

## Mitogen-Activated Protein Kinase Phosphatase-1 in Human Breast Cancer Independently Predicts Prognosis and Is Repressed by Doxorubicin

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**Abstract Purpose:** Mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) dephosphorylates mitogen-activated protein kinase [extracellular signal-regulated kinase (ERK), c-Jun NH<sub>2</sub>-terminal kinase (JNK), and p38], mediates breast cancer chemoresistance, and is repressible by doxorubicin in breast cancer cells. We aimed to characterize doxorubicin effects on MKP-1 and phospho-MAPKs in human breast cancers and to further study the clinical relevance of MKP-1 expression in this disease.

**Experimental Design:** Doxorubicin effects on MKP-1, phospho-ERK1/2 (p-ERK1/2), phospho-JNK (p-JNK), and phospho-p38 were assayed in a panel of human breast cancer cells by Western blot and in human breast cancer were assayed *ex vivo* by immunohistochemistry ( $n = 50$ ). MKP-1 expression was also assayed in a range of normal to malignant breast lesions ( $n = 30$ ) and in a series of patients ( $n = 96$ ) with breast cancer and clinical follow-up.

**Results:** MKP-1 was expressed at low levels in normal breast and in usual ductal hyperplasia and at high levels in *in situ* carcinoma. MKP-1 was overexpressed in ~50% of infiltrating breast carcinomas. Similar to what was observed in breast cancer cell lines, *ex vivo* exposure of breast tumors to doxorubicin down-regulated MKP-1, and up-regulated p-ERK1/2 and p-JNK, in the majority of cases. However, in a proportion of tumors overexpressing MKP-1, doxorubicin did not significantly affect MKP-1 or phospho-MAPKs. With regard to patient outcome, MKP-1 overexpression was an adverse prognostic factor for relapse both by univariate ( $P < 0.001$ ) and multivariate analysis ( $P = 0.002$ ).

**Conclusions:** MKP-1 is overexpressed during the malignant transformation of the breast and independently predicts poor prognosis. Furthermore, MKP-1 is repressed by doxorubicin in many human breast cancers.

There is a need to find novel targets to improve the therapeutic options for breast cancer patients (1, 2). A recently proposed target is the mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1; refs. 3, 4). MAPKs, the substrates of MKPs, play important roles in proliferation, stress responses, apoptosis, and immune response (3–7). There are three well-known

MAPK subfamilies: extracellular signal-regulated kinases (ERK), c-Jun NH<sub>2</sub>-terminal kinases (JNK), and p38 MAPK isoforms. MAPKs are activated through a cascade of sequential phosphorylation events. The phosphorylation of MAPKs on threonine and tyrosine residues by specific upstream MAPK kinases (MEKs or MKKs) leads to their activated state.

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## Translational Relevance

There is increasing evidence on a role of the mitogen-activated protein kinase phosphatase-1 (MKP-1) as a mediator of *de novo* or acquired breast cancer resistance in human cell lines and the search of MKP-1 inhibitors is actively pursued. Here, we provide evidence on MKP-1 biology in human breast cancer at three levels: (a) MKP-1 is overexpressed during the malignant transformation of the breast, (b) MKP-1 overexpression is linked with poor patient outcome, and (c) MKP-1 is a repressible enzyme by doxorubicin in many human breast cancers. This repression is associated with an increase in the levels of phospho-extracellular signal-regulated kinase 1/2 and c-Jun NH<sub>2</sub>-terminal kinase. These results add clinical support to the concept of MKP-1 as a novel target for breast cancer therapy and justify further studies of MKP-1 as a promising prognostic marker.

Conversely, MKPs, also known as dual-specificity phosphatases, dephosphorylate MAPKs on tyrosine and threonine residues (3, 5, 8). The prototypic member of the family, MKP-1 is an inducible nuclear phosphatase able to dephosphorylate ERK, JNK, and p38. MKP-1 is induced by many of the same stimuli that activate MAPKs, including growth factors and stress (5). Furthermore, MAPKs can increase MKP-1 protein activity in two distinct ways: firstly, MKP binding to its MAPK target causes a subtype-specific enhancement of its catalytic activity (9), and secondly, by phosphorylation by ERK, which inhibits MKP-1 degradation through the ubiquitin pathway (10). This mechanism is viewed as a feedback control to attenuate MAPK signaling (4, 5, 11, 12). MKP-1 seems to play an important role in tumorigenesis (4, 13, 14) and counterbalances the cytotoxicity of various anticancer drugs (4, 14–21). In this regard, anthracyclines, alkylating agents, taxanes, cisplatin, or proteasome inhibitors induce apoptosis in part by activation of the JNK pathway (15, 16). Notably, high levels of MKP-1 may dephosphorylate JNK and therefore limit the cytotoxicity of these agents (14, 16, 17, 19–24). Conversely, down-modulation of MKP-1 might be proapoptotic by facilitating a persistent JNK phosphorylation (4, 17).

Several reports suggest MKP-1 as a potential target in breast cancer. In breast cancer cells, MKP-1 was a significant mediator of chemoresistance to anthracyclines, alkylating agents, and taxanes (15, 18, 19). Proteasome inhibitors induce MKP-1 and this induction played an antiapoptotic role (16, 20–23). Dexamethasone also induced MKP-1 and limited paclitaxel cytotoxicity (18, 19). In contrast, anthracyclines decrease expression of MKP-1 (15, 24). This repression is involved in the potentiation of the cytotoxicity of alkylating agents by anthracyclines (15). Aplidin also down-modulates MKP-1 (25). Complementing preclinical data, MKP-1 is overexpressed in human breast cancers (26, 27). These observations justify an interest in MKP-1 inhibitors for cancer therapy (3, 4, 19, 28, 29).

Here, we aimed to further characterize MKP-1 and phospho-MAPK regulation by doxorubicin in breast cancer cells and in an *ex vivo* model (30, 31). We also analyzed the

expression of MKP-1 in breast malignant transformation and in breast cancer patients with clinical follow-up.

## Materials and Methods

**Reagents, antibodies, and cells.** Doxorubicin and Ro-31-8220 (both from Calbiochem; ref. 32) were purchased for use. Doxorubicin was freshly dissolved in water and Ro-31-8220 was dissolved in DMSO at stock concentrations of 10 mmol/L. Elite avidin-biotin complex method kit was from Vector Laboratories. Enhanced chemiluminescence detection kit was from Amersham Pharmacia Biotech. All tissue culture materials were from Life Technologies. The following antibodies were used: anti-phospho-ERK1/2 (p-ERK1/2; Thr<sup>202</sup>/Tyr<sup>204</sup>), anti-ERK1/2, anti-JNK, anti-p38 MAPK, and anti-phospho-p38 (p-p38) MAPK (all from Cell Signaling Technology). Anti-phospho-JNK (p-JNK; Thr<sup>183</sup>/Tyr<sup>185</sup>) from Promega and Cell Signaling Technology was used for immunohistochemical and Western blot analysis, respectively. To detect MKP-1, two antibodies were used: one for Western blot and one for immunohistochemical assays. Antibodies to detect expression of estrogen receptor, progesterone receptor, and HER2 (HercepTest) were purchased from Dako. HER2 amplification was assayed by HER2 fluorescence *in situ* hybridization pharmDx (Dako). The human breast cancer cell lines BT-474, SK-BR3, MDA-MB-468, MDA-MB-453, MDA-MB-231, and MCF-7 (from the American Type Culture Collection) were maintained as previously reported (9).

