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Increased MAPK Activity and MKP-1 Overexpression in Human Gastric Adenocarcinoma

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Mitogen-activated protein kinase (MAPK) has been known to play a critical role in the regulation of the carcinogenesis in human cancers. In an effort to understand the functional role of the MAPK in the carcinogenesis of human gastric tissues, we examined the changes of MAPK levels in human gastric adenocarcinoma. We found that increased MAPK activity was accompanied by overexpression of mitogen-activated protein kinase phosphatase-1 (MKP-1), suggesting that signaling pathways leading to the activation of MAPK and the induction of MKP-1 expression are associated with carcinogenesis of human gastric adenocarcinoma.

Mitogen-activated protein kinase (MAPK), also known as extracellular signal-regulated kinase (ERK) has been known to play a pivotal role in the cell proliferation and differentiation in many cells and tissues (1, 2). Signaling pathway leading to the activation of MAPK constitutes protein kinases cascades in which a series of phosphorylation occurs by Raf (MAPKK), MEK (MAPKK), and MAPK (3, 4). These signaling modules are executed via p21 ras activation in response to various extracellular stimuli, and thus transmit the primary extracellular signals from the cytoplasmic to the nuclear compartment. Recently it has been reported that the constitutive activation of MAPK occurs in a wide variety of human tumor tissues (5). In particular, it was suggested that MAPK activation plays a critical role in the initiation and progression of human breast cancer (6). In contrast, Atten *et al.* reported that decreased MAPK activity was associated with human gastric adenocarcinoma (7). MKP-1 protein, a p42 MAPK-specific phosphatase, acts negatively on the regulation of MAPK activity by dephosphorylating and inactivating p42 MAPK (8). It has been suggested to be a tumor suppressor gene product in cultured cells (9). However, it is also known that MKP-1 does not act to dephosphorylate and inactivate p42 MAPK, despite of overexpression of MKP-1 in primary tumor tissues (5). MKP-1 is originally identified as an immediate early gene and its expression is inducible in serum-treated cells (9, 10). In the present study we reassessed the changes of MAPK and MKP-1 levels in human gastric adenocarcinoma. We found an increase in both MAPK activity and MKP-1 expression, suggesting that signaling pathways leading to the activation of MAPK and the induction of MKP-1 expression may play a critical role in the carcinogenesis of human gastric adenocarcinoma.

MATERIALS AND METHODS

Preparation of gastric tissues extracts. Primary gastric tumor tissues were surgically resected from patients with gastric adenocarcinomas. For control samples, primary normal gastric tissues were obtained from the surrounding tumor-free tissues. These tissues were stored in −70°C before use. After thawing, tissues extracts were prepared as described before (11). The tissues were homogenized with a Teflon glass homogenizer in 1 ml of ice-cold extract buffer consisting of 20 mM Tris HCl (pH 8.0), 10 mM EGTA, 5 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1% Triton-X 100, 1 mM sodium orthovanadate, l mM sodium fluoride, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml), and aprotinin (10 μg/ml). The total tissues extracts were centrifuged at 12,000g for 15 min at 4°C and then centrifuged at 100,000g for 30 min at 4°C. The supernatants were collected and the protein amounts were measured by the BCA method (12).

Western blotting analysis. The soluble extracts were mixed with Laemmli sample buffer (2×) and boiled for 1 min. After the proteins were separated by SDS–PAGE and transferred to the nitrocellulose membrane, immunoblot analysis was performed as described before (11). Monoclonal anti-ERK1 and anti-ERK2 antibodies (Transduction Laboratories), polyclonal anti-phospho-MAPK antibody (Santa Cruz Biotechnology), and polyclonal anti-MKP-1 antibody (Santa Cruz Biotechnology) were, respectively, used to probe immunoblots to detect ERK, p-MAPK and MKP-1.

MAPK assay. As described before (11), aliquots of either soluble fractions or immunoprecipitated samples were mixed to a total volume of 50 μl with the assay buffer containing 20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 150 μM [γ-^{32}P] ATP (Amersham; 100–200 cpm/pmol), 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, l

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mM sodium fluoride, 1 mM dithiothreitol, 1 mg/ml BSA, with or without 50 μg MBP (Sigma). After the reaction mixtures were incubated for 15 min at 37°C, 10 μl aliquots were applied to P81 Whatman filter paper, washed with 1% phosphoric acid, and counted for radioactivity. The radioactivity observed in the absence of MBP was subtracted from that obtained in the presence of MBP. These values were triplicated and averaged.

