

Featured Article

Mitogen-Activated Protein Kinase Phosphatase-1 Is Overexpressed in Non-Small Cell Lung Cancer and Is an Independent Predictor of Outcome in Patients

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ABSTRACT

Purpose: An increase in the activity of the mitogen-activated protein kinases (MAPKs) has been correlated with a more malignant phenotype in several tumor models *in vitro* and *in vivo*. A key regulatory mechanism of the MAPKs [extracellular signal-regulated kinase (ERK); c-jun NH₂-terminal kinase (JNK); and p38] is the dual specificity phosphatase CL100, also called MAPK phosphatase-1 (MKP-1). This study was designed to examine the involvement of CL100/MKP-1 and stress-related MAPKs in lung cancer.

Experimental Design: We assessed the expression of CL100/MKP-1 and the activation of the MAPKs in a panel of 18 human cell lines [1 primary normal bronchial epithelium, 8 non-small cell lung cancer (NSCLC), 7 small cell lung cancer (SCLC), and 2 carcinoids] and in 108 NSCLC surgical specimens.

Results: In the cell lines, CL100/MKP-1 expression was substantially higher in NSCLC than in SCLC. P-ERK, P-JNK, and P-p38 were activated in SCLC and NSCLC, but the degree of their activation was variable. Immunohistochemistry in NSCLC resection specimens showed high levels

of CL100/MKP-1 and activation of the three MAPK compared with normal lung. In univariate analysis, no relationship was found among CL100/MKP-1 expression and P-ERK, P-JNK, or P-p38. Interestingly, high CL100/MKP-1 expression levels independently predicted improved survival in multivariate analysis. JNK activation associated with T₁₋₂ and early stage, whereas ERK activation correlated with late stages and higher T and N. Neither JNK nor ERK activation were independent prognostic factors when studied for patient survival.

Conclusions: Our data indicate the relevance of MAPKs and CL100/MKP-1 in lung cancer and point at CL100/MKP-1 as a potential positive prognostic factor in NSCLC. Finally, our study supports the search of new molecular targets for lung cancer therapy within the MAPK signaling pathway.

INTRODUCTION

The mitogen-activated protein kinase (MAPK) pathway is one of the most important intracellular signaling cascades conserved from yeast to mammals (1). MAPKs are a family of serine/threonine kinases in which three main components are the extracellular signal-regulated kinase (ERK), c-jun NH₂-terminal kinase (JNK), and p38 (2, 3). Activation of any of the MAPK family members requires phosphorylation of two regulatory residues (a threonine and a tyrosine) by their upstream kinases (1, 4). Dephosphorylation of one or both of these regulatory residues induces deactivation of MAPK family members. MAPK dephosphorylation is carried out by some phosphatases, similar to the dual specificity MAPK phosphatases (MKPs), which simultaneously dephosphorylate both serine/threonine and tyrosine residues (5, 6). The first characterized member of this family was the MKP-1 (or CL100; Ref. 7). It is encoded by an early response gene, which is transiently induced by mitogens and stress signals such as serum, cytokines, UV radiation, heat shock, and hypoxia (7–11). MAPK phosphatase-1 (CL100/MKP-1) expression is induced by some of the members of the MAPK family (12) and other protein kinases (13–15). Although ERK, JNK, and p38 are known substrates of CL100/MKP-1, the ability to dephosphorylate each one of these kinases varies depending on the cell system and its environmental conditions (11, 16, 17).

CL100/MKP-1 has also been correlated with tumorigenesis. The reported expression patterns of CL100/MKP-1 in carcinogenesis change depending on the organ studied. Prostate, colon, and bladder tumors have high CL100/MKP-1 expression in the early phases of carcinogenesis and loss of expression levels in advanced stage (18). Besides, in prostate tumors, a correlation between an increased activation of ERK, JNK, or both and CL100/MKP-1 overexpression has

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been described previously (19). In other cancers such as breast carcinomas, a high expression of CL100/MKP-1 in poorly differentiated or late stages has been shown (18). More recently, we have observed a decreased CL100/MKP-1 expression in ovarian cancer as compared with normal ovarian epithelial tissue (20).

The MAPK pathway has been shown to be very relevant in human carcinogenesis (21, 22). Activation of ERK has been reported in lung (23, 24), kidney (25), breast (26, 27), head and neck (28), liver (29), prostate (30), salivary gland (31), and skin (32) tumors. We have recently shown that ERK activation in lung cancer is associated with late stage (33). Few studies on the activation of JNK and p38 have been carried out in clinical specimens of cancer patients. Activation of both JNK and p38 has been found in prostate carcinoma (34), and deactivation of these kinases has been reported in colon cancer (35, 36) and in advance stages of prostate carcinoma (34). P-p38 has also been observed in a few cases of non-small cell lung cancer (NSCLC; Ref. 37). All these data have contributed to the rationale of developing inhibitors of the MAPK pathway (38), which are now entering clinical trials.

CL100/MKP-1 mRNA has been found in normal lung extracts (10), but little is known about its cellular distribution and the role of this phosphatase in lung cancer. In addition, stress-related MAPKs (JNK and p38) have not yet been studied in lung cancer. Therefore, the aim of this study was to characterize the expression and localization of CL100/MKP-1 and to explore the possible correlation of CL100/MKP-1 overexpression and activation of any MAPK. Finally, we also analyzed the potential relevance of these markers as prognostic factors in NSCLC.

MATERIALS AND METHODS

Patient Specimens. Surgical specimens of human lung tumors were collected from a total number of 108 patients. Ninety-five clinical specimens were obtained from patients of the University Hospital of Navarra, and 13 samples were obtained from patients of the Canisius Wilhelmina Hospital (Nijmegen, the Netherlands). All of the samples were collected before any chemo- or radiotherapy schedule. We had clinical follow-up of 70 of the 108 NSCLC specimens. We also had normal samples of distant tissue to the tumor or adjacent tissue to the tumor from approximately one-third of the lung cancer patients studied. Each specimen was fixed in 10% formalin and embedded in paraffin. Five- μ m sections were stained with H&E for tumor morphology assessment. Two pathologists in our group evaluated the tumors (M. Lozano and G. Toledo): 43 tumors were adenocarcinomas, and 65 were squamous cell carcinomas.

