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Tumor-specific Activation of Mitogen-activated Protein Kinase in Human Colorectal and Gastric Carcinoma Tissues

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To search for the signaling events in colorectal carcinoma relevant to its tumorigenesis, we investigated the activity of mitogen-activated protein kinase (MAPK) in human colorectal carcinoma tissues and paired normal tissues. Of 64 cases examined, approximately 75% (48 cases) showed tumor-specific activation of MAPK by *in situ* kinase renaturation assay, as well as *in vitro* kinase assay with immunoprecipitated MAPK. In addition, tumor-specific activation of MAPK was associated with the activation of MAPK kinase in the cases we examined. However, no clear correlation of MAPK activation with lymph node involvement, metastatic rate, stage, histological classification, age or sex was observed. These results suggest that the MAPK pathway is involved in colorectal tumor development, but its activation alone is not sufficient for malignant conversion. In contrast to colorectal carcinoma, gastric carcinoma tissues showed a lower rate of MAPK activation, suggesting that the signaling pathway activated in colorectal carcinoma tissues may differ in part from that of gastric carcinoma.

Key words: MAP kinase — Human colorectal carcinoma — Gastric carcinoma — Signal transduction

Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases activated in response to a variety of external signals. Various receptor tyrosine kinases, cytokine receptors, G proteins and oncogene products activate MAPKs[1–3] through phosphorylation by MAPK kinase (MEK).[4–7] Thus, MAPKs are proposed to be a critical integrator of various signaling transduction systems. However, MAPKs appear to elicit opposite effects on cell growth. While MAPKs can stimulate tumorigenic growth in NIH3T3, activation of MAPKs is necessary and sufficient to induce neuronal differentiation in PC12 cells with concomitant arrest of cell growth.[8, 9] Thus, MAPKs appear to play a critical role in signaling, but have opposite effects on cell growth depending on the cellular context.

Tumorigenesis in human is a multistep process.[10, 11] Molecular events that underlie the process that can activate MAPK have been reported and some of these alterations appear to correlate with malignancy of the tumors. For example, point mutation of *ras* has been observed in a wide range of human cancers.[11] Activation of c-Src kinase was found in colorectal carcinoma.[12] Overexpression of *erbB*-2 was observed in a wide range of human cancers including breast,[13] ovarian,[14] gastric,[15] lung[16] and prostate,[17] and was associated with a poor prognosis.[13, 18] Our study showed that activation of tyrosine phosphorylation in lung cancer correlated with a poor prognosis.[19] Recently, we found that the Ras-MAPK pathway in ovarian cancer cells plays a critical role in the activation of matrix metalloproteinase-9 and invasion of the cells.[20, 21] Despite these observations, however, available evidence of the activation of MAPK in human cancer tissues is limited. Indeed, Atten *et al*.[22] reported that MAPK activity in human gastric adenocarcinoma was rather suppressed, though a high incidence of MAPK activation was observed in renal cell carcinomas.[23] Thus, evidence suggesting a role of MAPK in human tumorigenesis is scanty and contradictory compared to the numerous results accumulated through *in vitro* study.

To obtain more clues, we studied the activities of 41- and 43-kDa MAPKs (ERK2 and ERK1, respectively) and the activator, MEK, in surgically resected human colorectal carcinoma, compared with those of paired normal tissues. Here we show that colorectal carcinoma has a high incidence of MAPK activation in a tumor-specific manner, while gastric carcinoma showed a low incidence of activation as previously reported.[22] In addition, we present evidence that MEK of colorectal carcinoma is also activated in a tumor-specific manner.

MATERIALS AND METHODS

Tissues Tissue samples were obtained from surgical specimens of 64 patients diagnosed as colorectal carcinoma cases and 35 patients diagnosed as gastric carcinoma cases at the Nagoya University Hospital. Small amounts of resected tissues were frozen immediately with liquid nitrogen. Tumors were classified according to the

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histological subgroups recommended by the World Health Organization (WHO) and staged by the tumor-nodal involvement-metastasis (TNM) system.

