MKK4 Status Predicts Survival After Resection of Gastric Adenocarcinoma

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Hypothesis: Lack of expression of the tumor-suppressor gene MKK4 is significantly correlated with poor survival after resection of gastric adenocarcinoma.

Design: Retrospective review of medical records after construction and immunolabeling of tissue microarrays for clinical correlation.

Setting: The Johns Hopkins Hospital, Baltimore, Md.


Main Outcome Measure: Long-term survival and MKK4 status.

Results: Primary tumors (N=124) were scored as 0 (no labeling), 1+/H11001 (weak labeling), or 2+/H11001 (strong labeling) in 9 (7%), 80 (65%), and 35 (28%) patients, and 5-year survival in these patients was 0%, 21%, and 28%, respectively. Given the small size (7%) of the MKK4-negative group (as expected, given the 5%-10% incidence of genetic loss in carcinomas), a Cox proportional hazards analysis was performed, adjusting for age, sex, and tumor stage. This multivariate analysis revealed a 5-fold increased risk of death ($P<.001$) in patients whose primary tumors were MKK4-negative. Furthermore, the addition of MKK4 status significantly improved the Cox model, changing log likelihood from −1410 to −369, confirming that MKK4 status was truly the effector of the survival difference and not a bystander.

Conclusions: The lack of expression of the tumor-suppressor gene MKK4 in resected gastric adenocarcinoma is robustly associated with poor survival. This finding may provide a useful prognostic tool in patients with gastric adenocarcinoma.

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EACH YEAR IN THE UNITED States, an estimated 22,710 new gastric cancer cases are diagnosed, and approximately 11,780 of those patients will die of the disease.¹ Gastric adenocarcinoma (GA) remains the second leading cause of cancer death worldwide, accounting for approximately 10% of all newly diagnosed cancers.

MKK4 (also known as JNKJ1, MAP2K4, and SEK1), located on chromosome 17p11, is thought to be a tumor-suppressor gene because of its mutation in approximately 5% of pancreas, biliary, and breast cancers.²⁻⁴ Mitogen-activated protein kinase kinase 4 (MKK4) is a central mediator of the Jun N-terminal kinase cascade, whose members bear high similarity to the related extracellular signal-regulated kinase and p38 cascades in the mitogen-activated protein kinase family. The mitogen-activated protein kinase pathways determine cell fate in response to cellular stress: activation of ras may send a proliferative signal via the extracellular signal-regulated kinase cascade or may activate the Jun N-terminal kinase pathway to cause apoptosis.³

See Invited Critique at end of article

Reported data about MKK4 in GA are scant and conflicting. Multiple mutation reports document that 5% to 10% of tumors select for the loss of MKK4,²⁻⁴ indicating that it must be either a tumor-suppressor or genome-maintenance gene,⁶ but 1 report provides data that suggest an oncogenic role,⁷ and an older report detected no role for MKK4 in gastric carcinogenesis.⁸ Recently, Xin et al⁹ calibrated an immunohistochemistry (IHC) assay to reflect the actual known MKK4 genetic status in 24 patients with pancreatic ductal adenocarcinoma; no significant associa-
tion between survival and MKK4 status was detected in that small study. The purpose of the current study was to follow up on the work of Xin et al, using the same calibrated IHC assay but with a larger and independent group of patients, to test the hypothesis that tumor-suppressor MKK4 status will predict survival after resection of GA.

METHODS

PATIENTS

Patients who underwent resection of GA, American Joint Committee on Cancer stages I to IV, were selected consecutively from an institutional patient and specimen database. All 124 patients were operated on at The Johns Hopkins Hospital, Baltimore, Md, from April 15, 1984, to September 22, 1995; this period allowed for 10-year follow-up. After approval from The Johns Hopkins University Institutional Review Board, demographic characteristics, date of surgery, initial signs and symptoms, tumor stage, surgical and medical treatment methods, complications, survival time, and other relevant data were extracted from hospital records.

TISSUE MICROARRAYS

Paraffin-embedded tissue from patients undergoing surgical resection at The Johns Hopkins Hospital was used for this study. Seven tissue microarrays (TMA) were constructed using a manual tissue puncher/arrayer (Beecher Instruments, Silver Spring, Md), as previously described. For each sample, a 1.4-mm core was punched from the donor block, and 99 cores were arrayed per block. Several serial sections were cut from all TMA, 1 of which was stained with hematoxylin-eosin as reference. The TMA contained multiple samples for each of the 124 patients, including primary and metastatic cancer and adjacent nonneoplastic stomach tissue.