Polymerase chain reaction and single strand conformation polymorphism (PCR-SSCP) analysis. Ras gene mutation was analyzed as described before (13). Briefly, after genomic DNAs were isolated from primary gastric tumor tissues, amplified DNA fragments of the ras oncogene were obtained by PCR using oligonucleotide primers. Aliquots were denatured at 95°C for 5 min and electrophoresed in 6% nondenaturing acrylamide gel with 10% glycerol. Gels were dried and visualized by autoradiography.

RESULTS

In an effort to understand the functional role of MAPK in the carcinogenesis of human gastric carcinomas, we examined MAPK activity in primary gastric tissues, resected after surgery from 5 patients with gastric adenocarcinomas. Tumor and tumor-free adjacent normal tissues were prepared from the same individ-

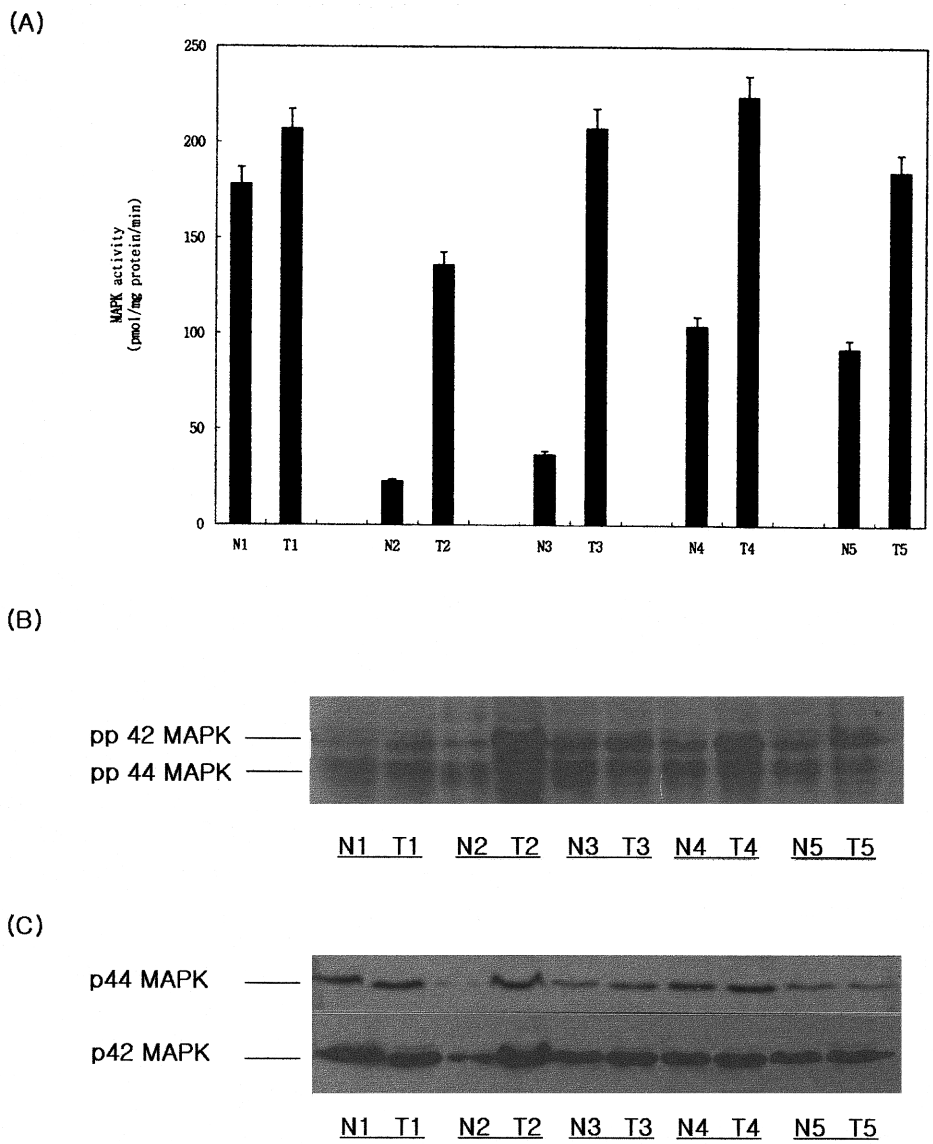

FIG. 1. (A) Changes of MAPK activities of the primary normal (N) and tumor (T) gastric tissues. Each samples were obtained from the same patient and MAPK activity was assayed as described in "Materials and Methods." (B) Western blot analysis with antibody specific for phospho-MAPK. Phosphorylated p44 and p42 MAPK were detected from the primary normal and tumor gastric tissues. (C) Western blot analysis with antibodies specific for p44/p42 MAPKs. In the tissues extracts p44/p42 MAPKs were detected as described in "Materials and Methods."

