	26 (57.8)
T3 (>51)	3 (6.7)
SBR grade	
SBR 2	11 (24.4)
SBR 3	34 (75.6)
Hormone receptor status	
ER^+	29 (64.4)
ER^-	16 (35.6)
PR^+	18 (40.0)
PR^-	27 (60.0)
HER2 status	
Positive	15 (33.3)
Negative	30 (66.7)
Triple-negative	10 (22.2)

ER, estrogen receptor; PR, progesterone receptor; SBR, Scarff-Bloom-Richardson; HER2, human epidermal growth factor receptor 2.

Expression of other phosphorylated-signaling proteins MEK1, GSK3β, S6K. Significant expression of p-MEK1, p-GSK3β and p-S6K was detected in 39/45 (87%), 31/45 (69%) and 37/45 (82%) of the protein extracts, respectively. No significant difference in p-MEK1, p-GSK3β and p-S6K expression was evidenced regarding either ER and TNBC status or SBR grade (data not shown). No significant correlation was found between the expression levels of any of the phosphorylated proteins.

Discussion

In breast cancer, P38 MAPK expression has previously been correlated with invasiveness and poor prognosis (8-11).

In the present study, we compared the expression of P38 MAPK and p-P38 MAPK in clinical specimens of invasive breast carcinomas in association with ER, HER2 and SBR grade and aimed to ascertain a correlation between P38 MAPK expression or activation of MEK/ERK and the AKT/mTOR signaling pathways.

In our series, expression of P38 MAPK and p-P38 MAPK was observed in nearly all tumor specimens. This was consis-

