MAP2K4/MKK4 Expression in Pancreatic Cancer: Genetic Validation of Immunohistochemistry and Relationship to Disease Course

Wei Xin,1 Ki J. Yun,4 Francesca Ricci,2 Marianna Zahrak,3 Wanglong Qiu,4 Gloria H. Su,4 Charles J. Yeo,5,6 Ralph H. Hruban,4,5 Scott E. Kern,4,5 and Christine A. Iacobuzio-Donahue4,5

1Department of Pathology, The University of Michigan Medical Center, Ann Arbor, Michigan; 2Department of Pathology, University La Sapienza, Rome, Italy; and the Departments of 3Biostatistics, 4Pathology, 5Oncology, and 6Surgery, The Johns Hopkins University Hospital, Baltimore, Maryland

ABSTRACT

MKK4 (MAP2K4/SEK1) is a member of the mitogen-activated protein kinase family, originally identified as a kinase involved in the stress-activated protein kinase pathway by directly phosphorylating c-Jun NH2-terminal kinase. MKK4 genetic inactivation has been observed in a subset of pancreatic carcinomas, implicating deregulation of the stress-activated protein kinase pathway in pancreatic carcinogenesis. We evaluated Mkk4 protein expression patterns by immunohistochemical labeling in a series of 60 resected primary infiltrating pancreatic adenocarcinomas (24 cases with known MKK4 genetic status), and 14 different tissue arrays representing the primary carcinoma and all of the gross metastases from 26 patients that died of metastatic pancreatic cancer. Among the surgically resected carcinomas, focal or diffuse-positive immunolabeling for Mkk4 protein was found in 52 of 60 cases (86.7%). Among the eight carcinomas with negative Mkk4 immunolabeling, three harbored a homozygous deletion or intragenic mutation of the MKK4 gene, in contrast to none of the 52 cases with positive Mkk4 immunolabeling (P < 0.01). Loss of Mkk4 immunolabeling showed a trend toward shorter survival, with Mkk4-positive carcinomas having half the risk of death than Mkk4-negative carcinomas (P = 0.09). Mkk4 immunolabeling patterns were also evaluated among unresectable primary and metastatic cancer tissues from autopsy specimens, indicating intact Mkk4 immunolabeling in 88.8% of the unresectable primary carcinomas as compared with 63.3% of distant metastases (P < 0.001). Our data indicate that the loss of Mkk4 protein expression in pancreatic carcinomas may be more frequent than suggested by the rates of genetic inactivation alone and that Mkk4 loss may contribute to disease progression. The correlation of MKK4 genetic status with immunolabeling patterns validate this approach for the evaluation of MKK4 status in routine histologic sections and may provide useful information regarding patient prognosis.

INTRODUCTION

The mitogen-activated protein (MAP) kinase cascades are multifunctional signaling pathways that are evolutionarily well conserved in all of the eukaryotic cells. One of the biological responses mediated through the MAP kinase pathways seems to be the decision of cell fate in response to stress-activation resulting in apoptosis. Specifically, three parallel MAP kinase cascades have been described that converge on extracellular signal-regulated kinases, c-Jun NH2-terminal kinases, or p38 MAP kinases, and each consists of three classes of serine/threonine kinases, the MAP kinase, the MAP kinase kinase (MAPKK, also known as MEK), and the MAPKK kinase (MAPKKK). MAPKKK phosphorylates and thereby activates MAPKK, and activated MAPKK in turn phosphorylates and activates MAP kinase (1–3).

MKK4 (MAP2K4/SEK1) is a member of the MAP kinase family specifically involved in the stress-activated protein kinase (SAPK) pathway by directly phosphorylating the c-Jun NH2-terminal kinase in response to Ask1 activation (4, 5). Genetic inactivation of the MKK4 gene on chromosome 17p has been reported in a subset of pancreatic, biliary, and breast carcinomas, suggesting dysregulation of the SAPK pathway may be selected for in carcinogenesis of these organs (6–8). In prosatic and ovarian carcinomas, Mkk4 is thought to function as a metastasis-suppressor gene in that loss of Mkk4 expression, but not genetic inactivation, is associated with metastasis formation in these organs (9, 10).

