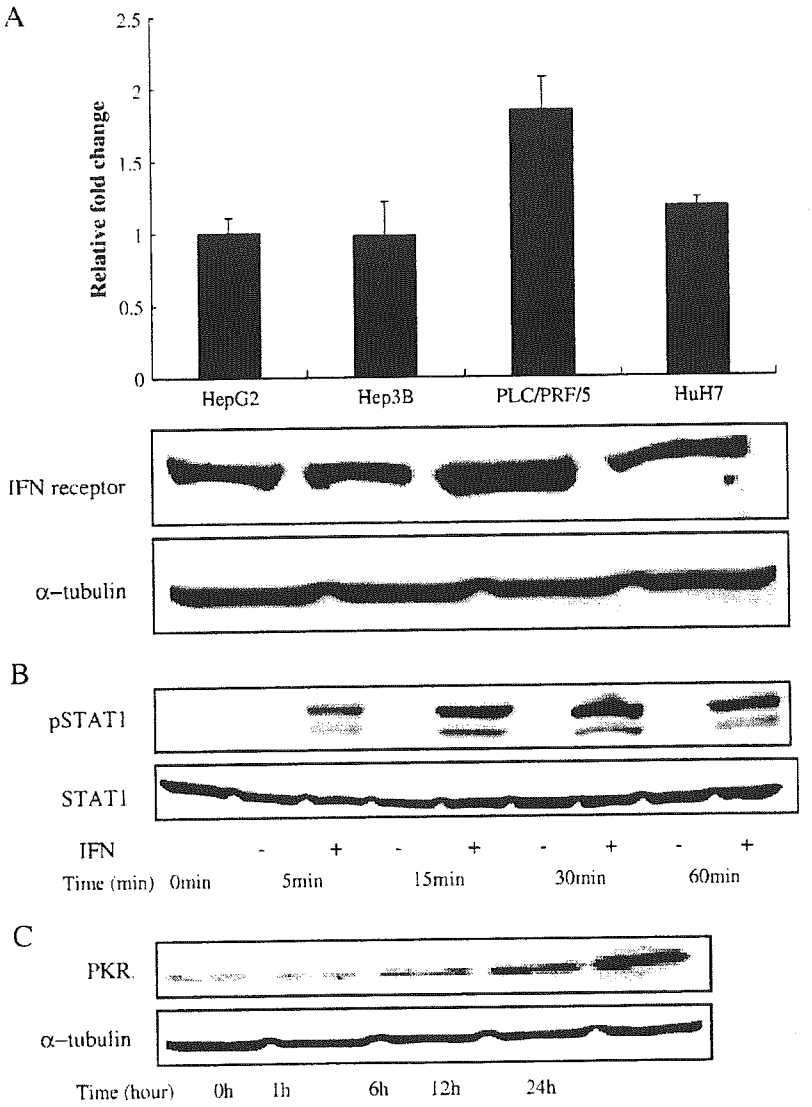

Fig. 3. Expression of the IFN receptor and activation of STAT signaling. (A) The PLC/PRF/5 cell line shows the prominent protein band of the interferon receptor. Equal protein amounts in the loaded samples were confirmed by the Lowry method and α-tubulin. (B) Activation of STAT signaling and (C) PKR gene expression induction by IFN-α. The PLC/PRF/5 cell line was treated with IFN-α and the subsequent STAT1 expression and phosphorylated STAT1 expression were detected with immunoblotting. Although the expression of STAT1 did not change with or without IFN-α, the expression of phosphorylated STAT1 increased 15 min after treatment and the peak intensity occurred at 30 min. Phosphorylated STAT1 had two bands: the upper band was STAT1-α (91 kDa) and the lower band was STAT1-β (84 kDa) (B). The expression of PKR is gradually increased by IFN-α up to 24 h (C).

HCC cells. We explored the molecular mechanisms of the tumor inhibitory potential of IFN-α. Human HCC cell lines HepG2, Hep3B, PLC/PRF/5, and HuH7 showed cell proliferation inhibition by IFN-α, and PLC/PRF/5 and HuH7 had statistically significant differences in the growth inhibitory effect by IFN-α. The cell line PLC/PRF/5, which showed the most abundant expression of IFN receptors, showed the most growth inhibition and rapid inhibition of MAPK signaling and anti-proliferation effect via JAK/STAT. Although we did not examine the affected signaling cascades in all cell lines, IFN-α may show an inhibitory effect on cell proliferation through inhibition of MAPK signaling.

