[CANCER RESEARCH 58, 2339–2342, June 1, 1998]

Advances in Brief

Alterations in Pancreatic, Biliary, and Breast Carcinomas Support *MKK4* as a Genetically Targeted Tumor Suppressor Gene[1]

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Abstract

Mitogen-activated protein kinase (MAPK) kinase 4 (MKK4) is a component of a stress and cytokine-induced signal transduction pathway involving MAPK proteins. The MKK4 protein has been implicated in activation of JNK1 and p38 MAPK on phosphorylation by conserved kinase pathways. A recent report on the deletion and mutation of the *MKK4* gene in human pancreatic, lung, breast, testicle, and colorectal cancer cell lines suggests an additional role for *MKK4* in tumor suppression. Both the gene function and the infrequency of mutations might be considered atypical for many human tumor suppressor genes, and constitutional DNA was not previously available to determine whether the reported sequence variants had preceded tumor development. Here, we report that homozygous deletions are detected in 2 of 92 pancreatic adenocarcinomas (2%), 1 of 16 biliary adenocarcinomas (6%), and 1 of 22 breast carcinomas (when combined with reported sequence alterations, 3 of 22 or 14%). In addition, in a panel of 45 pancreatic carcinomas prescreened for loss of heterozygosity, one somatic missense mutation of *MKK4* is observed and confirmed in the primary tumor (2%).

Mapping of the homozygous deletions further indicated *MKK4* to lie at the target of deletion. The finding of a somatic missense mutation in the absence of any other nucleotide polymorphisms or silent nucleotide changes continues to favor *MKK4* as a mutationally targeted tumor suppressor gene. Coexistent mutations of other tumor suppressor genes in *MKK4*-deficient tumors suggest that *MKK4* may participate in a tumor suppressive signaling pathway distinct from *DPC4*, *p16*, *p53*, and *BRCA2*.

Introduction

Tumor development is a gradual process involving the accumulation of gene mutations (1). One allele of a tumor suppressor gene is inactivated through germline transmission or a somatic mutation, and inactivation of the second allele is required for tumorigenesis (2). Biallelic inactivation of a tumor suppressor gene often involves a large chromosomal deletion that manifests as LOH[3] of the region. This accompanies a more subtle change that inactivates the other allele, either by a small intragenic change or by a nested deletion that leads to homozygous deletion of the gene and flanking sequences. The inactivations of the *p53* and *APC* genes often occur in the form of intragenic mutations (3–5). In contrast, homozygous deletions can be a means to inactivate the *p16* (6, 7), *BRCA2* (8), and *DPC4* (9) genes.

The discovery of some tumor suppressor genes has been facilitated by the identification of homozygous deletions in chromosomal regions with high frequencies of LOH. Chromosome 17p13 has been of persistent interest because of its high frequency of LOH in many cancer types (10). The inactivation of *p53* at 17p13 does not account for all cases of LOH, spurring speculation regarding other potential tumor suppressors within the region. Using a marker, D19S969, located approximately 10 cM centromeric of the *p53* locus, a homozygous deletion was identified in a pancreatic cancer (11). The *MKK4* gene was mapped within this homozygous deletion. Additional deletions and sequence variants that would inactivate MKK4 protein function were identified at a low rate (3%) in cancers of the breast, colon, testis, and pancreas (11). Two other candidate genes were also identified within the deleted region. Neither was found to harbor any nucleotide variations upon sequencing (11).

We were interested in studying *MKK4* in part because of its reported mutations in pancreatic cancer (11). LOH of distal 17p affected 90% of our pancreatic cancer series (12), a remarkably high figure even when compared with the *p53* mutation rate of 75% (3). An expanded set of pancreatic and distal biliary carcinomas were available to assess the role that *MKK4* may play in pancreatic tumorigenesis. This tumor panel has been well-studied for other known mutations (13) and, therefore, could enable us to infer pathway relationships if *MKK4* abnormalities were found. Because the previous report did not address whether the nucleotide changes and deletions of *MKK4* were acquired or germline variants (11), we hoped to compare the results from cancer-derived DNA and constitutional DNA at sites of abnormality.

