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# Mitogen-Activated Protein Kinase Expression and Activation Does Not Differentiate Benign from Malignant Mesothelial Cells

**Lina Vintman,** M.Sc.[1]
**Søren Nielsen,** C.T.[2]
**Aasmund Berner,** M.D., Ph.D.[3]
**Reuven Reich,** Ph.D.[1]
**Ben Davidson,** M.D., Ph.D.[3]

[1] Department of Pharmacology and Experimental Therapeutics, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel.

[2] Department of Pathology, Aalborg Hospital, Aalborg, Denmark.

[3] Department of Pathology, The Norwegian Radium Hospital, University of Oslo, Oslo, Norway.

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Reuven Reich is affiliated with the David R. Bloom Center for Pharmacy at the Hebrew University.

Address for reprints: Ben Davidson, M.D., Ph.D., Department of Pathology, The Norwegian Radium Hospital, Montebello N-0310 Oslo, Norway; Fax: (011) 47 22508554; E-mail: ben.davidson@radiumhospitalet.no

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**BACKGROUND.** In vitro studies of malignant mesothelioma (MM) cells have suggested activation of mitogen-activated protein kinase (MAPK) in response to asbestos exposure. The objective of this study was to investigate protein expression (level) and phosphorylation status (activity) of the extracellular-regulated kinase (ERK), the c-Jun amino-terminal kinase (JNK), and the high-osmolarity glycerol response kinase (p38) in vivo through the analysis of fresh frozen reactive mesothelium (RM) and MM specimens.

**METHODS.** MAPK levels were analyzed in 36 fresh-frozen MM specimens (32 effusions, 4 biopsies) and in 14 RM specimens (all effusions) using immunoblotting with antibodies detecting the total (pan-) and activated (phospho-) fraction (p-) of ERK, JNK, and p38. Values for pan-MAPK and p-MAPK expression and the p-MAPK/pan-MAPK ratio in MM and RM specimens were compared. Results were corroborated using immunocytochemistry for p-ERK, p-JNK, and p-38 in selected specimens.

**RESULTS.** Pan-ERK, pan-JNK, and pan-p38 expression was found frequently in both MM specimens (35 of 36 specimens) and RM specimens (14 of 14 specimens) using immunoblotting, with comparable findings for activated p-p38 (34 of 36 MM specimens, 13 of 14 RM specimens). Activation of p-ERK (27 of 36 MM specimens, 10 of 14 RM specimens) and p-JNK (25 of 36 MM specimens, 10 of 14 RM specimens) was less frequent. Pan-ERK ($P = 0.016$), pan-JNK ($P = 0.004$), pan-p38 ($P = 0.012$), and p-ERK ($P = 0.02$) expression levels were higher in MM specimens from female patients. Pan-p38 expression levels also were higher in peritoneal MM specimens ($P = 0.019$). MM and RM showed similar MAPK expression, activation, and activation ratios (Mann–Whitney test; $P > 0.05$). Immunocytochemistry localized MAPK to MM and RM cells.

**CONCLUSIONS.** The current results provided the first evidence of in vivo activation of MAPK in clinical MM and RM. The similar values in these two cell types suggest that MAPK may not be involved in the transformation of benign to malignant mesothelium, thus bringing into question the validity of using MAPKs as molecular therapeutic targets in patients with MM. ***Cancer* 2005;103:2427–33.**


**KEYWORDS: malignant mesothelioma, mitogen-activated protein kinases, immunocytochemistry, immunoblotting.**

In the process of tumor development and progression, malignant cells acquire aberrations that affect major cellular functions, including adhesion, proliferation, differentiation, and apoptosis.[1] Extracellular signals, including stress, growth factors, cytokines, and mitogens, affect many of these tumor-related processes, including the synthesis of metastasis-associated molecules (e.g., proteolytic en-


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zymes, angiogenic factors), refractoriness to apoptotic signals, and immortality. These signals are initiated at membrane receptors, such as tyrosine kinase receptors,[2,3] which, in turn, relay their messages to the nucleus using a complex network of intracellular signaling pathways.

