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Expression and mutation analyses of *MKK4*, a candidate tumour suppressor gene encoded by chromosome 17p, in human gastric adenocarcinoma

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Abstract

Homozygous deletion or somatic mutations of mitogen-activated protein kinase kinase 4 (*MKK4*), a candidate tumour suppressor gene located at 17p11, have been observed in many types of human tumours. To explore the likelihood that MKK4 acts as a suppressor in gastric tumorigenesis, we examined the expression and mutation status of *MKK4* in 144 gastric tissues and cell line specimens. Expression of the *MKK4* transcript was easily detectable in all normal and benign tumour tissues and none of 102 primary carcinomas and cell lines showed an abnormal reduction in *MKK4* expression. Expression levels of *MKK4* transcript showed no cancer-specific reduction in 43 matched sets and did not correlate with stage, grade and histopathological types of the tumours. Western blot analysis also revealed that MKK4 protein expression in carcinoma tissues and cell lines is comparable to non-cancerous tissues. A significant loss of heterozygosity (LOH) was detected at telomeric markers of the *MKK4*, locus. However, no allelic deletion of the *MKK4* gene or at the centromeric loci was identified. Moreover, no evidences for somatic mutations leading to amino acid substitutions or frameshifts of *MKK4* were identified in the carcinoma tissues and cell lines, whereas a substantial fraction of the same set showed allelic loss or mutations of the *TP53* gene located at 17p13, suggesting that LOH at telomeric loci or the *TP53* locus might not extend into the *MKK4* gene in gastric cancers. In this study, we also report the identification of a highly conserved *MKK4* processed pseudogene, which shares 95% homology with the coding region of the functional *MKK4* transcript. Collectively, our data demonstrate that genomic deletion or somatic mutation of *MKK4* is infrequent in gastric cancers, suggesting that *MKK4* might not be a critical target of genetic inactivation in gastric tumorigenesis.

Keywords: MKK4; Tumour suppressor gene; Gastric cancer; 17p; *TP53*; Loss of heterozygosity; Processed pseudogene

1. Introduction

Protein kinases participate in various signal transduction pathways and play a crucial role in the regulation of many cellular aspects such as cell growth, differentiation and apoptosis [1,2]. Oncogenic activation of tyrosine kinases and serine/threonine kinases, two major subgroups of protein kinases, has been implicated in the progression of a variety of human tumours [3,4]. It has been demonstrated that nearly all protein tyrosine kinases are involved in cell growth signalling and therefore have a high chance to be activated as oncogenes [3,5]. In gastric cancer, abnormal overexpression or gene amplification of receptor tyrosine kinases such as *erbB2/neu, EGFR, c-met* and *k-sam* has been frequently observed and often associated with uncontrolled cell growth and tumour progression [6–8].

Recently, mitogen-activated protein kinase (MAPK) kinase 4 (MKK4), a dual specificity kinase that is activated by dual phosphorylation on threonine and tyrosine residues, has been demonstrated to be aberrantly expressed in a substantial fraction of human gastric adenocarcinomas [6,9]. MKK4 acts upstream of MAPK and is a part of the Ras-dependent and independent MAPK signaling pathway [10,11]. In response to proinflammatory cytokines and cellular stresses, MKK4 directly phosphorylates and activates the c-Jun NH_2-terminal kinases (JNK), which play essential roles in the

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stress activated-protein kinase (SAPK) signalling pathway [12]. MKK4 is also known as JNK kinase and SEK1 (SAPK/ERK kinase-1) because it activates p38 MAPK and plays a central regulatory role in the cell stress response pathway [13]. Consistent with its potential roles as a key component of signalling pathways involving MAPK proteins, gastric adenocarcinoma patients with MKK4 overexpression in their cancer tissues showed a significantly shorter relapse-free survival and overall shorter survival compared with patients without MKK4 expression [9].

Interestingly, however, recent studies demonstrated genetic alterations of the *MKK4* gene in several types of human cancers, suggesting its additional role in tumour progression [14,15]. *MKK4* was initially suggested to be a tumour suppressor due to its chromosomal localisation at 17p11-12, from which a remarkably high frequency of loss of heterozygosity (LOH) has been detected in many cancers, including gastric adenocarcinomas [14–19]. Several allotyping studies utilising polymorphic markers revealed that homozygous deletions or LOH of 17p11-12 at the *MKK4* locus does not extend into the *TP53* locus at 17p13 in many tumours, indicating that allelic loss of *TP53* does not account for all cases of LOH at 17p and the existence of other critical target(s) of inactivation in this region [14,15]. The *MKK4* gene is located at 17p11.2, approximately 10 cM centromeric of *TP53* and LOH at 17p11-12 occurs in approximately 48% of human cell lines [14]. In addition, in a set of 88 human cell lines tested, homozygous deletions or point mutations of *MKK4* were detected in seven cell lines derived from pancreatic, breast, colon and testis cells, and four of these mutants were found to result in a loss of its kinase activity [14]. Deletions or somatic mutations of *MKK4* have also been observed in some types of human primary tumours including pancreatic, biliary and breast carcinomas [15]. Recently, the role for MKK4 as a suppressor has also been attributed to its capability to reduce the metastastic potential of cancer cells. Yoshida and colleagues [16] showed that in a nude mice model, the lung metastatic potential of rat prostate carcinoma cells is significantly reduced by the expression of wild-type MKK4. Thus, these observations strongly suggest that MKK4 might play a role as a tumour suppressor in some types of human cancers.

Although gastric adenocarcinoma is one of the most common malignancies and a leading cause of cancer mortality worldwide, the pathogenesis of this disease and the underlying molecular genetic events that contribute to its development are largely undefined [20]. LOH analyses have shown significant allelic loss on chromosomes 1q, 3p, 4p, 5q, 9p, 12q, 13q, 17p and 18q, suggesting the involvement of several tumour suppressor genes in gastric carcinogenesis [18,19,21–24]. Chromosome 17p is one of the most frequently lost regions in gastric cancers and 36–77% of sporadic gastric adenocarcinomas have been reported to show allelic deletion at this region [18,19]. 17p13 is known to harbour the *TP53* tumour suppressor gene, which has been well documented to be inactivated by mutation or deletion in the majority of gastric carcinomas [25–28]. A recent allelotype study also demonstrated 17p11-12 as the most common LOH locus in gastric adenocarcinomas, supporting the hypothesis that a potential tumour suppressor gene(s) which plays a critical role in gastric tumorigenesis might exist at these loci [19].