Materials and Methods

Tissue Samples and Cell Lines. Pancreatic and biliary cancers were resected at the Johns Hopkins Hospital. At the time of the surgery, normal duodenal mucosa was frozen and stored at −80°C and cancer xenografts were established and processed as described previously (6). Breast cell lines (BT20, BT474, BT483, BT549, Hs578T, MCF7, DU4475, MDA-MB134-VI, MDA-MB157, MDA-MB175-VII, MDA-MB231, MDA-MB415, MDA-MB361, MDA-MB453, MDA-MB436, MDA-MB468, SKBR3, T47D, UACC812P1, UACC893, ZR75-1, ZR-75-30) were purchased from American Type Culture Collection (Manassas, VA).

Homozygous Deletion Analysis. Genomic DNA samples (40 ng/sample) were screened for homozygous deletions using PCR analysis as described previously (6, 8). The primers used to amplify D19S969A and the *MKK4* exons were identical to those described previously (11). The integrin-β-4 primer sequences are INTB4-A-F 5′-gtgtccgtgtggataaggac-3′ and INTB4-A-R 5′-tccatgaccacgatgctctc-3′. The boundaries of homozygous deletions were determined by using the D17S954, D17S1303, WI-6478, WI-5743, WI-2437, WI-2335, and D17S947 dinucleotide repeat and sequence-tagged site markers (Research Genetics, Huntsville, AL).

LOH and Sequence Analyses. LOH was determined using four polymorphic markers (D17S969, D17S1303, D17S954, and D17S947; Research Genetics). LOH was conclusively present when analysis of the tumor DNA showed the loss of one allele in comparison with its corresponding normal DNA. When a normal DNA sample was unavailable, the LOH status was presumptively shown by the unambiguous presence of only a single allele size among all polymorphic markers evaluated. Forty-five samples were randomly selected for sequencing from the tumors having conclusive LOH. Each exon

Received 2/18/98; accepted 4/17/98.

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[1] Supported by the NIH SPORE (Specialized Program of Research Excellence) in Gastrointestinal Cancer CA62924, and Deutsche Krebshilfe (W. H.).

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[3] The abbreviations used are: LOH, loss of heterozygosity; MKK4, mitogen-activated protein kinase kinase 4; MAPK, mitogen-activated protein kinase.

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was amplified by PCR, treated with exonuclease I and shrimp alkaline phosphatase (USB, Cleveland, OH), and subjected to cycle-sequencing (Thermo-Sequenase, Amersham, Arlington Heights, IL). The sequencing primers were taken from genomic sequences of the previous report (11).

Results

Homozygous Deletions Detected in Pancreatic and Biliary Xenografts and a Breast Cancer Cell Line. Using the D17S969A marker and the primers specific for exon C of *MKK4* (CG2exC.FA and CG2exC.RB), we screened a panel of 92 pancreatic ductal adenocarcinomas, 16 distal common bile duct adenocarcinomas, and 19 other carcinomas of the region (predominantly duodenal and ampullary cancers) for homozygous deletions. Twenty-two breast cancer cell lines were also analyzed. Two pancreatic adenocarcinoma xenografts (PX91 and PX359), one biliary tumor xenograft (PX109), and one breast cancer cell line (DU4475) exhibited homozygous deletions at D17S969A and/or *MKK4* exon C (Fig. 1*A* and data not shown). Homozygous deletions were confirmed with duplex PCR, wherein a deleted marker fails to amplify in the same PCR reaction that allows amplification of a second nondeleted marker (Fig. 1*A* and data not shown; 6, 9). Of the 22 breast cancer cell lines examined for homozygous deletions, 2 have been reported previously to harbor sequence mutations (11), bringing the current and reported mutation rate of *MKK4* to 14% in this breast cancer series. Pancreatic cancer PX359 had a homozygous deletion at D17S969A, but not at *MKK4* exon C (Fig 1*A* and Table 1). DU4475 harbored a homozygous deletion at *MKK4* exon C, but not at D17S969A (Table 1). Further analyses revealed that the exon A of *MKK4* (the exon nearest to D17S969) was deleted from PX359 (Fig. 1*B*), but all other exons were present in the tumor. All other homozygous deletions spanned the entire exonic sequence of *MKK4* (Fig 1*B* and Table 1).