The mitogen-activated protein kinase (MAPK) intracellular signaling pathway is a four-level cascade in which each kinase activates the following kinase substrate through a complex network, enabling the cell to maintain diversity and specificity while responding to various extracellular stimuli. The first level consists of MAPK kinase kinase kinases (MAPKKKK), such as p21-activated kinase, that are phosphorylated through interaction with small guanine triphosphate-binding proteins (e.g., Ras, CDC42 and Rac-1).[2,4] MAPK kinase kinases (MAPKKK) in the second level of the cascade are a heterogeneous group, of which Raf molecules and MAPK/ERK kinase kinases 1–4 (MEKK1–MEKK4) are important members.[3,4] The third level of the cascade includes the 7 MKKK-activated MAPK kinases, MEK1, MEK2, and MKK3–MKK7, that are activated through double phosphorylation of serine and threonine residues.[3–5]

The final level consists of 12 MAPKs, including extracellular-regulated kinase 1–5 (ERK1–ERK5), c-jun amino-terminal kinases 1–3 (JNK1–JNK3), and the high-osmolarity glycerol response kinase (p38) in its 4 different isoforms (p38$\alpha$, p38$\beta$, p38$\gamma$, and p38$\delta$). The JNK and p38 subfamily of kinases is activated by a large spectrum of stress-related stimuli. These include osmotic shock, inhibition of protein synthesis, and formation of oxygen radical species.[6] Signaling by p38, for example, affects gene expression, signaling through the adrenergic, arachidonate, and nitric oxide pathways; apoptosis, proliferation, and differentiation and is involved in the pathology of ischemic injury, infection, and wound healing.[7] The ERK subfamily of kinases is activated largely by growth factor signals, like those mediated by receptor tyrosine kinases.[3] The net results are growth, differentiation, and proliferation.[3] Thus, it is believed that p38 and JNK largely mediate apoptotic signals, whereas ERK promotes the opposing effect.[8] However, it is now known that there are overlaps in these functions.

Tyrosine and threonine phosphorylation of MAPK occurs in a specific manner. Thus, MEK1 and MEK2 activate ERK, MKK5 activates ERK5, MKK4 and MKK7 activate JNK, and MKK3 and MKK6 activate p38 subfamily members.[3,4] Activation of MAPK is followed by phosphorylation of a variety of cytosolic substrates as well as their translocation to the nucleus, where they activate a large number of transcription factors, such as AP-1, p53, Elk-1, Ets-1, c-Myc, and STATs.[3] This results in a variety of biologic effects, some of which are induced by several members that belong to the three groups of MAPK. Pathologic modulation of MAPK has been recognized increasingly as a critical component of stress-induced cellular responses and diseases.[9–11]

Malignant mesothelioma (MM) is a tumor derived from mesothelial cells, which are native to the body cavities. The pleural cavity is the most common site, with a present ratio of 9:1 with peritoneal tumors. Exposure to asbestos can be documented in $\approx$ 80% of the tumors.[12] The incidence of MM appears to be rising steeply in western countries, a trend that is likely to continue.[12] MM is an aggressive and rapidly fatal disease, with a median survival of 8 months if untreated, although selected patients achieve survival $\geq$ 2 years when surgery is combined with adjuvant therapy.[13] The disease develops decades after exposure, during which period the neoplastic cells accumulate a variety of chromosomal aberrations.[14] The molecular correlates of these chromosomal changes in clinical tumors are defined poorly; therefore, the targets that are relevant in the malignant transformation of mesothelial cells largely remain unknown.

Molecular events that have been shown to occur as a result of exposure to asbestosis in vitro affect all major intracellular pathways, including MAPK signaling.[15] Specifically, it was shown that asbestos induced epidermal growth factor-dependent ERK activation,[16] whereas oxidative stress-sensitive activation of p38 was found in rat mesothelial cells that were exposed to asbestos.[17] It was shown recently that the activator protein-1 component Fra-1 was expressed in mesothelioma cell lines that developed in the peritoneal cavity of rats after exposure to asbestos.[18] Despite this expanding research effort in vitro, little or nothing is known about MAPK expression and activation in clinical specimens of MM and reactive mesothelium (RM).

In the current study, we evaluated MAPK expression in 36 MM specimens and 14 RM specimens using phosphorylation state-specific antibodies that were suited ideally for studying complex patterns of phosphoregulation.[19] Activation of MAPK requires that these enzymes will be phosphorylated dually by the respective MEKs on both the Thr and Tyr residues in the consensus sequences within the catalytic domains.[20] The phospho-MAPK (p-MAPK) antibodies used in the current study were developed as polyclonal antibodies in rabbit against dually phosphorylated, synthetic peptides that encompassed the consensus sequences described above. The antibodies were purified using a repetitive adsorption step to remove antibodies that recognize the nonphosphorylated peptide, followed by positive selection-affinity

purification with the dually phosphorylated peptide to select for antibodies that preferentially recognize ERK1/ERK2 (44/42 kiloDaltons [kDa]), JNK1 (49 kDa), and JNK2 (55 kDa) or p38$\alpha$, p38$\beta$, and p38$\gamma$. These antibodies were investigated intensively in a variety of cell lines.[21–23] In the current study, we observed similar MAPK expression and activation in MM and RM specimens, suggesting that the malignant transformation in mesothelial cells does not involve specific MAPK activation and, thus, is of questionable value as a therapeutic target in this disease.