In an attempt to define whether *MKK4* is a target gene of 17p11-12 LOH and whether inactivation of MKK4 is implicated in gastric tumorigenesis, we performed expression and mutation analyses of *MKK4* in 15 gastric carcinoma cell lines and 129 tissues, including 87 malignant adenocarcinomas.

2. Materials and methods

2.1. Tissue specimens and cell lines

A total of 129 gastric tissues including 87 adenocarcinomas, three adenomas, six hamartomas, seven hyperplastic polyps and 26 normal gastric tissues were obtained from 87 gastric cancer patients and 42 non-cancer patients by surgical resection in the Kyung Hee University Medical Center (Seoul, Korea). Tissue specimens were snap-frozen immediately in liquid N_2 and stored at -70 °C until use. Tissue slices were subjected to histopathological review and tumour specimens composed of at least 80% carcinoma cells were chosen for molecular analysis. Fifteen gastric carcinoma cell lines (SNU1, SNU5, SNU16, SNU216, SNU484, SNU601, SNU620, SNU638, SNU719, MKN1, MKN28, MKN45, MKN74, AGS and KATO-III) were obtained from the Korean Cell Line Bank (Seoul National University, Seoul, Korea) or American Type Culture Collection (Rockville, MD, USA). Total cellular RNA was extracted from tissues and cell lines by a single-step method described in Ref. [29]. Genomic DNA was extracted from the same cells by dialysis of the DNA phase after the RNA was extracted.

2.2. Semi-quantitative PCR analysis

Our polymerase chain reaction (PCR)-based strategies for quantitation were previously described in Ref. [30]. Briefly, 1 μg of DNase1-treated RNA was converted to cDNA by reverse transcription using random hexamer primers and MoMuLV reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD, USA). PCR was initially performed over a range of cycles (24, 26, 28, 30, 32, 34, 36 and 38 cycles) and 2 μl of 1:4 diluted cDNA (12.5 ng/50 μl PCR reaction) undergoing 28–36 cycles was observed to be within the logarithmic

phase of amplification with primers, MKK4–11 (sense; 5′-CCGAGTTTCATCAACTTTGT-3′) and MKK4–20 (antisense; 5′-GGATGAAAATTCTTTACGTC-3′) for *MKK4* and G3 (sense; 5′-AACCATGAGAAGTATGACAACAGC-3′) and G2 (antisense; 5′-CATGTGGGCCATGAGGTCCACCAC-3′) for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), an endogenous expression control [31]. PCR was performed for 34 cycles at 95 °C (1 min), 58 °C (0.5 min) and 72 °C (1 min) in 1.5 mM $MgCl_2$-containing reaction buffer (PCR buffer II; Perkin-Elmer). For quantitative genomic PCR, exon 7 of *MKK4*, exon 8 of *TP53*, and intron 5 of *GAPDH* were separately amplified with intron-specific primers MKK4-21 (sense; 5′-GCTTAGTGTTCTCTTTATGG-3′) and MKK4-8 (antisense; 5′-AGACATCAGAGCGGACATCA-3′), SG85 (sense; 5′-TCCTTACTGCCTCTTGCTTCTCTTT-3′) and SG83 (antisense: 5′-TCTCCTCCACCGCTTCTTGT-3′), and G3 (see above) and G5 (antisense; 5′-GAGTCCTTCCACGATACCAAAG-3′), respectively. Ten microlitres of the PCR products were resolved on 2% agarose gels. Quantitation was achieved by densitometric scanning of the ethidium bromide-stained gels and absolute area integrations of the curves representing each specimen were compared after adjustment for *GAPDH*. Integration and analysis were performed using Molecular Analyst software program (Bio-Rad, Hercules, CA, USA). Quantitative PCR was repeated at least three times for each specimen and the mean was obtained.

2.3. LOH studies

LOH was determined using three polymorphic STS markers (D17S969, D17S1303 and D17S947) localised at chromosome 17p11–12. PCR amplification was performed on each tumour and normal DNA sample pair obtained from 43 patients and subsequently electrophoresed on standard denaturating 8% polyacrylamide gels. If the two alleles appeared in the normal tissue DNA, the patient was considered an 'informative case' for the particular marker. Signal intensity of fragments and the relative ratio of both tumour and normal allele intensities were determined by scanning densitometry. Because a certain number of non-cancerous cells might be present in the tumour tissues, LOH was assigned when the intensity ratio of the two tumour alleles differed by at least 50% from that observed for the corresponding normal DNA.

2.4. Non-isotopic reverse transcriptase-polymerase chain reaction-single-strand conformation polymorphism (RT-PCR-SSCP) analysis

To detect sequence alterations in *MKK4*, we performed non-isotopic RT-PCR-SSCP analysis as previously described in Ref. [32]. The entire coding region of the *MKK4* transcript was amplified with six primer sets MKK4-1/MKK4-2 (exons 1–3), MKK4-3/MKK4-4 (exons 2–4), MKK4-5/MKK4-6 (exons 4–6), MKK4-7/MKK4-8 (exons 5–8), MKK4-9/MKK4-10 (exons 8–10), and MKK4-11/MKK4-20 (exons 9–11). Sequences of the primers used for our RT-PCR-SSCP analysis can be obtained upon request from the authors. RT-PCR-SSCP analysis of *TP53* transcripts was performed as previously described in Ref. [33]. Twenty microlitres of PCR products mixed with 5 μl of 0.5 N NaOH, 10 mM ethylene diamine tetra acetic acid (EDTA) and 10 μl of denaturing loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). After heating at 95 °C for 5 min, samples were loaded in wells precooled to 4 °C. SSCP was performed using 8% non-denaturating acrylamide gels containing 10% glycerol at 4–8 and 18–22 °C.