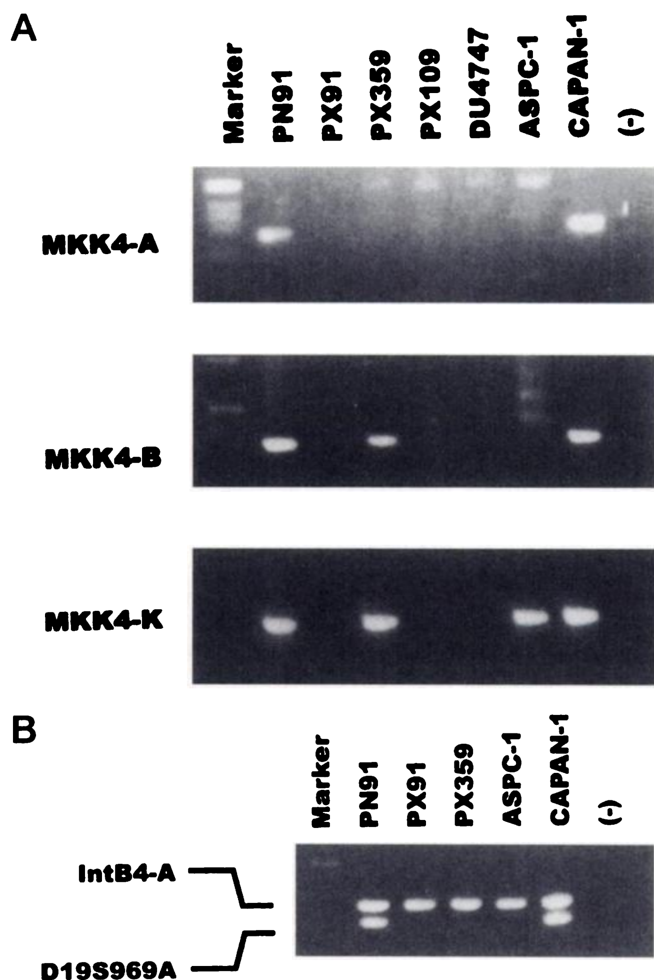

Fig. 1. Homozygous deletions in pancreatic, biliary, and breast cancers. ASPC-1 and CAPAN-1 pancreatic cancer cell lines serve as the negative and positive controls; CAPAN-1 has a nonsense mutation in *MKK4*, and ASPC-1 contains a homozygous deletion from D17S969 to *MKK4* exon C (11). *A*, detection of homozygous deletions in the genomic DNAs of pancreatic cancer xenografts, PX91 and PX359, by duplex PCR using primers for D17S969A and integrin-β-4 (internal control). *B*, mapping of homozygous deletions. The presence of each *MKK4* exon was examined by PCR. The entire coding region of *MKK4* was deleted in PX91, PX109 (biliary cancer), and DU4475 (breast cancer). Only exon A of *MKK4* was deleted in PX359.

Neighboring markers (D17S954, D17S1303, WI-6478, WI-5743, WI-2437, WI-2335, and D17S947) were used to further define the boundaries of the detected homozygous deletions. The distance between the most telomeric marker (D17S954) and the most centromeric marker (D17S947) is 10 cM, and all of the deletions were confined exclusively within the region defined by the two inner markers, WI-5743 and D17S947 (Table 1). Sequences at D17S954, D17S303, and WI-6478 were also retained in these cancer samples.

