

## Featured Article

# Characterization of Active Mitogen-Activated Protein Kinase in Ovarian Serous Carcinomas

Chih-Yi Hsu,<sup>1,3,4</sup> Robert Bristow,<sup>2</sup>  
Moon Seok Cha,<sup>2</sup> Brant G. Wang,<sup>1</sup>  
Chung-Liang Ho,<sup>1</sup> Robert J. Kurman,<sup>1,2</sup>  
Tian-Li Wang,<sup>2</sup> and Ie-Ming Shih<sup>1,2</sup>

Departments of <sup>1</sup>Pathology and <sup>2</sup>Gynecology and Obstetrics, Johns Hopkins Medical Institutions, Baltimore, Maryland; <sup>3</sup>Department of Pathology and Laboratory Medicine, Taipei Veterans General Hospital Taipei, Taiwan; and <sup>4</sup>Department of Pathology, National Yang-Ming University School of Medicine, Taipei, Taiwan

## ABSTRACT

**Purpose:** Mitogen-activated protein kinase (MAPK) plays a pivotal role in signal transduction. Activation of MAPK is regulated by upstream kinases including *KRAS* and *BRAF*, which are frequently mutated in low-grade ovarian serous carcinoma. This study evaluates the expression of active MAPK in ovarian serous carcinomas, with response to treatment and survival.

**Experimental Design:** Expression of active MAPK was assessed by immunohistochemistry in 207 cases of ovarian serous tumors. Immunoreactivity was correlated with tumor grade, mutational status of *KRAS* and *BRAF*, *in vitro* drug resistance, and clinical outcome.

**Result:** There was a lower frequency of expression of active MAPK in high-grade ovarian serous carcinomas as compared with low-grade serous tumors, including borderline tumors and low-grade serous carcinoma ( $P < 0.001$ ). Active MAPK was present in all of the 19 low-grade tumors with either *KRAS* or *BRAF* mutations as well as in 14 (41%) of 34 tumors with wild-type *KRAS* and *BRAF* in both low- and high-grade carcinomas. Expression of active MAPK alone served as a good survival indicator in the 2-year follow-up ( $P = 0.037$ ) but not in the 5-year follow-up ( $P = 0.145$ ). However, a combination of expression of active MAPK and *in vitro* sensitivity of paclitaxel significantly correlated with a better prognosis in 5-year survival rate ( $P = 0.048$ ) in patients with advanced-stage high-grade serous carcinoma.

**Conclusions:** Active MAPK is more frequently expressed in low-grade than in high-grade ovarian serous carcinoma. Active MAPK serves as a good prognostic marker in patients with high-grade serous carcinomas.

## INTRODUCTION

Ovarian cancer is the leading cause of death in women who suffer from gynecologic cancer. Of the various histologic subtypes of ovarian cancer, ovarian serous carcinoma is the most common and corresponds to what is generally referred to as ovarian cancer. Based on a number of clinicopathologic and molecular genetic studies (1), we have recently proposed a dualistic model for the development of ovarian serous carcinoma (2, 3). In this model serous carcinomas are subdivided into low- and high-grade tumors. Low-grade serous carcinoma, also known as invasive micropapillary serous carcinoma, develops in a step-wise fashion from an intraepithelial (*in situ*) low-grade serous carcinoma, which in turn arises from an atypical proliferative serous tumor. These two noninvasive neoplasms correspond to borderline tumors. Atypical proliferative serous tumor may develop either from a cystadenoma or directly from ovarian surface epithelium. High-grade serous carcinoma develops in a *de novo* fashion directly from ovarian surface epithelium or from small epithelial inclusion cysts as no intermediate stages have thus far been identified. Low-grade serous carcinomas typically have an indolent course with recurrences developing over many years, but eventually these patients develop intra-abdominal carcinomatosis, which proves to be fatal. High-grade serous carcinomas are highly aggressive, and most patients present with advanced stage disease at the time of diagnosis (4). Recent studies have shown that activating mutations of *BRAF* or *KRAS* are found in ~60% of serous borderline tumors (atypical proliferative tumors and intraepithelial low-grade serous carcinomas) and low-grade serous carcinomas but rarely in the high-grade serous carcinomas (1). This suggests that the signaling pathway involving *KRAS* and *BRAF* is important in the development of low-grade serous tumors.

