in tumour cells, resulting in the expression of repressed genes that cause growth arrest, terminal differentiation and apoptosis.[40] The expression of HDAC1 is associated with prognosis of various carcinomas. BCOR is BCL6 co-receptor and is regulated by p53 and its characteristic expression is reported in various cancer cells.[41] This gene contributes to carcinogenesis in various malignancies such as B cell lymphoma and breast cancer.[42] BIRC5 is one of the major apoptosis regulators and is reported to be a prognostic marker of urothelial carcinomas and breast cancer.[43,44] These genes may serve as diagnostic markers for the development of HCC and help in the resolution of molecular mechanism of recurrence of HCC. To gain biological insights from these informative gene sets, we also used network analysis using Ingenuity Pathway Analysis. This analysis revealed that a few canonical signalling pathways, P38 MAPK signalling and PPARa/RXRa Activation signalling that are reported to be related to metastasis in human cancer,[45,46] harboured many of the upregulated informative genes (Supplementary Figure 1).

Our group has investigated the prediction of recurrence of various malignancies by gene expression profiling.[12,13,15,20,33,47] Kurokawa and colleagues[20] also reported a prediction model for HCC recurrence using a small-scale PCR-array system. They reported that early recurrence (within 2 year) in HCC patients could be predicted using 20-gene set after comparing cases with recurrence within 2 years and cases without recurrence over 2 years from 3072 primers.[20] In our study, cases with early intrahepatic recurrence within 2 years and reference cases without recurrence over 3 years during the training phase were defined based on DFS time of the characteristic recurrence patterns.[24] Furthermore, cases with common clinical background were analysed during the training phase using more strict criteria than previously reported using whole gene analysis, and accordingly, our results should be more reliable.

The report of Iizuka and colleagues[21] showed a correlation between gene expression, using a predictive system consisting of 12 genes, with early (within 1 year) post-hepatectomy intrahepatic recurrence, with a prediction accuracy of 89.3%. In their study, the DFS time of the reference group was more than one year and it is possible that characteristic recurrence patterns coexisted in the reference group. The study of Ho and colleagues[22] identified a molecular signature associated with vascular invasion (VI) in HCC and concluded that the signature could serve as a surrogate marker for predicting early recurrence after surgical resection.[22] Conventional prognostic indicators for early intrahepatic recurrence are not limited to vascular invasion only. A more direct approach should be considered for the prediction of early intrahepatic recurrence. While we did not analyse the reasons for the discrepancy in the prediction genes among the reported studies, we suspect that differences in clinical end-point may affect the results of the analysis and that accumulation of subtle differences in the dynamic range due to the platform of array might influence selection of the prediction genes.

In conclusion, the results of the present study showed that a characteristic gene expression pattern for early intrahepatic recurrence is encoded in primary HCC tumour and that gene profiling can be potentially helpful in predicting the prognosis of patients. Prediction of early recurrence of HCC may allow tailored treatment of individual patients and improvement of prognosis.

Conflicts of interest statement

None declared.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2008.12.019.

