BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **236,** 54–58 (1997)
ARTICLE NO. RC976840

# Increased MAPK Expression and Activity in Primary Human Hepatocellular Carcinoma

C. Max Schmidt, Iain H. McKillop, Paul A. Cahill, and James V. Sitzmann
*Department of Surgery, Georgetown University Medical Center, Washington, DC 20007*

Received May 26, 1997

**We investigated the expression and activity of mitogen-activated protein kinase (MAPK) in human hepatocellular carcinoma (HCC). MAPK expression was determined in five human tumors and five normal tissues (adjacent non-neoplastic liver) by Western blotting using specific antisera raised against four MAPK pathway intermediates: Erk-1, Erk-2 (extracellular-signal regulated kinases), Mek-1 and Mek-2 (mitogen activated protein kinase kinases). There was a significant increase in Erk-1, Erk-2, Mek-1 and Mek-2 expression in particulate and cytosolic fractions prepared from tumor specimens as compared with the adjacent normal control tissues. The functional activity of both membrane and cytosolic Erk-2, determined by phosphorylation of myelin basic protein (MBP), was significantly increased in tumor specimens as compared to normal (membrane: 321%±50%, $p<0.05$; and cytosol: 597%±233%, $p<0.05$ percent of normal tissue). These data demonstrate for the first time a significant increase in MAPK expression and functional activity in human HCC. Because of the important role that the MAPK pathway plays in cellular growth and differentiation, overexpression of MAPK may be of critical importance to the formation and maintenance of human hepatocellular carcinoma.** 

Hepatocellular carcinoma (HCC) represents the 4th most frequent gastrointestinal cancer in the United States accounting for in excess of 23,000 deaths per year and one of the most common cancers worldwide (1,2). The design of treatment strategies for HCC has been compromised in the past by a poor understanding of its etiology and pathogenesis.

The regulation of cellular mitogenesis and proliferation under normal circumstances is dependent on a number of separate, yet integrated, signaling pathways responsible for the transmission of extracellular signals to the cell nucleus. In common with other tumorigenic cells, HCC is characterized by the normal growth-promoting and growth-arresting components of the signal transduction cascade becoming unbalanced, the net result of which leads to uncontrolled hepatocyte growth (3-4). Mitogen activated protein kinases (MAPKs) are activated in response to external stimuli in numerous cell types (5-8). Activation of the MAPK cascade plays a central role in many signal transduction pathways. MAPK phosphorylates and regulates numerous cellular proteins including growth factor receptors, transcriptional factors, cytoskeletal proteins, phospholipases and other protein kinases (10). In addition they are a convergence point essential to transmitting mitogenic and proliferative signals (9). The MAPK cascade is stimulated through receptor tyrosine kinases and G-protein linked receptors through both ras-dependent and ras-independent pathways (5-10). Activation of MAPK requires phosphorylation of both threonine and tyrosine residues which are catalyzed by a protein kinase known as MAPK kinase or Mek. The final intermediate in the cascade, Erk-2, traverses the nuclear membrane leading to the subsequent activation of immediate early genes through regulation of transcription (10).

Alterations in the expression and activity of intermediates in the MAPK pathway have been recently reported in human tumors (11-12). In studies performed on colon and gastric adenocarcinoma tumors, MAPK activity was significantly decreased (11-12). Presently, there is no information regarding MAPK expression or activity in human HCC. While several studies have documented mitogen stimulated MAPK in normal hepatocytes (13-14), the regulation of MAPK in transformed hepatocytes remains unclear. The aim of our study was to determine the activity and expression of MAPK isoforms in normal and transformed human hepatocytes (HCC).

## METHODS

*Tissue preparation.* Surgically resected specimens were frozen in liquid nitrogen after procurement and then stored at −70°C. They were then placed in ice cold homogenization buffer containing 10 mM Tris HCl and 1 mM EDTA (pH 7.5) and homogenized. Homogenates were centrifuged at 30,000 × g for 30 mins. Cytosol was collected and stored at −70°C. Pellets were resuspended in 10 mM Tris

0006-291X/97 $25.00

HCl containing 1 mM EDTA (pH 7.5), and washed at 30,000×g for 30 minutes at 4°C. The pellet was resuspended in 50 mM Tris HCl (pH 7.4) containing 1 mM EDTA, 0.3 mM PMSF, 1 mg/ml Leupeptin and 0.2 mg/ml benzamidine and stored at −70°C. Protein was measured by the method of Bradford with bovine serum albumin (BSA) as standard (15).