Immunoblotting Tissue lysates were prepared as described previously.[19] Frozen tissue samples were crushed into fine pieces, suspended in a buffer containing 2% sodium dodecyl sulfate (SDS) and 5% mercaptoethanol, and immediately homogenized. Lysates were boiled and stocked at −80°C. Assay of protein concentration, SDS-polyacrylamide gel electrophoresis (PAGE), and immunoblotting were described previously.[24, 25]

***In situ* kinase renaturation assay** Assay of MAPK activity by *in situ* kinase renaturation assay was performed as described previously.[26] Briefly, 50 μg aliquots of tissue extracts were subjected to 10% SDS-PAGE in gel containing myelin basic protein (MBP) (0.5 mg/ml) as a substrate for MAPK. After denaturation and renaturation, the kinase activity *in situ* was measured.

Kinase assay with immunoprecipitated MAPK Preparation of tissue lysates and immunoprecipitation with antibody were described previously.[19, 24] Kinase assay was performed as described previously.[27] Briefly, frozen tissue samples were crushed into fine pieces, suspended in IP buffer (50 m*M* Tris-HCl [pH 7.5], 150 m*M* NaCl, 10 m*M* sodium pyrophosphate, 1 m*M* ethylene glycol bis(2-aminoethyl ether)tetraacetic acid [EGTA], 10 m*M* NaF, 1 m*M* sodium vanadate, 1 m*M* phenylmethanesulfonyl fluoride [PMSF] and 10 μg/ml aprotinin) and clarified by centrifugation. Ten micrograms aliquots of the supernatants were immunoprecipitated with 1 μg of anti-ERK2 antibody (Santa Cruz Biotechnology Inc.) as previously described.[19] Immune complexes were washed with MBP incubation buffer (20 m*M* Tris-HCl [pH 7.5], 50 m*M* 2-glycerophosphate, 5 m*M* ethylenediaminetetraacetic acid [EDTA], 1 m*M* PMSF, 12 m*M* 2-mercaptoethanol, 1 m*M* Na_3VO_4 and 0.1 m*M* Na_2MoO_4), and suspended in kinase buffer (50 m*M* *N*-2-hydroxyethylpiperazine-2-ethanesulfonic acid [HEPES; pH 7.5], 1 m*M* dithiothreitol [DTT], 5 m*M* $MgCl_2$, 5 μg of MBP and 10 μCi of [^{32}P]ATP). Kinase reaction was performed at 30°C for 15 min. After the reaction, immune complexes were boiled and subjected to 12.5% SDS-PAGE followed by autoradiography.

Assay of MEK activity with glutathione *S*-transferase (GST)-ERK2 GST-ERK2 plasmid was kindly supplied by Dr. K. Kaibuchi of Nara Institute of Science and Technology. GST-ERK2 fusion protein was purified from bacteria with glutathione Sepharose 4B as described previously.[28] MEK activity was assayed with GST-ERK2 protein as described previously.[27] Briefly, tissues were lysed in IP buffer with a homogenizer and clarified by centrifugation. MEK activity was determined by adding 10 μg of the supernatant to 30 μl of the kinase buffer containing 10 μCi of [^{32}P]ATP, 10 m*M* HEPES [pH 7.4], 10 m*M* $MgCl_2$, 1 m*M* DTT and 5 μg of GST-ERK2. Reaction was performed at 30°C for 30 min, and samples were subjected to 10% SDS-PAGE followed by autoradiography.

Statistical analysis Non-parametric statistical tests were used to evaluate all of our studies. The χ^2 test was used to assess the relationship between categorical variables and the tumor-specific activation of MAPK to calculate *P*-values. The relationships between ERK1 activity and ERK2 activity, and between *in situ* kinase assay and kinase assay with immunoprecipitated ERK2 were determined by Spearman rank correlation.

RESULTS

MAPK activity in colorectal tumor tissues We first examined MAPK activities in colorectal tumor tissues by *in situ* kinase renaturation assay as described in "Materials and Methods." As shown in Fig. 1, two phosphoproteins of 43 and 41 kDa were identified by this assay. By immunoprecipitation with specific antibodies, we confirmed that the 43- and 41-kDa proteins were ERK1 and ERK2, respectively. Of 64 cases examined, tumor tissues of 42 cases (65.6% of total) showed more than twofold higher activities of either or both of the ERKs than paired normal tissues. In 48 cases (75.0%), tumor tissues had more than 1.5-fold higher activities than paired normal tissues. Of 64 cases of colorectal tumor, 26 cases were

