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 MAPKs1–3) 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, ovarian,13) gastric,14) lung15) and prostate,16) 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
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. 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 stored at ~80°C. Assay of protein concentration, SDS-polyacrylamide gel electrophoresis (PAGE), and immunoblotting were described previously.24, 25) Frozen tissue samples were crushed into fine pieces, suspended in a buffer containing 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 10 µg/ml aprotinin) and clarified by centrifugation. MEK activity was determined by adding 50 µg aliquots of tissue extracts 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.

**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 followed by autoradiography. In situ kinase renaturation assay was performed as described previously.24) Kinase assay was performed as described previously.27) Briefly, tissues were lyzed 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 [32P]ATP, 10 mM HEPES [pH 7.4], 10 mM MgCl₂, 1 mM 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 χ² 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. 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.
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 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).

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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 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 activity between normal and tumor tissues.
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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 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 found20, 21) that MAPK activation was involved in the activation of matrix metalloproteinase-9 and invasion of ovarian cancer cells.

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.

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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             | 0.58    |
| Female         | 8              | 12             |         |
| Lymph node metastasis |          |                |         |
| 0              | 6              | 18             |         |
| 1              | 8              | 9              |         |
| 2              | 2              | 3              |         |
| 3              | 4              | 8              | 0.52    |
| unknown        | 0              | 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              |         |
| Location       |                |                |         |
| colon          | 5              | 21             |         |
| rectum         | 17             | 21             | 0.03    |

In 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 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 found20, 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 consistent with the previous report that MAPK activity is reduced in gastric adenocarcinoma. 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. 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 by v-Raf in NIH 3T3 cells and in vitro. Science, 257, 1404–1407 (1992).
5) Howe, L. R., Leevers, S. J., Gomez, N., Nakiely, 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 byrl 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 p60<sup>tyr</sup> by 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. L., 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., Sugaumana, 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) Attan, 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., Kawauchi, M., Kohno, M. and Yoshiida, 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., Matsuoyoshi, N., Ohnishi, Y., Gotoh, B., Takeichi, M. and Nagai, Y. p60c-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 the MEK-1 in human neutrophils. *J. Biol. Chem.*, **269**, 19313–19320 (1994).

28) Itoh, T., Kikuchi, 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).