*Western blot analysis.* Particulate or cytosolic proteins (30 $\mu$g) were separated on 12% SDS-polyacrylamide, as described previously (16). Briefly, following SDS-PAGE, separated proteins are electrophoretically transferred to nitrocellulose membranes. Following transfer, membranes are incubated for 1-2hrs in blocking solution, washed, and then incubated with specific MAPK antisera (transduction laboratories) in TTBS. Following the washes, blots are incubated with the secondary antibody solution (horseradish peroxidase conjugated IgG diluted in TTBS). The blots are finally washed in TTBS, then incubated with a mixture of ECL detection solution (Amersham, Arlington Heights, IL) for one minute, covered in plastic wrap and placed in a film cassette to expose to film with an intensifying screen for 5-15 secs. Equal protein loading is confirmed by India ink-staining of protein in each lane of the same blot. The signal intensity (integral volume) of the bands are analyzed using a Personal Densitometer and Imagequant software. Student's t test was used for determination of statistical significance with significant values at $p<0.05$.

*MAPK phosphorylation assay.* The membrane or cytosolic fraction (100$\mu$g, prepared in MAPK buffer containing 12.5mM MOPS,12.5 mM $\beta$-glycerophosphate, 7.5 mM MgCl2, 0.5 mM EGTA, 0.5 mM sodium vanadate, 0.3 mM PMSF, 1 mg/ml Leupeptin and 0.2 mg/ml benzamidine) were incubated with 1$\mu$g of anti-Erk2 (Santa Cruz Labs, Santa Cruz, CA) for 1h at 4°C and then incubated with 30$\mu$l protein G-sepharose beads (Pharmacia Biotech, San Francisco, CA) for 15 minutes on a shaker at 4°C. After centrifugation, the beads and Erk-2 (immunocomplex) were pelleted, washed with PBS containing 1% NP-40, 2 mM vanadate, Tris buffer (Tris 100 mM, 0.5M LiCl) and kinase reaction buffer (12.5 mM MOPS, 12.5 mM $\beta$-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5mM sodium vanadate). They were then resuspended in 30$\mu$l kinase reaction buffer containing 1$\mu$Ci $\gamma$-$^{32}$P-ATP (NEN-Dupont), 20 mM cold ATP, 3.3 mM DTT, and 40 $\mu$g of MBP (Gibco/BRL). After 30 min at 30°C, the reaction was terminated by adding 10$\mu$l of Laemmli sample buffer. Samples were boiled for 5 minutes then run on 15% SDS-PAGE. The phosphorylated substrate (MBP) was identified by autoradiography at 21 kD.

## RESULTS

Liver tissue was obtained from individuals undergoing surgery for primary hepatocellular carcinoma. Demographically, these individuals were all Caucasian with a mean age 65±6 years. The male:female ratio was 4:1 and only one of the individuals had a known history of viral hepatitis. These tumors were all resected by a single surgeon at Georgetown University Medical Center.

Normal (control) and tumor tissue was obtained from the same individual. Normal tissue was removed from the adjacent non-neoplastic liver at least 1 cm beyond the margin for tumor resection and the lack of tumor infiltration was confirmed histologically. Tumor tissue was removed at least 1 cm within the margins for resection and both tumor infiltration and lack of tissue necrosis was confirmed histologically.