All patients with clinical follow-up (70 of 108) had an Eastern Cooperative Oncology Group performance status of 0 to 2, adequate baseline organ function and no severe co-morbid conditions. Written-signed informed consent was obtained from all patients before treatment. The treatment of the 70 patients was homogeneous as detailed below.

Tumor stage was determined according to the Tumor-Node-Metastasis staging system. Treatment was different

depending on the stage of the tumor and other characteristics of the patient. Most patients were treated after a multidisciplinary therapeutic approach. In short, surgery was the initial treatment for medically operable stage I to IIB and IIIA non- N_2 tumors. Lobectomy and ipsilateral mediastinal lymph node sampling were the treatments of choice. Postoperative radiotherapy consisting of 45 Gy using conventional fields and fractionation schedules was added depending on pathological findings. Patients with locally advanced NSCLC were treated with paclitaxel- and cisplatin-based chemotherapy previous to radical hyperfractionated radiotherapy (69.6 Gy) with concurrent chemotherapy. Stage IV patients were treated with cisplatin-based chemotherapy. Median follow-up time of patients was 29.5 months (range, 0.5–68 months).

Cell Culture. Small cell lung cancer (SCLC) cell lines (NCI-H69, NCI-H82, NCI-H187, NCI-H345, NCI-H510, and NCI-H774), NSCLC cell lines (NCI-H23, NCI-H157, NCI-H676, NCI-H1264, and NCI-A549), and carcinoid cell line (NCI-H720) were obtained from the Department of Cell and Cancer Biology (National Cancer Institute, NIH, Bethesda, MD). NCI-H446 (SCLC), NCI-H441, Calu3, HTB-58 (NSCLC), and NCI-H727 (carcinoid) were obtained from the American Type Culture Collection (Manassas, VA). Normal human bronchial epithelium (NHBE) primary cells were obtained from Clonetics Corp. (San Diego, CA). All cell lines were grown in RPMI 1640 with glutamax supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin, and 10% heat-inactivated fetal bovine serum (Life Technologies, Inc., Rockville, MD), except for NHBE 2006 that was grown in bronchial epithelial growth medium (Clonetics Corp.) according to the manufacturer's instructions.

Immunohistochemistry and Immunocytochemistry. Immunohistochemistry (IHC) was performed on 5- μ m sections of formalin-fixed, paraffin-embedded tissues. The polyclonal antibodies used in the immunohistochemical studies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) (sc-1109) to detect CL100/MKP-1, from Cell Signaling (Beverly, MA) to detect P-ERK and P-p38, and from Promega (Madison, WI) to detect P-JNK. Samples were incubated overnight at 4°C with the primary antibody. Immunocytochemical reaction was shown using the EnVision intensifying kit (Dako, Carpinteria, CA). Absorption controls with a specific peptide were performed to confirm for specificity of the CL100/MKP-1 immunostaining. Positive controls for phosphorylated antibodies were stress-activated HTB58 human lung cancer cells fixed in 10% formalin and treated with medium containing 20% FBS. MAPK activation was achieved in positive controls by culturing the cells during 16 h in a serum deprived medium (containing 0.4% FBS). The results were evaluated independently by two observers. The tumors were scored according to the proportion of nuclei or cytoplasm stained within the tumor. For CL100/MKP-1 and phospho-JNK, >50% of stained nuclei or cytoplasm of tumor cells within the specimens were considered positive. In the case of P-ERK and P-p38, >15% of the nuclei or cytoplasm in tumor cells was considered positive. In all cases, cutoff was chosen according to the median values and used only for clinical correlation purposes. Data regarding P-ERK immunostaining were part of a previous study (33), and they

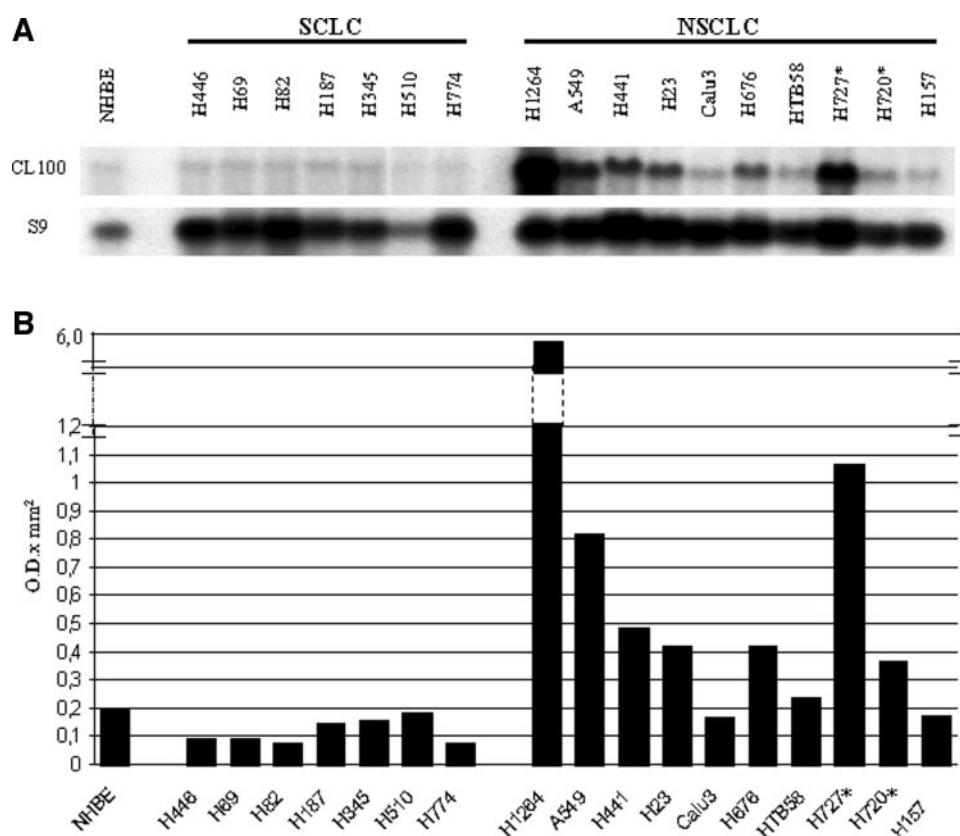


Fig. 1 mRNA expression of CL100/mitogen-activated protein kinase phosphatase 1 (MKP-1) in human lung cancer cell lines. **A**, CL100/MKP-1 mRNA of different cancer cell lines [small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC)] and of primary bronchial epithelial cells [normal human bronchial epithelium (NHBE)]. **B**, the table represents normalization of CL100/MKP-1 mRNA using ribosomal protein S9. The results shown are from one experiment of three performed, obtaining similar results. The *asterisk* indicates two carcinoid cell lines.

were used in the present study to analyze their correlation with CL100 expression levels and with activation of other MAPKs.