2.5. Western blot analysis

Cells were lysed in a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 1 mM EDTA, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenyl methyl sulphonyl fluoride (PMSF). The cell lysate was clarified by centrifugation and 30 μg of total protein was fractionated by 10% SDS-PAGE. MKK4 was detected by immunoblotting using a polyclonal antibody C-20 or K-18 (SantaCruz biotechnology, CA, USA). Antibody binding was detected by enhanced chemiluminescence (Pierce) using a secondary antibody conjugated to horseradish peroxidase. For stripping, the blots were incubated in a stripping buffer (0.2 M glycine (pH 2.2), 0.1% SDS, 1% Tween-20) at 50 °C for 60 min.

2.6. 5-Aza-2′ deoxycytidine treatment

Fifteen gastric carcinoma cell lines were placed in a six-well tissue culture plate and grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS). 5-aza-2′deoxycytidine (Sigma, St. Louis, MO, USA) was added to a final concentration of 2 μM for 4 days.

2.7. Cloning of a processed pseudogene

The putative processed pseudogene of *MKK4* was amplified by PCR using normal human lymphocyte genomic DNA as template and *MKK4* exon-specific primer sets. The PCR products were cloned into pCR2.1-TOPO vectors (Invitogen, San Diego, CA, USA) and five colonies were sequenced in both directions using Sequenase 2.0 (Amersham, Arlington Heights, IL, USA).

3. Results

3.1. Expression of MKK4 mRNA in normal and benign tumour tissues

To explore the likelihood that *MKK4* is a tumour suppressor gene involved in gastric tumorigenesis, we initially characterised the expression status of *MKK4* mRNA in 26 normal gastric tissues and 16 benign tumours including three adenomas, six hamartomas and seven hyperplastic polyps. For validation of our quantitative PCR approach, serially diluted cDNA was subjected to PCR amplification of *MKK4* and *GAPDH* over a range of cycles. Linearity of the cDNA dilution experiments demonstrated the ability of our PCR procedure to discriminate the various levels of *MKK4* mRNA expression (data not shown). As shown in Fig. 1, *MKK4* mRNA expression was easily detectable in all normal and benign tumour tissues we examined and no significant variation in the expression levels was identified among the specimens. The expression levels of *MKK4* in normal and benign tumour tissues were observed within 0.98–1.65 (mean 1.32) and 1.02–1.68 (mean 1.35), respectively (Fig. 2). RT-PCR was repeated at least three times for each specimen and the mean of the expression levels in non-cancerous tissues was determined as 1.33.

3.2. Expression of MKK4 in carcinoma cell lines and primary tumours

Next, we evaluated expression levels of *MKK4* mRNA in 15 gastric carcinoma cell lines and 87 primary tumours. Expression of *MKK4* transcript was detected in all primary tumours and cell lines we analysed and its expression levels were observed in a range of 0.91–1.61 (mean; 1.27) and 0.96–1.63 (mean 1.31), respectively (Figs. 1 and 2). Based on the expression levels in non-cancerous gastric tissues, we arbitrarily set less than one-half (0.67) of the mean of non-cancerous tissues as being abnormally low. However, none of the carcinoma cell lines and malignant primary tumours examined in this study was classified as an abnormally low expressor. In addition, no association of *MKK4* expression with stage, grade or histopathologic type of the tumours was observed (data not shown). Furthermore, none of 43 matched sets showed a detectable reduction of *MKK4* in cancer tissues compared with the adjacent non-cancerous tissues (Fig. 1). Although none of tumours examined showed loss or abnormal reduction of *MKK4*, variable expression levels were recognised among the specimens. To examine the possibility that the transcription of *MKK4* is downregulated in some cancer cells by epigenetic mechanism(s) such as abnormal