Reduction in the phosphorylation of MEK and ERK by IFN-α in CD4+ T lymphocytes, lymphoblastoid cell lines, and monocytoid cell lines has been shown by several groups [17,18], but there is no report describing the inhibition of the MAPK pathway in HCC cells. Furthermore, IFN-α reduced the phosphorylation of MEK and ERK1/2 within 5 min after IFN-α treatment. Our data show rapid inhibition of MEK and ERK1/2 activation but previous reports using immune cell lines showed at least 2 h was needed to inhibit the signaling of the MAPK pathway [17,18]. This difference may be due to tissue specificity. Knockdown of STAT1 and JAK1 with siRNA diminished the reduction of the phosphorylation of MEK

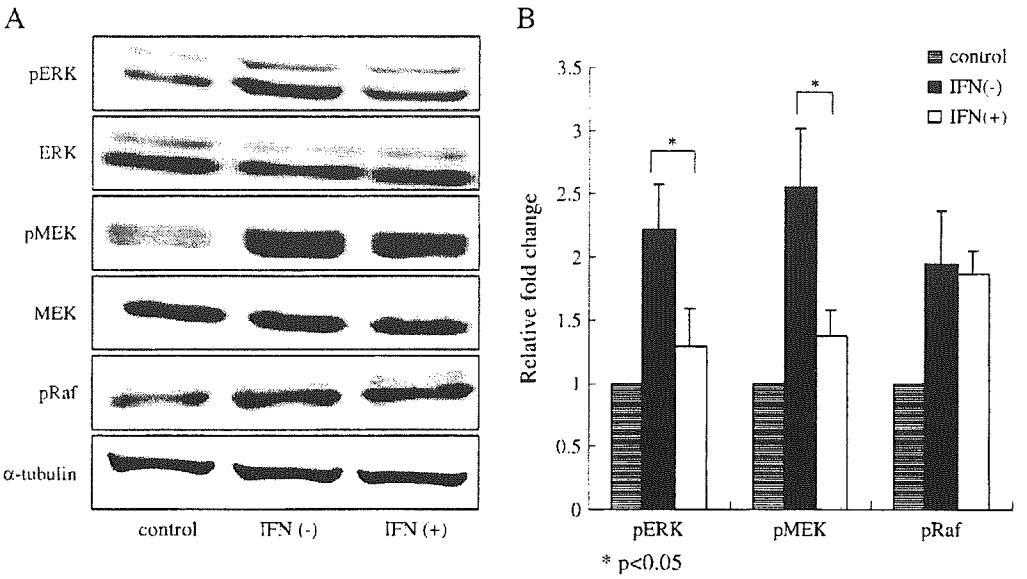

Fig. 4. Rapid inhibition of MEK/ERK phosphorylation but not Raf phosphorylation by IFN-α. (A) Immunoblotting of Raf, MEK, and ERK1/2 and their phosphorylated forms at the indicated time of incubation with [IFN (+)] or without [IFN (−)] IFN-α treatment. Control samples were not treated with IFN-α. Whole-cell lysates were collected 5 min after treatment. The results are expressed as arbitrary relative units of phosphorylation. The mean and S.E. of four trials are shown. (B) Phosphorylation status of ERK, MEK1/2, and Raf in functional time after IFN-α treatment are shown as ratios to the control (0 time). IFN-α reduced the phosphorylation of ERK1/2 (41% reduction), and MEK (47% reduction) at 5 min ($P<0.05$). However, phosphorylation of Raf-1 did not show any difference between cells treated with or without IFN-α.

and ERK1/2 by IFN-α. These results strongly suggest cross-talk between Raf-MAPK signaling and the JAK/STAT pathway because the inhibition of MAPK phosphorylation is too rapid to induce de novo protein synthesis and/or specific gene expression.