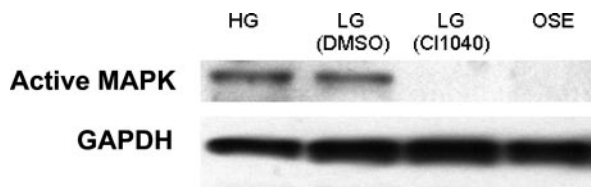
The mitogen-activated protein kinase (MAPK), also known as extracellular signal-regulated protein kinase (ERK), is a downstream target of the RAS, RAF, and MAP/ERK kinase and is crucial for transduction of growth signals from several key growth factors, cytokines, and proto-oncogenes (5). Mutations or overexpression of components, including *KRAS* and *BRAF*, in the MAPK pathway lead to constitutive activation of MAPK by phosphorylation. Activation of MAPK in turn activates downstream protein kinases, nuclear proteins, and transcription factors (6), which may contribute to the tumor development (7). Besides its role in oncogenesis, activation of MAPK may sensitize cancer cells to anticancer agents (5) as activation of MAPK is essential for the killing effect of CD437, a synthetic retinoid that has been reported to induce growth arrest and

Received 5/5/04; revised 6/21/04; accepted 6/25/04.

**Grant support:** United States Department of Defense grant OC010017. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Requests for reprints:** Ie-Ming Shih, Johns Hopkins Medical Institutions, 1503 E. Jefferson Street, Room B-315, Baltimore, MD 21231. Phone: (410) 502-774; Fax: (410) 502-7943; E-mail: ishih@jhmi.edu.

©2004 American Association for Cancer Research.



**Fig. 1** Western blot analysis of active MAPK (*top panel*) and glyceraldehyde-3-phosphate dehydrogenase (*bottom panel*) in ovarian serous tumors. Active MAPK ( $M_r$  42,000) is identified in a high-grade and a low-grade DMSO serous carcinoma cell cultures but not in ovarian surface epithelial culture. Expression of active MAPK is decreased to an undetectable level in the low-grade serous carcinoma cell culture treated with CI-1040, a compound that inhibits MEK and phosphorylation of MAPK. The same blot was probed with a glyceraldehyde-3-phosphate dehydrogenase antibody and showed a similar amount of protein loaded in each lane. (*GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *HG*, high-grade; *LG*, low-grade; *OSE*, ovarian surface epithelial).

apoptosis in ovarian cancer cell lines (8). It has been shown that constitutive activation of the MAPK signaling pathway occurs in a wide variety of human carcinomas including pancreatic, colorectal, lung, breast, and prostate carcinomas (9). In this study, we attempted to characterize the expression of active (phosphorylated) MAPK in ovarian serous tumors by assessing its differential expression in low-grade and high-grade serous tumors. In addition, we examined whether phosphorylated MAPK alone, or in combination with other clinical parameters, could serve as a prognostic marker.

## MATERIALS AND METHODS

**Tissue Samples.** Formalin-fixed, paraffin-embedded tissue samples of 207 ovarian serous tumors including 29 serous cysts, 7 atypical proliferative serous tumors, 15 intraepithelial low-grade serous carcinomas, 16 low-grade serous carcinomas, and 140 high-grade serous carcinomas were retrieved from the archival file of the Department of Pathology from the Johns Hopkins Hospital. The acquisition of paraffin tissues was approved by the Johns Hopkins Institutional Review Board. The paraffin tissues were organized into tissue microarrays, which were made by removing three 1.5-mm diameter cores of tumor