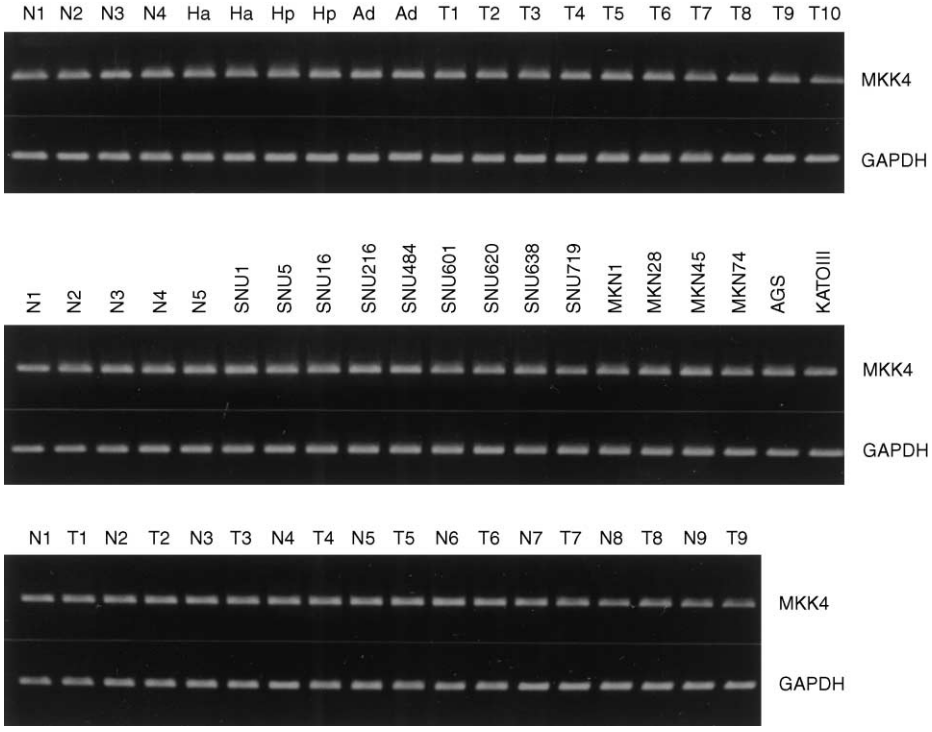

Fig. 1. Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *MKK4* expression in human gastric tissues and cell lines. Exons 9–11 region of *MKK4* transcript was amplified with primers MKK4-11 and MKK4-20 (256 bp). Ten microlitres of the PCR products were resolved on 2% agarose gels and stained with ethidium bromide. Negative controls without RNA (for RT) and cDNA (for PCR) were included in every assay to exclude the false positive by possible contamination (data not shown). N, normal gastric tissue; Ha, hamartoma; Hp, hyperplastic polyp; Ad, adenoma; T, carcinoma. *MKK4* mRNA expression in cancer and adjacent non-cancerous tissues of the same gastric cancer patients (#1–9) is shown.

hypermethylation, 15 gastric cell lines were treated with the demethylating agent 5-aza-2′ deoxycytidine for 2–4 days and the *MKK4* mRNA level determined by RT-PCR. However, any detectable increase in *MKK4* expression level was not recognised in the treated cell lines compared with untreated controls (data not shown).

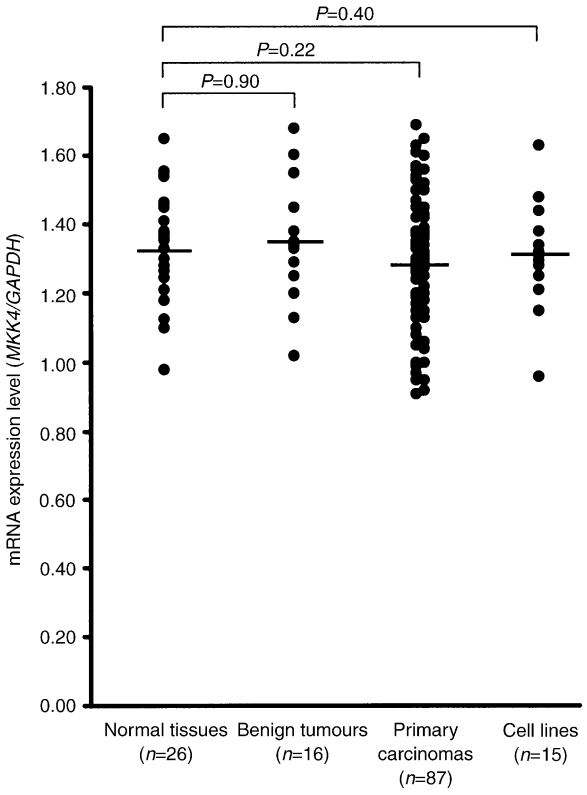

Fig. 2. Expression levels of *MKK4* mRNA in gastric tissues and cell lines. Quantitation was achieved by densitometric scanning of *MKK4* RT-PCR products in ethidium bromide-stained gels and absolute area integrations of the curves representing each specimen were compared after adjustment for *GAPDH*. Semi-quantitative PCR was repeated at least three times for each specimen and the means were obtained. Bar indicates the mean expression level of each specimen group.

Next, we examined the expression status of the MKK4 protein in gastric carcinoma cell lines and primary tumours using Western blotting analysis. As shown in Fig. 3, expression of the MKK4 protein was easily detectable in all of the 15 cell lines and 20 primary tumours that we analysed, whereas the AsPc-1 human pancreatic carcinoma cells with a homozygous deletion of the *MKK4* gene showed no MKK4 expression [17]. No significant variation in the expression level of MKK4 was identified among the cell lines and tissue specimens. Expression levels of the MKK4 protein in the 20 primary carcinomas were comparable to those of the adjacent non-cancerous tissues, indicating that MKK4 is normally expressed in a majority of gastric cancers at both the mRNA and protein levels.

3.3. *LOH at 17p11-12 and genomic status of* MKK4 *in gastric carcinomas*

To analyse the allelic status of *MKK4* in gastric cancers, we first determined the genomic level of *MKK4* using quantitative PCR. For comparison and validation of our PCR approach, genomic levels of *TP53* at 17p13 were also screened for in the 15 gastric cell lines, whose allelic status with regard to *T53* have been previously described in Refs. [26,27]. As shown in Fig. 4a, absence and marked reduction of *TP53* were detected in nine cell lines which have homozygous deletion or LOH of the gene while normal levels of *TP53* were detected in five wild-type *TP53* (wtp53)-carrying cell lines (SNU-1, SNU-719, MKN45, MKN74 and AGS), indicating that genomic level determined by our quantitative PCR is consistent with the allelic status of the gene. 24 of the 87 (28%) primary gastric carcinomas we analysed showed a detectable reduction of *TP53* levels, whereas none of the tumour tissues and cell lines had a reduced *MKK4* expression (Fig. 4a and data not shown).

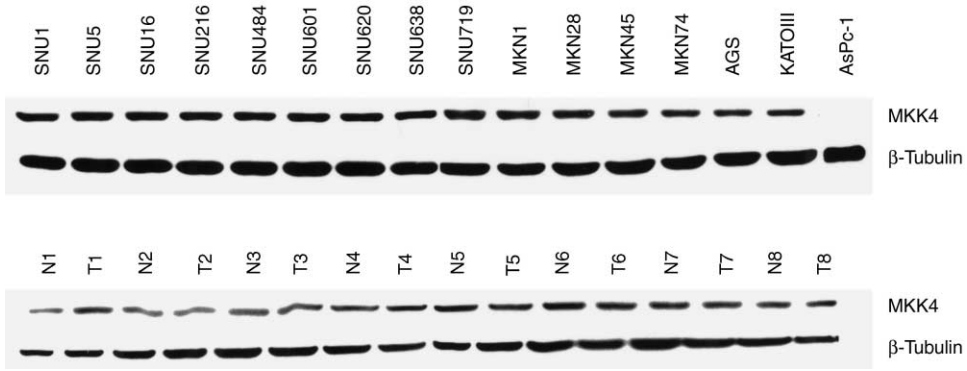

Fig. 3. Western blot analysis of MKK4 protein expression in gastric cell lines and primary tumours. Thirty μg of total protein was loaded in each lane and fractionated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). MKK4 protein was detected using a polyclonal antibody C-20 and enhanced chemiluminescence. β-tubulin was used as a loading control. AsPc-1 human pancreatic cancer cells, which do not express MKK4 due to a homozygous gene deletion, were used as a negative control. T1–T8, primary carcinomas; N1–N8, adjacent non-cancerous tissues.