REFERENCES

1. Yang Y, Nagano H, Ota H, Morimoto O, Nakamura M, Wada H, et al. Patterns and clinicopathologic features of extrahepatic recurrence of hepatocellular carcinoma after curative resection. *Surgery* 2007;**141**:196–202.
2. Tung-Ping Poon R, Fan ST, Wong J. Risk factors, prevention, and management of postoperative recurrence after resection of hepatocellular carcinoma. *Ann Surg* 2000;**232**:10–24.
3. Taura K, Ikai I, Hatano E, Fujii H, Uyama N, Shimahara Y. Implication of frequent local ablation therapy for intrahepatic recurrence in prolonged survival of patients with hepatocellular carcinoma undergoing hepatic resection: an analysis of 610 patients over 16 years old. *Ann Surg* 2006;**244**:265–73.
4. Park JH, Koh KC, Choi MS, Lee JH, Yoo BC, Paik SW, et al. Analysis of risk factors associated with early multinodular recurrences after hepatic resection for hepatocellular carcinoma. *Am J Surg* 2006;**192**:29–33.
5. Kosuge T, Makuuchi M, Takayama T, Yamamoto J, Shimada K, Yamasaki S. Long-term results after resection of hepatocellular carcinoma: experience of 480 cases. *Hepatogastroenterology* 1993;**40**:328–32.
6. Arii S, Tanaka J, Yamazoe Y, Minematsu S, Morino T, Fujita K, et al. Predictive factors for intrahepatic recurrence of hepatocellular carcinoma after partial hepatectomy. *Cancer* 1992;**69**:913–9.
7. Di Carlo V, Ferrari G, Castoldi R, Nadalin S, Marenghi C, Molteni B, et al. Surgical treatment and prognostic variables of hepatocellular carcinoma in 122 cirrhotics. *Hepatogastroenterology* 1995;**42**:222–9.
8. Armengol C, Boix L, Bachs O, Sole M, Fuster J, Sala M, et al. P27(Kip1) is an independent predictor of recurrence after surgical resection in patients with small hepatocellular carcinoma. *J Hepatol* 2003;**38**:591–7.
9. Kobayashi T, Sugawara Y, Shi YZ, Makuuchi M. Telomerase expression and p53 status in hepatocellular carcinoma. *Am J Gastroenterol* 2002;**97**:3166–71.
10. Kondo K, Chijiiwa K, Makino I, Kai M, Maehara N, Ohuchida J, et al. Risk factors for early death after liver resection in patients with solitary hepatocellular carcinoma. *J Hepatobiliary Pancreat Surg* 2005;**12**:399–404.
11. Arango D, Laiho P, Kokko A, Alhopuro P, Sammalkorpi H, Salovaara R, et al. Gene-expression profiling predicts recurrence in Dukes' C colorectal cancer. *Gastroenterology* 2005;**129**:874–84.
12. Takemasa I, Higuchi H, Yamamoto H, Sekimoto M, Tomita N, Nakamori S, et al. Construction of preferential cDNA microarray specialized for human colorectal carcinoma: molecular sketch of colorectal cancer. *Biochem Biophys Res Commun* 2001;**285**:1244–9.