MAPK expression was determined in tumor and normal tissue by Western blot analysis using specific anti-

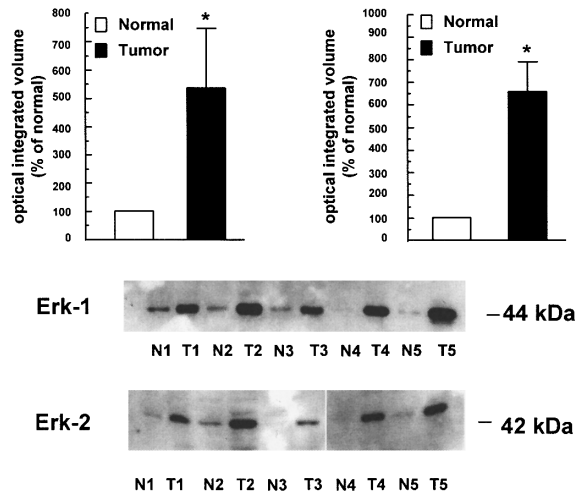


**FIG. 1.** Erk-1 and Erk-2 expression in normal human liver and hepatocellular carcinoma (HCC). Representative Western blots depicting the expression of Erk-1 and Erk-2 in membrane fractions prepared from 5 human tumors and the adjacent non-neoplastic normal livers are shown. Cumulative densitometric analysis was performed on each sample and the mean ± SEM of 5 normals and 5 tumors are presented. (* $p<0.05$).

sera raised against the MAPK pathway intermediates. Anti-Erk-1 and Erk-2 detected proteins of 44 and 42, kDa molecular weight respectively. The analysis was performed in the linear range (10-50 $\mu$g) for detection of these proteins using the specific antisera (data not shown). Differences in Erk-1 and Erk-2 protein expression were quantified by densitometry and expressed as percentage change from normal controls. In both tumor and normal tissue Erk-1 and Erk-2 were detected in both particulate and cytosolic fractions. As shown in Figure 1, there was a significant increase in the expression of Erk-1 and Erk-2 in particulate fractions prepared from tumor tissues as compared to normal (Erk1, 537%±210%, $p<0.05$; Erk2, 656%±133%, $p<0.05$). In a similar manner, cytosolic expression of Erk-1 and Erk-2 was significantly increased in tumor tissues as compared with control (Figure 2, Erk-1, 285%±24%, $p<0.05$ and Erk-2, 313%±107%, $p<0.05$).

Mitogen activated protein kinase kinase (Mek-1 and Mek-2) expression was also examined in membrane and cytosolic fractions. Anti-Mek-1 and Mek-2 detected proteins of 45 and 46 kDa, respectively. The analysis was performed in the linear range (10-50 $\mu$g) for detection of these proteins using the specific antisera (data not shown). There was a significant increase in Mek-1 and Mek-2 expression in membrane fractions prepared from tumor tissues (Figure 3, Mek-1, 418%±156%, $p<0.05$; and Mek-2, 356%±107%, $p<0.05$). In contrast, there was only a significant increase in cytosolic Mek-1 and Mek-2 expression in 40% of tumors examined.

The functional activity of Erk-2 was examined in particulate and cytosolic fractions following immunopre-

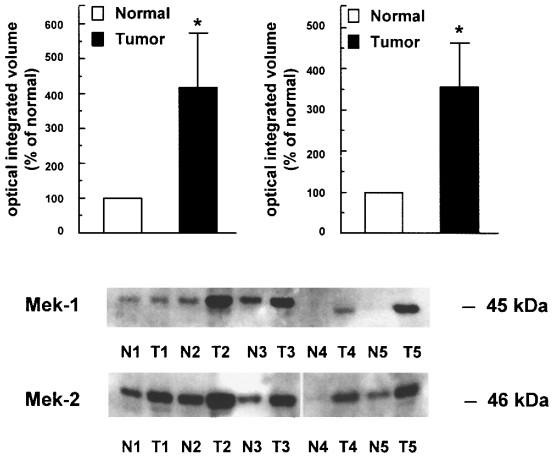


**FIG. 2.** Erk-1 and Erk-2 expression in normal human liver and hepatocellular carcinoma (HCC). Representative Western blots depicting the expression of Erk-1 and Erk-2 in cytosolic fractions prepared from a representative human tumor and the adjacent non-neoplastic normal liver are shown. Cumulative densitometric analysis was performed on each sample and the mean ± SEM of 5 normals and 5 tumors are presented. (* $p<0.05$).