**Real-time quantitative reverse transcription-PCR.** Specific PCR primer combinations and fluorogenic probes (5'-FAM, 3'-TAMRA) for the target MKP-1 mRNA and housekeeping RNA (endogenous control, 18S,  $\beta$ -actin, and RPLPO) were purchased from Applied Biosystems. Total RNA was isolated using RNeasy kit (Qiagen) and reverse transcribed to cDNA using SuperScript reverse transcriptase (Invitrogen). cDNAs were combined with primers and probes specific for each gene of interest along with predeveloped Taqman Gene Expression Master Mix (Applied Biosystems) for the following genes: *MKP-1* (Hs-00610256-g1), human  $\beta$ -actin (Hs99999903\_m1), human 18S (Hs99999901\_s1), and human *RPLPO* (large ribosomal protein PO; Hs99999902\_m1). The PCR protocol was 50°C for 2 min and 95°C for 10 min followed by 50 cycles at 95°C for 15 s and at 60°C for 1 min. Negative controls were included and yielded no products. Real-time PCR analysis was carried out on an ABI-7500HT. C<sub>t</sub> values were determined using SDS v2.2 software (Applied Biosystems) and compared using the C<sub>t</sub> method.

**Western blot analysis.** Western blot analysis in cultured cells was done following as previously reported (20). Briefly, cells were washed in PBS and scraped, and whole-cell lysates were prepared. Frozen breast tumor samples were analyzed by Western blot, also as described previously (33).

**Cell viability assay in vitro.** Cells were seeded into six-well plates at  $3 \times 10^5$  per well and allowed to adhere overnight. Cells were then treated as indicated in Results. The dishes were incubated for 21 h before trypsinization and counting using trypan blue and/or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (9).

**Patient data and tissue specimens for clinicopathologic analysis.** The study was approved by the ethics committee of the hospital and conducted following institutional guidelines. For clinicopathologic correlations and outcome analysis, retrospective samples were needed. To this end, formalin-fixed paraffin-embedded breast cancer samples obtained from surgical specimens from patients with biopsy-proven breast cancer were retrieved. Normal breast tissues, hyperplastic lesions, and *in situ* carcinomas from the same specimens were also assessed. In addition, selected paired formalin-fixed paraffin-embedded and frozen samples were obtained. Tumor-node-metastasis (TNM) staging was classified using the American Joint Committee on Cancer staging system for breast cancer (34). Histologic grades were defined according to Scarff-Bloom-Richardson modified by Elston criteria (35). Estrogen and progesterone receptors and HER2 status were determined by immunohistochemistry

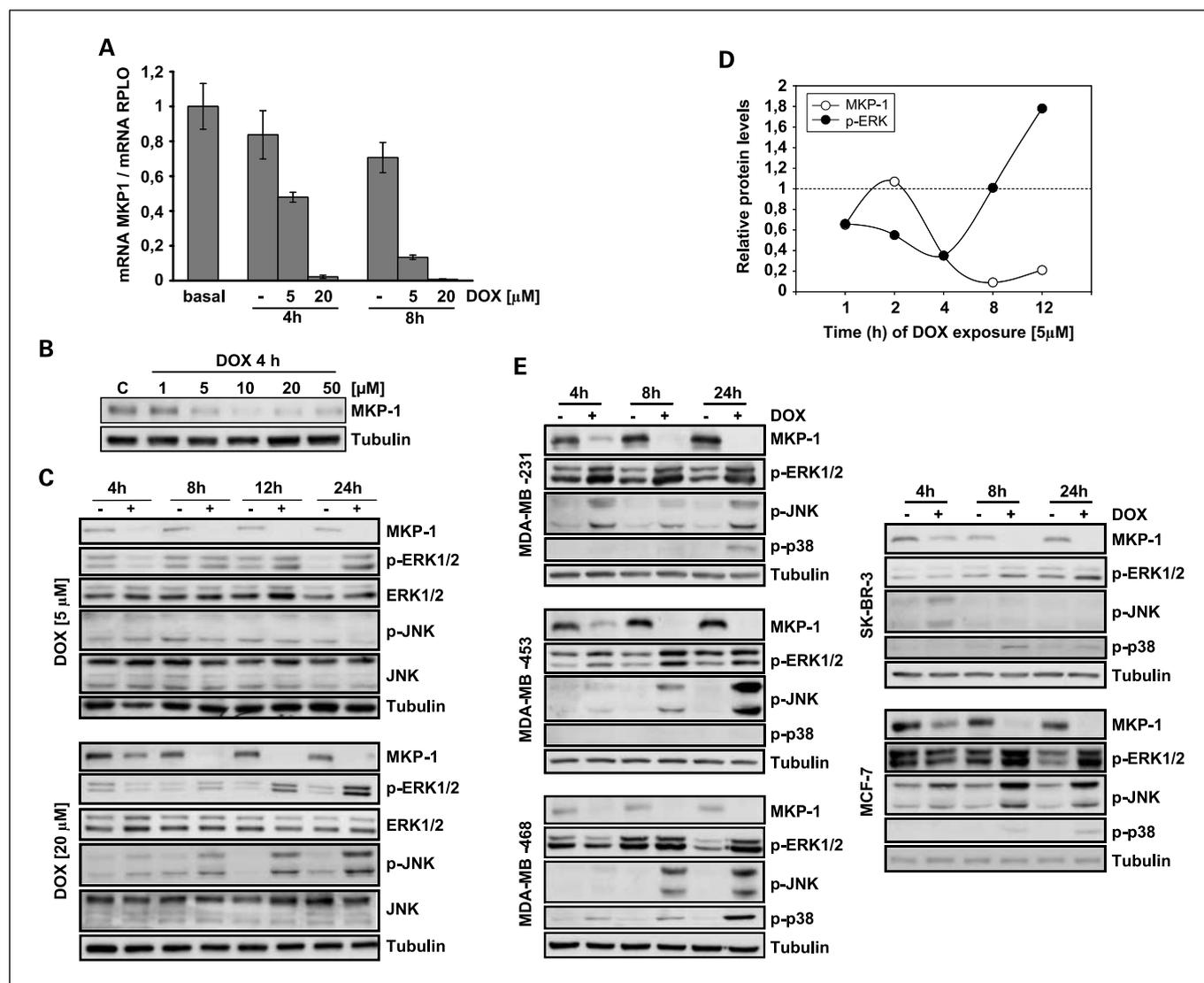
or fluorescence *in situ* hybridization (36). Clinical data and follow-up were obtained from review of patient's medical records. Disease relapse was considered as any primary, regional, or distant recurrence, as well as the appearance of a secondary tumor.