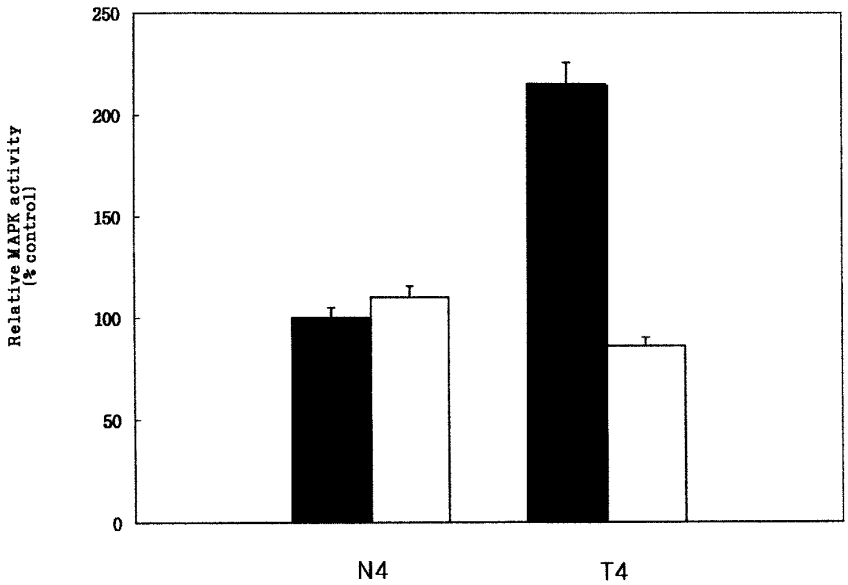

FIG. 2. MAPK activities in the presence and absence of phosphatase inhibitors. MAPK activities of N4/T4 tissues extracts were assayed in the presence (solid bars) and in the absence (open bars) of phosphatase inhibitors.

ual. We examined MAPK activity by measuring the ability of the tissues extracts to phosphorylate MBP as a substrate. MAPK activities of the normal tissues showed little variable values ranging from 22.6 pmol/mg protein/min to 180 pmol/mg protein/min. MAPK activities of the tumor tissues were increased, compared with those of the corresponding normal tissues (Fig. 1A). Western blotting analysis using antibody specific for phospho-MAPK revealed that elevated level of the phosphorylation of MAPK was observed in the tumor tissues, compared with that of the corresponding normal tissues (Fig. 1B), suggesting that an increase in immunoreactivity with phospho-MAPK antibody reflects an increase in the activity of MAPK. The amount of MAPK protein of the normal and tumor tissues remained almost constant except for the case of N2/T2 (Fig. 1C), suggesting that elevated MAPK activity is due to the increased phosphorylation of MAPK by its upstream activators, not by its overexpression. It was reported that the constitutive activation of MAPK was accompanied by its overexpression in the human hepatocellular carcinomas (14) and renal cell carcinomas (15). Our results were in contrast with previously reported data by Atten *et al.* in which decreased MAPK activity was associated with human gastric adenocarcinoma (7). Assuming that this difference would be caused by the omission of phosphatase inhibitors in their assay condition for measuring MAPK activity, we examined MAPK activity in the presence and absence of phosphatase inhibitors in our kinase assay system. When MAPK activity was assayed in the presence of β-glycerophosphate, sodium orthovanadate and sodium fluoride, increased MAPK activity was obtained in the tumor tissues, compared with that in the normal tissues. In contrast, when assayed in the absence of these phosphatase inhibitors, MAPK activity was decreased by ≈20% in the tumor tissues (Fig. 2). These results suggest an increase of phosphatase activity or expression in the tumor tissues. Dual specificity protein kinase to phosphorylate MAPK on both tyrosine and threonine residues is required for its activity, and dual specificity protein phosphatase to dephosphorylate MAPK leads to its inactivation. It has been known that MKP-1 is a p42 MAPK-specific phosphatase. Both MAPK and MKP-1 are known to be co-expressed in a wide variety of human epithelial tumor tissues (5). To test whether MKP-1 expression is increased in human gastric adenocarcinomas, we performed Western blotting analysis using a polyclonal anti-MKP-1 antibody after an immunoblot membrane was deprobed. Expression of MKP-1 was increased in the tumor tissues, compared with that in the normal tissues (Fig. 3). This induction of MKP-1 expression correlated well with an increase in MAPK activity. It is important to note that

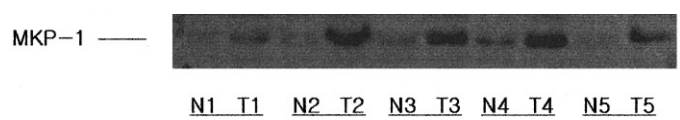

FIG. 3. Western blot analysis with anti-MKP-1 antibody. MKP-1 was detected from the primary normal and tumor gastric tissues after the same immunoblot membrane used in Fig. 1 was reprobed by polyclonal anti-MKP-1 antibody.