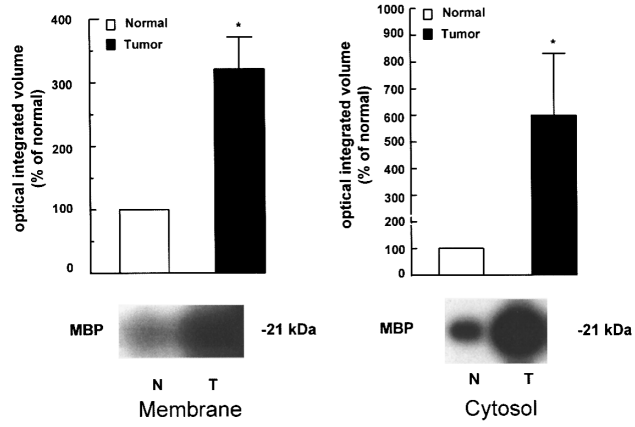


**FIG. 4.** Erk-2 dependent phosphorylation of myelin basic protein (MBP). Erk-2 immunoprecipitates were incubated with MBP before separation on SDS-PAGE as described in Methods. Representative autoradiograph depicting the phosphorylation of Erk-2 substrate (MBP) in membrane and cytosolic fractions prepared from a representative human tumor and its adjacent non-neoplastic normal liver are shown. Cumulative densitometric analysis was performed on each sample and the mean ± SEM of 5 normals and 5 tumors are presented. (* $p<0.05$).

cipitation and determination of Erk-2 mediated phosphorylation of myelin basic protein (MBP). Phosphorylation was linear with respect to protein concentration and maximal after 30 min (data not shown). There was a significant increase in the phosphorylation of MBP in both particulate and cytosolic fractions prepared from tumor tissues as compared to normal (membrane: 321%±50%, $p<0.05$; and cytosol: 597%±233%, $p<0.05$, Figure 4).

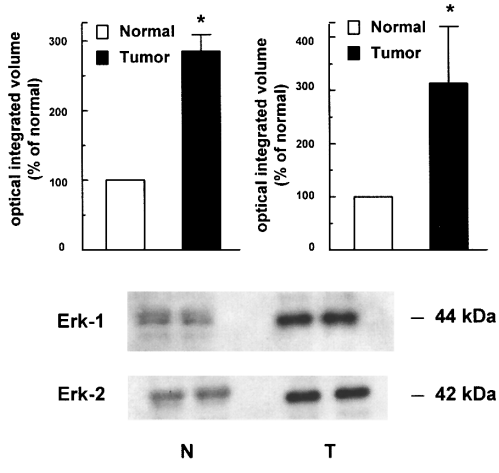


**FIG. 3.** Mek-1 and Mek-2 expression in normal human liver and hepatocellular carcinoma (HCC). Representative Western blots depicting the expression of Mek-1 and Mek-2 in membrane fractions prepared from 5 human tumors and the adjacent non-neoplastic normal livers are shown. Cumulative densitometric analysis was performed on each sample and the mean ± SEM of 5 normals and 5 tumors are presented. (* $p<0.05$).

## DISCUSSION

These data demonstrate for the first time an increase in the expression and activity of intermediates in the MAPK pathway in human primary hepatocellular carcinoma (HCC).

It is now evident that control of cellular growth and transformation is initiated by a mitogen stimulated phosphorylation cascade that regulates gene transcription (10). Since growth factors almost universally activate MAPK pathway in most cell types (5-10), the hypothesis has been advanced that MAPK activation is required for cell growth. In support of such a hypothesis, several studies have shown that mitogen-stimulated cell growth is strongly associated with mitogen-dependent activation of MAPK (17) and translocation of MAPK from the cytoplasm to the nucleus is evident in response to mitogenic stimulation (18). Several genes which are activated during carcinogenesis have also been identified as transformed stimuli that regulate the intracellular MAPK cascade pathway (10, 19, 20). For example the ras proto-oncogene encoded GTP associated protein, p21, regulates mitogenic stimulation of MAPK (21). It is now thought that mutations in the ras gene may represent one of the initial events in colon carcinogenesis that lead to alterations within the MAPK pathway in these tumors (22).