We confirmed the signaling pathway from IFN-α receptors and the phosphorylation of STAT1 to target gene expression. Cells deficient of STAT1 suggest that STAT1 has a growth effect in type I IFNs [24] and defects of the STAT pathway are observed in some human tumors [25]. The PLC/PRF/5 cells retained IFN-α-dependent signal transduction, and phosphorylation of STAT1 followed by expression of PKR was observed. The relationship between the high expression level of IFN-α receptor and the growth inhibition in HCC cells supports results found in a previous report [26]. IFN-α treatment prolonged the doubling times of HCC cells and FACS analysis revealed retardation in the G1-S transition by serum stimulation. In some cell lines, IFN-α induces apoptosis with or without apoptotic ligands or genotoxic reagents. In this study, no apoptotic cells were observed by annexin V staining and FACS analysis of IFN-α-treated PLC/PRF/5 cells (data not shown). Neither apoptosis nor cell cycle arrest was seen even at high concentrations of IFN-α (1000 units/ml was the maximum tested).

Genetic alterations, like the loss of the tumor suppressor p53, are implicated in HCC [27]. HepG2 expresses functionally intact p53, while Hep3B expresses no p53 and HuH7 has a mutation of p53 (Y220C). The PLC/PRF/5 cell line also has a mutation of p53 (R249S). Despite the genetic heterogeneity of the four HCC cell lines, IFN-α treatment inhibited proliferation in all cell lines at several concentrations, suggesting that p53 may not be involved in this rapid growth inhibition and cell cycle retardation by IFN-α.

Inhibition of protein phosphorylation or activation of dephosphorylation results in the reduction of phosphorylated MAPK. Phosphorylation plays an important role in the regulation of many diverse cellular processes and a delicate balance between protein kinases and protein phophatases regulates these responses. Protein phosphatase 2A is a family of mammalian serine/threonine phosphatases that accounts for most of the regulation of serine/threonine kinase activity in cells, including the negative regulation of ERK signaling [28]. We examined the activities of protein phosphatases 2A, 2B, and 2C in our experiment, but there was no induction by IFN-α (data not shown).

IFN-α did not reduce Ras/Raf activation at 5 min or other time points. The expression level of Raf-1 was significantly higher in HCC than in liver cirrhosis [29]. Raf-1 is a key upstream protein in the MAPK pathway that affects the cascade of phosphorylation involving other cytoplasmic protein kinases, including MAPK and PKC [30,31].

Although we did not show direct evidence of protein interaction or an affected signaling pathway between MEK and IFN-α activation whereby IFN-α reduced the phosphorylation of MEK and ERK1/2, decreased MEK/ERK activity may contribute to the molecular mechanism of anti-proliferative activity by IFN-α. The ERK1/2 pathways