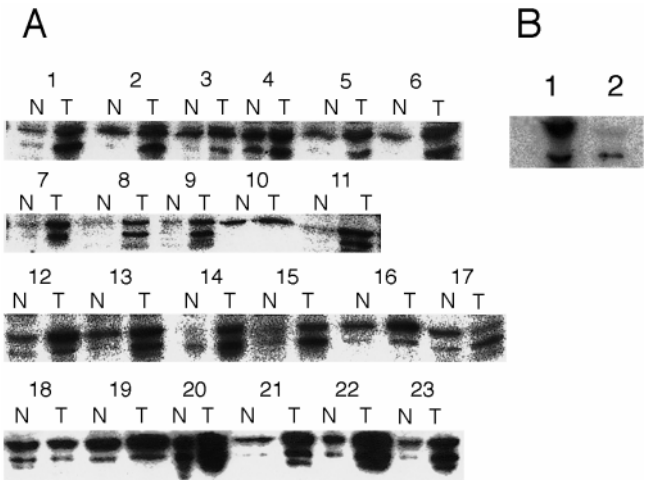


Fig. 1. Assay of MAPK activity in surgical specimens of colorectal carcinoma by *in situ* kinase renaturation assay. (A) MAPK activities in lysates from paired colorectal tumor (T) and normal (N) tissues were analyzed by *in situ* kinase renaturation assay as described in "Materials and Methods." Each number indicates the case number. (B) (1) MAPK activity of case 6 tumor tissue. (2) ERK1 and ERK2 in case 6 tumor tissue were immunoprecipitated and subjected to *in situ* kinase renaturation assay.

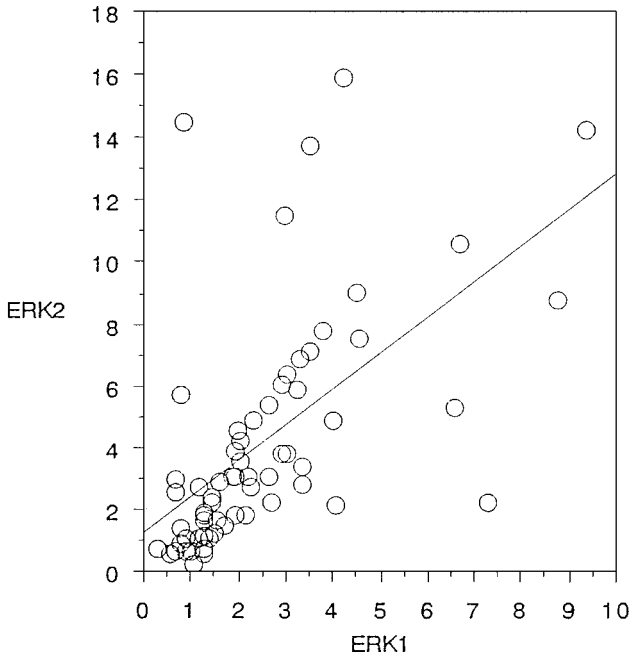


Fig. 2. Relative ratio of ERK1 and ERK2 activities in tumor tissues compared to those in paired normal tissues. Each value represents the relative ratio of activation of ERK1 and ERK2 in tumor tissues compared to those in paired normal tissues.

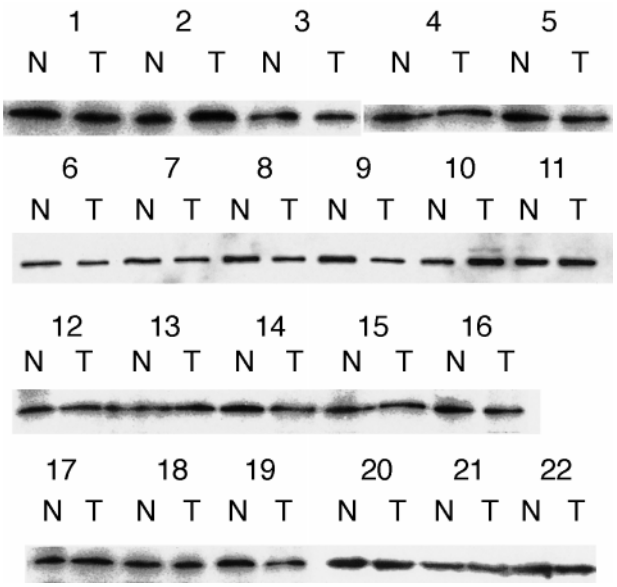
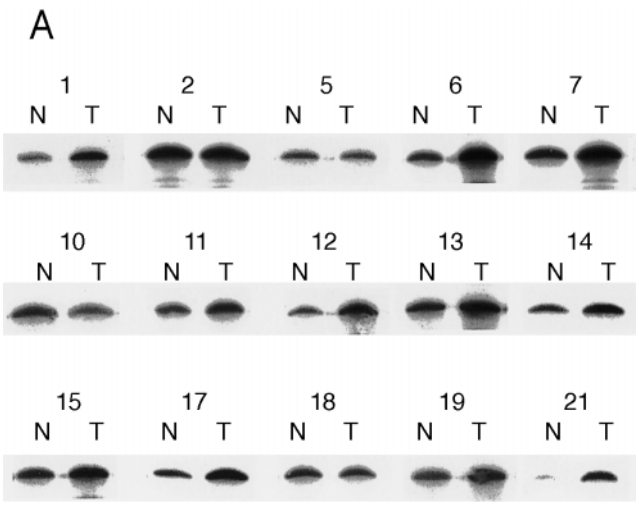