## MATERIALS AND METHODS

### Patients and Materials

The studied material consisted of 50 specimens (46 effusions, 4 biopsies) that were submitted to the Departments of Pathology at The Norwegian Radium Hospital and Aalborg Hospital during the period of from 1998 to 2002. These consisted of 36 MM specimens (32 effusions, 4 biopsies) and 14 RM specimens (all effusions). MM specimens were obtained from 36 patients (30 males and 6 females) ages 39–91 years (mean age, 67 years). Twenty-eight MM specimens were of pleural origin, and 8 MM specimens were peritoneal. All specimens with RM proliferations were obtained from patients who had a previously diagnosed malignancy or a clinical suspicion of malignancy. All specimens underwent morphologic evaluation by experienced pathologists and were characterized further using immunocytochemistry and flow cytometry with broad antibody panels against carcinoma and mesothelial epitopes.[24,25] The presence of malignant cells in reactive specimens was excluded beyond any doubt using these methods. Informed consent was obtained according to institutional guidelines.

### Immunoblotting

The protocol for this experiment was published previously.[26,27] Briefly, frozen specimens were thawed and subsequently lysed in 1% NP-40; 20 mM Tris HCl, pH 7.5; 137 mM NaCl; 0.5 mM ethylenediamine tetraacetic acid; 10% glycerol; 1 mM phenylmethylsulfonyl fluoride; 1 $\mu$g/mL aprotinin; 2 $\mu$g/mL leupeptin; 1 mM sodium orthovanadate; and 0.1% sodium dodecyl sulfate (SDS). Under reducing conditions, 15 $\mu$g from each sample were loaded into each lane and separated by electrophoresis through SDS-10% polyacrylamide gels. After electrophoresis, proteins were transferred to Immobilon transfer membranes (Millipore, Bedford, MA). Membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 (TBST) and incubated overnight at 4 °C in 5% bovine serum albumin (BSA) in TBST containing either antiphospho-ERK (anti-p-ERK), anti-p-JNK, or anti-p-p38 (all from BioSource, Camarillo, CA). After incubation, membranes were washed and incubated for 1 hour with peroxidase-conjugated AffiniPure goat antirabbit immunoglobulin G (Jackson ImmunoResearch, West Grove, PA) in TBST containing 5% BSA. Membranes were developed using the enhanced chemiluminescence kit (Pierce, Rockford, IL), according to the manufacturer's specifications. Membranes were then washed; stripped in 0.2 M glycine, 0.1% SDS, and 1% Tween 20, pH 2.2; blocked in TBST containing 5% BSA; and incubated overnight at 4 °C in 5% BSA in TBST containing anti-ERK (BioSource), anti-JNK (Biosource), or anti-p38 (StressGen, Victoria, BC, Canada). The above-described procedure was performed to visualize the signal. A375SM melanoma cells were used as controls in all the gels.

### Quantification of Blotting Results

Gels were photographed using the Kodak EDAS 290 system. Densitometer analysis of films was performed using a computerized image analysis program (NIH Image, version 1.62). The parameters analyzed were as follows: 1) enzyme level (using the "pan" antibody), 2) enzyme phosphorylated activity (using the phospho-directed antibody), and 3) enzyme activation ratio (the p-MAPK/pan-MAPK ratio).

### Immunocytochemical Analysis

Formalin fixed, paraffin embedded cell pellets from 8 MM effusions and 5 RM effusions were analyzed using antibodies against p-ERK, p-JNK, and p-p38 (Biosource). Pretreatment consisted of microwave oven using low-pH citrate buffer. Slides were immunostained at the Department of Pathology at the Norwegian Radium Hospital. Staining was performed using the EnVision™ + peroxidase system (DakoCytomation). Positive controls consisted of the melanoma cell line WM35. Nuclear and cytoplasmic immunoreactivity were scored as positive. The extent of staining was scored using the following scale: 0 = no staining, 1 = staining of 0–5% of tumor cells, 2 = staining of 6–25% of tumor cells, 3 = staining of 26–75% of tumor cells, and 4 = staining of 76–100% of tumor cells.