The purpose of this study was to perform a survey of Mkk4 expression among a set of genetically well-characterized surgically resected primary infiltrating pancreatic cancers as well as among unresectable metastatic pancreatic cancers with an immunohistochemical approach. In doing so, we hoped to determine the specificity and sensitivity of this assay for the MKK4 gene status, the relative rates of MKK4 loss among primary infiltrating and metastatic pancreatic cancers, and the relationship of MKK4 to long-term prognosis.
MATERIALS AND METHODS

Tissues and Cell Lines. Paraffin-embedded blocks of 60 surgically resected primary infiltrating pancreatic adenocarcinomas resected between 1992 and 1996 were collected from the Surgical Pathology Files of The Johns Hopkins Hospital. Clinical and pathological data were also obtained from the Surgical Pathology Files, including age, gender, race, tumor size, tumor location, lymph node status, histologic subtype of invasive carcinoma, and patient survival. The mean patient age was 63.3 years old and included 34 males and 26 females. The H&E-stained slides from each case of primary infiltrating carcinoma were screened by light microscopy, and representative sections containing infiltrating adenocarcinoma were selected for immunolabeling.

Construction of Metastatic Pancreatic Cancer Tissue Microarrays. The paraffin-embedded tissues from 26 patients who died of histologically confirmed metastatic pancreatic cancer between 1972 to 1993 were collected from the Autopsy Pathology Files of The Johns Hopkins Hospital and used to construct 14 different tissue arrays representing the primary carcinoma and all of the gross metastases from these patients. Patient ages ranged from 35 to 89 years with a mean of 62.8. Fifteen patients were male and 11 were female. A total of 20 different target organs were represented with a mean number of metastatic sites per patient of 3.4. The three major metastatic sites were liver, lung, and lymph node. For tissue microarray construction, representative paraffin-embedded sections containing primary infiltrating or metastatic pancreatic ductal adenocarcinoma were circled on the glass slides and used as a template. The tissue microarray was constructed with a manual Tissue Puncher/Arrayer (Beecher Instruments, Silver Spring, MD). For each individual primary or metastatic sample, up to 4 1.4-mm cores were punched from the donor block to account for tissue heterogeneity (depending on the size of the lesion). A total of 99 cores each were included on each of the 14 recipient blocks, representing the primary infiltrating or metastatic pancreatic cancers, the matched metastases, and a variety of normal control tissue cores from each patient.

Immunohistochemical Labeling for Mkk4 Protein. Unstained 5-micron sections were cut from the paraffin blocks selected for each case or the tissue microarrays and deparaffinized by routine techniques. Slides were treated with 1X sodium citrate buffer (diluted from 10X heat-induced epitope retrieval buffer; Ventana-Bio Tek Solutions, Tucson, AZ) before steaming for 20 minutes at 80°C. Slides were then cooled 5 minutes before incubating with antihuman Mkk4 monoclonal antibody [NCL-MKK4 (1:80 dilution), Novacastra, Newcastle, United Kingdom] with a Dako automated stainer. Finally, Mkk4 primary antibody was detected by adding secondary antibody followed by avidin-biotin complex and 3,3'-diaminobenzidine chromagens. Sections were counterstained with hematoxylin. Immunohistochemical labeling of Mkk4 was evaluated by two chromagens. Sections were counterstained with hematoxylin.

Relationship of Mkk4 Protein Expression to Mkk4 Genetic Status. The status of the Mkk4 gene was previously determined for 24 of the 60 pancreatic cancers that were also immunolabeled (7), providing the opportunity to correlate Mkk4 immunolabeling patterns to the genetic status for each case (Table 1). Among these 24 pancreatic adenocarcinomas, 23 showed loss of heterozygosity at 17p. Mkk4 was genetically

Determinations of Loss of Heterozygosity and Sequencing of Mkk4. The genetic status of Mkk4 was available for 24 of the resected primary infiltrating pancreatic adenocarcinomas analyzed in the current study. These genetic analyses have been reported previously (7).

Statistical Analysis. The frequencies of Mkk4 immunolabeling among cancer samples with known genetic status were analyzed by the \( \chi^2 \) test with modification by the Fisher’s exact test to account for frequency values <.5. For purposes of statistical analysis, focal and diffuse-positive labeling carcinomas were combined for comparison to negative labeling cancers. For determinations of overall survival in relation to immunohistochemical labeling of Mkk4 protein, event time distributions were estimated with the Kaplan-Meier method (11) and compared with the log-rank statistic (12) or the proportional hazards regression model (13). Other factors tested for prognostic value included age, race, gender, size of tumor, nodal status, and tumor differentiation. Hazard ratios were expressed relative to a baseline reference category. Computations were done with the Statistical Analysis System or EGRET (14). All \( P \)s reported are two-sided. For all of the statistical analyses, \( P \)s of \( \leq 0.05 \) were considered significant.