Fig. 3. Detection of ERK2 in tumor tissues. Expression of ERK2 in paired normal (N) and colorectal tumor (T) tissues was examined with anti-ERK2 antibody.



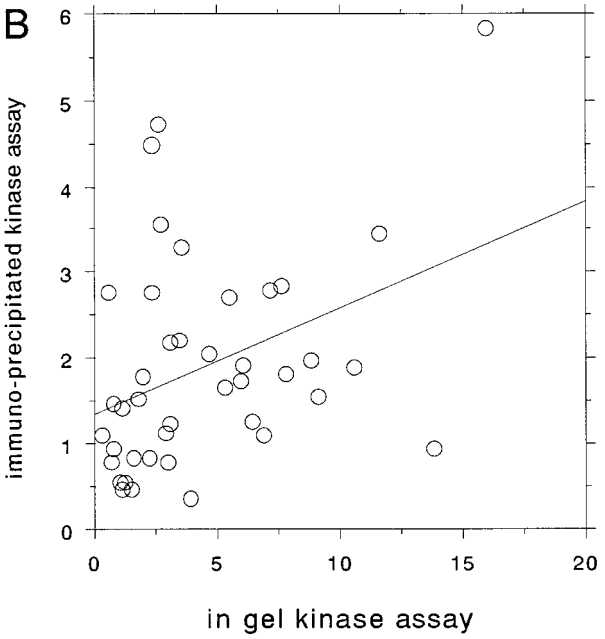


Fig. 4. Assay of ERK2 activities in colorectal tumor tissues with immunoprecipitated ERK2. (A) ERK2 in tissue lysates of paired normal (N) and colorectal tumor (T) tissues was immunoprecipitated with anti-ERK2 antibody and subjected to kinase assay as described in "Materials and Methods." (B) Relative ratios of ERK2 activities examined by *in situ* kinase renaturation assay or kinase assay with immunoprecipitated ERK2. Relative ratios of ERK2 activities in tumor tissues to those in paired normal tissues assayed by *in situ* kinase renaturation assay or kinase assay with immunoprecipitated ERK2 were plotted.

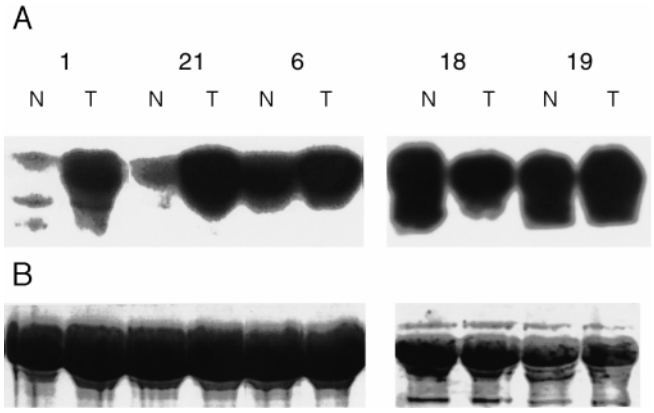


Fig. 5. MEK activities in colorectal carcinoma. MEK activities in paired normal (N) and colorectal carcinoma (T) tissues were assayed with recombinant GST-ERK2 as a substrate as described in "Materials and Methods" (A). Cases which showed tumor-specific activation of MAPK (cases 1, 6 and 21) and no specific activation (cases 18 and 19) were examined. Relative amounts of GST-ERK2 in each reaction were confirmed by Coomassie blue staining (B).