A tissue microarray was also constructed. Three tissue cores (1-mm diameter) were obtained from each specimen. In addition, 30 complete sections were assayed for same markers to correlate with observed tissue microarray expression. The tissue cores were precisely arrayed into a new paraffin block guided by a defined x-y position using a tissue microarray workstation (T1000; Chemicon) as reported in the literature (37).

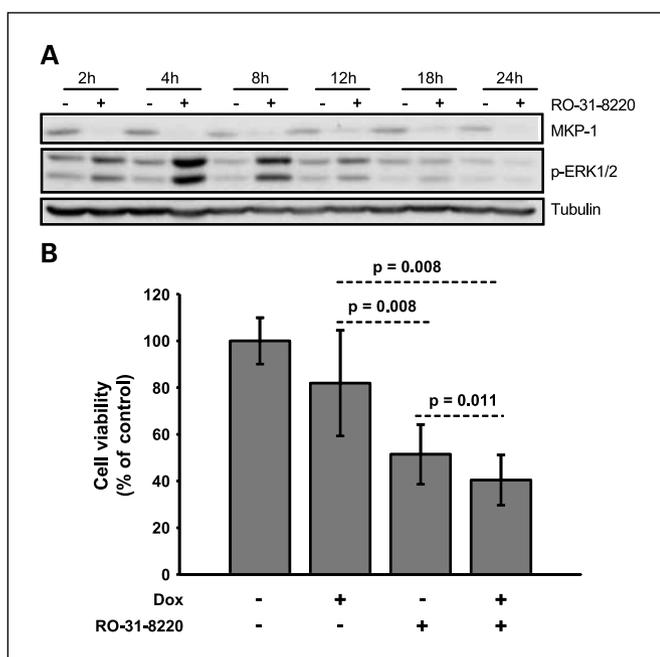
**Exposure of human breast cancers to doxorubicin ex vivo.** In addition to retrospective formalin-fixed paraffin-embedded specimens, fresh breast cancer specimens were obtained to add *ex vivo* doxorubicin and assess its molecular effects (30, 31). Tissue slices, which were not needed for diagnostic purposes, from primary breast tumors larger than

1.5 cm were obtained from surgical specimens of patients newly diagnosed with invasive breast cancer. One slide (control sample) was put into culture medium, and a second slice (treated sample) was put on the same culture medium plus doxorubicin at 5  $\mu\text{g}/\text{mL}$  (38). Incubation was done in 24-well plates at 37°C in a constant atmosphere of 5% CO<sub>2</sub> for 24 h. At 24 h, specimens were fixed in 10% neutral-buffered formalin for 16 h and embedded in paraffin under vacuum conditions. A full report of this method will be reported separately. Specimens were then assayed by immunohistochemistry.

**Immunohistochemistry.** Immunostaining was done using 3- $\mu\text{m}$  tissue sections, placed on plus charged glass slides. After deparaffinization in xylene and graded alcohols, heat antigen retrieval was done in buffered solutions: pH 9 EDTA-based buffer (Dako) was used to detect MKP-1, JNK, and p-JNK; pH 8 citrate-based buffer (Ventana) was used to detect ERK1/2, p-ERK1/2, p-p38, and p38. Endogenous peroxidase was blocked by immersing the sections in 0.03% hydrogen peroxide for 5 min. Slides



**Fig. 1.** Doxorubicin (DOX) effects on MKP-1 and phospho-MAPK expression in BT-474 breast cancer cells. **A**, levels of MKP-1 mRNA after doxorubicin exposure, measured by quantitative multiplex real-time reverse transcription-PCR. cDNA was synthesized from RNA samples from control- and doxorubicin-exposed cells. MKP-1 expression values are relative to the levels of a control RNA (RPLPO). Similar results were obtained with  $\beta$ -actin and 18S RNA. **B**, levels of MKP-1 protein in BT-474 cells exposed to increasing concentrations of doxorubicin, as assayed by Western blot. Tubulin was used as loading control. **C**, time course effects of doxorubicin (5 and 20  $\mu\text{mol}/\text{L}$ ) on MKP-1 and total and phosphorylated protein levels of MAPKs (ERK1/2 and JNK), as assayed by Western blot. No effects on p-p38 were seen (data not shown). Tubulin was used as loading control. **D**, graphic representation of a densitometric analysis of MKP-1 and p-ERK1/2 protein levels. **E**, time course effects of doxorubicin on MKP-1 and phospho-MAPKs (ERK1/2, JNK, and p38) in a panel of breast cancer cell lines, as assayed by Western blot. Tubulin was used as loading control.



**Fig. 2.** A, time course of Ro-31-8220 (a nonspecific MKP-1 inhibitor) 3 h before treatment followed by a washout on MKP-1 and p-ERK1/2 in BT-474 cells, as assayed by Western blot. Please note that at 24 h the effects on p-ERK1/2 are reversed. B, effect of Ro-31-8220 before treatment on cell viability after 48 h of doxorubicin treatment, as assayed by manual trypan blue exclusion method.

were washed for 5 min with TBS solution containing Tween 20 at pH 7.6 and incubated with the primary antibodies for 60 min at room temperature followed by incubation with the appropriate anti-Ig horseradish peroxidase-conjugated EnVision polymer (Dako) or peroxidase-conjugated OmniMap multimer (Ventana) to detect antigen-antibody complexes. Sections were then visualized with 3,3'-diaminobenzidine as a chromogen for 5 min and counterstained with hematoxylin. All immunohistochemical stainings were done in Dako Link or Discovery XT platform and the same sections incubated with nonimmunized serum were used as negative controls. As positive control, sections of a breast human tumor with known marker expression were stained. In addition to human specimens, tissues (kidney and liver) for validation of MKP-1 immunohistochemical assay were obtained from wild-type and MKP-1 gene knockout mice (39), generously provided by Bristol-Myers Squibb Co., and processed in the same way.

Expression of markers was assessed in a blinded fashion by two investigators. For MKP-1 and MAPKs (ERK1/2, p-ERK1/2, JNK, p-JNK, p38, and p-p38), nuclear staining was required for considering a cell as positive. As we previously reported for phosphorylated forms of kinases, a tumor was scored as positive for a given phosphorylated protein when any percentage of tumor cells was stained (33, 40). In addition, a semiquantitative histoscore (Hscore) was calculated. The Hscore was determined by estimation of the percentage of tumor cells positively stained with low, medium, or high staining intensity. The final score was determined after applying a weighting factor to each estimate. The formula used was  $Hscore = (low\%) \times 1 + (medium\%) \times 2 + (high\%) \times 3$ , and the results ranged from 0 to 300.

**Statistics.** Statistical analysis was carried out with Statistical Package for the Social Sciences version 13.0 (SPSS, Inc.). To analyze correlations between MKP-1 expression and clinicopathologic variables, we used the  $\chi^2$  test (Fisher's exact test) or Mann-Whitney test. A paired *t* test was used to compare molecular marker scores (immunohistochemistry) between control-treated and *ex vivo*-treated breast cancers. Effects of doxorubicin in breast specimens *ex vivo* were also expressed by means of unsupervised hierarchical clustering using the agglomeration rule

average linkage that placed cases and marker results next to each other if they were most similar in their immunohistochemical profiles (41, 42). Disease recurrence was analyzed by the Kaplan-Meier method. Curves were compared by the log-rank test. Multivariate analysis, including continuous quantitative and qualitative clinicopathologic parameters, was done using the Cox proportional hazards model. All the statistical tests were conducted at the two-sided 0.05 level of significance.