IMMUNOHISTOCHEMISTRY STUDIES

Unstained 5-µm sections were deparaffinized by routine techniques. Slides were treated with 1/100 sodium citrate buffer (diluted from 1/10 heat-induced epitope retrieval buffer; Ventana-BioTek Solutions, Tucson, Ariz) before steaming for 20 minutes at 80°C. Slides were then cooled for 5 minutes before incubating with antihuman MKK4 monoclonal antibody (NCL-MKK4, 1:80 dilution; Novocastra, Newcastle, England) using an automated stainer (Dako Corp, Carpenteria, Calif). Finally, MKK4 primary antibody was detected by adding secondary antibody followed by avidin-biotin complex and 3,3'-diaminobenzidine chromogens. Sections were counterstained with hematoxylin. Immunohistochemical labeling of MKK4 was evaluated by 3 blinded individuals (S.C.C., C.I.-D., and E.M.). The labeling of each sample was recorded as 0 if no cytoplasmic labeling of MKK4 protein was observed, 1 if weak, or 2 if strong labeling was present (Figure 1). Since MKK4 functions in a stress-activated pathway, the TMA samples were evaluated for histologic evidence of repair (edema, lymphocytic infiltrate, damaged epithelium, and pseudostratified nuclei). Our laboratory recently reported the ability of this MKK4 IHC assay to detect the genetic status of tumor specimens.

DATA ANALYSIS

We used χ² tests to compare categorical variables (gender, histologic findings, and microscopic margins), and Wilcoxon rank sum tests to compare continuous variables (age and survival months) and ordinal variables (stage) between patients with MKK4-negative and MKK4-positive tumors. The Kaplan-Meier method was used to calculate survival. Survival data were compared using log-rank tests and Cox proportional hazards models. In bivariate analyses, the results of log rank tests and Cox proportional hazards models were almost identical. Therefore, only results from the Cox model are reported. The Cox proportional hazards model was also used to test the significance between survival after adjustment for age, sex, tu-

Figure 1. MKK4 labeling pattern in normal and neoplastic gastric tissues. A, Healthy gastric mucosa shows no MKK4 labeling of the normal gastric pits or of the surrounding stromal cells. B, Primary lesion of gastric adenocarcinoma shows 2+ (strong) labeling of cytoplasmic MKK4 protein, with sparing of surrounding stroma. C, Gastric adenocarcinoma metastatic to a lymph node shows 2+ labeling of cytoplasmic MKK4 protein.
Table 1. MKK4 Immunolabeling of Healthy Stomach, Reparative Mucosa, and Primary and Metastatic Gastric Adenocarcinoma

<table>
<thead>
<tr>
<th>Immunolabeling Score†</th>
<th>Healthy Stomach (n = 76)</th>
<th>Reparative Mucosa (n = 18)</th>
<th>Primary Cancer (n = 124)</th>
<th>Metastatic Cancer (n = 73)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>70 (92)</td>
<td>0</td>
<td>9 (7)</td>
<td>3 (4)</td>
</tr>
<tr>
<td>&gt;1</td>
<td>6 (8)</td>
<td>18 (100)</td>
<td>80 (65)</td>
<td>58 (80)</td>
</tr>
<tr>
<td>&gt;2</td>
<td>0</td>
<td>0</td>
<td>35 (28)</td>
<td>12 (16)</td>
</tr>
</tbody>
</table>

*Data are given as number (percentage) adapted from Cunningham et al.15
†See “Immunohistochemistry” section for description of scoring system.

RESULTS

PATIENTS

Of all patients undergoing GA resection at The Johns Hopkins Hospital from May 16, 1984, to July 25, 2002, 124 operated on before September 22, 2005, were selected for this study. Detailed characteristics of The Johns Hopkins Hospital’s GA experience have been previously reported.16 In brief, the patients in the current study were predominantly male (66%), with a median age at surgery of 65 years. Most patients had advanced disease at the time of resection: in 53%, tumors were diagnosed at stage III or IV. The primary tumors were distributed throughout the anatomical areas of the stomach: 26% in the antrum, 23% in the cardia, 7% along the lesser curvature, 5% in the body, 4% in the fundus, 4% in the pylorus, 2% along the greater curvature, and 30% elsewhere or overlapping in the stomach. Most (72%) of these patients had tumors of diffuse Lauren histologic type.

MKK4 PROTEIN EXPRESSION

Results of MKK4 IHC are given in Table 1.15 Nonneoplastic gastric mucosa distant from the primary cancer was available for immunolabeling in the same TMA (Figure 1A). Normal nonneoplastic mucosa was MKK4-negative in 70 (92%) of 76 tumors, but nonneoplastic mucosa labeling histologic evidence of repair (edema, damaged epithelium, lymphocytic infiltrate, or pseudostratified nuclei) was positive in 18 (100%) of 18 tumors (P < .001).