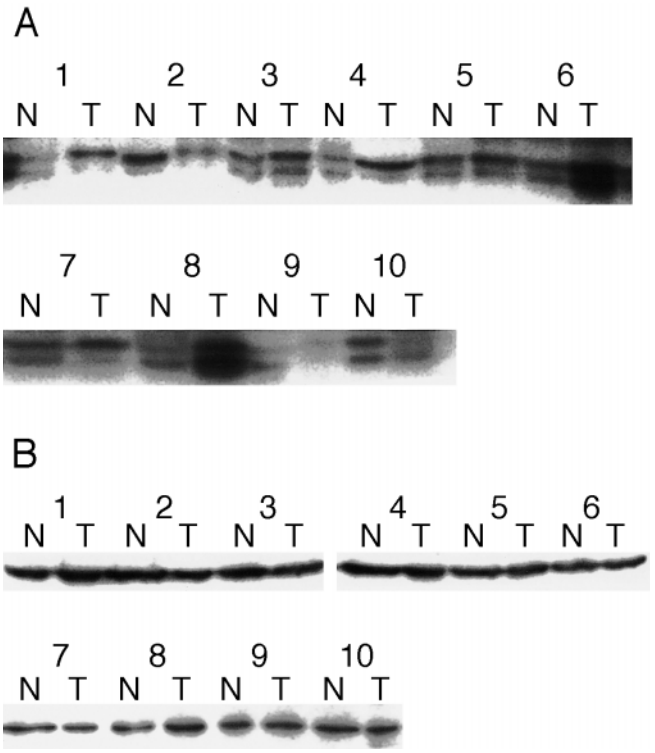


Fig. 6. Expression and activities of MAPK in gastric carcinoma. (A) MAPK activities in lysates from paired normal (N) and gastric tumor (T) tissues were analyzed by *in situ* kinase renaturation assay as described in "Materials and Methods." Each number indicates the case number. (B) Expression of ERK2 in paired normal (N) and gastric tumor (T) tissues was assayed by immunoblotting with anti-ERK2 antibody.

colon tumor, of which 21 cases (80.8%) showed twofold higher activation of MAPK, while 35 cases were rectal tumor, of which 21 cases (55.3%) showed activation. In case No. 22, colon tumor (Fig. 1) with typical tumor-specific activation of MAPK, ERK1 and ERK2 of tumor tissue showed 9.4- and 14.3-fold higher activities than those of paired normal tissues, respectively. We compared the relative rate of activation between ERK1 and ERK2 in cases that showed tumor-specific activation (Fig. 2). Although the relative activation rates of ERK1 and ERK2 differed from case to case, they showed a statistically significant proportional correlation ($P<0.0001$). ERK2 showed 1.2-fold higher activity than ERK1 in general.

We next examined the steady-state levels of ERK2 expression in these tissues by immunoblotting with specific antibody (Fig. 3). We found no detectable difference in ERK2 levels between tumor tissues and paired normal tissues, although the relative amounts of MAPK differed among the cases.

To confirm the tumor-specific activation of MAPK, we next examined ERK2 activity by *in vitro* kinase assay with immunoprecipitated ERK2 and MBP as described in "Materials and Methods" (Fig. 4A). Because of the limited availability of tumor lysates and relatively large requirement of tumor lysates for kinase assay, we compared ERK2 kinase activity in 41 cases both by *in situ* kinase renaturation assay and by *in vitro* kinase assay with immunoprecipitated kinase (Fig. 4B). We found a statistically significant proportional relationship between *in situ* kinase renaturation assay and *in vitro* kinase assay with immunoprecipitated ERK2 ($P=0.0096$), and confirmed the tumor-specific activation of ERK2 by *in vitro* kinase assay in most of the cases that showed activation by *in situ* kinase renaturation assay.

MEK activity in colorectal tumor tissues Since MAPK activity is regulated by MEK, we examined MEK activities with recombinant ERK2 as a substrate (Fig. 5). Although examined cases were restricted because of the limited availability of tumor lysates, we found that tumor tissue showed 3- to 10-fold higher activity of MEK than that of paired normal tissue in cases (case No. 1, 6 and 21) that showed tumor-specific activation. In contrast, activation of MEK was undetectable in cases (case No. 18 and 19) that showed no tumor-specific activation.