Immunocytochemistry for CL100/MKP-1 of cultured cells was performed on cell lines fixed in Saccamano's fixative. Cells growing attached to the substrate (H23, H441, H446, A549, H727, H1264, and HTB58) were seeded on glass slides and grown at a confluence of 60–70%, washed twice with PBS, and fixed. Cells growing in suspension (H82, H187, H345, H460, H510, and H720) were centrifuged at 1500 rpm at 4°C in 50-ml Falcon tubes, washed twice with PBS, and resuspended in Saccamano's fixative. Before the immunocytochemistry, the floating cells were spun down to the slides using a cytospin (Cytospin 3; Shandon Scientific Limited, Pittsburgh, PA). Slides were immersed in methanol/acetone (1:1 V/V) for 5 min and air dried. The staining results for CL100/MKP-1 in the cell lines were evaluated according to the following criteria: for each cell line, five high power fields randomly selected were assessed. When the average percentage of the five fields was >50%, immunostaining was considered positive. To avoid variability, the staining and the study of all cell lines were performed at the same time, with the same reagents and incubation times.

RNA Isolation and Northern Blot Analyses. Total RNA was extracted from cells growing in exponential phase with guanidinium isothiocyanate and isolated using a CsCl

gradient protocol. Ten μ g of total RNA were separated on a 1% agarose gel containing 2.2 M formaldehyde, transferred by capillary action overnight onto a nitrocellulose membrane (Schleiner & Schuell, Inc., Dassen, Germany) using a 10 \times SSC blotting buffer (1.5 M NaCl and 0.15 M trisodium citrate). After the transfer, the membrane was exposed to UV light for 2 min to immobilize the RNA and hybridized with a ³²P-labeled CL100/MKP-1 cDNA probe corresponding to a *SalI/XhoI* fragment of human CL100/MKP-1 (from bp 644 to 1001 of CL100 cDNA; GenBank accession no. X68277). Hybridization was carried out overnight at 42°C in a hybridization buffer containing 40% formamide. The hybridized isotopic signal was detected using Kodak XAR Film, quantitated with a densitometer (JX-325; SHARP, Buenos Aires, Argentina) and analyzed with Imagemaster 1D software (Amersham Biosciences, Piscataway, NJ).

Western Blot Analyses. Cells were collected in log phase and lysed with lysis buffer that contained 25 mM sodium β -glycerophosphate, 25 mM NaF, 1 mM Na₃VO₄, 1 mM Na₂P₃O₄, 5 mM EDTA, 5 mM EGTA, 1 mM benzamidine, 0.05% sodium deoxycholate (w/v), sodium SDS 0.01% (w/v), 1% Triton X-100 (v/v), 0.1% β -mercaptoethanol (v/v), and a protease inhibitors mixture (Roche, Basel, Switzerland). The final protein concentration was determined using the BCA kit (Pierce, Rockford, IL). Sixty μ g of each protein lysate were electrophoresed in 4–12% polyacrilamide NuPAGE gels (In-