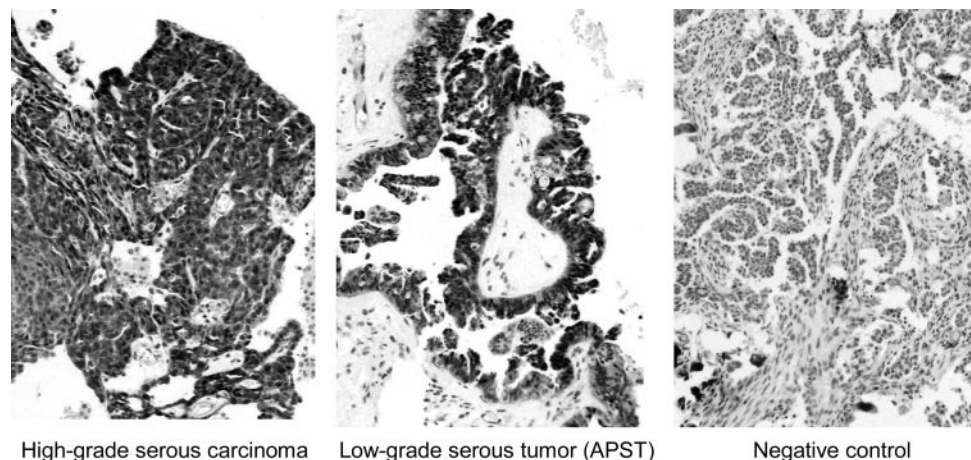
from each block. The selection of areas for core was made by two pathologists (Chih-Yi Hsu and Ie-Ming Shih) based on review of the H&E slides. To evaluate whether expression of active MAPK was a prognostic marker in patients with ovarian serous carcinoma, we analyzed 117 advanced stage high-grade serous carcinomas of which 96 were Fédération Internationale des Gynaecologues et Obstétristes (FIGO) stage III and 21 were FIGO stage IV.

### Immunohistochemistry and Western Blot Analysis.

Expression of the active (phosphorylated) form of MAPK was assessed by immunohistochemistry and Western blot analysis. The antibody used was a rabbit polyclonal antibody, pTEpY, that specifically reacts with the phosphorylated but not the unphosphorylated MAPK (Promega, Madison, WI). Immunohistochemistry was done on tissue microarrays at a dilution of 1:500 followed by the EnVision+System with the peroxidase method (DAKO, Carpinteria, CA). The percentage of positive cells was estimated by randomly counting ~500 tumor cells from three different high-power fields ( $\times 40$ ) within one specimen. For the negative control, an isotype (IgG1) matched antibody, MN-4, was used in parallel (10). A positive reaction was defined as discrete localization of the brown chromagen in the nucleus or cytoplasm. Cases in which more than 5% of the tumor cells showed detectable immunoreactivity were scored as positive.

Western blot analysis was done with the same antibody (1:3,000) on 2 representative ovarian serous carcinomas and one specimen of an ovarian surface epithelial cell culture. One of the ovarian serous carcinoma cell cultures was previously treated with CI-1040, a compound that specifically inhibits the activity of MEK which activates (phosphorylates) MAPK (7). For positive control, the same blot was incubated with an antibody that reacts to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Research Diagnostics, Inc., Flanders, NJ) at a dilution of 1:2,000. Similar amounts of total protein from each lysate were loaded and separated on 12% Tris-glycine-SDS polyacrylamide gels (Novex, San Diego, CA) and electroblotted to Millipore Immobilon-P polyvinylidene difluoride membranes. Western blots were developed by chemiluminescence (Pierce, Rockford, IL).

**Fig. 2** Immunohistochemistry of active MAPK in ovarian serous tumors. An intense immunoreactivity is present in both nuclei and cytoplasm of a high-grade and a low-grade serous tumor, atypical proliferative serous tumor. The negative control did not show any detectable immunoreactivity. (*APST*, atypical proliferative serous tumor).



High-grade serous carcinoma

Low-grade serous tumor (APST)

Negative control

Table 1 Frequency of positive MAPK immunostaining cases in ovarian serous tumors.

Tumor type	Case no.	MAPK+ n (%)	P
High-grade serous carcinoma	140	58 (41)	<0.001
Low-grade serous carcinoma	16	13 (81)	
Serous borderline tumor			
Intraepithelial low-grade serous carcinoma	15	12 (80)	
Atypical proliferative serous tumor	7	5 (71)	

Table 2 Status of *in vitro* drug resistance and active MAPK staining in high-grade serous carcinoma

Chemotherapy drug	n	MAPK+ n (%)	P
Paclitaxel			0.893
Sensitive	46	24 (52)	
Resistant	12	6 (50)	
Cisplatin			0.071*
Sensitive	50	29 (58)	
Resistant	9	2 (22)	
Carboplatin			1.000*
Sensitive	44	23 (52)	
Resistant	6	3 (50)	

\* Fisher's exact test.