Studies of MAPK activity and expression in human tumor tissues are only now being elucidated. MAPK activity has been shown to be integral to growth factor signaling in hepatocytes (13, 14). In normal hepatocytes, MAPK activity is stimulated by epidermal growth factor (EGF) and hepatocyte growth factor

(HGF), both potent mitogens for hepatocytes (13,14). However, the consequences of hepatocyte transformation on MAPK signaling and subsequent alterations in this signaling pathway in hepatocellular carcinoma have not been investigated. Studies performed on transformed cells from gastric carcinoma and colorectal adenocarcinoma have shown decreased MAPK activity in these tumors as compared to control tissues (11, 12). The authors concluded that the reductions in MAPK expression and activity were indicative of the loss of normal growth control. In contrast, overexpression of receptor tyrosine kinases that activate MAPK has been shown in human gastric tumors (23).

In the present study, we have observed a significant increase in Erk-1 and Erk-2 expression in HCC with a corresponding increase in Erk-2 activity in both membrane and cytosolic fractions. Membrane Mek-1 and Mek-2 were also both significantly overexpressed in tumor. Activation of MAPK intermediates are often followed by their subcellular translocation, and it is the final destination of a particular kinase that determines its physiological effects. Translocation of MAPK from the cytoplasm to the nuclear membrane has been closely associated with cell proliferation (18). In the present study all MAPK intermediates were significantly enhanced in membrane fractions prepared from tumor specimens supporting a role for the MAPK pathway in the growth of primary human hepatocellular carcinoma.

Increased MAPK activity could result from an alteration in the signal transduction systems upstream that modulate their expression and activity. Tyrosine kinases, protein kinase C and ras all contribute to MAPK activation by hepatic mitogens in normal hepatocytes (14, 24). Recent studies have shown that the proto-oncogene ras (p21) is overexpressed in regenerating rat liver, preneoplastic hepatocytes, cirrhotic livers and in HCC (25, 26). Moreover, the c-met proto-oncogene product which is the tyrosine kinase receptor for HGF, is closely associated with regeneration and carcinogenesis of the liver (27). Tyrosine kinase activity is also significantly enhanced following chemical induced carcinogenesis of rat liver (28). Taken together there is ample evidence for enhanced expression of signal transduction systems upstream from MAPK that could modulate the expression and activity of this phosphorylation cascade system in HCC.

Alternatively, modulation of mitogen-activated protein kinase phosphatase (MKP-1) may contribute to the enhanced MAPK activity. In proliferating cells, MKP-1 levels are low concomitant with abundant MAPK expression and activity (29). SV40 small tumor antigen (small t) has also been shown to induce growth in cells by inhibiting protein phosphatases 2A activity and thereby preventing inactivation of Erk-1 and MEk-1 (30). Interestingly a recent study described how an alteration in the balance between MAPK and MKP-1 activities could contribute to the age-related decline in the growth of hepatocytes (31). Future experiments will concentrate on defining the mechanisms responsible for the enhanced MAPK expression and activity in HCC.

In summary these data demonstrate for the first time a significant alteration in MAPK expression and functional activity in human HCC. Because of the important role that the MAPK pathway plays in regulating proliferation in normal hepatocytes, it is possible that this alteration in signal transduction may contribute to the proliferative phenotype characteristic of these tumors.