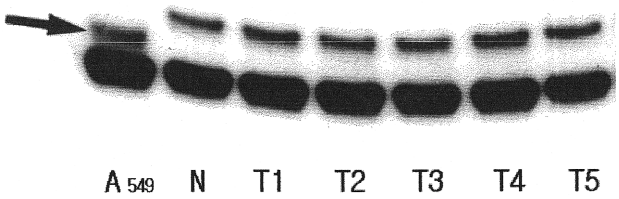

FIG. 4. PCR-SSCP analysis in exon 1 of K-ras gene from the primary normal and tumor gastric tissues. A549, lung carcinoma cell line, serves as a positive control for K-ras gene mutation, and band with a shifted mobility detected in the SSCP analysis is indicated by arrow.

elevated MAPK activity was retained in the tumor tissues, despite of MKP-1 overexpression. This would probably arise from its inability to gain access to the phosphorylated p42 MAPK in intact cells. Thus, expressed MKP-1 appears to be functionally inactivated *in vivo*. On the basis of the fact that MKP-1 is an immediate early gene product and its expression is induced by serum or growth factors in cultured cells, our results suggest that induced MKP-1 expression following increased MAPK activity may be involved in the carcinogenesis of human gastric adenocarcinomas. Signaling pathway leading to the activation of MAPK constitutes the protein kinases cascades, in which Raf (MAPKKK), MEK (MAPKK), and MAPK are sequentially phosphorylated and activated upon p21 ras activation (4). Mutations in 12, 13, or 61 of ras genes convert the normal ras genes into the active oncogenes. This ras gene mutations were found in a variety of human tumor tissues (16). To examine the effect of ras gene mutations on MAPK activity we performed PCR-SSCP assay after purifying genomic DNA from the primary gastric tissues. Neither K-, N-, nor H-ras gene mutation was found in these tumor tissues examined, suggesting that MAPK activity was activated through a ras-independent pathway in primary human gastric adenocarcinoma (Fig. 4, and data not shown).

DISCUSSION

In the present study we showed that an increase in both MAPK activity and MKP-1 expression occurs in human gastric adenocarcinoma. Consistent with other investigators (17), these results support that increased proliferative capacity of cells and tissues is associated with elevated level of MAPK activity. Our results were inconsistent with previously reported data showing that decreased MAPK activity was associated with human gastric adenocarcinoma (7). This difference might be due to the omission of phosphatase inhibitors in their assay condition for measuring MAPK activity. Elevated protein phosphatase activity appears to be linked with the induction of MKP-1 expression, suggesting that signaling pathways leading to the induction of MKP-1 expression is involved in the carcinogenesis of human gastric adenocarcinomas. Importantly, despite of MKP-1 overexpression, increased MAPK activity was detected. These results imply that MKP-1 does not functionally act to dephosphorylate and thereby inactivate MAPK activity *in vivo*, probably due to the different subcellular compartmentation between an active, phosphorylated MAPK in the nucleus and MKP-1 in the cytoplasm. Or MKP-1 would be improperly positioned to target MAPK for dephosphorylating it somehow. Contrast to primary tumor tissues, MKP-1 expression causes an inhibition of DNA synthesis induced by oncogenic ras in rat embryonic fibroblast REF-52 cells (9). A protein complex between MKP-1 and phosphorylated p42 MAPK exists, but its physiological relevance remains to be elucidated. During signal transduction, the differential regulation in the protein kinases cascades appears to be achieved by protein–protein interactions to form a signaling network (18, 19). Signaling pathway of receptor tyrosine kinase stimulation leads to the activation of MAPK via signaling elements including ras, Raf, MEK, etc. Expression of TGF-α and erbB-2 (HER-2/neu) may be involved in regulating MAPK activity in gastric carcinomas. No ras gene mutation was found in primary gastric adenocarcinoma examined here, suggesting that MAPK activity was activated through a ras-independent pathway. Our current interest is to study the changes of MAPK activity and MKP-1 expression, depending on tumor types, stages, grades and differentiations of the primary gastric tissues.

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