LOH Analysis. We screened pancreatic cancer xenografts for LOH with the highly polymorphic markers D17S969, D17S1303, D17S954, and D17S947. LOH of 17p at the *MKK4* locus was seen in 73 of 83 xenografts (88%). Conclusive LOH was found in 48 of the 55 cases for which normal DNA was available (87%); presumptive LOH was inferred in 25 of the 28 cases wherein normal DNA was not available (89%), as defined in "Materials and Methods." Larger and smaller alleles of each pair were lost at a ratio of 1:1. We also noted evidence of *MKK4* as a target of allelic loss. PX359 had two alleles at marker D17S954, the most telomeric marker. DU4475 had two alleles at D17S954, D17S1303, and WI-6478, the three most telomeric markers. Thus, the LOH accompanying the homozygous deletion of these tumors did not extend into the (more telomeric) p53 locus.

A Missense Mutation Detected in a Pancreatic Cancer. We examined the exonic sequences and splice junctions of the *MKK4* gene in 45 xenografts exhibiting conclusive LOH. One mutation was discovered at codon 12 within exon A, creating a missense mutation from glycine to serine in tumor sample PX17 (Table 1). The corresponding normal sample for PX17 did not harbor the same nucleotide change, indicating a somatic mutation origin, presumably during tumor development. DNA of the corresponding primary cancer tissue was prepared using cryostat-dissection, sequenced, and the mutation was verified. Two tumor samples were found to share the same polymorphism (T to C) in the 5'-untranslated region of exon A, 82 bp upstream of the ATG start site (data not shown). Due to the technical difficulty of sequencing a G-C rich domain, we were able to examine exon A sequences in only 36 of 45 xenografts (80%). All other exons were sequenced in entirety, and no other mutation was identified.

Clinicopathological Review. Review of the histological features of the original resected tissues and of the history and clinical courses of the four patients revealed no distinctive features among the pancreatic and biliary cancers harboring *MKK4* gene inactivation. All four patients had a history of tobacco smoking.

Discussion

MKK4 belongs to the MAPK kinase family and has been shown to specifically phosphorylate JNK1 and p38, but not ERK1 (14, 15). C-jun and the closely related jun family members are the known substrates for JNK1 (16). MKK4 protein can be activated by MEKK, which is part of the Ras-dependent and cytokine/stress-induced signaling cascades (14, 15). Given the proto-oncogene Ras or Ras-like proteins upstream and C-jun downstream, MKK4 holds an unusual pathway relationship for a tumor suppressor gene. The role that

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Table 1 *MKK4 genetic alterations in pancreatic, biliary, and breast cancers*[a]

Sample	Carcinoma type	Sequence alteration[b]	D17S954 D17S1303 WI6478, WI5743	D17S969	*MKK4* Exon A	*MKK4* Exons B–K	WI2437	WI2335	D17S947
PX17	Pancreatic	Codon 12 GGC to AGC	Ret.	Ret.	Ret.	Ret.	Ret.	Ret.	Ret.
PX91	Pancreatic	N.A.	Ret.	H.D.	H.D.	H.D.	Ret.	Ret.	Ret.
PX359	Pancreatic	N.A.	Ret.	H.D.	H.D.	Ret.	Ret.	Ret.	Ret.
PX109	Biliary	N.A.	Ret.	H.D.	H.D.	H.D.	H.D.	H.D.	Ret.
DU4475	Breast	N.A.	Ret.	Ret.	H.D.	H.D.	H.D.	Ret.	Ret.

[a] Ret., one allele retained; H.D., homozygous deletion. All markers and exons are placed in chromosomal map order, from telomeric (left) to centromeric (right).
[b] N.A., not applicable due to homozygous deletion.

MKK4 plays in the known stress-induced pathway may indeed prove to be important for tumor suppression (11). However, it is also possible that MKK4 possesses other uncharacterized biological functions *in vivo*.