***In vitro* Drug Resistance Assay.** Drug sensitivity was assessed by an agar-based cell culture system with radioactive thymidine incorporation as the end point (Oncotech, Tustin, CA). There were 59 cases in which the results of this assay were available for study. The protocol of the assay has been detailed online.<sup>5</sup> Briefly, fresh tumor specimens immediately removed at the time of surgery were placed into the RPMI (Life Technologies, Inc., Carlsbad, CA) and shipped to Oncotech, Inc. for analysis. The tumor tissue was minced and enzymatically digested to disaggregate the tumor cells, which were then plated in soft agar. Tumor cells were then exposed to antineoplastic agents at a concentration of a maximum tolerated dose for 5 days *in vitro*. Tritiated thymidine was introduced during the last 2 days of culture as a measure of cell proliferation, and the drug treated cells were compared with the untreated controls. Assay results were divided into three categories: extreme, intermediate, and low drug resistance with the criteria described in the company's web site. The extreme drug resistance was interpreted as drug resistant, and the intermediate and low drug resistance were interpreted as drug sensitive in this study as reported previously (11, 12).

**Mutational Analysis of *KRAS* and *BRAF* Genes.** For nucleotide sequencing, genomic DNA was purified from microdissected tumors as described previously (1). The mutational analysis was done with the software of Lasergene (DNASTar, Madison, WI).

**Statistical Analysis.** The significance of age for high-grade *versus* low-grade ovarian serous tumors was tested by the

Mann-Whitney test. The significance of expression of active MAPK and patient age, FIGO stage, and *in vitro* drug resistance was determined using the  $\chi^2$  or the Fisher's exact test. Overall survival was defined as the time between diagnosis and death or the most recent follow-up. The survival curves were established using the Kaplan-Meier method, and their differences were tested by the log-rank test. All of the patients with advanced high-grade serous carcinomas were treated with a combined chemotherapy including paclitaxel, carboplatin, or cisplatin.

## RESULTS

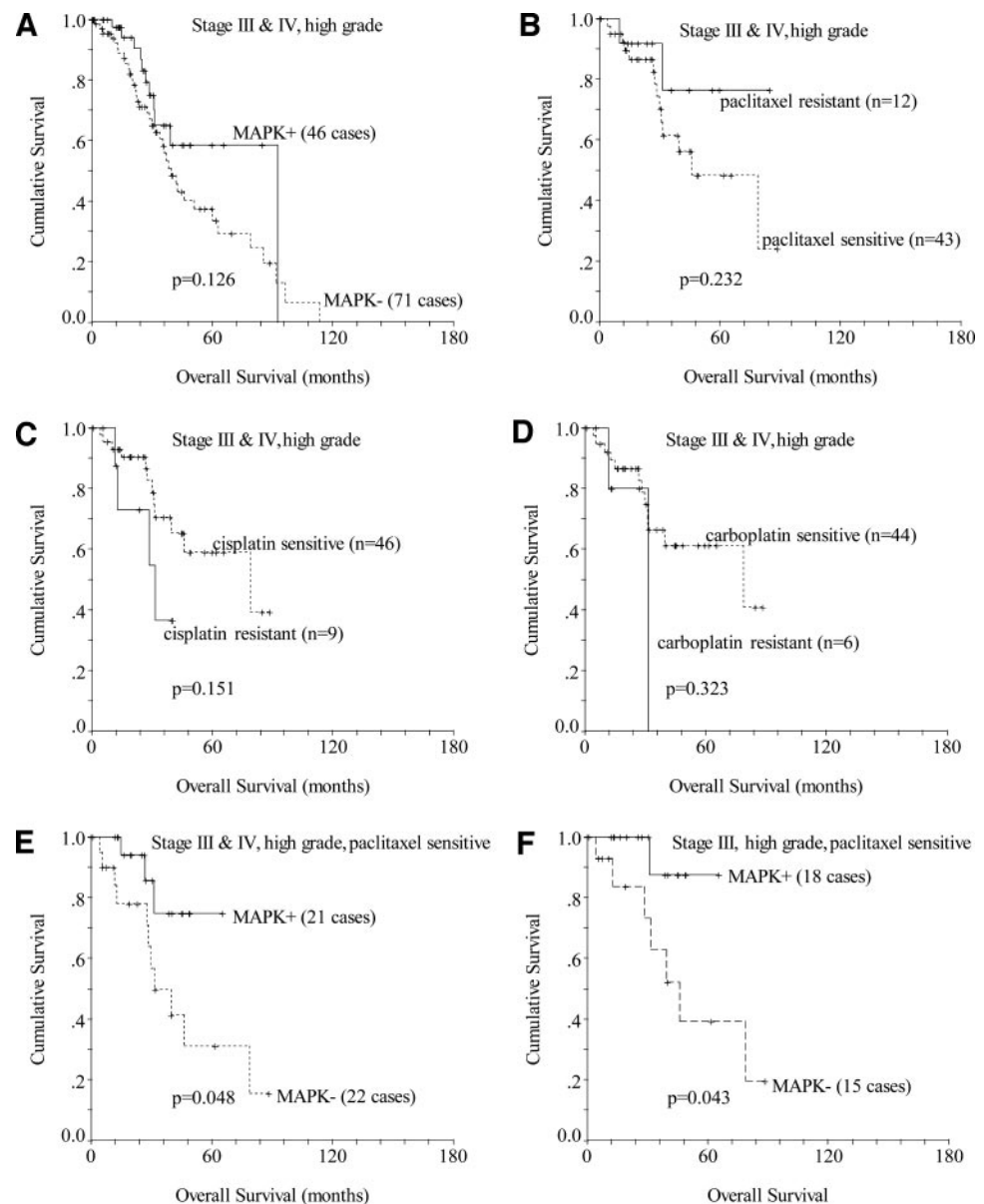
Patients with high-grade serous carcinoma [median (range), 61 (33 to 86) years] were older than patients with low-grade serous carcinoma [39 (24 to 65) years; Mann-Whitney test,  $P < 0.001$ ]. Expression of the active MAPK in ovarian serous tumors was assessed with immunohistochemistry. The specificity of the antibody for binding with the active (phosphorylated) form of MAPK was shown by Western blot analysis. As shown in Fig. 1, a single band of  $M_r$  42,000 corresponding to the phosphorylated MAPK is present in both the high-grade and low-grade serous carcinoma cell cultures but not in ovarian surface epithelial cells. The band from the low-grade tumor cells was not detectable in cells pretreated with CI-1040 that inhibited phosphorylation of MAPK, confirming the specificity of the antibody.