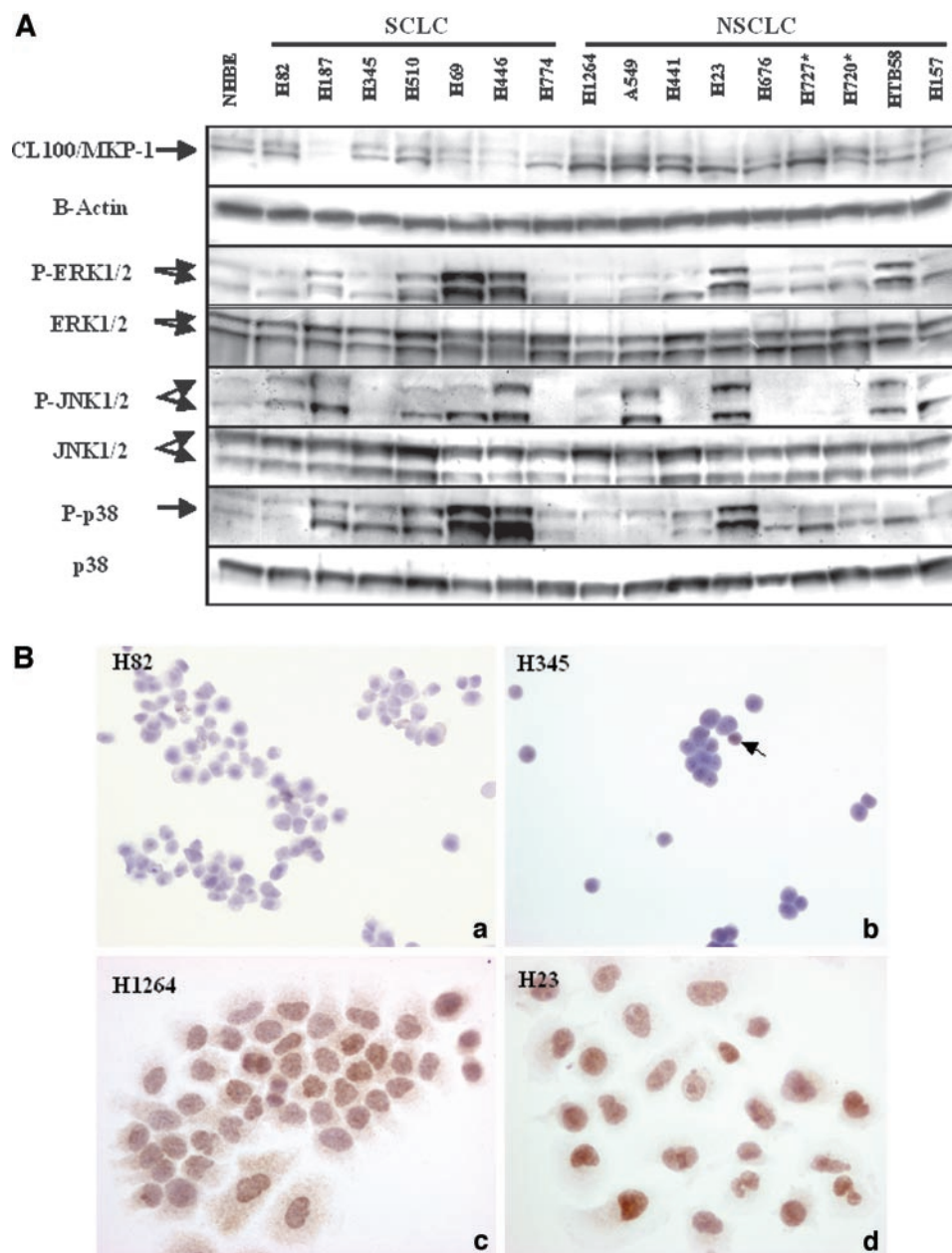


Fig. 2 CL100/mitogen-activated protein kinase phosphatase 1 (MKP-1) protein expression and mitogen-activated protein kinase activation in small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) cell lines. **A**, Western blots of SCLC cell lines as compared with NSCLC cell lines. The *asterisk* indicates two carcinoid cells. **B**, **a** and **b** are SCLC cell lines immunostained for CL100/MKP-1 showing low levels of the phosphatase. *Arrows* point at a single weak immunostained cell in **B**. **c** and **d** are NSCLC cell lines showing strong CL100/MKP-1 staining.

vitrogen) and transferred to a polyvinylidene difluoride Immobilon P membrane (Millipore, Bedford, MA) according to the manufacturer's instructions. Membranes were probed with a specific anti-MKP-1 antibody (Santa Cruz Biotechnology), anti-phospho-ERK, anti-total-ERK, anti-phospho-JNK, anti-total-JNK, anti-phospho-p38 anti-total-p38 (Cell Signaling), and anti- β -actin (Sigma, St. Louis, MO). The Lumilight kit (Roche) was used to visualize immunoreaction

in Hyperfilm enhanced chemiluminescence films (Amersham Biosciences).

Statistical Analysis. Statistical analysis was performed using the SPSS Data Analysis Program, version 9.0. The tests used were the χ^2 test, two tailed, for dicotomous variables; the Mann-Whitney test, two tailed, for continuous variables; and the Kaplan-Meier survival plot, the log-rank test, and the Cox regression for survival analysis.

Table 1 CL100/mitogen-activated protein kinase phosphatase-1 expression in lung cancer cell lines

Cell lines	% positive cells	Staining localization
Small cell lung cancer (<i>n</i> = 6)		
H69	20%	Nuclear
H82	20%	Nuclear
H187	10%	Nuclear
H345	40%	Nuclear
H446	55%	Nuclear/cytoplasmic
H510	30%	Nuclear/cytoplasmic
Non-small cell lung cancer (<i>n</i> = 9)		
H23	55%	Nuclear
H441	80%	Nuclear/cytoplasmic
A549	75%	Nuclear/cytoplasmic
H676	60%	Nuclear/cytoplasmic
H720 ^a	60%	Nuclear/cytoplasmic
H727 ^a	80%	Nuclear/cytoplasmic
H1264	85%	Nuclear/cytoplasmic
H1385	70%	Nuclear
HTB58	70%	Nuclear

^a H720 and H727 are carcinoid cells.

RESULTS

CL100/MKP-1 mRNA Differential Expression in Lung Tumor Cell Lines. We evaluated CL100/MKP-1 gene expression levels in NHBE cells and in lung cancer cell lines by Northern blot analysis. A 2.4-Kb band corresponding to the CL100/MKP-1 mRNA was detected in all cell lines. mRNA expression levels were different among SCLC (*n* = 7) and NSCLC (*n* = 8) cell lines. SCLC cells showed equal or lower CL100/MKP-1 mRNA levels than control NHBE cells. In contrast, NSCLC cell lines studied expressed higher levels of CL100/MKP-1 mRNA (at least twice the levels of NHBE), except for Calu3, HTB58, and H157, in which the levels remained similar to those of NHBE (Fig. 1).

CL100/MKP-1 Protein Expression in Lung Tumor Cell Lines. Total protein extracts of SCLC and NSCLC cell lines were assessed by Western blot. Similar to what we found for CL100/MKP-1 mRNA levels, NSCLC and SCLC showed different patterns of CL100/MKP-1 protein expression. In most SCLC cell lines, protein levels were low (H345, H446, H69, and H774) or undetectable (H82). CL100/MKP-1 expression was found in all NSCLC cell lines, and 5 of 9 cell lines (H1264, A549, H441, H23, H441, and H727) showed high protein expression. In general, protein levels in NSCLC and carcinoid cell lines correlated with mRNA (Fig. 2A).

CL100/MKP-1 Protein Localization in Lung Tumor Cell Lines. Intracellular localization of CL100/MKP-1 protein was assessed in 15 cell lines (6 SCLCs and 9 NSCLCs) by immunohistochemistry (Table 1 and Fig. 2B). Higher protein levels were found in NSCLC cell lines when compared with those of SCLC. In NSCLC and SCLC cells immunostaining was located both in the nucleus and in the cytoplasm.

CL100/MKP-1 Protein Expression in Normal and Tumor Tissues: Overexpression in NSCLC. CL100/MKP-1 protein expression in normal lung areas and lung tumor tissues was evaluated by IHC. Firstly, we assessed 30 specimens of distant normal lung obtained from surgical specimens of lung cancer patient tumors. The normal epithelial

tissue was considered negative regarding the cutoff used for this molecular marker. In normal alveoli, CL100/MKP-1 protein expression was undetectable in type I and type II pneumocytes (Fig. 3A). However, in the normal bronchial epithelium, present in 12 of 30 biopsies, some ciliated cells (20–40%) showed nuclear staining (12 of 12 cases studied; Fig. 3B). In 10 cases, a light cytoplasmic staining was also found in ciliated cells. Macrophages, endothelial cells, and smooth muscle cells were also stained. Smooth muscle staining was used as an internal positive control for the IHC technique.

We also assessed the expression of CL100/MKP-1 in type II cell hyperplasias found in alveolar fields close to the tumor. Alveolar hyperplasias (*n* = 14) were positive in all specimens studied (the proportion of type II cells that were positive was 50–70%). Immunostaining was found predominantly in the nucleus (Fig. 3C).