## REFERENCES


1. Sitzmann, J. V. (1992) *in* Current Therapy in Oncology (Niederhuber, J., Ed.), Dekker, New York.
2. Sitzmann, J. V., and Order, S. (1989) *Surg. Clin. N. Am.* **69,** 393–400.
3. Mays, E. T., Christopherson, W. M., and Mahr, M. M. (1976) *JAMA* **235,** 730–735.
4. Blumberg, B. S., and London, W. T. (1981) *NEJM* **304,** 732–735.
5. Cobb, M. H., Boulton, T. G., and Robbins, D. J. (1991) *Cell. Regul.* **2,** 965–978.
6. Thomas, G. (1992) *Cell* **68,** 3–6.
7. Sturgill, T. W., and Wu, J. (1991) *Biochem Biophys Acta* **1092,** 350–357.
8. Pelech, S. L., Sanghera, J. S. (1992) *TIBS* **17,** 233–238.
9. Lenormand, P., Pages, G., Sardet, C., L'Allemain, G., Meloche, S., and Pouyssegur, J. (1993) *Advances in Second Messenger and Phosphoprotein Research* **28,** 237–244.
10. Guan, K. L. (1994) *Cell Signal.* **6,** 581–589.
11. Atten, M. J., Attar, B. M., and Holian, O. (1995) *Biochem. Biophys. Res. Commun.* **212,** 1001–1006.
12. Attar, B. M., Atten, M. J., and Holian, O. (1996) *Anticancer Research* **16,** 395–400.
13. Gines, P., Li, X., Brown, S. E., Nakamura, K., Guzellan, P. S., Haesley, L. E., Shrier, R. W., and Nemenoff, R. A. (1996) *Hepatilogy* **23,** 1167–11173.
14. Adachi, T., Nakashima, S., Saji, S., Nakamura, T., and Nozawa, Y. (1996) *Hepatology* **23,** 1244–1253.
15. Bradford, M. M. (1976) *Ann. Biochem.* **72,** 248–254.
16. Cahill, P., Wu, Y., and Sitzmann, J. V. (1994) *J. CIin. Invest.* **93,** 2691–2700.
17. Pages, G., Lenormand, P., L'allemain, G., Cahn, J. C., Meloche, S., and Pouyssegur, J. (1993) *PNAS* **90,** 8319–8323.
18. Mii, S., Khalil, R. A., Morgan, K. G., Ware, J. A., and Kent, K. C. (1996) *Am. J. Physiol.* **270,** 39:H142–H150.
19. Howe, L. R., Leevers, J. J., Gomez, N., Nakileny, S., Cohen, P., and Marshall, C. J. (1992) *Cell* **71,** 335–343.
20. Kryiakis, J. M., App, H., Zhang, X., Baneerjee, P., Brautigan, D. L., Rapp, U. R., and Avruch, J. (1992) *Nature* **358,** 417–421.
21. Moodie, S. A., Willumsen, B. M., Weber, M. J., and Wolfman, A. (1993) *Science* **260,** 1658–1661.
22. Wright, P. A., and Williams, G. T. (1993) *Gut* **34,** 145–147.
23. Kiyokawa, E., Takai, S., Tanaka, M., Iwase, T., Suzuki, M., Xiang, Y., Naito, Y., Yamada, K., Sugimura, H., and Kino, I. (1994) *Cancer Res.* **54,** 3645–3650.
24. Arakaki, N., Hirono, S., Kawakami, S., Tsubouchi, H., Ishii, T.,

Hara, H., and Daikuhura, T. (1992) *Biochem. Biophys. Res. Commun.* **185,** 22–28.

25. Annen, K., Nojima, T., Hata, Y., Uchino, J., Tanaka, S., Matsuda, M., Tahara, E., and Nagashima, K. (1996) *Gen. Diagn. Pathol.* **141,** 179–186.
26. Nonomura, A., Ohta, G., Hayashi, M., Izumi, R., Watanabe, K., Takayanagi, N., Mizukami, Y., and Matsubara, F. (1987) *Am. J. Gastroenterol.* **82,** 512–518.
27. Selden, C., Farnaud, S., Ding, S. F., Habib, N., Foster, C., and Hodgson, H. J. (1994) *J Hepatol.* **21,** 227–234.
28. Bombick, D. W., and Matsumura, F. (1987) *Life Sci.* **41,** 429–436.
29. Lai, K., Wang, H., Lee, Ws., Jain, M. K., Lee, M. E., and Haber, E. (1996) *J. Clin. Invest.* **98,** 1560–1567.
30. Sontag, E., Federov, S., Kamibayashi, C., Robbins, D., Cobb, M., and Mumby, M. (1993) *Cell* **75,** 887–897.
31. Liu, Y., Guyton, K. Z., Gorospe, M., Xu, Q., Kokkonen, G. C., Mock, Y. D., Roth, G. S., and Holbrook, N. J. (1996) *J. Biol. Chem.* **271,** 3604–3607.