To further elicit the allelic status of *MKK4*, we surveyed 43 gastric carcinomas for LOH of 17p11-12 region using D17S1303, D17S969 and D17S947. Among 43 matched sets tested, 33 (77%) were informative for at least one of the two telomeric markers (D17S1303 and D17S969), which are located approximately 10 cM centromeric of the *TP53* locus. LOH at D17S1303 and D17S969 was observed in 13 of 21 (62%) and 6 of 19 (31%) informative cases, respectively. Fig. 4b illustrates typical LOH or retention of heterozygosity (ROH) for each marker. Among seven tumours that are informative for both markers, LOH and ROH at both loci were detected in 1 and 2 cases, respectively, and the other 4 cases showed LOH only at the more telomeric D17S1303 (Table 1). In contrast to frequent LOH at telomeric loci, all of the 33 informative tumours at centromeric D17S947 were found to retain heterozygosity. In addition, in PCR amplification of these marker DNAs, relatively low levels of D17S1303 or D17S969 were observed in 9 of 13 LOH tumours whereas all of these tumours showed normal levels of *MKK4* and D17S947 (data not shown). Collectively, these results suggest that LOH at the telomeric region of the *MKK4* locus is a frequent event, but rarely extends into the *MKK4* gene in gastric adenocarcinoma.

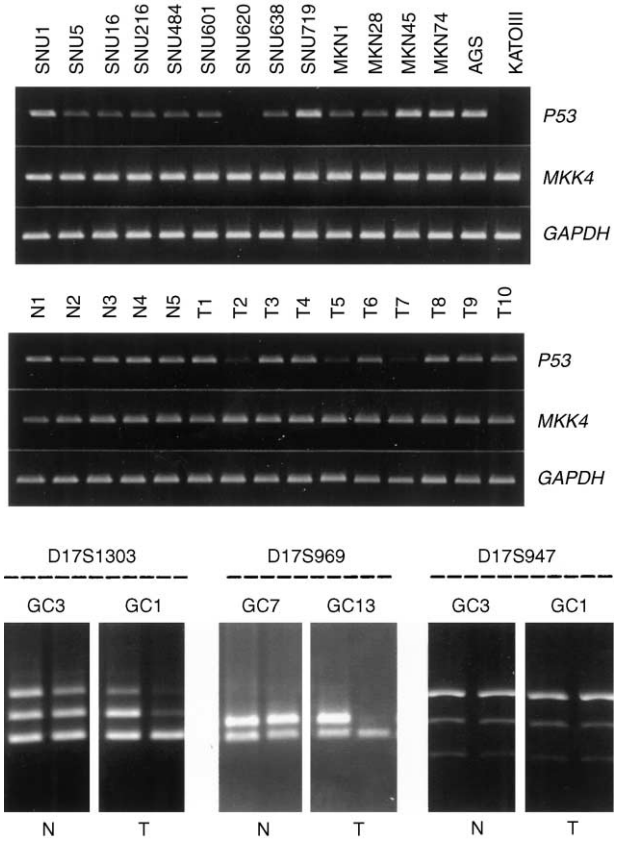

Fig. 4. Semi-quantitative genomic PCR analysis of *MKK4* and *TP53* in gastric carcinoma cell lines and tissues (a). Exon 7 of *MKK4* and exon 8 of *TP53* were amplified by intron-specific primers MKK4-21 and MKK4-8 (269 bp) and SG85 and SG83 (210 bp), respectively. N, normal gastric tissue; T, primary carcinoma. Representative examples of LOH analysis (b). At marker D17S1303, specimen GC1 exhibits loss of heterozygosity (LOH) in the tumour DNA (T) compared with their corresponding normal DNA (N), whereas GC3 shows retention of heterozygosity (ROH) at this locus. At marker D17S969, specimens GC13 and GC7 exhibit LOH and ROH, respectively. At centromeric marker D17S947, both GC1 and GC3 show ROH.

3.4. *No sequence alterations of* MKK4 *in gastric carcinomas*

For detection of mutational alterations of *MKK4*, we performed RT-PCR-SSCP analysis of *MKK4* transcripts expressed in the carcinoma tissues and cell lines. The entire coding region of the *MKK4* transcripts was amplified by PCR with 6 different primer sets and the RT-PCR products were subjected to SSCP analysis. For comprehensive screening, the same transcript regions were repeatedly amplified using different primer sets, digested with several different endonuclease(s), and

Table 1
Results of LOH analysis at the 17p11-12 region in gastric cancers

Markers (Cen→Tel)	D17S947	D17S969	D17S1303	*TP53* status
GC1	ROH	ROH	LOH	MT
GC2	ROH	ROH	LOH	MT
GC3	ROH	NI	ROH	WT
GC4	ROH	LOH	NI	WT
GC5	NI	NI	LOH	MT
GC6	ROH	ROH	ROH	WT
GC7	ROH	ROH	ROH	WT
GC8	NI	NI	LOH	WT
GC9	ROH	LOH	NI	MT
GC10	ROH	NI	LOH	MT
GC11	NI	NI	ROH	WT
GC12	NI	ROH	NI	WT
GC13	ROH	LOH	LOH	MT
GC14	ROH	NI	LOH	WT
GC15	ROH	NI	LOH	MT
GC16	ROH	ROH	NI	WT
GC17	ROH	ROH	NI	WT
GC18	ROH	NI	ROH	WT
GC19	NI	NI	LOH	MT
GC20	ROH	LOH	NI	WT
GC21	ROH	NI	ROH	WT
GC22	NI	ROH	NI	WT
GC23	ROH	ROH	LOH	MT
GC24	ROH	NI	ROH	WT
GC25	NI	NI	LOH	WT
GC26	ROH	ROH	NI	WT
GC27	ROH	ROH	LOH	MT
GC28	ROH	NI	ROH	WT
GC29	NI	LOH	NI	MT
GC30	ROH	ROH	NI	WT
GC31	ROH	NI	LOH	MT
GC32	ROH	LOH	NI	WT
GC33	NI	ROH	NI	WT