Finally, we evaluated NSCLC clinical specimens (*n* = 108). CL100/MKP-1 immunostaining was widely distributed in this type of tumor. All tumor specimens showed expression of the phosphatase. Seventy-eight percent of cases (93% of the adenocarcinomas and 74% of the squamous cell carcinomas) were considered positive for clinical correlation purposes according to the cutoff selected previously. Generally, immunostaining was found both in the nucleus and in the cytoplasm (Fig. 3C).

MAPK Activation in Lung Cancer Cell Lines. Analysis of MAPK activation in the cell lines by Western blot analysis showed that all three proteins were activated both in SCLC and NSCLC cell lines, although activation levels were different among cell lines (Fig. 2A). Concerning SCLC, ERK activation was shown in all cell lines, P-JNK in 5 of 7 and P-p38 in 7 of 7. In NSCLC, P-ERK was found in all cell lines, P-JNK in 5 of 7 cell lines and P-p38 in 5 of 7. We did not observe any evident correlation between CL100/MKP-1 protein levels and activation of ERK, JNK, and p38 when individual cell lines were compared, the only exception being an apparent inverse correlation between CL100/MKP-1 expression and p38 phosphorylation in SCLC.

P-JNK and P-p38 Protein in Normal and NSCLC Tissues: Activation in Tumor Tissue. We also assessed the phosphorylation of the stress-activated MAPKs, JNK and p38 (Figs. 4 and 5). Data for P-ERK are published in a separate study (33) and will be mentioned in the discussion for comparison purposes. JNK activation was found predominantly in the ciliated cells of the normal bronchiolar epithelium (60–85% of cytoplasm and 25–50% of the nuclei of these cells) but not in the alveolar epithelial cells (Fig. 4, A and C). P-p38 was not found either in the alveolar or in the bronchiolar epithelial cells (Fig. 4, B and D). Other cell types such as fibroblasts, endothelial cells, and smooth muscle cells showed JNK but no p38 activation. In type II alveolar hyperplasia, we found that JNK was activated in the nucleus in 5–10% of type II pneumocytes, whereas p38 was activated in 30% of these cells (Fig. 4, E and F). Fig. 5 shows the incidence of positive cases in every stage analyzed for the nuclear activation of JNK and p38, according to the cutoff used for each MAPK.

When assessing NSCLC specimens, we observed acti-

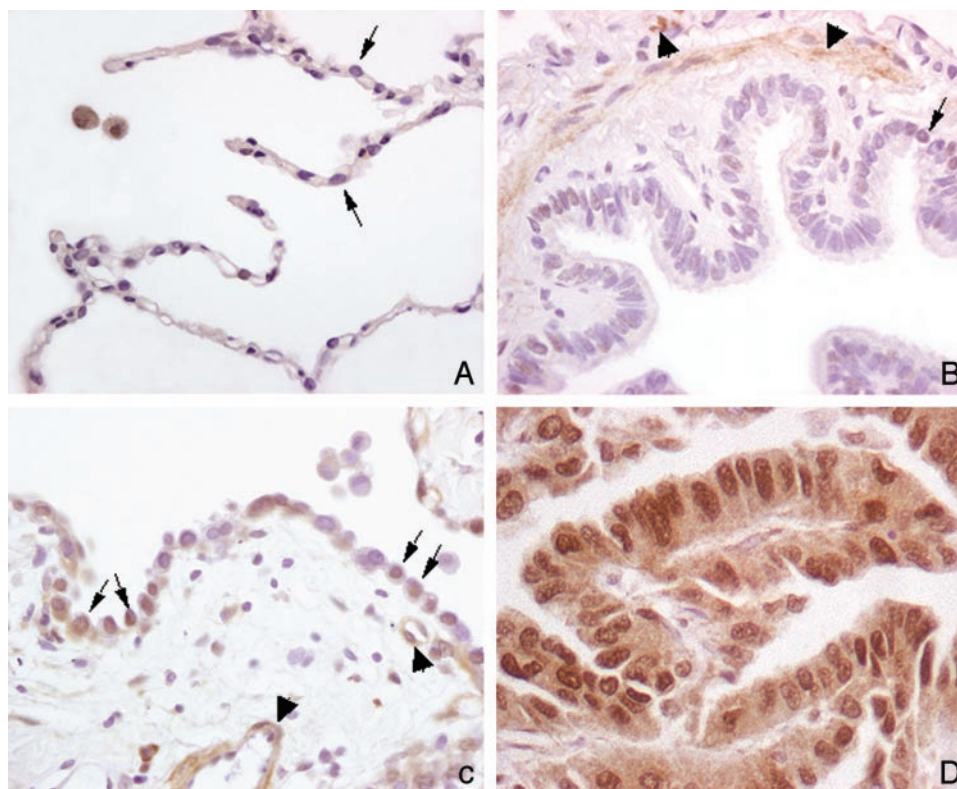


Fig. 3 Immunohistochemistry for anti-CL100/mitogen-activated protein kinase phosphatase 1 (MKP-1). Examples of normal lung tissue areas immunostained with an anti-CL100/MKP-1 antibody. Alveoli (A) and bronchus (B) are shown. Alveolar cells are negative for CL100/MKP-1 staining (arrows). Some ciliated and basal cells are positive in bronchial epithelium (arrows), as well as smooth muscle and fibroblasts (arrowheads). C, type II hyperplasia is shown. Nuclei of hyperplastic pneumocytes are immunostained (arrows), as well as nonepithelial cells (arrowheads). D, adenocarcinoma with nuclear and cytoplasmic staining.

vation of both JNK and p38 in 57 of 70 and 45 of 70 of the tumor specimens, respectively (Fig. 4, G and H). In 15 of P-p38-positive specimens, most of the immunostained carcinoma cells were found at the periphery of the tumor, as previously had been found for P-ERK activation (33). The immunocytochemical signal of phosphorylation of these MAPKs was found both in the nucleus and the cytoplasm of the tumor cells.

Prognostic Value of CL100/MKP-1 in NSCLC. The characteristics of the 70 patients with clinical follow-up are shown in Table 2. The relationships between CL100/MKP-1 nuclear expression, P-ERK (nuclear and cytoplasmic activation), P-JNK, and p38 (nuclear activation), and several clinicopathological factors are shown in Table 3. CL100/MKP-1 expression was associated with tumor type (adenocarcinoma; $P = 0.04$) in an univariate analysis. No association between the phosphatase expression and either clinicopathological variables or MAPKs IHC patterns was observed.