Because of the frequent mutations in *KRAS* and *BRAF* in ovarian low-grade serous tumors as compared with high-grade serous carcinoma (1, 13), we examined whether there is a differential expression of activated MAPK in low-grade and high-grade serous tumors. The immunoreactivity of active (phosphorylated) MAPK was detected in both the nucleus and the cytoplasm of the tumor cells (Fig. 2), which is consistent with a previous report (14). The frequency of expression of active MAPK in high-grade serous carcinomas (41%) was significantly lower than in low-grade serous carcinomas (81%), intraepithelial low-grade serous carcinomas (80%), and atypical proliferative serous tumors (71%;  $\chi^2$  test,  $P < 0.001$ ; Table 1). Among the high-grade serous carcinomas, the expression of active MAPK was more frequent in younger patients (53% in patients  $< 60$  years *versus* 31% in patients  $\geq 60$  years old;  $\chi^2$  test,  $P = 0.010$ ). Expression of active MAPK did not correlate with FIGO stage ( $P = 0.398$ ) nor with drug resistance for paclitaxel ( $P = 0.893$ ), cisplatin ( $P = 0.071$ ), and carboplatin ( $P = 1.000$ ) that are commonly used in treating ovarian cancer

Table 3 Correlation of MAPK immunoreactivity and mutational status of *BRAF* and *KRAS*.

	High-grade	Low-grade
Mutation in <i>BRAF</i> or <i>KRAS</i>		
MAPK+	0	19
MAPK-	0	0
No mutation in <i>BRAF</i> or <i>KRAS</i>		
MAPK+	9	5
MAPK-	16	4
Total cases	25	28

<sup>5</sup> Web address: <http://www.oncotech.com/innovation/PublicationDetails.asp?id=28>.



**Fig. 3** The significance of active MAPK and *in vitro* drug sensitivity in survival of patients with high-grade advanced ovarian serous carcinoma. Overall survival in relation to (A) active MAPK expression ( $n = 117$ ); B, status of paclitaxel resistance ( $n = 55$ ); C, status of cisplatin resistance ( $n = 55$ ); D, status of carboplatin resistance ( $n = 50$ ); E, active MAPK expression in paclitaxel sensitive cases ( $n = 43$ ); and F, active MAPK expression in paclitaxel sensitive cases at FIGO stage III ( $n = 33$ ).

patients (Table 2). In low-grade serous tumors, expression of activated MAPK was independent of age (Mann-Whitney test,  $P = 0.368$ ) and FIGO stage ( $\chi^2$  test,  $P = 0.777$ ).