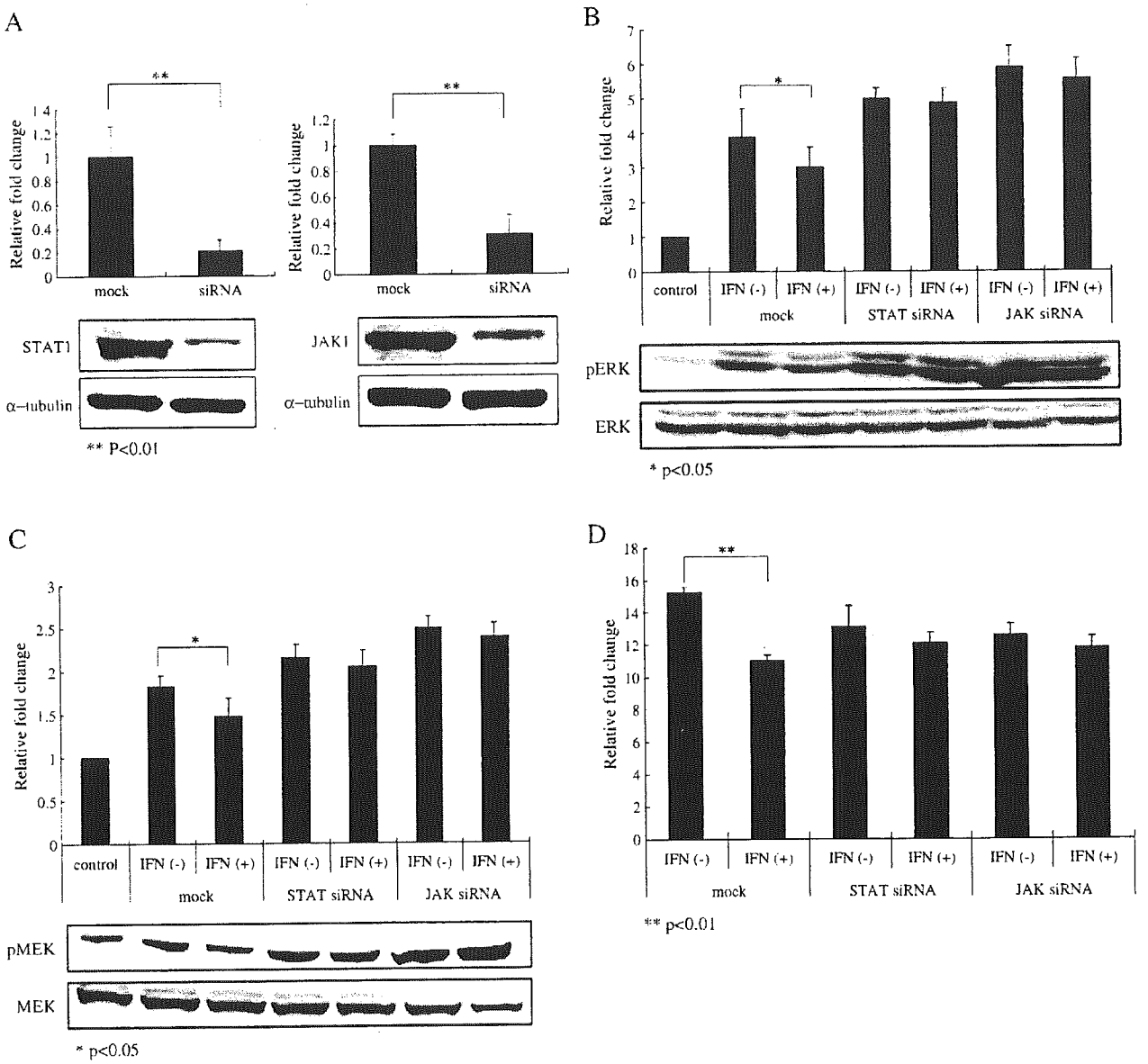

Fig. 5. Knockdown of STAT1 and JAK1 reduces the effect of IFN-α on cell proliferation. (A) Knockdown of STAT1 and JAK1 with siRNA treatment was examined by immunoblotting. (B, C) Phosphorylation of MEK and ERK1/2 and its reduction by suppression of IFN-α occurred with the knockdown of STAT1 and JAK1 siRNA. Relative change means the ratio of phosphorylation of ERK and MEK in the siRNA and IFN-α-treated samples relative to control cells without IFN-α and serum stimulation. (D) Assayed by WST-1, mock-transected cells had 28% reduction at Day 8, which was statistically significant ($P<0.01$), but knockdown of STAT1 and JAK1 diminished the anti-proliferative effect of IFN-α treatment. The mean and S.E. of four trials are shown.

were frequently activated in rat HCC [32] and human HCC [33]. The MAPK pathway is activated in liver cirrhosis and HCC and may be a target for therapy to prevent the progression of HCC. Constitutive administration of IFN-α to patients with hepatitis and liver cirrhosis may have an advantageous pharmacological action for preventing carcinogenesis. Defining the exact target for IFN-α signaling in the reduction of MEK and ERK1/2 activation of cell growth is still necessary and will improve the effectiveness of treating and preventing liver carcinogenesis and improving the prognosis.

Acknowledgement

This work was supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) and for New Energy and Industrial Technology Development Organization (NEDO), Ministry of International Trade and Industry. I thank Dr. Masanao Miwa (University of Tsukuba) for helpful comments and discussion, and Ms. Miu Kimura (MCBI, Inc.) and Ms. Ayako Kabayama (University of Tsukuba) for cell cultures.

References

[1] B. Sangro, M. Herraiz, M.A. Martinez-Gonzalez, I. Bilbao, I. Herrero, O. Beloqui, M. Betes, A. de-la-Pena, J.A. Cienfuegos, J. Quiroga, J. Prieto, Prognosis of hepatocellular carcinoma in relation to treatment: a multivariate analysis of 178 patients from a single European institution, Surgery 124 (1998) 575–583.

[2] S. Tada, H. Saito, S. Tsunematsu, H. Ebinuma, K. Wakabayashi, T. Masuda, H. Ishii, Interferon regulatory factor-1 gene abnormality and loss of growth inhibitory effect of interferon-alpha in human hepatoma cell lines, Int. J. Oncol. 13 (1998) 1207–1216.