We also performed the patient survival analysis. Kaplan-Meier plots of all patients with clinical follow-up whose tumors had positive nuclear staining for CL100/MKP-1 ($\geq 50\%$ tumor nuclei stained) and of those whose tumors were negative ($< 50\%$ tumor nuclei stained) were carried out. The survival analysis showed a better outcome of those

patients with positive staining. As Fig. 6A shows, the median survival of patients with positive nuclear staining has not been reached, whereas in patients with negative nuclear staining tumors, the median survival was 30 months ($P = 0.009$; log rank). An additional subgroup analysis limited to patients with early-stage (I or II) tumors showed a trend between the CL100/MKP-1 expression and an improved survival of borderline significance ($P = 0.059$; Fig. 6B). Multivariate Cox regression analysis showed that the association between survival and phosphatase expression was maintained when adjusted for tumor stage ($P = 0.014$). Hazard ratio of death for negative staining was 3.12 (95% confidence interval 1.25–8.33).

JNK Activation Is Associated with Early Stages and Lower T. We also studied the influence of MAPKs activation on the clinical outcome of the tumors (Table 3). First, we found that ERK was associated with advanced stage and also with higher T and N. Second, nuclear JNK activation was associated with early-stage ($P = 0.006$) tumors and lower T ($P = 0.001$) and also with histology ($P = 0.02$). Finally, P-p38 directly associated with nuclear ($P = 0.001$) and cytoplasmic ERK activation ($P = 0.002$).

We also performed Kaplan-Meier plots to assess the influence of each MAPK activation in NSCLC patients. Uni-

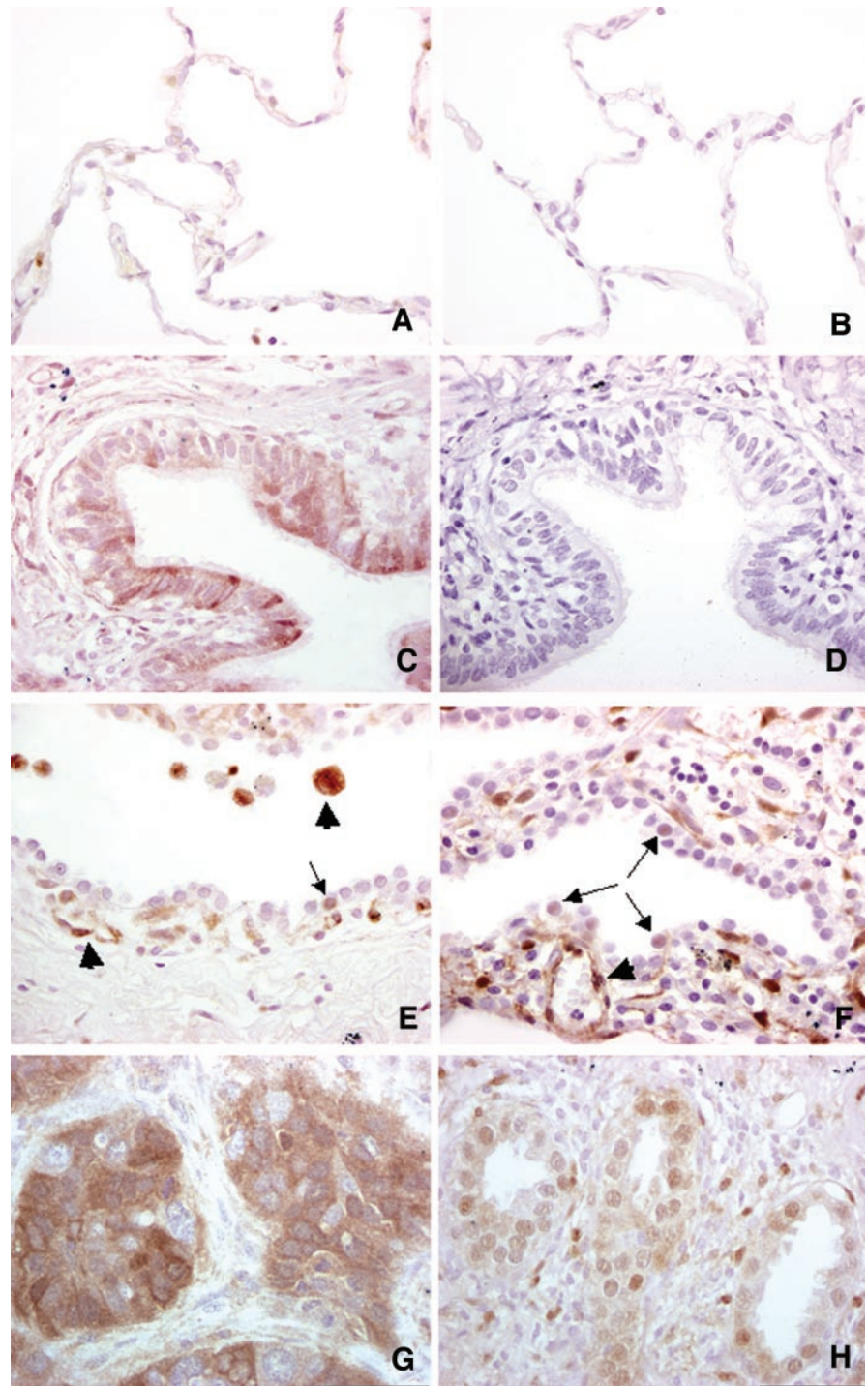


Fig. 4 Immunohistochemistry for anti-P-c-jun NH₂-terminal kinase (P-JNK) and anti-P-p38. Normal alveolar and bronchiolar epithelium immunostained for P-JNK (A and B) and P-p38 (C and D). C, immunostaining is only observed in bronchial epithelium stained with P-JNK. E and F, type II hyperplasias for P-JNK and P-p38. Arrows point to some positive pneumocytes and arrowheads to nonepithelial-positive cells. G, squamous carcinoma immunostained for P-JNK. H, adenocarcinoma immunostained for P-p38.

variate analysis showed that nuclear and cytoplasmic P-JNKs were potential positive prognostic factors of NSCLC patients ($P = 0.03$). No influence on survival was observed for any of the three MAPK in the multivariate analysis, although nuclear P-JNK showed a trend toward significance ($P = 0.12$).