To assess if there was a correlation of expression of active MAPK and mutation in *KRAS* or *BRAF*, we compared the immunoreactivity of active MAPK in 28 cases of low-grade serous carcinomas and 25 cases of high-grade serous carcinomas that were available for mutational analysis (Table 3). Mutational status of *KRAS* and *BRAF* in 38 of the 53 cases has been reported in our previous study (1). In low-grade serous tumors, all of the 19 cases with mutant *KRAS* or *BRAF* were MAPK positive, whereas 5 of 9 low-grade tumors with wild-type *KRAS* and *BRAF* were MAPK positive. Thus, mutations in *KRAS* or *BRAF* correlated with the immunoreactivity of active MAPK (100 versus 55.6%; Fisher's exact test,  $P = 0.006$ ). In high-

grade serous carcinomas, all of the 25 cases contained wild-type *KRAS* and *BRAF*. Nine of the high-grade serous carcinomas were positive for activated MAPK.

Next, we evaluated whether expression of active MAPK was a prognostic marker in patients with ovarian serous carcinoma. We focused on 117 advanced stage high-grade serous carcinomas because this is the most common clinical presentation in patients with ovarian cancer. As compared with high-grade serous carcinomas, low-grade serous carcinomas are rare, and the case number is not sufficient for statistical analysis of cumulative survival. The median survival for stage III patients was 51 months, and the median survival for stage IV patients was only 30 months (log-rank test,  $P = 0.002$ ). The 2-year and 5-year survival rates in patients whose tumors expressed active MAPK appeared better than those not expressing it (90.4 versus 71.1% and 58.6 versus 37.3%, respec-

tively) but was statistically significant only for the 2-year survival (log-rank test,  $P = 0.037$ ; Fig. 3A). Patient age (log-rank test,  $P = 0.490$ ) and the status of *in vitro* drug resistance (paclitaxel  $P = 0.232$ , cisplatin  $P = 0.151$ , and carboplatin  $P = 0.323$ ) did not reveal a significant difference in the survival for advanced stage high-grade serous carcinoma (Fig. 3, B–D) but was significantly better for survival when tumors expressed active MAPK and were sensitive to paclitaxel (5-year survival 74.9 versus 31.0%; log rank test,  $P = 0.048$ ; with age adjusted,  $P = 0.014$ ; Fig. 3E). In contrast, expression of active MAPK failed to show such significant prognostic difference in combination with cisplatin and carboplatin. When the patients at advanced stages were stratified into stage III and stage IV groups, 33 patients at stage III with active MAPK expression and paclitaxel sensitivity had even better overall survival with a 5-year survival of 87.5 versus 39.2% in MAPK-positive cases (log rank test,  $P = 0.043$ ; with age adjusted,  $P = 0.027$ ; Fig. 3F). It lacked a statistical power to assess the significance in stage IV patients because of a limited sample size ( $n = 10$ ).

## DISCUSSION

In this study, active MAPK was more frequently expressed in low-grade as compared with high-grade ovarian serous carcinomas. This finding is consistent with molecular genetic studies showing that activating mutations in *KRAS* and *BRAF*, the upstream regulators of MAPK, are common (>60%) in low-grade carcinomas and their precursors, serous borderline tumors (atypical proliferative serous tumor and intraepithelial low-grade serous carcinomas) but rare in high-grade serous carcinomas (1) and provides additional support for a new model of ovarian carcinogenesis (2). Expression of active MAPK correlated with mutations in *KRAS* or *BRAF* but was also detected in some low-grade tumors and high-grade carcinomas with wild-type *KRAS* and *BRAF*, suggesting that activation of MAPK could be because of other events besides mutation of *KRAS* and *BRAF* such as overexpression of genes upstream to MAPK. Therefore, this study provides additional support to our hypothesis of dual pathways for ovarian carcinogenesis (1, 2).

Analysis of MAPK expression with clinical outcome revealed that among the patients with stage III and IV high-grade serous carcinoma, expression of active MAPK was associated with better overall survival—a finding that was previously reported with ascites samples of ovarian serous carcinomas (15). Furthermore, our finding showed a good prognosis in patients whose tumors were sensitive to paclitaxel and expressed active MAPK. This finding contrasts to breast carcinoma in which expression of active MAPK is a poor prognostic marker (16). This difference in ovarian and breast carcinoma is intriguing and probably reflects different roles of the MAPK signaling pathway in different tumor types (5). Recent studies have shown that MAPK activation is necessary for cancer cell death initiated by a variety of anticancer agents (5, 8). For example, activation of MAPK is essential for the killing effect of CD437, a synthetic retinoid that has been reported to induce growth arrest and apoptosis in ovarian cancer cell lines (8). Additional studies are necessary to determine whether constitutive expression of active MAPK sensitizes ovarian cancer cells to paclitaxel *in vivo*.