13. Motoori M, Takemasa I, Yano M, Saito S, Miyata H, Takiguchi S, et al. Prediction of recurrence in advanced gastric cancer patients after curative resection by gene expression profiling. *Int J Cancer* 2005;**114**:963–8.
14. Hannemann J, Oosterkamp HM, Bosch CA, Velds A, Wessels LF, Loo C, et al. Changes in gene expression associated with response to neoadjuvant chemotherapy in breast cancer. *J Clin Oncol* 2005;**23**:3331–42.
15. Kurokawa Y, Matoba R, Nagano H, Sakon M, Takemasa I, Nakamori S, et al. Molecular prediction of response to 5-fluorouracil and interferon-alpha combination chemotherapy in advanced hepatocellular carcinoma. *Clin Cancer Res* 2004;**10**:6029–38.
16. Okabe H, Satoh S, Kato T, Kitahara O, Yanagawa R, Yamaoka Y, et al. Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. *Cancer Res* 2001;**61**:2129–37.
17. Honda M, Kaneko S, Kawai H, Shirota Y, Kobayashi K. Differential gene expression between chronic hepatitis B and C hepatic lesion. *Gastroenterology* 2001;**120**:955–66.
18. Chen X, Cheung ST, So S, Fan ST, Barry C, Higgins J, et al. Gene expression patterns in human liver cancers. *Mol Biol Cell* 2002;**13**:1929–39.
19. Cheung ST, Chen X, Guan XY, Wong SY, Tai LS, Ng IO, et al. Identify metastasis-associated genes in hepatocellular carcinoma through clonality delineation for multinodular tumor. *Cancer Res* 2002;**62**:4711–21.
20. Kurokawa Y, Matoba R, Takemasa I, Nagano H, Dono K, Nakamori S, et al. Molecular-based prediction of early recurrence in hepatocellular carcinoma. *J Hepatol* 2004;**41**:284–91.
21. Iizuka N, Oka M, Yamada-Okabe H, Nishida M, Maeda Y, Mori N, et al. Oligonucleotide microarray for prediction of early intrahepatic recurrence of hepatocellular carcinoma after curative resection. *Lancet* 2003;**361**:923–9.
22. Ho MC, Lin JJ, Chen CN, Chen CC, Lee H, Yang CY, et al. A gene expression profile for vascular invasion can predict the recurrence after resection of hepatocellular carcinoma: a microarray approach. *Ann Surg Oncol* 2006;**13**:1474–84.
23. Dupuy A, Simon RM. Critical review of published microarray studies for cancer outcome and guidelines on statistical analysis and reporting. *J Natl Cancer Inst* 2007;**99**:147–57.
24. Sakon M, Umeshita K, Nagano H, Eguchi H, Kishimoto S, Miyamoto A, et al. Clinical significance of hepatic resection in hepatocellular carcinoma: analysis by disease-free survival curves. *Arch Surg* 2000;**135**:1456–9.
25. Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science* 1999;**286**:531–7.
26. Ueno H, Mochizuki H, Hatsuse K, Hase K, Yamamoto T. Indicators for treatment strategies of colorectal liver metastases. *Ann Surg* 2000;**231**:59–66.
27. Kemeny N, Huang Y, Cohen AM, Shi W, Conti JA, Brennan MF, et al. Hepatic arterial infusion of chemotherapy after resection of hepatic metastases from colorectal cancer. *N Engl J Med* 1999;**341**:2039–48.
28. Morimoto O, Nagano H, Sakon M, Fujiwara Y, Yamada T, Nakagawa H, et al. Diagnosis of intrahepatic metastasis and multicentric carcinogenesis by microsatellite loss of heterozygosity in patients with multiple and recurrent hepatocellular carcinomas. *J Hepatol* 2003;**39**:215–21.
29. Kumada T, Nakano S, Takeda I, Sugiyama K, Osada T, Kiriyama S, et al. Patterns of recurrence after initial treatment in patients with small hepatocellular carcinoma. *Hepatology* 1997;**25**:87–92.
30. Ramaswamy S, Ross KN, Lander ES, Golub TR. A molecular signature of metastasis in primary solid tumors. *Nat Genet* 2003;**33**:49–54.
31. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. *Nature* 2000;**406**:747–52.
32. van't Veer LJ, Dai H, Van de Vijver MJ, He YD, Hart AA, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002;**415**:530–6.
33. Yamasaki M, Takemasa I, Komori T, Watanabe S, Sekimoto M, Doki Y, et al. The gene expression profile represents the molecular nature of liver metastasis in colorectal cancer. *Int J Oncol* 2007;**30**:129–38.
34. Hoshida Y, Villanueva A, Kobayashi M, Peix J, Chiang DY, Camargo A, et al. Gene expression in fixed tissues and outcome in hepatocellular carcinoma. *N Engl J Med* 2008;**359**:1995–2004.
35. Frade R, Balbo M, Barel M. RB18A regulates p53-dependent apoptosis. *Oncogene* 2002;**21**:861–6.
36. Thiagalingam A, De Bustros A, Borges M, Jasti R, Compton D, Diamond L, et al. RREB-1, a novel zinc finger protein, is involved in the differentiation response to Ras in human medullary thyroid carcinomas. *Mol Cell Biol* 1996;**16**:5335–45.
37. Qin LX, Tang ZY. The prognostic molecular markers in hepatocellular carcinoma. *World J Gastroenterol* 2002;**8**:385–92.
38. Ikeguchi M, Hirooka Y, Kaibara N. Quantitative analysis of apoptosis-related gene expression in hepatocellular carcinoma. *Cancer* 2002;**95**:1938–45.
39. Farinati F, Marino D, De Giorgio M, Baldan A, Cantarini M, Cursaro C, et al. Diagnostic and prognostic role of alpha-fetoprotein in hepatocellular carcinoma: both or neither? *Am J Gastroenterol* 2006;**101**:524–32.
40. Pathil A, Armeanu S, Venturelli S, Mascagni P, Weiss TS, Gregor M, et al. HDAC inhibitor treatment of hepatoma cells induces both TRAIL-independent apoptosis and restoration of sensitivity to TRAIL. *Hepatology* 2006;**43**:425–34.
41. Huynh KD, Fischle W, Verdin E, Bardwell VJ. BCoR, a novel corepressor involved in BCL-6 repression. *Genes Dev* 2000;**14**:1810–23.
42. Phan RT, Dalla-Favera R. The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. *Nature* 2004;**432**:635–9.
43. Yin W, Chen N, Zhang Y, Zeng H, Chen X, He Y, et al. Survivin nuclear labeling index: a superior biomarker in superficial urothelial carcinoma of human urinary bladder. *Mod Pathol* 2006;**19**:1487–97.
44. Span PN, Tjan-Heijnen VC, Heuvel JJ, de Kok JB, Foekens JA, Sweep FC. Do the survivin (BIRC5) splice variants modulate or add to the prognostic value of total survivin in breast cancer? *Clin Chem* 2006;**52**:1693–700.
45. Hsieh YH, Wu TT, Huang CY, Hsieh YS, Hwang JM, Liu JY. P38 mitogen-activated protein kinase pathway is involved in protein kinase Calpha-regulated invasion in human hepatocellular carcinoma cells. *Cancer Res* 2007;**67**:4320–7.
46. Knapp P, Jarzabek K, Blachnio A, Zbroch T. The role of peroxisome proliferator-activated receptors (PPAR) in carcinogenesis. *Ginekol Pol* 2006;**77**:643–51.
47. Motoori M, Takemasa I, Doki Y, Saito S, Miyata H, Takiguchi S, et al. Prediction of peritoneal metastasis in advanced gastric cancer by gene expression profiling of the primary site. *Eur J Cancer* 2006;**42**:1897–903.