DISCUSSION

CL100/MKP-1 regulates the activity of the different MAPKs, but little is known about its expression and biological role in lung tumorigenesis. In the present study, we reported a differential expression of CL100/MKP-1 *in vitro* between the two major types of lung cancer. In general,

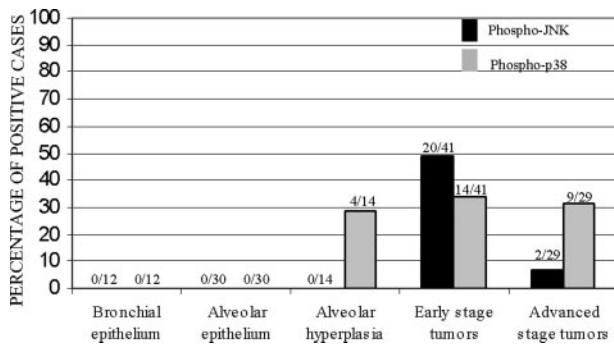


Fig. 5 Percentage of positive cases for P-c-jun NH₂-terminal kinase (P-JNK) and P-p38 in normal, hyperplastic, and tumor lung tissues. The final value for each bar accounts for the number of positive specimens divided by the total of analyzed specimens in each stage of tissue.

constitutive levels of the phosphatase in NSCLC were higher than those found in SCLC cell lines and in normal cells in basal growth conditions.

In clinical specimens, previous data had suggested an overexpression of the CL100/MKP-1 protein in tumors such as breast, colon, and prostate (18) and a decrease from normal to tumor tissue in ovarian cancer (20). Our present results in NSCLC were similar to what had been published in breast tumors where an overexpression of the phosphatase was maintained in late stages of the disease (18).

In our IHC survey of normal lung tissue, we found most of the CL100/MKP-1 expression in nonepithelial cells, whereas normal bronchiolar and alveolar epithelial cells show limited or no expression of the phosphatase. These results suggest that the previous study of high CL100/MKP-1 levels in normal lung extracts (10) may be mainly related to the relative abundance of nonepithelial cells, stressing the importance of studying cell type localization when assessing gene expression. In addition, no expression of CL100/MKP-1 in preneoplastic lesions had been described thus far. In this work, we showed that CL100/MKP-1 proteins increased in epithelial hyperplastic lesions such as type II hyperplasia with respect to normal tissue. Depending on its nuclear atypia, type II cell hyperplasia has been considered either an early event in NSCLC carcinogenesis (39) or a reactive lesion (40).

MAPK activation had been previously studied in lung tumor cells *in vitro* (41–46), but only basal ERK activation was analyzed in detail in a large panel of lung cancer cell lines (47). Thus far, simultaneous evaluation of ERK, JNK, and p38 activation has not been performed in lung cancer either in a relevant number of cell lines or in clinical specimens. In a previous study, Greenberg *et al.* (37) reported p38 activation in a short number of NSCLC patients ($n = 19$), but they did not observe either ERK or JNK activation in these specimens. In the present work, we observed the activation patterns of the three MAPKs in a panel of 17 lung cancer cell lines and 70 NSCLC biopsies. In the clinical specimens, we found an increase in the nuclear activation of both JNK and p38 in the tumor when compared with normal lung in the same way as we recently described for ERK (33).

We have identified an association between high CL100/MKP-1 expression levels and better survival in NSCLC (Table 4 and Fig. 6), even for early-stage patients. Our results are not what would be expected if CL100/MKP-1 would exclusively be involved in the down-regulation of JNK-mediated apoptosis (17). It has been shown in several cell types, including tumor cells, that CL100/MKP-1 down-regulates the activation of the three MAPKs in the absence (20) or presence of several extracellular stimuli (17, 48, 49). Better survival of patients with higher levels of CL100/MKP-1 may be because of CL100/MKP-1 down-regulating mitogenic-inducing ERK activity (20). On the other hand, biological responses attributed to JNK activation include not only apoptosis but DNA repair, apoptosis inhibition, and neoplastic transformation (50). In this regard, CL100/MKP-1 could also inhibit the phospho-JNK-dependent growth factor-stimulated cell growth (51), as has been described by Lee *et al.* (49), who have suggested that in NSCLC an increased expression of MKP-1 is involved in the retinoid acid inhibition of serum-induced growth-promoting JNK phosphorylation.

Deactivation of JNK has been previously described in prostate (34) and colon cancers (35). In the case of prostatic tumors, the authors suggested that JNK deactivation in advanced stage tumors could be related to a lower apoptotic rate. Our data agree with these findings as we showed an association between JNK activation and early-stage tumors. In addition, a trend toward significance when comparing P-JNK and survival has also been described in our work. In this regard, phosphorylation of JNK in some NSCLC could increase the sensitivity of the tumor cell to chemo- or radiotherapy-induced apoptosis. A larger number of patients is needed to confirm the relevance of activated JNK in the survival of NSCLC patients.

One interesting finding in our study has been the lack of association between CL100/MKP-1 and MAPKs activation. This fact suggests that the important balance MAPK activation/dephosphorylation is controlled by more than one factor and subjected to complex regulation. First, the potential

Table 2 Characteristics of the patients

	No. of patients ($n = 70$)	Percentage of cases
Age (yrs)		
Median	62	
Range	31–87	
Gender		
Male	61	87.1%
Female	9	12.9%
Histology		
Adenocarcinoma	32	45.7%
Squamous cell carcinoma	38	53.3%
Stage		
Early (I/II)	41	58.6%
Advanced (III/IV)	29	41.4%
T		
T ₁ -T ₂	45	64.3%
T ₃ -T ₄	25	35.7%
N		
N ₀	39	55.7%
N ₁ -N ₃	31	44.3%

Table 3 Clinicopathological factors and their relationship to nuclear CL100/MKP-1^a staining

	CL100/MKP-1	P-JNK nucleus	P-p38 nucleus
Age (yrs)	$P = 0.47$	$P = 0.56$	$P = 0.18$
Sex	$P = 0.47$	$P = 0.51$	$P = 1$
Histology (adenocarcinomas versus squamous cell carcinoma)	$P = 0.04^b$	$P = 0.02$	$P = 0.79$
Stage (I/II versus III/IV)	$P = 0.62$	$P = 0.006$	$P = 0.61$
T (T ₁₋₂ versus T ₃₋₄)	$P = 0.81$	$P = 0.001$	$P = 0.38$
N (N ₀ versus N ₁₋₃)	$P = 0.8$	$P = 0.018$	$P = 0.8$
P-ERK nucleus	$P = 1$	$P = 0.59$	$P = 0.002$
Cytoplasm	$P = 0.63$	$P = 0.6$	$P = 0.001$
P-JNK nucleus	$P = 0.41$		
P-p38 nucleus	$P = 0.28$	$P = 0.77$	

^a MKP-1, mitogen-activated protein kinase phosphatase-1; JNK, c-jun NH₂-terminal kinase; ERK, extracellular signal-regulated kinase.