### Statistical Analysis

Statistical analysis was performed applying the SPSS-PC package (version 10.1; SPSS, Chicago, IL). A probability $< 0.05$ was considered statistically significant. The associations between MAPK expression, activation, the activation ratio and specimen type (MM vs. RM), the tumor site (pleural vs. pleural), and patient gender were evaluated using the Mann–Whitney test. Because MAPK values are continuous variables, this

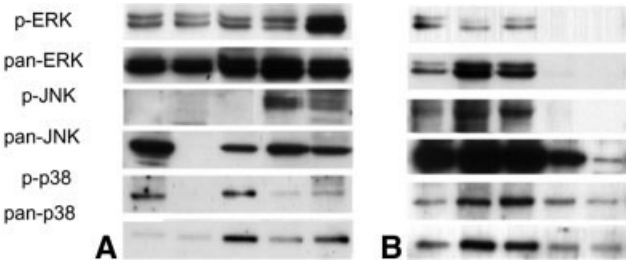


**FIGURE 1.** Mitogen-activated protein kinase expression and activation are frequent in malignant mesothelioma (MM) and reactive mesothelium (RM). (A) MM specimens: Expression and phosphorylation of total (pan-) extracellular-regulated kinase (pan-ERK) is seen in five MM effusions. Note the presence of 2 bands corresponding to the p42/p44 ERK1/ERK2 isoforms. Total c-Jun amino-terminal kinase (pan-JNK) expression is strong in 4 effusions, with minimal expression (1% of control) in the remaining specimen that was detectable only by densitometric analysis. Phosphorylation is evident in lanes 4 and 5 and is evident weakly in lane 2, whereas lanes 1 and 3 are negative for phosphorylation. Variable expression of the high-osmolarity glycerol response kinase (p38) is seen in all specimens, with the activated (phosphorylated) fraction (p-) of p38 (p-p38) expressed in 4 of 5 effusions. (B) RM specimens: Expression of pan-ERK, pan-JNK, and pan-p38 is seen in 5 RM effusions but is minimal for pan-ERK in lanes 4 and 5 (1% of control in lane 5; detectable only by densitometric analysis). Activated p-p38 is seen in all lanes, whereas only lanes 1–3 show activated p-ERK and p-JNK.

test provided the most accurate analysis of potential differences between these groups.

## RESULTS

### MAPKs Are Expressed and Activated Frequently in both MM and RM

MAPK signaling mediates critical biologic events in both normal and tumor cells, but tumor cells alone are characterized by dysregulation of death and survival pathways. To define cellular events that potentially may be unique for tumor cells, we compared MAPK signaling in benign and malignant mesothelial cells. All 36 MM specimens showed expression of pan-JNK (range, 1–308%; mean, 59% of control values) and pan-p38 (range, 1–180%; mean, 57% of control values) (Fig. 1A). Pan-ERK was expressed in 35 of 36 specimens (range, 1–368%; mean, 89% of control values). Phosphorylation of p38 was the most frequent (34 of 36 specimens; range, 1–345%; mean, 34% of control values), with less pronounced phosphorylation of p-ERK (27 of 36 specimens; range, 0–58%; mean, 9% of control values) and p-JNK (25 of 36 specimens; range, 0–69%; mean, 13% of control values) (Fig. 1A). The ERK, JNK, and p38 mean activation ratios were 0.17%, 2.4%, and 0.8%, respectively. Immunocytochemistry showed nuclear expression of p-ERK and p-JNK in all 8 MM specimens, with p-p38 expression found in 2 of 8 specimens (Fig. 2A–F).