## Results

**Doxorubicin affects MKP-1 and phospho-MAPK in breast cancer cells.** We first assessed doxorubicin effects in BT-474 cells. As assayed by MTS at 48 hours, doxorubicin  $IC_{25}$  was  $\sim 5 \mu\text{mol/L}$ ,  $IC_{50}$  was  $\sim 20 \mu\text{mol/L}$ , and  $IC_{75}$  was  $\sim 50 \mu\text{mol/L}$  in BT-474 (data not shown). A concentration- and time-dependent reduction of MKP-1 transcripts was observed on doxorubicin exposure, as assayed by reverse transcription-PCR (Fig. 1A) MKP-1 protein levels also decreased, with an evident effect at doxorubicin concentrations  $\geq 5 \mu\text{mol/L}$  (Fig. 1B). MKP-1 protein down-regulation was observed at 4 hours (no effects were detected at earlier time points; data not shown) and was maximal at 8 hours (Fig. 1C). This inhibition was maintained at 24 hours, the latest time point tested. The decrease in MKP-1 preceded an increase in phosphorylated forms of MAPK family members. p-ERK1/2 expression increased in a time- and dose-dependent manner (Fig. 1C). Induction of p-JNK was observed at doxorubicin concentrations of  $\geq 20 \mu\text{mol/L}$  (Fig. 1C, bottom, and experiments at  $50 \mu\text{mol/L}$  not shown). p-p38 was undetected in BT-474 cells in all the conditions tested (data not shown). No evident changes were observed in total ERK1/2, JNK, and p38 proteins. A subtle reduction in p-ERK1/2 levels was observed at early time points (1-4 hours; Fig. 1D), which may decrease MKP-1 protein stability and perhaps contribute to MKP-1 down-modulation (10, 43). These kinetic effects were also observed at higher concentrations (data not shown). We next assayed five additional breast cancer cell lines (SK-BR3, MDA-MB-231, MDA-MB-468, MDA-MB-453, and MCF-7). In all of them, MKP-1 was down-modulated by doxorubicin and p-ERK1/2 and p-JNK were induced (Fig. 1E), as observed in BT-474 cells. In four of these cell lines, p-p38 was induced by doxorubicin (Fig. 1E).

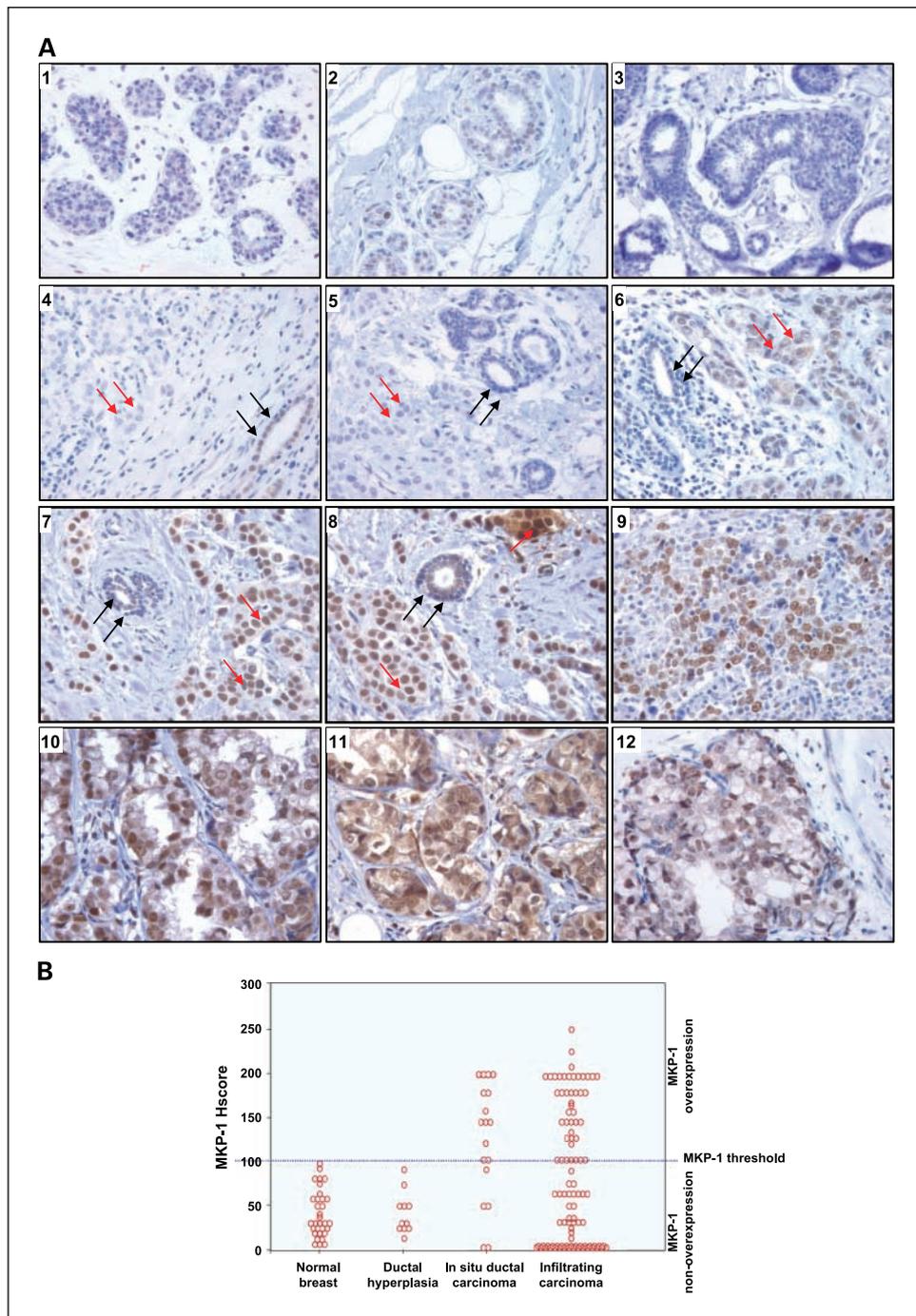
We then combined doxorubicin with Ro-31-8220, an agent originally synthesized as a protein kinase C inhibitor, which reduces MKP-1 expression (32). A short exposure to Ro-31-8220 (3 hours followed by a washout) markedly reduced MKP-1 and induced p-JNK and p-ERK1/2. p-ERK1/2 levels returned to baseline at 24 hours (Fig. 2A). A combined treatment of doxorubicin and Ro-31-8220 resulted in a modest, but significant, additional reduction of cell viability compared with each agent used alone (Fig. 2B).