^b Values in boldface indicate significance levels <0.05.

activity of other MKPs such as MKP-3 or MKP-7 (52, 53); second, the fine-tuned feedback loop consisting of concurrent CL100/MKP-1 deactivation of the MAPKs (16) and stimulation of the phosphatase transcription by its activated MAPKs substrates (12); and third, the regulation of CL100/MKP-1 expression by kinases other than the MAPKs (such as protein kinases A and C; Refs. 13, 14). This delicate balance between MKP-1 and its substrates may also lead to cell-to-cell differences in the phosphatase threshold levels required for CL100/MKP-1-dependent deactivation of the MAPKs. This could also explain the lack of association between the groups of patients classified according to our arbitrary phosphatase cutoff values and the clinicopathological variables.

The best known substrates for JNK and p38 are located in the nucleus (1). We have clearly found nuclear and cytoplasmic immunolocalization of phosphorylated forms of these two MAPKs in NSCLC. Nuclear and cytoplasmic activation of the stressed-related kinases has also been described in prostate and colon cancer (34, 35). These results suggest the existence of noncharacterized cytoplasmic substrates for these MAPKs. In addition, as shown by previous studies (19, 20), our immunocytochemical results for CL100/MKP-1 show not only nuclear but also cytoplasmic localization of the phosphatase. This finding suggests that CL100/MKP-1 may be deactivating the MAPKs both in the nucleus (5) and in the cytoplasm.

In summary, in our work, we have described the involvement of a downstream regulator of MAPKs (CL100/MKP-1) in NSCLC patient survival, as well as the activation of the ERK, JNK, and p38 and their relationship with the clinical outcome. Moreover, these clinical associations reflect the role of MAPKs in key regulatory mechanisms for NSCLC *in vivo* that have already been suggested *in vitro* (45, 46, 49). Our observations support the view of CL100/MKP-1 and the MAPKs as potential targets for lung cancer treatment. It will be useful in the future to explore whether the phosphatase expression might account for the response of lung cancer treatments targeting MAPK activation (38) or upstream elements of the MAPK cascade like the epidermal growth factor receptor signaling pathway (54).

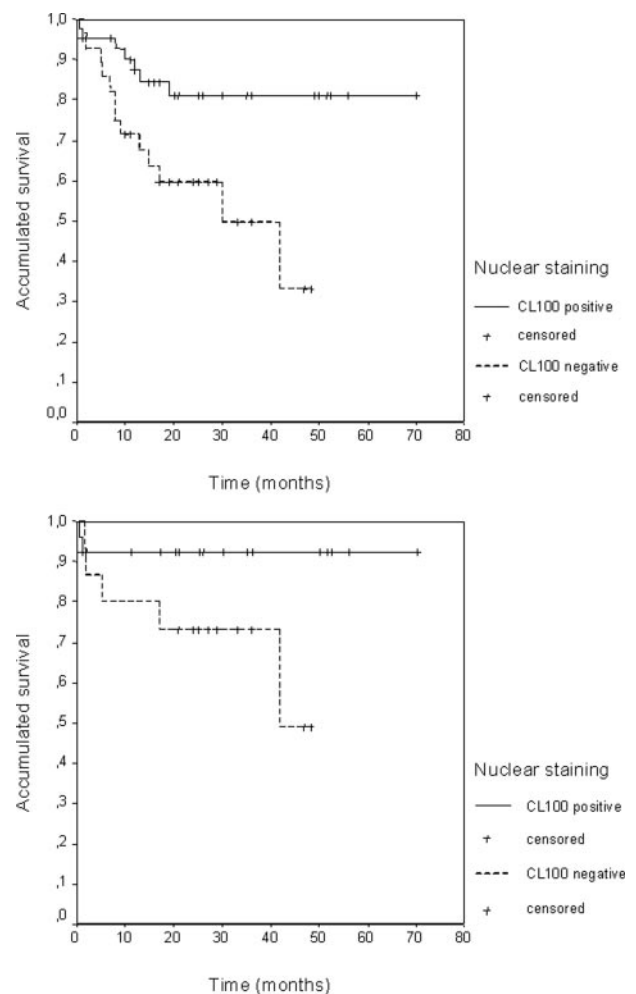


Fig. 6 Prognostic relevance of CL100/mitogen-activated protein kinase phosphatase 1 (MKP-1) expression in non-small cell lung cancer. A, survival plot from patients with clinical follow-up ($n = 70$), representing CL100/MKP-1 expressing cases (continuous line) versus non-expressing cases (dashed line). Differences between the two groups are statistically significant ($P = 0.009$). B, survival plot from early stage patients that shows CL100/MKP-1-positive cases (continuous line) and CL100/MKP-1-negative cases (dashed line). Differences between the two groups are at borderline significance ($P = 0.059$).

Table 4 Results of univariate and multivariate analysis assessing the influence of clinical and molecular variables on survival

Parameter	Univariate <i>P</i>	Adjusted <i>P</i> ^a	Hazard ratio ^b (confidence interval)
Stage	0.001 ^c		4.1 (1.8–9.2)
Negative nuclear mitogen-activated protein kinase phosphatase-1	0.009	0.015	3.17 (1.25–8.03)
Negative nuclear P-c-jun NH ₂ -terminal kinase	0.03	0.12	3.38 (0.72–15.87)
Negative nuclear P-p38	0.23	0.15	2.29 (0.72–7.27)

^a Significance of different parameters after controlling for stage.

^b Hazard ratio values of patients at higher risk in their survival according to the molecular markers.

^c Bold face indicates significance level <0.05.

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