In conclusion, the results of this study showing a differential

expression of active MAPK in low-grade serous carcinoma compared with high-grade serous carcinoma together with previous molecular genetic findings of frequent mutations of *KRAS* and *BRAF* in low-grade serous tumors support the view that low-grade and high-grade serous tumors develop along different pathways. Active MAPK in combination with *in vitro* paclitaxel sensitivity may serve as a prognostic marker in patients with advanced stage high-grade serous carcinoma. This finding would have important implications for clinical management of ovarian cancer patients.

## ACKNOWLEDGMENTS

We thank the immunohistochemistry laboratory for excellent immunostaining and the members in the Molecular Genetics of Ovarian Cancer Laboratory at Johns Hopkins Medical Institutions for previewing the manuscript.

## REFERENCES

- Singer G, Oldt R III, Cohen Y, et al. Mutations in BRAF and KRAS characterize the development of low-grade ovarian serous carcinoma. *J Natl Cancer Inst* (Bethesda) 2003;95:484–6.
- Shih I-M, Kurman RJ. Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. *Am J Pathol* 2004;164:1511–8.
- Singer G, Kurman RJ, Chang H-W, Cho SKR, Shih I-M. Diverse tumorigenic pathways in ovarian serous carcinoma. *Am J Pathol* 2002;160:1223–8.
- Seidman JD, Horkayne-Szakaly I, Haiba M, et al. The histologic type and stage distribution of ovarian carcinomas of surface epithelial origin. *Int J Gynecol Pathol* 2004;23:41–4.
- Olson JM, Hallahan AR. p38 MAP kinase: a convergence point in cancer therapy. *Trends Mol Med* 2004;10:125–9.
- Peyssonnaud C, Eychene A. The Raf/MEK/ERK pathway: new concepts of activation. *Biol Cell* 2001;93:53–62.
- Allen LF, Sebolt-Leopold J, Meyer MB. CI-1040 (PD184352), a targeted signal transduction inhibitor of MEK (MAPKK). *Semin Oncol* 2003;30(5 Suppl 16):105–16.
- Holmes WF, Soprano DR, Soprano KJ. Early events in the induction of apoptosis in ovarian carcinoma cells by CD437: activation of the p38 MAP kinase signal pathway. *Oncogene* 2003;22:6377–86.
- Hoshino R, Chatani Y, Yamori T, et al. Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. *Oncogene* 1999;18:813–22.
- Shih IM, Nesbit M, Herlyn M, Kurman RJ. A new Mel-CAM (CD146)-specific monoclonal antibody, MN-4, on paraffin-embedded tissue. *Mod Pathol* 1998;11:1098–106.
- Fruehauf JP, Alberts DS. Assay-assisted treatment selection for women with breast or ovarian cancer. *Recent Results Cancer Res* 2003;161:126–45.
- Eltabbakh GH, Piver MS, Hempling RE, et al. Correlation between extreme drug resistance assay and response to primary paclitaxel and cisplatin in patients with epithelial ovarian cancer. *Gynecol Oncol* 1998;70:392–7.
- Sieben NL, Macropoulos P, Roemen G, et al. In ovarian neoplasms, BRAF, but not KRAS, mutations are restricted to low-grade serous tumors. *J Pathol* 2004;202:336–40.
- Blackhall FH, Pintilie M, Michael M, et al. Expression and prognostic significance of kit, protein kinase B, and mitogen-activated protein kinase in patients with small cell lung cancer. *Clin Cancer Res* 2003;9:2241–7.
- Givant-Horwitz V, Davidson B, Lazarovici P, et al. Mitogen-activated protein kinases (MAPK) as predictors of clinical outcome in serous ovarian carcinoma in effusions. *Gynecol Oncol* 2003;91:160–72.
- Sivaraman VS, Wang H, Nuovo GJ, Malbon CC. Hyperexpression of mitogen-activated protein kinase in human breast cancer. *J Clin Invest* 1997;99:1478–83.