Of the 124 primary cancers, 9 (7%) were MKK4-negative (score 0) and 115 (93%) were MKK4-positive, of which 80 (65%) were 1+ (weak) and 35 (28%) were 2+ (strong) at immunolabeling. Seventy (96%) of 73 metastatic lesions were MKK4-positive. MKK4 labeling of the primary GA and metastatic lesions occurred in a predominantly cytoplasmic pattern, with scattered nuclear labeling (Figure 1B and 1C).

MKK4 EXPRESSION AND PATIENT SURVIVAL

Ten-year follow-up data were available for 114 of 124 patients, of whom 7 (6%) were MKK4-negative and 107 (94%) were MKK4-positive (75 weak [1+] and 32 strong [2+] at immunolabeling). The MKK4-positive and MKK4-negative groups did not show any statistically significant difference with respect to relevant clinicopathologic variables (Table 2). However, survival was significantly lower in patients with MKK4-negative tumor samples. The Cox proportional hazards model adjusting for age, sex, and tumor stage revealed that patients whose primary tumors were MKK4-negative had a 5-fold increased risk of death (hazard ratio, 5.3; P = .001). Furthermore, the addition of MKK4 status significantly improved the Cox model, with a change in log likelihood from −1410 to −369.

The 5-year survival rate in patients with primary tumor MKK4 immunolabeling scores of 0, 1+, and 2+ were 0%, 21%, and 28%, respectively (Figure 2A). A dose-response is suggested by the 3 curves, but the difference between the 1+ and 2+ curves was not statistically significant. A similar pattern was observed for metastatic lesions (Figure 2B); 5-year survival was 17% in patients with MKK4-positive metastatic lesions but 0% in those with MKK4-negative metastatic lesions. Median survival in the MKK4-positive group was 17.3 months (7.4-fold) longer than in the MKK4-negative group.

Because margin status and Lauren histologic type have also been shown to predict mortality in this population,17 we performed the regression analysis with mar-

Table 2. Comparison of Patients With MKK4-Positive and MKK4-Negative Primary Cancers

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>MKK4-Positive (n = 107)</th>
<th>MKK4-Negative (n = 7)</th>
<th>P Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, y</td>
<td>62 (68)</td>
<td>67 (4)</td>
<td>.17</td>
</tr>
<tr>
<td>Male sex</td>
<td>73 (68)</td>
<td>4 (57)</td>
<td>.68</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>14 (13)</td>
<td>0 (0)</td>
<td>.42</td>
</tr>
<tr>
<td>II</td>
<td>25 (24)</td>
<td>1 (14)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>36 (34)</td>
<td>4 (57)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>31 (29)</td>
<td>2 (29)</td>
<td></td>
</tr>
<tr>
<td>Histologic findings</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffuse</td>
<td>36 (34)</td>
<td>4 (57)</td>
<td>.46</td>
</tr>
<tr>
<td>Intestinal</td>
<td>18 (17)</td>
<td>1 (14)</td>
<td></td>
</tr>
<tr>
<td>Undetermined</td>
<td>53 (49)</td>
<td>2 (29)</td>
<td></td>
</tr>
<tr>
<td>Positive microscopic margin</td>
<td>31 (29)</td>
<td>3 (43)</td>
<td>.42</td>
</tr>
<tr>
<td>5-Year survival rate, %</td>
<td>24 (23)</td>
<td>0 (0)</td>
<td>.02</td>
</tr>
</tbody>
</table>

*Data are given as number (percentage) unless otherwise indicated.
†P values are derived from the Fisher exact test (sex, tumor margins, and histologic findings) or the Wilcoxon rank sum test (age, survival, and tumor stage).
been shown to predict mortality in this population, we controlled for age, sex, and tumor stage (nonegativity that persisted after multivariate analysis had yielded similar results and both confirmed highly significant survival differences between patients who tested MKK4-positive and MKK4-negative. We observed MKK4-negative immunolabeling in 92% of normal stomach samples. Both we and Xin et al found that low MKK4 expression characterized the wild-type MKK4 gene in normal gastric mucosa, whereas higher and detectable expression characterized cancer tissues expressing wild-type MKK4. The highly reliable overexpression in cancer facilitates detection of the mutant state of MKK4.

Previous reports in the literature provide conflicting data about MKK4. For instance, ample evidence of inactivating somatic mutations of MKK4 in cancers defines this gene as a candidate tumor-suppressor gene. Given that MKK4 is reliably somatically mutated in approximately 5% of surveyed patient tumors and cell lines, it must function in human oncogenesis as either a tumor-suppressor gene or a genome-maintenance gene because there is no reasonable alternative explanation for the selection of MKK4 mutants by the tumors. However, there are conflicting data about the role of MKK4 in carcinogenesis. Wang et al, for example, recently claimed evidence for MKK4 having pro-oncogenic activity, but the interpretation of those findings is debatable, given that a known proportion of tumors select for the loss of MKK4.