RESULTS

Mkk4 Protein Expression in Normal Pancreas and Pancreatic Cancers. Mkk4 protein expression was noted in 52 of 60 (86.7%) infiltrating pancreatic ductal adenocarcinomas (Fig. 1, A and B). In all of the cases, Mkk4 labeling had a cytoplasmic distribution with scattered nuclear labeling. Of these 52 adenocarcinomas with positive immunolabeling, 45 (87%) showed diffuse labeling and 7 (13%) showed focal labeling. Adjacent normal pancreatic tissue was also available for study within the same sections of infiltrating carcinoma. Immunolabeling for Mkk4 was detected within normal acini, islets, and duct epithelium (Fig. 1C).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Immunohistolabeling of Mkk4 in pancreatic cancers with known genetic status</th>
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<tbody>
<tr>
<td>MKK4 genetic status *</td>
<td>Mkk4 IHC-positive</td>
</tr>
<tr>
<td>Mutant †</td>
<td>0</td>
</tr>
<tr>
<td>LOH with wild-type allele</td>
<td>18</td>
</tr>
<tr>
<td>No LOH</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
</tr>
</tbody>
</table>

Abbreviations: IHC, immunohistochemistry; LOH, loss of heterozygosity.
* Reported by Su et al. (7).
† Codon exons were sequenced in all of the cases with LOH, and mutations confirmed by repeat sequencing of an independently derived PCR product.
‡ \( P < 0.01 \).
inactivated in three of these 23 cancers with 17p loss of heterozygosity. Two of the 23 carcinomas contained a homozygous deletion of the \textit{MKK4} gene, and one carcinoma with allelic loss contained a missense mutation (G12S) of the remaining allele. Immunohistochemical labeling of these 24 cancers indicated that six were negative and 18 were positive for Mkk4 protein. Of the six negative cancers, two contained a homozygous deletion, one contained a missense mutation, and three were wild-type for Mkk4. In contrast, all 18 of 18 carcinomas with positive Mkk4 immunolabeling had wild-type \textit{MKK4} genetic status ($P < 0.01$, Fisher’s exact test).

**Relationship of Mkk4 Expression to Patient Survival.** Long-term, follow-up information was available for 45 of the 60 patients for which Mkk4 immunolabeling was done on the resected primary carcinoma. Thirty-seven of these 45 patients (82\%) showed positive Mkk4 immunolabeling, and eight patients (18\%) were negative for Mkk4. No significant difference in age, gender distribution, tumor size, tumor location, differentiation, or lymph node status among the Mkk4 immunonegative and immunopositive groups were found (Table 2). The only factor marginally associated with survival was Mkk4 immunolabeling status (Table 2; Fig. 2). Patients with positive MKK4 had half the risk of death compared with MKK4 negative patients, Hazard Ratio $= 0.5$ (95\% confidence interval: 0.22, 1.12), $P = 0.09$.

**Mkk4 Protein Expression in Metastatic Pancreatic Cancer.** Loss of Mkk4 expression has been reported in association with metastatic ability (9, 10). Therefore, we also determined the Mkk4 immunohistochemical labeling patterns among a variety of metastatic pancreatic adenocarcinoma tissues from archival autopsy material of 26 different patients with widespread disease, 18 for which the primary pancreatic carcinoma was also available for study.

Intact Mkk4 immunolabeling was noted within the neoplastic epithelium of 16 of 18 (88\%) primary pancreatic carcinomas analyzed, similar to the rate of Mkk4 immunolabeling found for surgically resected pancreatic cancers. In contrast, among 460 different metastases from these 26 patients, only 291 (63.3\%) showed intact Mkk4 immunolabeling ($P < 0.001$). Mkk4 immunolabeling patterns of the metastatic lesions were also evaluated with respect to the matched primary carcinomas also available for study. For the 16 patients whose primary carcinoma showed intact Mkk4 immunolabeling, the matched metastatic disease for 15 of these patients showed heterogeneity of Mkk4 expression in that metastases with both positive and negative Mkk4 immunolabeling were seen.