Cen, centromere; Tel, telomere; ROH, retention of heterozygosity; NI, not informative; LOH, loss of heterozygosity; MT, mutant type; WT, wild-type.

examined under two different running conditions. However, we failed to detect any types of mutation leading to amino acid substitutions or frameshifts of *MKK4* in all 102 carcinomas including the 15 cell lines, whereas 32 (31%) of the same tumour set showed mutational alterations of *TP53* (Fig. 5). *TP53* mutations were found in 12 of 18 (67%) tumours with LOH at 17p11-12 whereas only 3 of 15 (20%) tumours with no LOH showed *TP53* alterations (Table 1).

3.5. Identification of a processed pseudogene

During PCR amplification of the *MKK4* gene, we observed smaller-than-expected genomic fragments, the structure of which was consistent with a processed pseudogene. Cloning of these fragments from normal human genomic DNA and subsequent sequencing analysis verified the presence of a *MKK4* pseudogene that completely lacks intronic sequences of the gene (Fig. 6, GeneBank Accession number AF332564). This processed pseudogene has 63 sequence substitutions and one single nucleotide insertion. Sixty of the 63 mismatches were within the coding region, which represents 5.0% (60/1200) of the protein-coding sequences of *MKK4* transcript. Two new termination codons were generated as a result of the alterations. Expression analysis by pseudogene-specific PCR and restriction endonuclease(s) digestion assay for RT-PCR products showed that this pseudogene is not transcribed (data not shown).

4. Discussion

LOH at chromosome 17p is frequently encountered in a variety of human neoplasias including gastric adenocarcinoma [18,19]. 17p13 is known to harbour the well-characterised *TP53* tumour suppressor gene, but allelic loss of *TP53* does not account for all cases of LOH at 17p in many cancer types [15]. Several allotyping studies identified frequent homozygous deletions at 17p11-12, approximately 10 cM cetromeric of the *TP53* locus, suggesting the presence of other potential tumour suppressor gene(s) in this region [19,34]. Recently, the *MKK4* gene was mapped within this region and homozygous deletions and somatically acquired missense mutations of *MKK4* were observed in cancer cell lines and tissue specimens. Teng and colleagues [14] first reported that six of 213 (2.8%) human cell lines derived from pancreatic, lung, breast and colon carcinomas harbour homozygous deletions or sequence variants of *MKK4*, which were verified as loss of function mutants that lack the ability to phosphorylate SAPK. Deletions or somatic missense mutations of *MKK4* were also observed in some primary tumours or xenografts derived from pancreatic and biliary adenocarcinomas [15]. Interestingly, Yoshida and colleagues [16] observed that transfection of wild-type *MKK4* significantly reduces the metastatic ability of rat prostate cancer cells and this phenotype is consistent with the metastasis suppressor activity conferred by human chromosome 17p, implicating *MKK4* as a metastasis suppressor gene encoded by 17p. Recently, it was also reported MKK4 expression is inversely related to the histopathological pattern in advancing human prostate cancers [17]. Collectively, these observations strongly suggest that MKK4 may function as a suppressor of tumorigenesis or metastasis in certain types of cells.

LOH on chromosome 17p11-13 is one of the most common molecular abnormalities associated with gastric tumorigenesis [18–20]. Allele loss or mutation of the *p53* gene localised at 17p13.1 occurs in 23–65% of gastric cancers, while LOH at markers on 17p11-12 affects approximately 70–77% of gastric adenocarcinomas [19,25,26]. However, the interrelationship between LOH on these two regions and the likelihood of *MKK4* acting as a tumour suppressor have been poorly understood in gastric cancer. In this study, we demonstrate that *MKK4* is expressed in all types of gastric tissues and not deleted or mutated in malignant adenocarcinomas, suggesting that *MKK4* might not be a critical target of genetic alteration at 17p in gastric carcinogenesis. Expression of MKK4 mRNA and protein was easily detectable in all carcinoma tissues and cell lines we examined and no cancer-specific reduction of *MKK4* was identified in 43 matched sets. In addition, expression levels of *MKK4* showed no association with stage, grade and the histopathological types of the tumours. Moreover, no evidences for allelic deletion or somatic mutations of the gene were recognised in 102 primary carcinomas and cell lines, whereas a substantial fraction of the same set of samples showed allelic loss or mutations of the *TP53* gene located at 17p13.

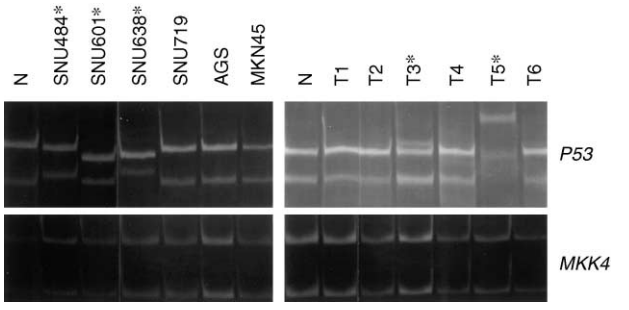

Fig. 5. Reverse transcriptase-polymerase chain reaction-single strand conformation polymorphism (RT-PCR-SSCP) analysis of *MKK4*. Exons 4–6 region of *MKK4* and exons 7–8 region of *TP53* were amplified by RT-PCR and 20 μl of the PCR products were subjected to non-isotopic SSCP analysis using 8% non-denaturating acrylamide gels containing 10% glycerol. After 4 h run at 18–22 °C, the gels were visualised by staining with ethidium bromide. N, normal gastric tissue; T1–T6, primary gastric carcinomas. Cell lines and tumor tissues showing migration shifts of *TP53* PCR products are indicated by an asterisk.