[3] R.Y. Shyu, H.L. Su, J.C. Yu, S.Y. Jiang, Direct growth suppressive activity of interferon-alpha and -gamma on human gastric cancer cells, J. Surg. Oncol. 75 (2000) 122–130.

[4] H. Yoshida, Y. Arakawa, M. Sata, S. Nishiguchi, M. Yano, S. Fujiyama, G. Yamada, O. Yokosuka, Y. Shiratori, M. Omata, Interferon therapy prolonged life expectancy among chronic hepatitis C patients, Gastroenterology 123 (2002) 483–491.

[5] K. Ikeda, Y. Arase, S. Saitoh, M. Kobayashi, Y. Suzuki, F. Suzuki, A. Tsubota, K. Chayama, N. Murashima, H. Kumada, Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of the primary tumor-A prospective randomized study of hepatitis C virus-related liver cancer, Hepatology 32 (2000) 228–232.

[6] L. Wang, W.-Z. Wu, H.-C. Sun, X.-F. Wu, L.-X. Qin, Y.-K. Liu, K.-D. Liu, Z.-Y. Tang, Mechanism of interferon alpha on inhibition of metastasis and angiogenesis of hepatocellular carcinoma after curative resection in nude mice, J. Gastrointest. Surg. 7 (2003) 587–594.

[7] T.W. Leung, Y.Z. Patt, W.Y. Lau, S.K. Ho, S.C. Yu, A.T. Chan, T.S. Mok, W. Yeo, C.T. Liew, N.W. Leung, A.M. Tang, P.J. Johnson, Complete pathological remission is possible with systemic combination chemotherapy for inoperable hepatocellular carcinoma, Clin. Cancer Res. 5 (1999) 1676–1681.

[8] S. Kaneko, T. Urabe, K. Kobayashi, Combination chemotherapy for advanced hepatocellular carcinoma complicated by major portal vein thrombosis, Oncology 62 (2002) 69–73.

[9] T.W.T. Leung, A.M.Y. Tang, B. Zee, S.C.H. Yu, P.B.S. Lai, W.Y. Lau, P.J. Johnson, Factors predicting response and survival in 149 patients with unresectable hepatocellular carcinoma treated by combination cisplatin, interferon-alpha, doxorubicin and 5-fluorouracil chemotherapy, Cancer 94 (2002) 421–427.

[10] T. Suou, A. Mitsuda, M. Koda, H. Matsuda, S. Maruyama, H. Tanaka, Y. Kishimoto, M. Kohno, Y. Hirooka, H. Kawasaki, Interferon alpha inhibits intrahepatic recurrence in hepatocellular carcinoma with chronic hepatitis C: a pilot study, Hepatol. Res. 20 (2001) 301–311.

[11] G.R. Klimpel, A.J. Infante, J. Patterson, C.B. Hess, M. Asuncion, Virus-induced interferon alpha/beta (IFN-alpha/beta) production by T cells and by Th1 and Th2 helper T cell clones: a study of the immunoregulatory actions of IFN-gamma versus IFN-alpha/beta on functions of different T cell populations, Cell. Immunol. 128 (1990) 603–618.

[12] J.E. Darnell Jr., I.M. Kerr, G.R. Stark, Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins, Science 264 (1994) 1415–1421.

[13] K. Shuai, G.R. Stark, I.M. Kerr, J.E.J. Darnell, A single phosphotyrosine residue of Stat91 required for gene activation by interferon-gamma, Science 261 (1993) 1744–1746.

[14] E. Meurs, K. Chong, J. Galabru, N.S. Thomas, I.M. Kerr, B.R. Williams, A. Hovanessian, Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon, Cell 62 (1990) 379–390.

[15] A. Takaoka, S. Hayakawa, H. Yanai, D. Stoiber, H. Negishi, H. Kikuchi, S. Sasaki, K. Imai, T. Shibue, K. Honda, T. Taniguchi, Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence, Nature 424 (2003) 516–523.

[16] A.M. Gardner, R.R. Vaillancourt, G.L. Johnson, Activation of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase by G protein and tyrosine kinase oncoproteins, J. Biol. Chem. 268 (1993) 17896–17901.

[17] F. Romerio, A. Riva, D. Zella, Interferon-alpha2b reduces phosphorylation and activity of MEK and ERK through a Ras/Raf-independent mechanism, Br. J. Cancer 83 (2000) 532–538.

[18] F. Romerio, D. Zella, MEK and ERK inhibitors enhance the antiproliferative effect of interferon-alpha2b, FASEB J. 16 (2002) 1680–1682.