\begin{table}
\begin{center}
\begin{tabular}{llcc}
\hline
Clinicopathologic variable & Hazard ratio & Confidence interval & $P$  \\
\hline
Mkk4 immunolabeling pattern & & &  \\
Negative ($n = 8$) & 1.00 & (0.22, 1.12) & 0.09  \\
Positive ($n = 37$) & 0.50 & &  \\
Age (y) & 66.6 ± 3.5 & 1.00 & (0.97, 1.02) & 0.77  \\
Race & & &  \\
Other ($n = 6$) & 1.00 & (0.22, 1.29) & 0.16  \\
White ($n = 39$) & 0.53 & &  \\
Gender & & &  \\
Female ($n = 17$) & 1.00 & (0.32, 1.20) & 0.16  \\
Male ($n = 28$) & 0.62 & &  \\
Nodal status & & &  \\
Negative ($n = 9$) & 1.00 & (0.49, 2.57) & 0.79  \\
Positive ($n = 36$) & 1.12 & &  \\
Tumor size & & &  \\
$\leq 3$ cm ($n = 15$) & 1.00 & (0.35, 1.34) & 0.28  \\
$\geq 3$ cm ($n = 30$) & 0.69 & &  \\
Tumor differentiation & & &  \\
Well/Moderately ($n = 32$) & 1.00 & (0.57, 2.35) & 0.69  \\
Poor ($n = 13$) & 1.16 & &  \\
\hline
\end{tabular}
\end{center}
\caption{Univariate Cox regression hazard ratios for survival}
\end{table}

* Values shown are based on 45 patients for which complete clinicopathologic information was available.
negative immunolabeling patterns noted. In one of the 16 patients with positive Mkk4 labeling of the primary tumor, all of the matched metastases showed loss of Mkk4 expression (Fig. 3). In the remaining two patients with negative labeling of the primary tumor, all of the matched metastases were also Mkk4 negative.

DISCUSSION

The ability to correlate Mkk4 immunohistochemical labeling patterns to \textit{MKK4} genetic status previously determined for these same pancreatic cancers provides a unique opportunity to directly correlate gene status, gene expression, and morphology. This is particularly advantageous in studies of pancreatic cancer, a tumor type well known for its low neoplastic cellularity that often hinders genetic analyses of this tumor type. We now present data that relate Mkk4 immunolabeling to the genetic status previously determined for those same cases, indicating the rate of Mkk4 loss in pancreatic cancers to be higher than reflected by genetic alterations alone [13\% in the current study versus 5\% reported by Su et al. (7)]. Epigenetic inactivation of tumor suppressor genes has been reported for other genes known to play a role in pancreatic carcinogenesis, such as \textit{CDKN2A/p16} (15). \textit{CDKN2A/p16} has been shown to be inactivated by a variety of mechanisms in pancreatic cancer, including homozygous deletion, allelic loss coupled with genetic alterations of the remaining allele, and methylation of the gene promoter (15, 16). Thus, we cannot rule out that mechanisms such as hypermethylation of the \textit{MKK4} gene may also occur in pancreatic cancers.

Our data also indicate a possible relationship among loss of Mkk4 expression and the poor prognosis often associated with pancreatic cancers. We found a trend toward worse survival for those patients with carcinomas that showed a loss of Mkk4 expression as compared with those with carcinomas with intact Mkk4 expression ($P = 0.09$). Thus, the evaluation of Mkk4 immunolabeling status may have prognostic value for patients with pancreatic cancer as has been shown for \textit{MADH4} (\textit{DPC4}), another marker of genetic status in patients with pancreatic cancer (17).

Immunohistochemical labeling for Mkk4 also indicate that Mkk4 loss may correlate with metastasis formation in target organs. Loss of Mkk4 immunolabeling was significantly more common in distant metastases (36.7\% of cases negative) than in the primary carcinomas in these same patients ($P < 0.001$). These findings were not related to differences in immunogenicity among surgical and autopsy materials or the number of metastases per patient, as identical patterns and rates of immunolabeling of primary pancreatic cancers were found among surgically resected or autopsy material. The \textit{MKK4} gene has been reported to function as a metastasis-suppressor gene in breast and prostatic carcinomas, in that the loss of \textit{MKK} gene expression facilitates metastasis formation without affecting primary tumor growth (9, 10). Our findings support the concept of Mkk4 as playing a role in metastatic behavior and suggest that MKK4 may have metastasis-suppressor gene properties in pancreatic cancer as well.

In summary, we propose that immunolabeling for the \textit{MKK4}
gene product may provide useful information regarding genetic status of pancreatic cancer, as well as provide prognostic information. Additional studies of Mkk4 expression on a larger, independent series of pancreatic carcinomas is warranted to evaluate this possibility, as well as for potential therapies aimed at restoration of the SAPK pathway in patients with advanced disease.

REFERENCES
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