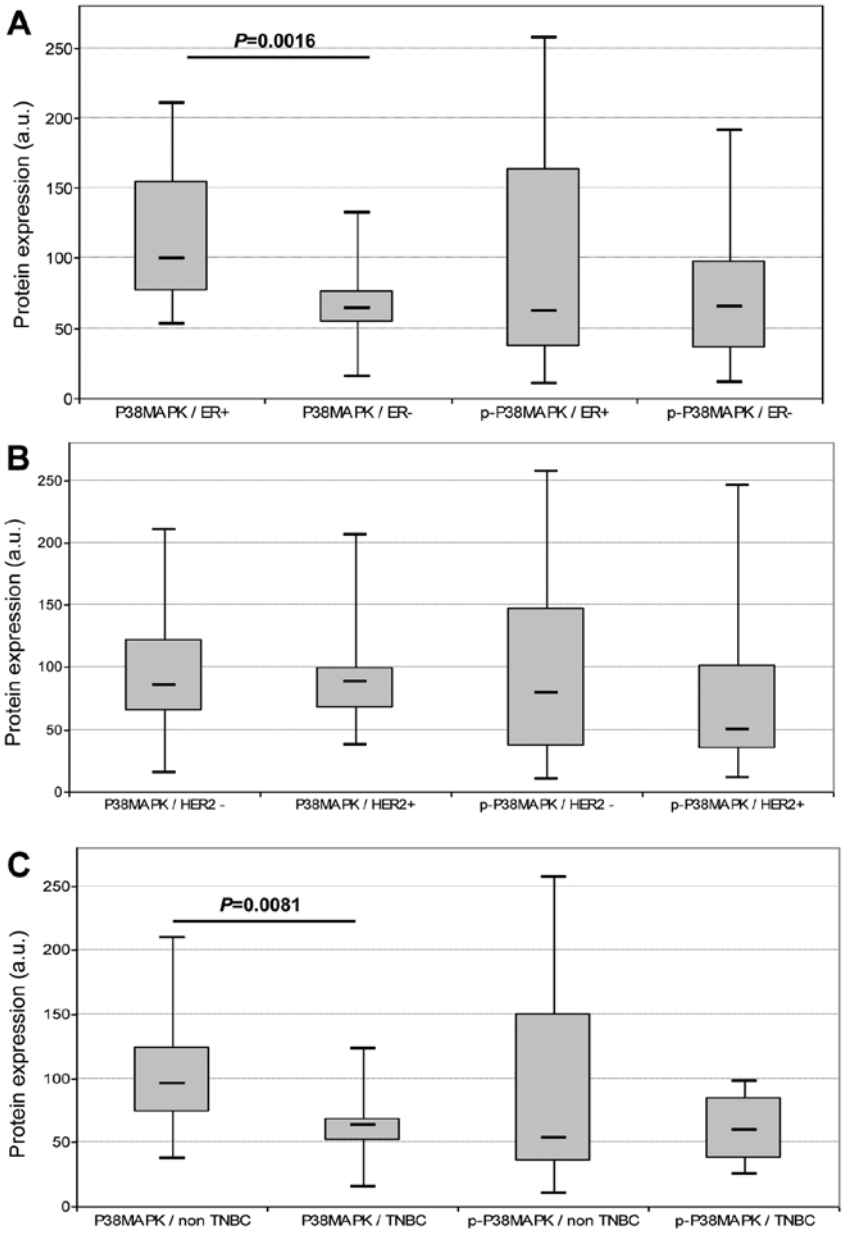

Figure 1. Expression of P38 MAPK and its phosphorylated form (p-P38 MAPK) in clinical specimens of invasive breast cancers. The results are presented as box-and-whisker plots, illustrating the median (central bar), 1st and 3rd quartiles (bottom and top of the grey box), minimum and maximum values (lower and upper bars). Expression in (A) estrogen-positive (ER^+) and ER-negative (ER^-) tumors; (B) HER2-positive ($HER2^+$) and HER2-negative ($HER2^-$) tumors; (C) triple-negative breast cancer (TNBC) and non-TNBC.

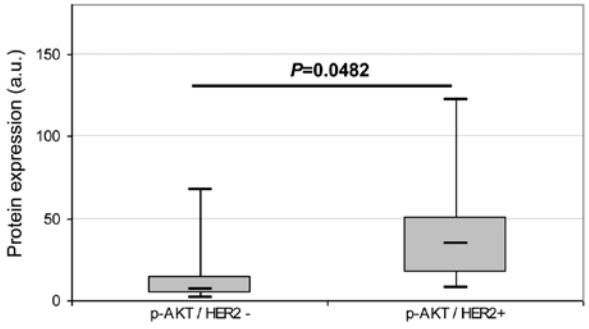

Figure 2. Expression of phosphorylated-AKT (p-AKT) in HER2-positive ($HER2^+$) and HER2-negative ($HER2^-$) tumors. The results are presented as box-and-whisker plots, illustrating the median (central bar), 1st and 3rd quartiles (bottom and top of the grey box), minimum and maximum values (lower and upper bars).

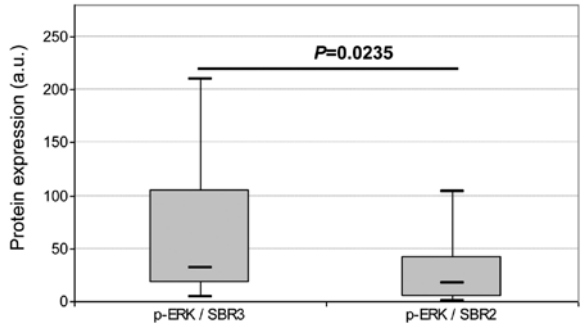

Figure 3. Expression of phosphorylated-ERK1/2 (p-ERK) in SBR grade 2 (SBR2) and grade 3 (SBR3) tumors. The results are presented as box-and-whisker plots, illustrating the median (central bar), 1st and 3rd quartiles (bottom and top of the grey box), minimum and maximum values (lower and upper bars).

tent with previously published data (11) reporting P38 MAPK and p-P38 MAPK expression in 100 and 89% of specimens, respectively, using western blot analysis, and 70% when IHC was used.

We report here that P38 MAPK was expressed at a higher level in ER^+ when compared with ER^- tumors without post-transductional activation since no variation in the phosphorylation rate of P38 MAPK was evidenced. This is consistent with previously published data (10,27) revealing the great interest in P38 MAPK in ER^+ tumors. P38 MAPK has been reported to be activated by anti-estrogens apart from ER their main target, resulting in a switch in ER signaling from its classical pathway, involving the estrogen response element (ERE) DNA domain, to the AP1-dependent non-classical pathways; therefore, activation of P38 MAPK can ultimately decrease the cellular response to endocrine therapy. Based on this concept, P38 MAPK has been proposed as a biomarker for resistance to endocrine therapy, and quantitative assessment of P38 MAPK expression and the detection of its activation in breast tumors may represent a new approach to predict the resistance of breast cancer to endocrine therapy. Furthermore, inhibition of P38 MAPK in ER^- tumors could restore ER expression and therefore restablish the sensivity to endocrine therapy (28).