MAPK activity in gastric tumor tissues We next examined MAPK activities in gastric tumor tissues by *in situ* kinase renaturation assay (Fig. 6A). In contrast to colorectal tumor, only 6 of 35 cases examined (17.1%) showed more than 2-fold higher activation of MAPK in tumor tissues. There was no clear difference in MAPK

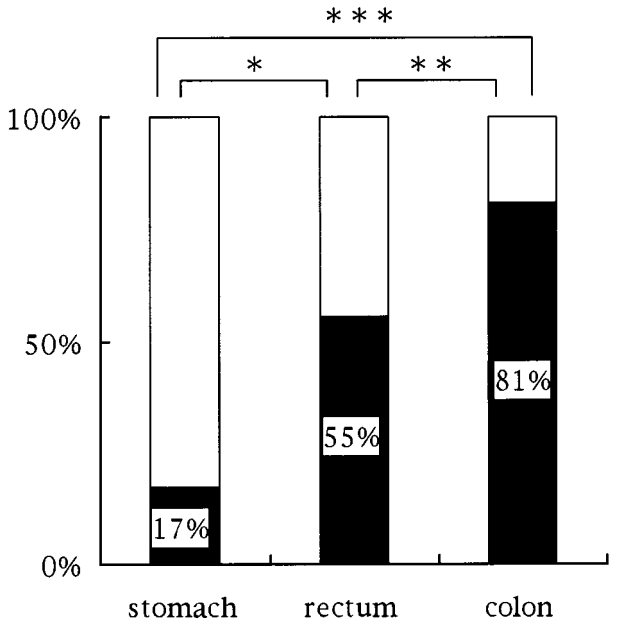


Fig. 7. Ratios of MAPK-active cases in gastric, rectal and colon carcinoma. Black boxes, cases that showed tumor-specific activation of MAPK; open boxes, cases that showed no specific activation of MAPK. □ negative, ■ positive. * P=0.0008, ** P=0.0349, *** P=0.0001.

expression between tumor tissues and paired normal tissues (Fig. 6B). Thus, gastric carcinoma tissues showed a lower rate of tumor-specific MAPK activation than did colorectal carcinoma. We found that the differences in MAPK activation rate among colon, rectum and gastric tumor were statistically significant (Fig. 7).

MAPK activity and clinicopathological manifestations We examined the correlation of relative MAPK activities with clinicopathological manifestations of colorectal carcinoma as summarized in Table I. These tumors consisted of 3 well differentiated and 59 moderately differentiated adenocarcinoma, and 3 mucinous carcinoma, and were obtained from 44 male and 20 female patients with an age range of 22 to 91 years (average 62 years). We found that 42 carcinoma tissues (66%) showed more than twofold higher activation of MAPK compared with paired normal tissues, while 22 cases (34%) showed no specifc activation. Tumor-specific activation of MAPK was not associated with sex (P=0.58), lymph nodal involvement (P=0.52), metastasis rate (P>0.99), stage (P=0.73), histological classification or age (P=0.11).

Table I. Tumor-specific Activation of MAPK and Clinicopathological Variables for Patients with Colorectal Carcinoma

	Negative (n=22)	Positive (n=42)	P-value
Male	14	30	
Female	8	12	0.58
Lymph node metastasis			
0	6	18	
1	8	9	
2	2	3	
3	4	8	0.52
unknown		6	
Metastasis			
0	17	33	
1	5	9	>0.99
Stage			
I	4	12	
II	3	7	
III	9	13	
IV	6	9	0.73
unknown		1	
Histology			
well	1	2	
mod	22	37	
muc	0	3	
Age (years)±SD	64.7±11.2	60.0±10.8	0.11
Location			
colon	5	21	
rectum	17	21	0.03

DISCUSSION

Evidence has been accumulated that MAPK functions as a critical integrator of cell growth and differentiation. In this report, we demonstrate a high frequency of tumor-specific activation of MAPKs in human colorectal carcinoma tissues both by *in situ* kinase renaturation assay and by *in vitro* kinase assay with immunoprecipitated MAPK. Of 64 cases of colorectal carcinoma that we examined, tumor tissues of 42 cases (65.6% of total) showed more than twofold higher activities of ERKs compared with those of paired normal tissues. MAPK activation is a result of the sequential activation of signaling molecules that consist of growth factors and their receptors, proto-oncogene products and kinases. We found that activation of MAPK in colorectal carcinoma tissues was associated with activation of MEK, although the availability of tumor tissues was limited. Our results suggest that constitutive activation of the MEK-MAPK signaling pathway is highly associated with tumorigenesis of colorectal carcinoma.