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ψ       1  CCCAACAATG GCGGCTCCGA GCCtGAGCGG CGGCtGCGGC TCCGGGGGCa GCAGCGGCAG CctCACCCCg
MKK4       CCCAACAATG GCGGCTCCGA GCCCGAGCGG CGGCGGCGGC TCCGGGGGCG GCAGCGGCAG CGGCACCCCC

ψ      71  GGaCCtGTAG GaTCCCtGaa GCCAGGCCAC CCaGCCaTCA GCAGCATGCA GGGTAAACGC AAAGCACTGA
MKK4       GGCCCCGTAG GGTCCCCGGC GCCAGGCCAC CCGGCCGTCA GCAGCATGCA GGGTAAACGC AAAGCACTGA

ψ     141  AGTTGAATTT TGCAAATCCA CCTTTCAAAT CTACAGCAAG GTTTACTCTG AATCCCAATC CTACAGGAGT
MKK4       AGTTGAATTT TGCAAATCCA CCTTTCAAAT CTACAGCAAG GTTTACTCTG AATCCCAATC CTACAGGAGT

ψ     211  TCAAAACCCA CACATAGAGA GACTGAGAtC ACACAGCATT GAGTCATCAG GAAAACTGAA GATCTCCCCT
MKK4       TCAAAACCCA CACATAGAGA GACTGAGAAC ACACAGCATT GAGTCATCAG GAAAACTGAA GATCTCCCCT

ψ     281  GAACAACACT GGGATTTCAC TGCAGAGGAC TTGAAAGACC TTGGAGAAAT TGGAtGAGGA GtTTATGGTT
MKK4       GAACAACACT GGGATTTCAC TGCAGAGGAC TTGAAAGACC TTGGAGAAAT TGGACGAGGA GCTTATGGTT

ψ     351  CTGcCAACAA AATGGTCCAC AAACCAAGTG GGCAAATAAT GGCAGTTAAA AGAATTCGGT CAACAGTGaA
MKK4       CTGTCAACAA AATGGTCCAC AAACCAAGTG GGCAAATAAT GGCAGTTAAA AGAATTCGGT CAACAGTGGA

ψ     421  TGAAAAAGAA CAAAAACAAC cTCTTAcGGA TTTGGATGaA GTAATGCaGA GTAGTGATTG CCCtTACATT
MKK4       TGAAAAAGAA CAAAAACAAC TTCTTATGGA TTTGGATGTA GTAATGCGGA GTAGTGATTG CCCATACATT

ψ     491  GTTCAGTTTT ATGaTGCACT CTaCAGAcAG GGTGACaGTa GGAcCTGTAT GGAACTCATG TCTAtCTCGT
MKK4       GTTCAGTTTT ATGGTGCACT CTTCAGAGAG GGTGACTGTT GGATCTGTAT GGAACTCATG TCTACCTCGT

ψ     561  TTGATAAGTT TTACAAATAT GTgTATAGTG TATTAGATGA TGTTATTCCA GAAGAAATTT TAGGCAgAAT
MKK4       TTGATAAGTT TTACAAATAT GTATATAGTG TATTAGATGA TGTTATTCCA GAAGAAATTT TAGGCAAAAT

ψ     631  CACTTTAGCA tCTGTGAAAG CAtTAAACCA CTTAAAAGAA AACTTGAAAA TTATTCACAG AGATATCAAA
MKK4       CACTTTAGCA ACTGTGAAAG CACTAAACCA CTTAAAAGAA AACTTGAAAA TTATTCACAG AGATATCAAA

ψ     701  CCTTCCAATA TTCTTCTGGA CAGAAGTGaA AATATTAAaC TCTGTGACTT tGGCATCAGT GGgCAGCTTG
MKK4       CCTTCCAATA TTCTTCTGGA CAGAAGTGGA AATATTAAGC TCTGTGACTT CGGCATCAGT GGACAGCTTG

ψ     771  TGGACTCTAT TGCCAAGACA gaAGATGCTG GCTGTAGGCC ATACATGGCA CCTGAAAGAA TAGACCCAAG
MKK4       TGGACTCTAT TGCCAAGACA AGAGATGCTG GCTGTAGGCC ATACATGGCA CCTGAAAGAA TAGACCCAAG

ψ     841  tGCATCACGA CAAGGATATG ATGTCtGCTC TGATGTCTGG AGTTTGGGGA TCACATTGTg TGAGTTGGCC
MKK4       CGCATCACGA CAAGGATATG ATGTCCGCTC TGATGTCTGG AGTTTGGGGA TCACATTGTA TGAGTTGGCC

ψ     911  ACAGGCtGAT TTCCTTATCC AAAGTGGAAT AGTGTATTTG ATCAACTAAC ACAAGTCGTG AAAGGAGATC
MKK4       ACAGGCCAGT TTCCTTATCC AAAGTGGAAT AGTGTATTTG ATCAACTAAC ACAAGTCGTG AAAGGAGATC

ψ     981  CTCCGCAGCT GAGTAATTCT GAGGAAAacG AATTCTCCCC aAGTTTCATC AACTTTGTCA ACTTGTGCCT
MKK4       CTCCGCAGCT GAGTAATTCT GAGGAAAGGG AATTCTCCCC GAGTTTCATC AACTTTGTCA ACTTGTGCCT

ψ    1051  TACGAAGGAT GAATCCAAAA GaCCAcAGcA TAAAGAGCTT CTGAAACATC ttTTTATTTT GATtTATGAA
MKK4       TACGAAGGAT GAATCCAAAA GGCCAAAGTA TAAAGAGCTT CTGAAACATC CCTTTATTTT GATGTATGAA