[19] A. Moro, S.E. Perea, C. Pantoja, A. Santos, M.D. Arana, M. Serrano, IFNalpha 2b induces apoptosis and proteasome-mediated degradation of p27Kip1 in a human lung cancer cell line, Oncol. Rep. 8 (2001) 425–429.

[20] O. Sangfelt, S. Erickson, J. Castro, T. Heiden, A. Gustafsson, S. Einhorn, D. Grander, Molecular mechanisms underlying interferon-alpha-induced G0/G1 arrest: CKI-mediated regulation of G1 Cdk-complexes and activation of pocket proteins, Oncogene 18 (1999) 2798–2810.

[21] A.G. Hovanessian, The double stranded RNA-activated protein kinase induced by interferon: dsRNA-PK, J. Interferon Res. 9 (1989) 641–647.

[22] L.R. Howe, S.J. Leevers, N. Gomez, S. Nakielny, P. Cohen, C.J. Marshall, Activation of the MAP kinase pathway by the protein kinase Raf, Cell 71 (1992) 335–342.

[23] J.M. Kyriakis, H. App, X.F. Zhang, P. Banerjee, D.L. Brautigan, U.R. Rapp, J. Avruch, Raf-1 activates MAP kinase-kinase, Nature 358 (1992) 417–421.

[24] J.F. Bromberg, C.M. Horvath, Z. Wen, R.D. Schreiber, J.E.J. FAU-Darnell, Transcriptionally active Stat1 is required for the antiproliferative effects of both interferon alpha and interferon gamma, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 7673–7678.

[25] D.H. Kaplan, V. Shankaran, A.S. Dighe, E. Stockert, M. Aguet, L.J. Old, R.D. Schreiber, Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 7556–7561.

[26] H. Yano, A. Iemura, M. Haramaki, S. Ogasawara, A. Takayama, J. Akiba, M. Jojiro, Interferon alfa receptor expression and growth inhibition by interferon alfa in human liver cancer cell lines, Hepatology 29 (1999) 1708–1717.

[27] L.Q. Jia, M. Osada, C. shioka, M. Gamo, S. Ikawa, T. Suzuki, H. Shimodaira, T. Niitani, T. Kudo, M. Akiyama, N. Kimura, M. Matsuo, H. Mizusawa, N. Tanaka, H. Koyama, M. Namba, R. Kanamaru, T. Kuroki, Screening the p53 status of human cell lines using a yeast functional assay, Mol. Carcinog. 19 (1997) 243–253.

[28] L.-G. Yu, L.C. Packman, M. Weldon, J. Hamlett, J.M. Rhodes, Protein phosphatase 2A, a negative regulator of the ERK signaling pathway, is activated by tyrosine phosphorylation of putative HLA class II-associated protein I (PHAPI)/pp32 in response to the antiproliferative lectin, jacalin, J. Biol. Chem. 279 (2004) 41377–41383.

[29] Y.H. Hwang, J.Y. Choi, S. Kim, E.S. Chung, T. Kim, S.S. Koh, B. Lee, S.H. Bae, J. Kim, Y.M. Park, Over-expression of c-raf-1 proto-oncogene in liver cirrhosis and hepatocellular carcinoma, Hepatol. Res. 29 (2004) 113–121.

[30] L.C. Cantley, K.R. Auger, C. Carpenter, B. Duckworth, A. Graziani, R. Kapeller, S. Soltoff, Oncogenes and signal transduction, Cell 64 (1991) 281–302.

[31] S.L. Pelech, J.S. Sanghera, MAP kinases: charting the regulatory pathways, Science 257 (1992) 1355–1356.

[32] I.H. McKillop, C.M. Schmidt, P.A. Cahill, J.V. Sitzmann, Altered expression of mitogen-activated protein kinases in a rat model of experimental hepatocellular carcinoma, Hepatology 26 (1998) 1484–1491.

[33] Y. Ito, Y. Sasaki, M. Horimoto, S. Wada, Y. Tanaka, A. Kasahara, T. Ueki, T. Hirano, H. Yamamoto, J. Fujimoto, E. Okamoto, N. Hayashi, M. Hori, Activation of mitogen-activated protein kinases/extracellular signal-regulated kinases in human hepatocellular carcinoma, Hepatology 27 (1998) 951–958.