**Validation of MKP-1 immunohistochemical assay and pattern of expression in normal and pathologic breast lesions.** We tested the sensitivity and specificity of several anti-MKP-1 commercially available antibodies by immunocytochemistry (data not shown) and immunohistochemistry. A polyclonal anti-MKP-1 antibody was chosen based on results on tissues from wild-type and knockout MKP-1 mice (data not shown; ref. 39) as well as results in human tumor samples. Preabsorption with a specific antigen peptide resulted in a negative staining (data not shown). In addition, frozen human tumor samples assayed

by Western blot had expression levels that corresponded closely to the levels observed by immunohistochemistry (data not shown).

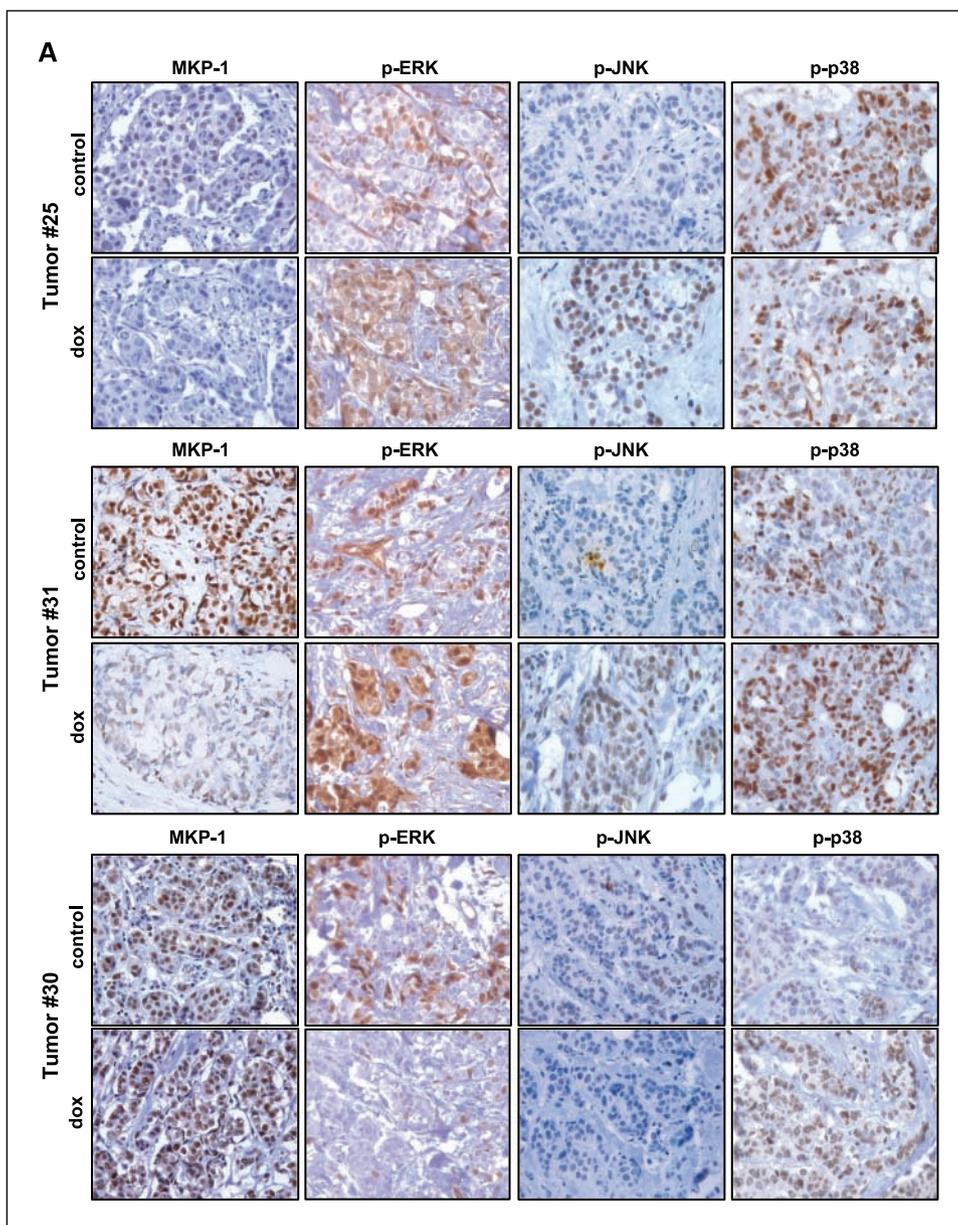
We then assayed 30 complete tissue sections that included histologic normal breast ( $n = 30$ ) as well as hyperplastic ( $n = 11$ ), *in situ* ( $n = 18$ ), and infiltrating ( $n = 30$ ) malignancies (Fig. 3A). Histologically normal breast ductal and lobular epithelial cells exhibited diffuse and weak MKP-1 staining in the nuclei. In myoepithelial cells, MKP-1 was undetected and a low level of MKP-1 expression was seen in fibroblasts and endothelial cells. In usual ductal hyperplasia,

the expression was similar to normal breast epithelium. All cases of benign breast tissue (normal and hyperplasia) had a MKP-1 Hscore  $<100$ . This Hscore value was selected to define the threshold of MKP-1 overexpression (Fig. 3B). In contrast, in all *in situ* carcinomas, MKP-1 was overexpressed (Fig. 3A and B). In infiltrating carcinoma, MKP-1 was overexpressed in up to 50% (Fig. 3A and B). In some specimens that had both *in situ* and infiltrating carcinoma, there was overexpression of MKP-1 in the *in situ* carcinoma, whereas it was not overexpressed in the infiltrating areas. Similar results in MKP-1 staining were observed in tissue microarray



**Fig. 3.** A, representative pictures showing a representative range of MKP-1 expression levels observed by immunohistochemistry in human breast tissues. Histologically normal epithelial cells (1 and 2), as well as usual ductal hyperplasia (3), exhibited weak and diffuse nuclear MKP-1 staining. In infiltrating carcinoma cells, there was a wide range of expression levels, from undetected/low (4 and 5) to increasingly high MKP-1 staining (4-11). In pictures 4 to 8, infiltrating carcinoma areas are pointed by red arrows and adjacent histologically normal breast by black arrows. In pictures 9 to 11, only infiltrating carcinoma areas are shown. Picture 12 shows an *in situ* carcinoma with high MKP-1 expression. B, scatter plot by categories, displaying the levels of MKP-1 expression (Hscore) in normal breast, ductal hyperplasia, *in situ* ductal carcinoma, and infiltrating carcinoma.