British Journal of Cancer (2009) 100, 1647–1658

www.bjcancer.com

Activation of Wnt/β-catenin signalling pathway induces chemoresistance to interferon-α/5-fluorouracil combination therapy for hepatocellular carcinoma

T Noda[1], H Nagano*[,1], I Takemasa[1], S Yoshioka[1], M Murakami[1], H Wada[1], S Kobayashi[1], S Marubashi[1], Y Takeda[1], K Dono[1], K Umeshita[2], N Matsuura[3], K Matsubara[4], Y Doki[1], M Mori[1] and M Monden[1]

[1]Department of Surgery, Graduate School of Medicine and Health Science, Osaka University, Osaka, Japan; [2]Department of Health Science, Graduate School of Medicine and Health Science, Osaka University, Osaka, Japan; [3]Department of Molecular Pathology, Graduate School of Medicine and Health Science, Osaka University, Osaka, Japan; [4]DNA Chip Research Inc., Kanagawa, Japan

Type I IFN receptor type 2 (IFNAR2) expression correlates significantly with clinical response to interferon (IFN)-α/5-fluorouracil (5-FU) combination therapy for hepatocellular carcinoma (HCC). However, some IFNAR2-positive patients show no response to the therapy. This result suggests the possibility of other factors, which would be responsible for resistance to IFN-α/5-FU therapy. The aim of this study was to examine the mechanism of anti-proliferative effects of IFN-α/5-FU therapy and search for a biological marker of chemoresistance to such therapy. Gene expression profiling and molecular network analysis were used in the analysis of non-responders and responders with IFNAR2-positive HCC. The Wnt/β-catenin signalling pathway contributed to resistance to IFN-α/5-FU therapy. Immunohistochemical analysis showed positive epithelial cell adhesion molecule (Ep-CAM) expression, the target molecule of Wnt/β-catenin signalling, only in non-responders. *In vitro* studies showed that activation of Wnt/β-catenin signalling by glycogen synthesis kinase-3 inhibitor (6-bromoindirubin-3′-oxime (BIO)) induced chemoresistance to IFN-α/5-FU. BrdU-based cell proliferation ELISA and cell cycle analysis showed that concurrent addition of BIO and IFN-α/5-FU significantly to hepatoma cell cultures reduced the inhibitory effects of the latter two on DNA synthesis and accumulation of cells in the S-phase. The results indicate that activation of Wnt/β-catenin signalling pathway induces chemoresistance to IFN-α/5-FU therapy and suggest that Ep-CAM is a potentially useful marker for resistance to such therapy, especially in IFNAR2-positive cases.
British Journal of Cancer (2009) **100,** 1647–1658. doi:10.1038/sj.bjc.6605064 www.bjcancer.com
Published online 28 April 2009