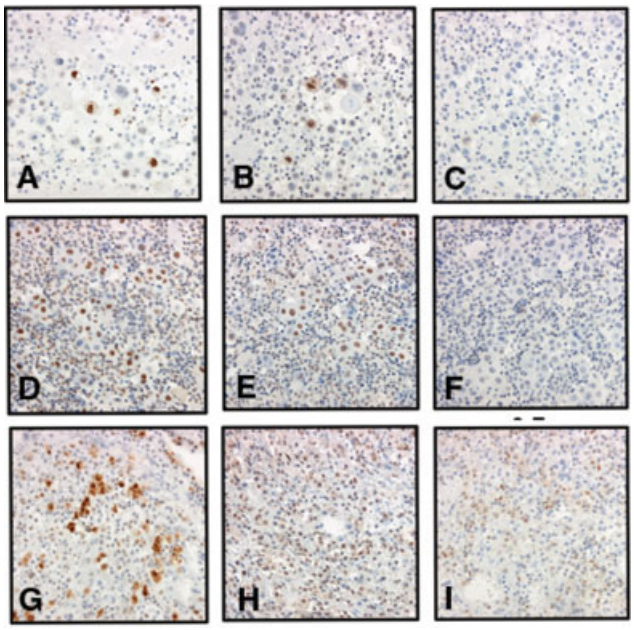


**FIGURE 2.** Malignant mesothelioma (MM) and reactive mesothelium (RM) specimens show nuclear expression of activated mitogen-activated protein kinase. (A–C) MM cells in a pleural effusion from a male patient age 91 years show distinct nuclear immunoreactivity for the activated (phosphorylated) fraction (p-) of extracellular-regulated kinase (p-ERK) (A) and phosphorylated c-Jun amino-terminal kinase (p-JNK) (B) with focal cytoplasmic expression of the phosphorylated form of the high-osmolarity glycerol response kinase (p-p38) (C). Note the p-ERK expression in cells undergoing mitosis. (D–F) Most of the MM cells in this pleural effusion from a female patient age 54 years show nuclear immunoreactivity for p-ERK (D) and p-JNK (E), with no expression of p-p38 (F). (G–I) Most of the RM cells in this benign reactive effusion show nuclear immunoreactivity for p-ERK (G), p-JNK (H), and p-p38 (I).

An analysis of 14 RM specimens showed expression of pan-ERK (range, 1–151%; mean, 54% of control values), pan-JNK (range, 1–106%; mean, 47% of control values), and pan-p38 (range, 2–153%; mean, 34% of control values) in all specimens (Fig. 1B). Like what was observed in the MM specimens, phosphorylation of p38 was found in 13 of 14 RM specimens (range, 1–457%; mean, 57% of control values), with less pronounced phosphorylation of p-ERK (10 of 14 specimens; range, 1–20%; mean, 6% of control values) and p-JNK (10 of 14 specimens; range, 1–114%; mean, 23% of control values) (Fig. 1B). The ERK, JNK, and p38 mean activation ratios were 0.15%, 0.41%, and 1.12%, respectively. Immunocytochemistry showed expression of p-ERK and p-JNK in all 5 specimens and expression of p-p38 in 4 of 5 specimens (Fig. 2G–I). Statistical analysis showed similar MAPK expression,

**TABLE 1**
**Mitogen-Activated Protein Kinase Expression, Phosphorylation, and Activation Ratio in Patients with Malignant Mesothelioma ($N = 36$) and Patients with Reactive Mesothelium ($N = 14$)**

| Parameter | MM[a] | RM[a] | *P* value |
|---|---|---|---|
| p-ERK | 25 | 26 | 0.8 |
| pan-ERK | 23 | 26 | 0.5 |
| ERK activation ratio | 25 | 25 | 1.0 |
| p-JNK | 26 | 25 | 0.8 |
| pan-JNK | 24 | 26 | 0.8 |
| JNK activation ratio | 27 | 25 | 0.6 |
| p-p38 | 25 | 26 | 0.9 |
| pan-p38 | 22 | 27 | 0.3 |
| p38 Activation ratio | 27 | 25 | 0.6 |

MM: malignant mesothelioma; RM: reactive mesothelium; p-: activated (phospho-) fraction; ERK: extracellular-regulated kinase; pan-: total; JNK: c-Jun amino-terminal kinase; p38: high-osmolarity glycerol response kinase.
[a] Mean rank values (Mann–Whitney test).

**TABLE 2**
**Mitogen-Activated Protein Kinase Expression, Phosphorylation, and Activation Ratio in Malignant Mesothelioma Specimens from Female Patients ($N = 6$) and from Male Patients ($N = 30$)**

| Parameter | Males[a] | Females[a] | *P* value |
|---|---|---|---|
| p-ERK | 17 | 27 | 0.02 |
| pan-ERK | 17 | 28 | 0.016 |
| ERK activation ratio | 18 | 19 | 0.7 |
| p-JNK | 17 | 25 | 0.09 |
| pan-JNK | 16 | 29 | 0.004 |
| JNK activation ratio | 19 | 18 | 0.9 |
| p-p38 | 17 | 26 | 0.06 |
| pan-p38 | 17 | 28 | 0.012 |
| p38 Activation ratio | 19 | 18 | 1.0 |

p-: Activated (phospho-) fraction; ERK: extracellular-regulated kinase; pan-: total; JNK: c-Jun amino-terminal kinase; p38: high-osmolarity glycerol response kinase.
[a] Mean rank values (Mann–Whitney test).

activation, and activation ratios in MM and RM specimens (Mann–Whitney test; $P > 0.05$) (Table 1).