Several lines of evidence suggest that activation of the tyrosine kinase-Ras signaling pathway activates malignant conversion of tumor cells. Recently, we found[20, 21] that MAPK activation was involved in the activation of matrix metalloproteinase-9 and invasion of ovarian cancer cells.

In contrast to these observations, however, we did not find any clear association of MAPK activation with the clinicopathological manifestations in the cases that we examined, although a high incidence of MAPK activation was observed. These results suggest that the MAPK signaling pathway may have multifarious roles in tumor cells depending on the cellular context. In colorectal carcinoma, activation of the MAPK pathway may be insufficient for malignant conversion, but may be required for the tumorigenic growth of the cells. Our results are consonant with the notion[11] that point mutation of k-*ras* is one of the early genetic changes widely observed in colorectal tumor.

In contrast to colorectal carcinoma, we found that only 6 of 35 cases of gastric carcinoma (17.1%) showed tumor-specific activation of MAPK. Our results are consistent with the previous report that MAPK activity is reduced in gastric adenocarcinoma.[22] It is tempting to argue that the signaling pathway activated in colorectal carcinoma may differ in part in terms of MAPK activation from that of gastric carcinoma. In gastric carcinoma, MAPK might have a more specific role than in colorectal carcinoma. In this report, however, we only examined the subtypes of MAPK, ERK1 and ERK2. In addition to these MAPKs, other types of MAPK as well as MAPK-independent pathways such as Jak-STAT have been identified. The activities of these signaling molecules in gastric carcinoma tissues remain to be examined.

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REFERENCES

1) Nishida, E. and Gotoh, Y. The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends. Biochem. Sci.*, **18**, 128–131 (1993).
2) Blenis, J. Signal transduction via the MAP kinases: proceed at your own RSK. *Proc. Natl. Acad. Sci. USA*, **90**, 5889–5892 (1993).
3) Marshall, C. J. MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr. Opin. Genet. Dev.*, **4**, 82–89 (1994).
4) Dent, P., Haser, W., Haystead, T. A. J., Vincent, L. A., Roberts, T. M. and Sturgill, T. W. Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and *in vitro*. *Science*, **257**, 1404–1407 (1992).
5) Howe, L. R., Leevers, S. J., Gomez, N., Nakielny, S., Cohen, P. and Marshall, C. J. Activation of the MAP kinase pathway by the protein kinase Raf. *Cell*, **71**, 335–342 (1992).
6) Hughes, D. A., Ashworth, A. and Marshall, C. J. Complementation of *byr*1 in fission yeast by mammalian MAP kinase kinase requires coexpression of Raf kinase. *Nature*, **364**, 349–352 (1993).
7) Macdonald, S. G., Crews, C. M., Wu, L., Driller, J., Clark, R., Erikson, R. L. and Mccormick, F. Reconstitution of the Raf-1-MEK-ERK signal transduction pathway *in vitro*. *Mol. Cell. Biol.*, **13**, 6615–6620 (1993).
8) Cowley, S., Paterson, H., Kemp, P. and Marshall, C. J. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell*, **77**, 841–852 (1994).
9) Mansour, S. J., Matten, W. T., Hermann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Vande Woude, G. F. and Ahn, N. G. Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science*, **265**, 966–970 (1994).
10) Foulds, L. The natural history of cancer. *J. Chronic Dis.*, **8**, 2–37 (1958).
11) Fearon, E. R. and Vogelstein, B. A genetic model for colorectal tumorigenesis. *Cell*, **61**, 759–767 (1990).
12) Bolen, J. B., Veillette, A., Schwartz, A. M., Deseau, V. and Rosen, N. Activation of pp60$^{c\text{-}src}$ protein kinase activity in human colon carcinoma. *Proc. Natl. Acad. Sci. USA*, **84**, 2251–2255 (1987).
13) Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A. and Press, M. F. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science*, **244**, 707–712 (1989).
14) Berchuck, A., Kamel, A., Whitaker, R., Kerns, B., Olt, G., Kinney, R., Soper, J. T., Dodge, R., Clarke-Pearson, D. L., Marks, P., Mckenzie, S., Yin, S. and Bast, R. C. J. Overexpression of HER-2/neu is associated with poor survival in advanced epithelial ovarian cancer. *Cancer Res.*, **50**, 4087–4091 (1990).
15) Kameda, T., Yasui, W., Yoshida, K., Tsujino, T., Nakayama, H., Ito, M., Ito, H. and Tahara, E. Expression of ERBB2 in human gastric carcinomas: relationship between p185ERBB2 expression and the gene amplification. *Cancer Res.*, **50**, 8002–8009 (1990).
16) Kern, J. A., Schwartz, D. A., Nordberg, J. E., Weiner, D. B., Greene, M. I., Torney, L. and Robinson, R. A. p185neu expression in human lung adenocarcinomas predicts shortened survival. *Cancer Res.*, **50**, 5184–5187 (1990).
17) Ware, J. L., Maygarden, S. J., Koontz, W. W., Jr. and