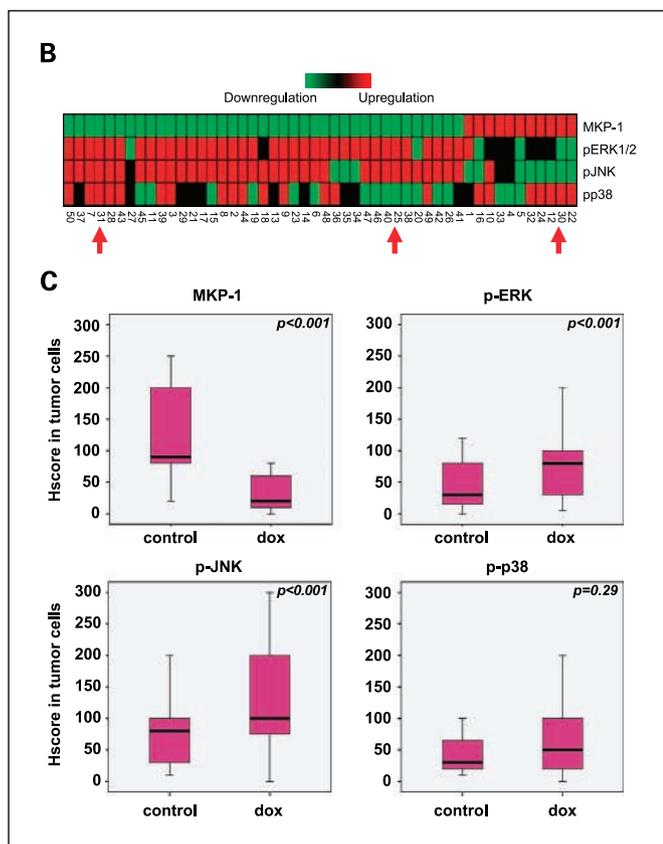
**Fig. 4.** Effects of doxorubicin, added *ex vivo* to fresh human breast cancer sections, on MKP-1 and phospho-MAPKs, as assayed by immunohistochemistry. A, three representative examples are shown. In tumor 25, doxorubicin resulted in an almost complete loss of detectable MKP-1 expression. p-ERK1/2 and p-JNK expression increased, whereas p-p38 expression was reduced by doxorubicin. In tumor 31, doxorubicin down-modulated MKP-1. p-ERK1/2, p-JNK, and p-p38 were up-regulated. In tumor 30, MKP-1 expression was slightly increased on doxorubicin exposure. p-ERK1/2 and p-JNK were down-regulated and p-p38 was up-regulated.



cores compared with the corresponding full tissue sections (data not shown).

**Doxorubicin represses MKP-1 expression in a large subset of human breast cancers.** Doxorubicin effects, as a single agent, on MKP-1 and phospho-MAPKs were assayed *ex vivo* in a series of 50 fresh breast cancer specimens under controlled experimental conditions (Fig. 4). In the whole series, doxorubicin induced a significant decrease in MKP-1 [mean Hscore MKP-1,  $117 \pm 64$  (SD) in control versus  $72 \pm 104$  in treated specimens;  $P < 0.001$ ]. p-JNK and p-ERK1/2 expression increased significantly by doxorubicin (Hscore p-ERK1/2,  $49 \pm 47$  in control versus  $87 \pm 65$  in treated specimens;  $P < 0.001$ ; Hscore p-JNK,  $72 \pm 50$  in control versus  $126 \pm 82$  in treated specimens;  $P < 0.001$ ). p-p38 expression was numerically higher in doxorubicin-treated samples, but differences were not statistically significant ( $P = 0.29$ ). Overall, this pattern (Fig. 4B and C) resembled the one observed in breast cancer cell lines (Fig. 1).

Effects, however, were not uniform; MKP-1 levels decreased on doxorubicin exposure in 39 specimens, whereas in the remaining 11 specimens the levels slightly increased (Fig. 4). Only in tumors that down-modulated MKP-1 levels there was a significant increase in p-ERK1/2 ( $P < 0.001$ ) and p-JNK ( $P < 0.001$ ). We then explored whether MKP-1 baseline levels might be linked to the ability of doxorubicin to down-regulate this protein. In the 27 tumors nonoverexpressing MKP-1, doxorubicin further down-modulated MKP-1 (MKP-1 Hscore,  $69 \pm 21$  in control versus  $13 \pm 14$  in treated specimens;  $P = 0.03$ ). In this subset, a significant up-regulation of p-ERK1/2 and p-JNK was observed (p-ERK1/2 Hscore,  $38 \pm 44$  in control versus  $96 \pm 71$  in treated specimens;  $P < 0.001$ ; p-JNK Hscore,  $58 \pm 44$  in control versus  $150 \pm 65$  in treated specimens;  $P < 0.001$ ). In contrast, in the series of 23 tumors overexpressing MKP-1, doxorubicin did not significantly change MKP-1 expression ( $P = 0.24$ ) and did not



**Fig. 4.** Continued. B, hierarchical cluster analysis (41, 42) of doxorubicin effects on MKP-1 and phospho-MAPKs in a total of 50 human breast cancers. Each column represents an effect on expression of the paired control- and doxorubicin-treated samples, and each row, a single immunohistochemical marker, including MKP-1, p-ERK1/2, p-JNK, and p-p38. Down-regulation of expression is displayed in green, up-regulation in red, and no effect in black. Red arrows point the breast tumors displayed in A. C, box plot graphs indicating the immunohistochemical results for MKP-1 and phospho-MAPKs in control- and doxorubicin-treated conditions from *ex vivo* human breast cancers in the entire series ( $n = 50$ ). The horizontal bar indicates median Hscore, the box represents the interquartile range, and the whiskers above and below the box show the minimum and maximum within a category. A  $P$  value for each marker is shown.

significantly affect p-ERK1/2 ( $P = 0.23$ ), p-JNK ( $P = 0.72$ ), or p-p38 ( $P = 0.32$ ).

**Expression of MKP-1, phospho-MAPKs, and clinicopathologic breast cancer features.** Tumor specimens from paraffin-embedded blocks, with enough available tissue for the studies, were retrospectively selected from consecutive newly diagnosed breast cancer patients that had the following criteria: infiltrating ductal or lobulillar carcinomas, nonmetastatic disease at diagnosis, diagnosed between 1998 and 2000, and available clinical follow-up. A total of 96 patients with this criteria were selected (Table 1). There were 78 ductal and 18 lobulillar infiltrating carcinomas. Stage was I in 42 patients, II in 28 patients, and III in 20 patients (unknown in 6). Additional clinicopathologic characteristics are shown in Table 1. All patients had surgical treatment. Forty-four patients received systemic treatment (chemotherapy, antihormonal therapy, or both) according to our clinical guidelines (patients were not part of any clinical trial). Patients were followed at the medical oncology department (median follow-up, 69 months; range, 9-128). A total of 18 patients had a relapse.