### MAPK Expression Is Higher in MM Specimens from Female Patients

In our cohort, most peritoneal MM tumors were diagnosed in women. We wanted to analyze the potential differences in MAPK expression and activation in tumors from female and male patients and to compare peritoneal MM with tumors in the pleural cavity. Pan-ERK (Mann–Whitney test; $P = 0.016$), pan-JNK (Mann–Whitney test; $P = 0.004$), pan-p38 (Mann–Whitney test; $P = 0.012$), and p-ERK (Mann–Whitney test; $P = 0.02$) expression levels were higher in MM specimens from female patients (Table 2). Similar trends were seen for p-JNK ($P = 0.09$) and p-p38 ($P = 0.06$). Pan-p38 expression levels also were higher in peritoneal MM compared with pleural MM (Mann–Whitney test; $P = 0.019$).

## DISCUSSION

RM cells exhibit morphologic changes that often are seen in tumor cells, such as a higher nuclear-cytoplasmic ratio, large nucleoli, and brisk mitotic activity.[28] Tumor therapy, including radiation directed to the serosal cavities and systemic or intracavital chemotherapy, often results in enhancement of these characteristics rather than elimination of these cells. Added to the postulated role of MAPK signaling in asbestos-induced tumorigenesis, these observations raise the question of whether MAPK activation can serve as a diagnostic molecular marker for MM in vivo.

We showed previously that MAPK expression and phosphorylation were associated with clinical parameters of better outcome in patients who had ovarian carcinoma with effusions.[26] In addition, pan-ERK, pan-JNK, and p-ERK expression predicted improved outcomes, and that predictive ability was independent for pan-ERK and pan-JNK in a Cox multivariate analysis.[26] These data were in agreement with the current hypothesis favoring less categoric separation between ERK and JNK/p38 in mediating proliferation and apoptosis, respectively. They also suggest that, at least in some tumors, efforts directed at targeting MAPK in clinical trials[29] may be counterproductive.

Expression of all 3 MAPK families was found in the current study using immunoblot analysis, but the activation of p38 was more frequent and more pronounced compared with the activation of JNK or ERK. This may be related to the cellular stress caused by the reduced availability of oxygen and nutrients in effusions but, as we reported in patients with ovarian carcinoma, results in only minimal cell death.[30] Immunocytochemistry in general confirmed these findings, with the exception of the infrequent p-p38 immunoreactivity in MM specimens, a difference that may be related to lower sensitivity of this antibody in paraffin-embedded material. The similar expression and activation of all three MAPK families in MM and RM cells suggest that these kinases do not mediate the biochemical progression from benign to malignant mesothelium. Because this transformation predicts extremely poor outcome, the lack of relevance of MAPK in this process is in agreement with our aforementioned clinical findings in patients who had ovarian carcinoma with effusions. We therefore hypothesize that MAPK signaling may mediate cellular events that reduce the aggressiveness of some tumor types, a

theory that we also are pursuing currently in metastatic breast carcinoma (unpublished results).

Peritoneal MM is much less frequent than pleural MM and afflicts younger patients, and a relatively higher proportion of women are diagnosed with the disease.[31] Molecular studies with the objective of elucidating differences that may be underlying this clinical division largely are unavailable to date. In a recent study, we found that expression of the activated high-affinity nerve growth factor tyrosine kinase receptor p-TrkA was higher in peritoneal MM compared with pleural MM.[32] In the current study, higher expression of pan-p38 was found in peritoneal MM. In addition, the total expression of all three MAPKs and p-ERK activation were higher in specimens from female patients, a patient group with tumors that constituted six of the eight peritoneal MM samples in our study. These findings suggest that MAPK expression may be one of the factors behind the generally less aggressive behavior of peritoneal MM.

In conclusion, MAPK expression and activation is a frequent event in both MM and RM, an observation that argues against a major role for this pathway in the malignant transformation of mesothelial cells. Our data highlight the ubiquitous presence of these molecules in benign and malignant cells and question their validity as therapeutic targets.

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