Nonetheless, genetic evidence strongly suggests that *MKK4* is a candidate tumor suppressor gene. Homozygous deletions of *MKK4* were observed previously in one pancreatic and one lung cancer cell line (11). In addition, sequence variants of *MKK4* were detected in two cell lines of breast cancer and one each of pancreatic, colorectal, and testicular cancers, resulting in a 3% total frequency of genetic alteration in the examined cell lines (11). Here, we report homozygous deletions in two pancreatic (2%) and one biliary (6%) tumor xenografts. A somatically acquired missense mutation of *MKK4* was detected in a pancreatic cancer (2%). A homozygous deletion of *MKK4* was observed in one breast cancer cell line. When combined with the results of cell lines in the previous report, the total frequency of genetic alteration of *MKK4* in the 22 breast cancer cell lines is at least 14%. The combined homozygous deletion map derived from the two studies indicates a consensus, with breakpoints within or near *MKK4*, strongly suggesting that *MKK4* represents the target gene of the deletions. Indeed, the minimal consensus deletion is defined by the span of the *MKK4* gene itself, as the deletions do not all overlap a particular marker. A similar pattern of deletion was originally used to justify the tumor suppressor candidate, *DPC4* (9). Two other genes cloned from a homozygous deleted region between *MKK4* and D19S969A were shown to lack any sequence variants in 89 miscellaneous cancer cell lines (11), and these genes lie outside the minimal consensus of deletion. Together, both groups have found no silent mutations of *MKK4*. We also found evidence, in tumors having an *MKK4* genetic alternation, that *MKK4* can be an independent target for LOH; the LOH contributing to *MKK4* inactivation is not merely a byproduct of the large deletions that target the nearby p53 gene.

In addition to the purely genetic data, nearly all mutated *MKK4* sequences were shown to code for truncated or altered proteins that do not exhibit normal kinase activity (11). Furthermore, there are two potential start sites of translation for the MKK4 protein, and the location of our missense mutation at codon 12 might be seen to favor one of them as the biological start site of translation. Only the 5′ start site would produce a protein that incorporates the mutation identified.

It might seem puzzling that the mutational frequency of *MKK4* is relatively low, but there are many examples of previously reported tumor suppressor genes with low mutation frequency. SMAD2 has a low mutation frequency in colorectal cancer (17). DPC4 is inactivated frequently among pancreatic cancers (9, 18), but only at a low rate in other cancer types (19–21). The incidence of somatic mutations of BRCA2 is extremely low in many cancer types examined (22–26). In the absence of a mismatch repair deficiency, the transforming growth factor-β type II receptor has a low mutation rate, thus far reported only in colorectal and head/neck cancers (27, 28).

Even at low mutational rates, the discovery of each new tumor suppressor gene aids the recognition of new regulatory pathways or facilitates the progressive elaboration of known suppressive pathways, member by member. For example, based on the presence of coexistent mutations in the same tumor samples, the *MKK4* suppressive pathway would be predicted to be distinct from the *p53*, *p16*, *DPC4*, and *BRCA2* pathways. Genetic inactivation of the *p53*, *p16*, and *DPC4* genes are known to coexist in PX91, ASPC-1, and CAPAN-1 (6, 9, 13). In addition, *BRCA2* is mutated in CAPAN-1 (24).

Homozygous deletion appears to be a common mechanism for inactivation of tumor suppressor genes in pancreatic cancer. Over 60% of pancreatic cancers harbor such deletions, and nearly 20% have at least two (13). Previously, the *DPC4*, *BRCA2*, and *p16* genes were found to be inactivated via homozygous deletion in pancreatic tumors (6, 8, 9, 18). *MKK4* is the fourth independent site of homozygous deletion characterized in pancreatic cancer, and the third site to aid the isolation of a novel suppressor gene (8, 9). Additional efforts to identify and map the homozygously deleted regions in pancreatic tumors should further facilitate the isolation of additional tumor suppressor genes.

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