Forty-three (45%) specimens overexpressed MKP-1 and 53 (55%) were nonoverexpressors (Fig. 3B). Mean MKP-1 Hscore was  $85.3 (\pm 79.5 \text{ SD})$ . The following characteristics were numerically more frequent in tumors with MKP-1 overexpression: lobular infiltrating carcinoma, grade III, negative estrogen receptors, negative progesterone receptors, HER2 overexpression/amplification, large primary tumors, lymph node metastasis, and advanced TNM stage. However, none of these characteristics was significantly correlated with MKP-1 overexpression (Table 1). Twenty-four of 44 (54%) patients that received systemic treatment had MKP-1-overexpressing tumors, whereas in the 52 that did not receive adjuvant treatment, 17 (33%) had MKP-1 overexpression ( $P = 0.16$ ). The more frequent use of adjuvant treatment in patients with MKP-1 overexpression may reflect that this was more common (albeit without reaching statistical significance) in patients with adverse prognostic features (Table 1). We also assessed the interplay between MKP-1 and phospho-MAPKs. All infiltrating breast cancers expressed some degree of p-ERK1/2, p-JNK, and p-p38. The mean Hscores and SD for these MAPKs were as follows: p-ERK1/2,  $58.3 \pm 58.7$ ; p-JNK,  $32.7 \pm 47.7$ ; and p-p38,  $120.6 \pm 76.7$ . Breast cancers overexpressing MKP-1 had slightly higher Hscore values of p-ERK1/2 ( $61.4 \pm 54.2$  in MKP-1 overexpressors versus  $55.8 \pm 62.4$  in nonoverexpressors;  $P = 0.020$ ) but had lower Hscore values of p-p38 ( $95.5 \pm 71.6$  versus  $140.9 \pm 75.4$ ;  $P = 0.005$ ). There were no significant differences in p-JNK ( $29.6 \pm 29.7$  versus  $35.2 \pm 41.6$ ;  $P = 0.98$ ) with regard to MKP-1 expression.

**Overexpression of MKP-1 and breast cancer relapse.** Disease-free survival analysis showed a higher risk of relapse in patients with MKP-1 overexpression (Fig. 5;  $P < 0.001$ , log-rank test). Kaplan-Meier survival curves for relapse and log-rank test comparisons also showed that high primary tumor stage ( $P = 0.035$ ), tumor grade ( $P = 0.013$ ), tumor size ( $P = 0.025$ ), axillary lymph node involvement ( $P < 0.001$ ), negative estrogen receptor status ( $P = 0.008$ ), negative progesterone receptor status ( $P = 0.004$ ), and low p-p38 expression ( $P < 0.001$ ) were associated with a higher risk of relapse. A multivariate analysis was done including all the baseline clinicopathologic factors (Table 1) and MKP-1. In this analysis, MKP-1 overexpression ( $P = 0.016$ ) retained its adverse prognostic role. For the clinicopathologic factors, age ( $P = 0.15$ ), tumor size ( $P = 0.50$ ), and nodal status ( $P = 0.10$ ) were also significant in this multivariate analysis, whereas estrogen receptor ( $P = 0.60$ ), progesterone receptor ( $P = 0.99$ ), HER2 ( $P = 0.94$ ), TNM stage ( $P = 0.72$ ), grade ( $P = 0.49$ ), and menopausal status ( $P = 0.19$ ) were not significant. Because p-p38 was also significant by univariate analysis, we did a second multivariate analysis, including MKP-1, p-p38, and all the clinicopathologic factors mentioned above. In this analysis, MKP-1 again retained its independent value ( $P = 0.015$ ), whereas p-p38 lost the significance ( $P = 0.13$ ). None of the patients with tumors nonoverexpressing MKP-1 had died at the time of analysis, thus precluding comparisons of overall survival.

## Discussion

We report that in human breast cancers, exposed *ex vivo* to doxorubicin, MKP-1 is a regulable enzyme, and in the majority of specimens, doxorubicin significantly reduced its expression. This event was coupled with phosphorylation of JNK and

ERK1/2, thus resembling the pattern reported in breast cancer cell lines. Results in a range of breast lesions also pointed to MKP-1 overexpression as a common event during breast malignant transformation. Finally, MKP-1 overexpression was an independent adverse prognostic factor in breast cancer patients.

Induction of MKP-1 by chemotherapeutic drugs, such as cisplatin or alkylating agents, and proteasome inhibitors limits their cytotoxicity, mainly by inactivation of JNK (3, 4, 14, 15, 44). However, other agents, such as doxorubicin (15) or aplidin (25), repress MKP-1, and this is viewed as part of their antitumor mechanisms of action by allowing a more efficient induction of p-JNK. We confirmed that doxorubicin decreased in a concentration- and time-dependent manner MKP-1 transcripts and protein levels in breast cancer cells. In all the cell lines tested, doxorubicin induced p-JNK and p-ERK1/2. The role of p-JNK as a mediator of apoptosis is well established, whereas the consequences of induction of p-ERK1/2 by doxorubicin are less clear. Although p-ERK1/2 is generally coupled with cell proliferation, a persistent p-ERK1/2 induction may be linked to apoptosis (45). It has to be noted that MKP-1 down-

modulation is not the sole mechanism of ERK1/2 activation by doxorubicin. In this regard, the generation of oxygen free radicals by doxorubicin is involved in ERK1/2 activation (29). p-p38 increased in four of the six lines tested. It is possible that in some breast cancer cells, p38 activity is minimally affected by MKP-1 (18).

We evaluated doxorubicin effects, added *ex vivo*, on MKP-1 in 50 fresh human breast cancers (30, 31). Doxorubicin induced a significant decrease in MKP-1 and an increase in p-JNK and p-ERK1/2. In contrast, p-p38 did not change significantly. This pattern resembles the one observed in breast cancer cell lines. In another study, dexamethasone induced MKP-1 and inhibited p-ERK1/2 and p-JNK. However, p-p38 levels were not significantly affected (18). These observations indicate that the correlation between MKP-1 and p-ERK1/2, p-JNK, and p-p38 in breast cancer is not always straightforward. This variability may be related to doxorubicin effects on MAPKs other than via MKP-1 (16), and also because depending on cell type and context, MKP-1 might dephosphorylate and inactivate any of the three MAPKs (4, 5). Interestingly, only in the tumors that down-modulated MKP-1 level there was a significant increase in p-ERK1/2 and p-JNK. Furthermore, doxorubicin decreased proliferation rates only in tumors with MKP-1 down-modulation (data not shown). Full data on proliferation will be reported in a separate methods manuscript.<sup>9</sup> There was a subset of tumors with high basal MKP-1 expression in which doxorubicin did not down-regulate, or slightly increased, MKP-1, for as yet unknown reasons. Of note, in breast cancer cells genetically expressing high, irrepressible levels of MKP-1, the ability of doxorubicin to enhance the cytotoxicity of alkylating agents was lost (15). Regardless of mechanisms, we hypothesized that lowering the level of MKP-1 might enhance the ability of doxorubicin to increase its cytotoxicity. Supporting this notion, BT-474 cells pretreated with Ro-31-8220, an agent that reduces MKP-1 expression, were more sensitive to doxorubicin. This finding agrees with an enhanced cytotoxicity of doxorubicin in breast cancer cells with genetic suppression of MKP-1 and in MKP-1 knockout mouse embryo fibroblasts (15). However, due to the nonspecific effects of Ro-31-8220, a variety of mechanisms might explain our result (32).