Wu et al, using a polyclonal antibody raised against mouse MKK4, performed IHC in 96 GAs and, using a non-calibrated scale, classified 55% as negative for MKK4 immunolabeling. Because MKK4 mutational studies reliably show the rate of MKK4 loss to be about 5% to 10%, the classification in that study may be arbitrary. A more accurate reflection of MKK4 status is possible by first calibrating the IHC assay to known genetic status and then applying the calibrated test to unknown samples. Such calibration, that is, the use of genetically defined cancers to set the interpretive guidelines for MKK4 IHC assays, was recently performed: Xin et al, using a monoclonal MKK4 antibody raised against human MKK4 and verifying the IHC data with genetic analysis, found that only 13% of pancreatic ductal adenocarcinomas were MKK4-negative.

Chae et al, using polymerase chain reaction and Western blotting, performed expression and mutational analyses of MKK4 in 87 human GA tissues and in 15 cell lines but did not include IHC analysis. No mutation was identified in any case, leading the authors to conclude that MKK4 inactivation may not be important in gastric tumorigenesis. However, given a pretest expectation for a rate approximating the low rate found in other foregut cancers (~5%), the failure of this analysis to find a mutation in 15 cell lines was not surprising. With respect to their mutational analysis of tissues, it was not reported whether their tissues were procedurally enriched for neoplastic cells, which has been shown

Figure 2. Five-year survival rates. Kaplan-Meier survival graphs for primary (A) and metastatic (B) gastric adenocarcinoma showing the degree of labeling of cytoplasmic MKK4 protein. 0 indicates no labeling; 1+, weak; and 2+, strong.

Our data suggest that lack of protein expression of the tumor-suppressor gene MKK4 in resected GA independently predicts poor survival. Using the same antibody and IHC method, we recently validated the ability of this calibrated IHC to accurately detect genetic MKK4 status. In that study, the MKK4 IHC assay was 100% sensitive in detecting genetic status; the rate of MKK4 loss, 13%, was consistent with previous reports. The patients with MKK4-negative cancers in that study, as in the current study, had lower survival rates (2-fold increased risk of death) compared with their MKK4-positive counterparts, although that finding did not reach statistical significance and the sample size was smaller (n=24). In the current, larger population of patients with GA with MKK4 labeling data (n=114), we detected a 5.3-fold increase in mortality conferred by MKK4 immunonegativity that persisted after multivariate analysis that had controlled for age, sex, and tumor stage (P=.001). Because margin status and Lauren histologic type have also been shown to predict mortality in this population, we performed another regression analysis with margin status and Lauren histologic findings as covariates, and significance persisted.

For this multivariate analysis, there were 107 subjects in the MKK4-positive group and 7 in the MKK4-negative group (6%, which is consistent with the observed incidence of MKK4 mutations). The probability of committing a type I (false-positive) error was fixed at the beginning of our analysis (α=.05) and did not depend on sample size. The probability of a type II (false-negative) error was irrelevant owing to our positive result. Furthermore, both the Cox proportional hazards model and the relatively assumption-free log-rank test yielded similar results and both confirmed highly significant survival differences between patients who tested MKK4-positive and MKK4-negative. We observed MKK4-negative immunolabeling in 92% of normal stomach samples. Both we and Xin et al found that low MKK4 expression characterized the wild-type MKK4 gene in normal gastric mucosa, whereas higher and detectable expression characterized cancer tissues expressing wild-type MKK4. The highly reliable overexpression in cancer facilitates detection of the mutant state of MKK4.

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to be required to detect the true frequency of mutations.\(^{17}\)

We have shown that lack of expression of the tumor-suppressor gene MKK4 in resected GA predicts poor survival in genetically controlled IHC studies. This is a finding not only robustly statistically significant but also clinically significant: median survival in the MKK4-positive group was 17.3 months (7.4-fold) longer than in the MKK4-negative group. This increase in median survival is almost 2-fold higher than that achieved with chemoradiation of gastric cancer.\(^{18}\) Most patients in this study had advanced disease at the time of GA resection, and the clinical relevance observed in these patients with advanced disease may be greater or less than in a population of patients with less advanced disease.

Our findings underscore potential diagnostic, therapeutic, and prognostic applications of MKK4 in GA. Should our results be replicated in other studies, the most immediate use of this finding may be in providing patients and surgeons with information about prognosis. However, as targeted therapies directed, for example, at restoration of MKK4 function become available, our findings may provide additional benefit in identifying patients with a treatable defect in the MKK4 tumor-suppressor pathway. Furthermore, in individual patients, identification of MKK4 status, for example, by performing a preoperative needle biopsy, may identify patients with more aggressive disease who may benefit from neoadjuvant therapy or from an extended resection procedure. At the population level, because this test has been calibrated to actual genetic status,\(^9\) it may be used to increase the power of clinical trials by increasing the ability to stratify enrolled patients. Larger multicenter studies are warranted to confirm our findings.

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