Moreover, evaluation of the molecular pathway may even be proposed for specimens obtained at recurrence since the molecular pathways driving tumor growth could be altered along with tumor progression (10).

An incomplete understanding of the complex mechanisms exists concerning the relationship between MAPK activation and expression of hormone receptors and HER2 in breast carcinoma *in vitro*. In effusion specimens, p38 activation was reported to be inversely associated with the intensity of HER2 membrane expression (11).

In this context, although we did not observe any inverse relationship between HER2 and p-P38 MAPK expression, our results revealed that expression of P38 MAPK was significantly lower in TNBCs than in the other tumor subtypes. This may be reconsidered if a more specific approach of selective inhibition of P38 MAPK isoforms can be envisaged, as recent preclinical studies have demonstrated the important role played by the P38 MAPK γ isoform in TNBCs in relation with its marked induction of cell cycle arrest in the G(2)/M phase (29) and its effect on the cellular sensitivity to topoisomerase II inhibitors (30). Stimulation of topo IIα gene expression by P38 MAPKγ may contribute to increased topo IIα levels and enhanced antitumor activity of topo II inhibitors (24), therefore opening the field for the investigation of selective inhibitors of the P38 MAPK γ isoform in combination with chemotherapy.

p-ERK was detected in 73% of the tumor specimens, consistent with data reporting significant p-ERK expression in 69 to 96% of breast tumors (11,31,32). A low expression rate (35%) was only reported in one cohort (33). In our study, the expression of p-ERK was higher in high grade tumors (SBR3) consistent with data linking the activation of ERK with breast cancer cell proliferation (33).

p-AKT was detected in 38% of the tumor specimens and at a higher level in $HER2^+$ tumors, consistent with data previously published using IHC which reported p-AKT cytoplasmic and nuclear expression rates of 36 and 29%, respectively, in invasive ductal breast tumors and higher activation of AKT in $HER2^+$ tumors (34). In this study (34), a correlation was observed between nuclear p-AKT and nuclear ER and PR expression while no difference was observed for cytoplasmic p-AKT expression and cytoplasmic ER and PR expression. This is consistent with our data showing an absence of a correlation between either ER or PR and p-AKT expression since when using total protein extract analysis no difference can be determined between cytoplasmic and nuclear compartments.

As a whole, we did not find any correlation between p-AKT, p-ERK expression and P38MAPK or p-P38MAPK expression either in the total population of this study or in ER, HER2 or TNBC subgroups. Similar findings have been reported and no significant correlations were evidenced between ER and levels of p-P38MAPK, p-AKT, or p-ERK (10,11). Only several correlations have been reported between p-P38 MAPK and p-AKT and between p-P38 MAPK and p-ERK in a global population of tumor specimens from untreated patients analyzed using IHC (10). Similar to other research (11), we did not find any relationship between HER2 and p-ERK.

Collectively, our data indicate that the regulation of P38 MAPK was not directly linked to any of the investigated signaling pathways and could be considered as an independent biomarker in breast cancer. This also confirms the complexity of breast cancer oncogenesis, involving the recruitment of multiple signaling pathways.

In conclusion, P38 MAPK expression and activation are frequently observed in breast carcinoma and appear to be positively associated with the expression of the ER. Our results confirm the capability of breast cancer cells to activate P38 MAPK-mediated stress mechanisms and that P38 MAPK may represent a biological target for ER^+ breast cancer. The frequent concomitant activation of P38 MAPK, ERK and AKT indicates that breast tumor growth involves the activation of multiple signaling pathways, probably explaining the multiple mechanisms by which tumor cells develop resistance. Control of tumor growth should therefore entail the inhibition of all signaling pathways by combining multiple targeted therapies either in concomitant or in sequential use.

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