Strom, S. C. Immunohistochemical detection of c-erbB-2 protein in human benign and neoplastic prostate. *Hum. Pathol.*, **22**, 254–258 (1991).
18) Seshadri, R., Firgaira, F. A., Horsfall, D. J., Mccaul, K., Setlur, V. and Kitchen, P. Clinical significance of HER-2/neu oncogene amplification in primary breast cancer. *J. Clin. Oncol.*, **11**, 1936–1942 (1993).
19) Nishimura, M., Machida, K., Imaizumi, M., Abe, T., Umeda, T., Takeshima, E., Watanabe, T., Ohnishi, Y., Takagi, K. and Hamaguchi, M. Tyrosine phosphorylation of 100–130 kDa proteins in lung cancer correlates with poor prognosis. *Br. J. Cancer*, **74**, 780–787 (1996).
20) Shibata, K., Kikkawa, F., Nawa, A., Suganuma, N. and Hamaguchi, M. Fibronectin secretion from human peritoneal tissue induces Mr 92,000 type IV collagenase expression and invasion in ovarian cancer cell lines. *Cancer Res.*, **57**, 5416–5420 (1997).
21) Shibata, K., Kikkawa, F., Nawa, A., Thant, A. A., Naruse, K., Mizutani, S. and Hamaguchi, M. Both focal adhesion kinase and c-Ras are required for the enhanced matrix metalloproteinase-9 (MMP-9) secretion by fibronectin in the ovarian cancer cells. *Cancer Res.*, **58**, 900–903 (1998).
22) Atten, M. J., Attar, B. M. and Holian, O. Decreased MAP kinase activity in human gastric adenocarcinoma. *Biochem. Biophys. Res. Commun.*, **212**, 1001–1006 (1995).
23) Oka, H., Chatani, Y., Hoshino, R., Ogawa, O., Kakehi, Y., Terachi, T., Okada, Y., Kawaichi, M., Kohno, M. and Yoshida, O. Constitutive activation of mitogen-activated protein (MAP) kinases in human renal cell carcinoma. *Cancer Res.*, **55**, 4182–4187 (1995).
24) Hamaguchi, M., Grandori, C. and Hanafusa, H. Phosphorylation of cellular proteins in Rous sarcoma virus-infected cells: analysis by use of anti-phosphotyrosine antibodies. *Mol. Cell. Biol.*, **8**, 3035–3042 (1988).
25) Hamaguchi, M., Matsuyoshi, N., Ohnishi, Y., Gotoh, B., Takeichi, M. and Nagai, Y. $p60^{v\text{-}src}$ causes tyrosine phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system. *EMBO J.*, **12**, 559–564 (1993).
26) Hattori, S., Fukuda, M., Yamashita, T., Nakamura, S., Gotoh, Y. and Nishida, E. Activation of mitogen-activated protein kinase and its activator by *ras* in intact cells and in a cell-free system. *J. Biol. Chem.*, **267**, 20346–20351 (1992).
27) Grinstein, S., Butler, J. R., Furuya, W., Lallemain, G. and Downey, G. P. Chemotactic peptides induce phosphorylation and activation of MEK-1 in human neutrophils. *J. Biol. Chem.*, **269**, 19313–19320 (1994).
28) Itoh, T., Kaibuchi, K., Masuda, T., Yamamoto, T., Matsuura, Y., Maeda, A., Shimizu, K. and Takai, Y. A protein factor for *ras* p21-dependent activation of mitogen-activated protein (MAP) kinase through MAP kinase kinase. *Proc. Natl. Acad. Sci. USA*, **90**, 975–979 (1993).