ψ    1121  GAACGTaCCa TTGAaGTCGC ATGCTATGtT TTGTcAAATC CTGGATCAAA TGCCAGCTAC TCCCAGCTtT
MKK4       GAACGTGCCG TTGAGGTCGC ATGCTATG T TTGTAAAATC CTGGATCAAA TGCCAGCTAC TCCCAGCTCT

ψ    1191  CCCATGTATG TCGtTTGATA TCGCTGCTAC ATCAGACTAa AAAAAAGGGC TGAGAGGAAG CAAaAgGTAA
MKK4       CCCATGTATG TCGATTGATA TCGCTGCTAC ATCAGACTAG AAAAAAGGGC TGAGAGGAAG CAAGACGTAA

ψ    1261  AGAATTTTCA TCCCG
MKK4       AGAATTTTCA TCCCG
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Fig. 6. Nucleotide sequences of the processed *MKK4* pseudogene (ψ). Sixty-three nucleotide substitutions and one nucleotide insertion are indicated (small letters in bold). Start and stop codons in the *MKK4* transcript and new termination codons in the pseudogene are underlined.

Our allelotyping study demonstrated that LOH occurs frequently at the telomeric markers (D17S1303 and D17S969) of the *MKK4* locus, but not at the centromeric marker (D17S947) in gastric carcinomas. In addition, compared with LOH (62%) at D17S1303, a significantly low rate of LOH (31%) was found at the more centromeric D17S969. Among seven tumours that are informative for both telomeric markers, 4 cases (GC1, GC2, GC23 and GC27) showed LOH only at D17S1303, whereas none of the tumours showed LOH exclusively at D17S969 (Table 1). It is also noteworthy that allelic deletion or mutations of *TP53* were found in 12 of the 18 (67%) tumours showing LOH at the telomeric markers, but in only 3 of the 15 (20%) tumours with ROH at these loci. With no evidence for allelic deletion or mutational alterations of *MKK4* in gastric carcinoma cell lines and primary tumours, these observations strongly suggest that LOH at 17p11-12 is more likely to be related to *TP53* alterations and might not extend into the *MKK4* locus in gastric cancer. It was previously reported that LOH of distal 17p affected 90% of pancreatic cancers, a remarkably high figure when compared with the *TP53* mutation rate of 75% in this cancer type, whereas LOH rate of 17p is not significantly high compared with the *TP53* mutation rate in gastric cancers [34]. In this context, *MKK4* may act as a tumour suppressor in a tissue type-specific manner [14,15]. Collectively, our data suggest that genetic abnormality of *MKK4* might be an extremely rare event in gastric tumorigenesis.

Although previous mutational analyses lend support a tumour suppressive role of MKK4 in some types of human cancers, MKK4 would be an unusual tumour suppressor gene in the context that it belongs to the MAPK kinase family and is part of the Ras-dependent and cytokine- or stress-induced signalling cascades [10–13]. Using an immunohistochemical assay, Wu and colleagues [9] showed that MKK4 immunoreactivity is much higher in gastric tumour cells compared with the intensity in the normal epithelium, indicating a higher expression level of MKK4 in cancer cells. In addition, gastric adenocarcinoma patients with MKK4 present in their gastric cancer tissues showed a significantly shorter relapse-free survival and overall shorter survival compared with patients without MKK4 expression. In contrast, they also recognised that approximately 48% of gastric cancers showed loss of MKK4 immunoreactivity, while their matching normal tissues were positive for MKK4 expression. Based on these observations, it was suggested that some of these tumours might harbour deletion or mutation of *MKK4*. Alternatively, it was hypothesised that the MKK4-JNK or MKK4-p38 pathway might be activated in these normal tissues in response to a cancerous micro-environment, which is supported by finding that MKK4 expression correlates with the size of tumour and lymph node involvement features [9]. In our study, however, we found the non-specific immunoreactivity of MKK4 antibodies used for previous immunohistochemical studies and our Western blot analysis revealed no marked down- or upregulation of MKK4 protein expression in the gastric cell lines and tumour tissues. In addition, expression of MKK4 protein was not activated by stresses such as serum starvation, heat shock, or H_2O_2 treatment, while the NIH3T3 cells used as a control showed strong expression of the MKK4 protein (data not shown). Although we cannot exclude the possibility that MKK4 is abnormally regulated at the post-translational level, our molecular data strongly suggests that MKK4 expression is not altered at both transcription and translation levels in a majority of gastric cancers. Further studies will be required to understand the biological significance of MKK4 protein expression and the molecular basis of its regulation in normal and tumour cells of the stomach.

In this work, we also discovered a putative pseudogene of *MKK4* which completely lacks the intronic sequences of the *MKK4* gene, which leads us to the conclusion that this is a processed pseudogene that results from reinsertion into the genome of a reverse transcribed *MKK4* transcript [35]. Sixty-one of 64 nucleotide differences were found within the *MKK4* coding region. Most of the differences were nucleotide transitions (68, 43/63) and C to T (or G to A) substitution was the most commonly observed (51%, 32/63). This pattern of mutation is consistent with that found in other pseudogenes such as ψ*PTEN* [36]. Through a search of a human genome database, we also found that gemonic sequences at chromosome Xq22 show a strong homology (624/628; 99.4%) to the *MKK4* pseudogene that we identified (nucleotides 1–628, Fig. 6), indicating the existence of another processed pseudogene of a truncated form. The high sequence homology of the pseudogene with the functional *MKK4* transcript and the lack of intronic sequences may potentially cause misinterpretation in attempts to discover pathogenic mutations in studies using cDNAs as templates. Hence, caution should be exerted when a sequence variant is noted in such screening approaches.

In conclusion, our study demonstrates that the *MKK4* gene is expressed normally in all types of gastric tumours and not altered in malignant adenocarcinomas, strongly indicating that *MKK4* is not a critical target of genetic alteration in gastric tumorigenesis.

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