Keywords: hepatocellular carcinoma; combination therapy; interferon-α; 5-fluorouracil; chemoresistance; Wnt signalling

Interferon (IFN) is a regulatory cytokine with various cellular activities, such as anti-proliferative, immunomodulatory and anti-angiogenic activities (Baron and Dianzani, 1994; Gutterman, 1994). Several studies emphasised the strong anti-tumour activity of IFN in hepatocellular carcinoma (HCC), when used in combination with other chemotherapeutic agents (Patt *et al*, 1993; Obi *et al*, 2006). We also reported the clinical efficacy of IFN-α/5-fluorouracil (5-FU) combination therapy for advanced HCC (Miyamoto *et al*, 2000; Sakon *et al*, 2002; Ota *et al*, 2005; Nagano *et al*, 2007a, b) and the mechanism of its anti-tumour effects (Eguchi *et al*, 2000; Yamamoto *et al*, 2004; Kondo *et al*, 2005; Nakamura *et al*, 2007; Wada *et al*, 2007). Further studies showed that the expression of IFN receptor type 2 (IFNAR2) in HCC tissue samples correlates significantly with clinical response to IFN-α/5-FU combination therapy (Ota *et al*, 2005; Nagano *et al*, 2007a). In an earlier study, we reported that 66% of those who responded to such treatment were IFNAR2-positive, but half of these 20 patients showed no clinical response (Nagano *et al*, 2007a). Therefore, finding novel biological markers of resistance to IFN-α/5-FU combination therapy is desirable, not only so that non-responders receive other potentially more successful treatments, but also to avoid their suffering caused by debilitating side effects.

Development of microarray technology has facilitated analysis of genome-wide expression profiles (Zembutsu *et al*, 2002; Yang *et al*, 2005). It can generate a large body of information concerning genetic networks related to pathological subtype, prognosis and resistance to anticancer drugs of neoplasm. We have reported many studies using PCR array or oligonucleotide microarray system in various human malignancies, particularly in gastrointestinal and HCCs (Komori *et al*, 2008; Kurokawa *et al*, 2004a, b; Motoori *et al*, 2005, 2006). To understand the complex biological processes, such as cancer progression and drug resistance, it is also important to consider differential gene expression by the gene network analysis (Kittaka *et al*, 2008). A detailed human interactive network that captures the entire cellular network would be invaluable in interpreting cancer signatures (Calvano *et al*, 2005; Rhodes and Chinnaiyan, 2005).

*Correspondence: Dr H Nagano, Department of Surgery, Graduate School of Medicine, Osaka University, 2-2, Yamadaoka E-2, Suita, Osaka 565-0871 Japan; E-mail: hnagano@gesurg.med.osaka-u.ac.jp
Received 5 January 2009; revised 9 March 2009; accepted 30 March 2009; published online 28 April 2009

In this study, we applied the methods of oligonucleotide microarray system and gene network analysis to identify informative gene set(s) and signalling pathway(s) related to resistance to IFN-α/5-FU combination therapy, especially in patients with IFNAR2-positive HCC. The results showed that Wnt/β-catenin signalling influenced resistance to IFN-α/5-FU combination therapy. The study also investigated the potential importance of epithelial cell adhesion molecule (Ep-CAM), which is encoded by the TACSTD1 gene and confirmed as one of the target genes of Wnt/β-catenin signalling (Yamashita *et al*, 2007), as a biological marker for resistance to IFN-α/5-FU combination therapy.