A role of MKP-1 in chemoresistance has been reported for cisplatin in lung and ovarian cancer (43, 44, 46). We have observed an enhancement of cisplatin cytotoxic effects in human non-small cell lung cancer cells when the activity of both MKP-1 and nuclear factor- $\kappa$ B was blocked.<sup>10</sup> MKP-1 is also induced in response to radiation and limits its apoptotic effects (47). Adding to the complexity of the field, other members of the MKP family are also involved in drug resistance. In this regard, MKP-3 is overexpressed in breast cancer and mediates resistance to tamoxifen (48).

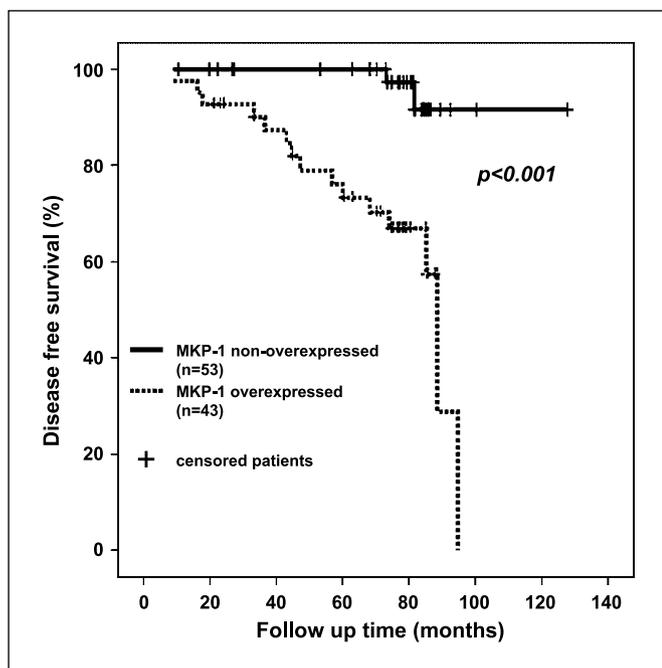
To date, there were two reports on MKP-1 expression in human breast cancer (26, 27). In one study, MKP-1 was overexpressed in the early phases of prostate, colon, and bladder carcinogenesis, with progressive loss of expression with higher histologic grade and in metastases (26). In contrast, breast carcinomas showed significant MKP-1 expression even when poorly differentiated or in late stages of the disease. MKP-1 and

**Table 1.** Patient clinicopathologic characteristics and MKP-1 overexpression

Characteristic (no. patients)	Overexpressing MKP-1, n (%)	P
Total series of patients (n = 96)	43 (45)	
Menopausal status		0.98
Premenopausal (n = 13)	6 (46)	
Postmenopausal (n = 72)	33 (46)	
Unknown (n = 11)	4 (36)	
Infiltrating tumor type		0.12
Ductal (n = 78)	32 (41)	
Lobullillar (n = 18)	11 (61)	
Tumor grade		0.55
1 (n = 24)	10 (42)	
2 (n = 46)	19 (41)	
3 (n = 26)	14 (54)	
Estrogen receptor		0.59
Negative (n = 20)	10 (50)	
Positive (n = 76)	33 (43)	
Progesterone receptor		0.08
Negative (n = 28)	16 (57)	
Positive (n = 68)	27 (40)	
HER2		0.72
Negative (n = 77)	33 (43)	
Positive (n = 19)	10 (53)	
Primary tumor size		0.45
T1 (n = 48)	19 (40)	
T2 (n = 37)	17 (46)	
T3 (n = 7)	5 (71)	
T4 (n = 4)	2 (50)	
Node stage		0.14
N0 (n = 57)	22 (39)	
N1 (n = 18)	10 (56)	
N2 (n = 10)	4 (40)	
N3 (n = 6)	5 (83)	
NX (n = 5)	2 (40)	
TNM stage		0.22
I (n = 42)	15 (36)	
II (n = 28)	14 (50)	
III (n = 20)	12 (60)	
Unknown (n = 6)	2 (33)	

<sup>9</sup> F. Rojo et al., in preparation.

<sup>10</sup> Cortes et al., submitted for publication.



**Fig. 5.** Actuarial disease-free survival for patients with tumors overexpressing MKP-1 ( $n = 43$ ) versus those nonoverexpressing MKP-1 ( $n = 53$ ), calculated by Kaplan-Meier analysis. See Fig. 3B for numerical data of MKP-1 expression in the entire series.

ERK1/2 were coexpressed in most tumors (26). ERK1 enzymatic activity was elevated despite MKP-1 overexpression. No loss of 5q35-ter (containing the MKP-1 locus) was detected by PCR in metastases compared with primary tumors and no mutations were found in the catalytic domain of MKP-1. MKP-1 was overexpressed in advanced disease stages of breast cancer (26, 27). Our work supports the notion that overexpression of MKP-1 occurs during the malignant transformation of the breast (26). Histologically normal breast ductal and lobular epithelial cells typically exhibited faint and diffuse MKP-1 nuclear staining. In contrast, in *in situ* carcinoma, MKP-1 was almost uniformly overexpressed. In infiltrating carcinoma, MKP-1 overexpression occurred in ~50% of the specimens. Notably, in some specimens, there was overexpression of MKP-1 in the *in situ* carcinoma, whereas it was not overexpressed in the infiltrating areas. This finding agrees with a prior study (26) and is also similar to the pattern reported for HER2 (49). In infiltrating breast cancers, several adverse clinicopathologic features were more

frequent in MKP-1-overexpressing tumors, but there were no statistically significant associations. We also assessed the interplay between MKP-1 and phospho-MAPKs. A significant association was found between MKP-1 overexpression and low p-p38 as well as with high p-ERK1/2. Similarly to our findings, high expression and activity of ERK has been reported in MKP-1-positive breast cancers, suggesting that ERK might be driving, at least in part, MKP-1 expression in unperturbed conditions (10, 26). Decreased activity of JNK1 has been also observed in breast cancers with MKP activity (27). We did not find statistically significant differences in p-JNK, albeit median Hscores were numerically lower in tumors overexpressing MKP-1.

Studies on the prognostic role of MKP-1 in human malignancies are as yet limited. In ovarian cancer, MKP-1 overexpression was associated to a shorter time to disease progression (50). In contrast, in non-small cell lung cancer, MKP-1 overexpression was linked to an improved prognosis (51). The possible reasons to explain this difference include diverse roles of MKP-1 in different malignancies, methodologic issues, or differences inherent to each population study. Here, we report an association between MKP-1 overexpression and high risk of breast cancer relapse by univariate and multivariate analysis. Taking all these considerations into account, it seems that the prognostic role of MKP-1 should be further assessed in larger series with clearly defined patient populations.

In summary, overexpression of MKP-1 occurs during the malignant transformation of the breast and was an independent adverse prognostic factor. Furthermore, MKP-1 is a regulable enzyme in human breast cancers. MKP-1 repression by doxorubicin was associated with a significant increase of phospho-MAPKs, including ERK1/2, JNK, and, in a nonsignificant manner, p-p38. This finding, in clinical specimens, is consistent with the biological role of MKP-1 and may underlay, at least in part, the cytotoxic effects of doxorubicin. Taken together, we believe that these results justify further studies of MKP-1 as a potential target or marker in breast cancer.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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