MATERIALS AND METHODS

Cell lines

The human HCC cell lines, PLC/PRF/5, HuH7, HLE, HLF and HepG2, were purchased from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The Hep3B cell line was obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). They were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% foetal bovine serum, 100 U ml^{-1} penicillin and 100 $\mu g\,ml^{-1}$ streptomycin at 37°C in a humidified incubator with 5% CO_2 in air.

Drugs and reagents

Purified human IFN-α was kindly supplied by Otsuka Pharmaceutical Co. (Tokyo, Japan) and 5-FU was obtained from Kyowa Hakko Co. (Tokyo, Japan). The small molecule of 6-bromoindirubin-3′-oxime (BIO), a specific inhibitor of glycogen synthesis kinase-3 (GSK-3), activating the Wnt/β-catenin signalling pathway (Sato *et al*, 2004), was purchased from Calbiochem (San Diego, CA, USA) and was dissolved in dimethyl sulphoxide (DMSO) (Wako Pure Chemical Industries, Osaka, Japan). We used the following antibodies for immunohistochemistry and western blot analysis: monoclonal mouse anti-human Ep-CAM antibody (Abcam, Cambridge, UK), polyclonal rabbit anti-human c-MYC antibody (Cell Signaling Technology, Beverly, MA, USA) and polyclonal rabbit anti-human β-actin (Sigma, St Louis, MO, USA).

Patients and specimens

In total, 30 patients with multiple liver tumours spreading to both lobes with tumour thrombi in the major branches of the portal vein, underwent palliative reduction surgery at the Osaka University Hospital as described in our earlier report (Nagano *et al*, 2007a). All 30 patients had visible tumours in the remnant liver, and received combination chemotherapy with 5-FU and IFN-α as described earlier (Sakon *et al*, 2002; Ota *et al*, 2005). The chemotherapeutic response was evaluated clinically according to the criteria of the Eastern Cooperative Oncology Group (Oken *et al*, 1982). In this study, responders were defined as patients with complete response or partial response; non-responders were defined as patients with stable disease or progressive disease. All aspects of our study protocol were approved by the Human Ethics Committee of Graduate School of Medicine, Osaka University, Japan. Surgical specimens were obtained from these patients. Appropriate informed consent was obtained from all patients.

For microarray analysis, we used samples of 18 cases that were positive for IFNAR2 expression, whereas no samples were available from 2 cases with insufficient quality of RNA. For reference in microarray experiment, we obtained a mixture of RNA from normal parts of the liver specimens of seven patients with liver metastases from intestinal carcinomas who were free of HBV and HCV infections. All tissues were snap-frozen into liquid nitrogen and stored at −80°C. Other samples were fixed in 10% buffered formalin, embedded in paraffin and stained with haematoxylin–eosin to study the pathological features of HCC in accordance with the classification proposed by the Liver Cancer Study Group of Japan.

Microarray experiments

The microarray experiments were conducted according to the method described earlier (Kittaka *et al*, 2008). In brief, total RNA was purified by TRIzol agent (Invitrogen, San Diego, CA, USA), according to the instructions provided by the manufacturer. The integrity of RNA was assessed by Agilent 2100 Bioanalyzer and RNA 6000 LabChip kits (Yokokawa Analytical Systems, Tokyo, Japan). Only high-quality RNA was used for analysis. For control reference, RNAs from normal liver tissues were mixed. The reference and sample were mixed and hybridised on a microarray covering 30 336 human probes (AceGene Human 30K; DNA Chip Research Inc. and Hitachi Software Engineering Co., Kanagawa, Japan). The ratio of expression level of each gene was converted to a logarithmic scale (base 2) and the data matrix was normalised. Genes with >10% missing data values in all samples were excluded from analysis; a total of 14 473 genes out of 30 336 were available for analysis.

To detect the significant genes for resistance, we used permutation testing (Kurokawa *et al*, 2004b). The original score of each gene (signal-to-noise ratio (S2N), $Si = (\mu A - \mu B)/(\sigma A + \sigma B)$, where μ and σ represent the mean and standard deviation of expression for each class, was calculated without permuting labels (responder or non-responder). The labels were randomly swapped and the values of S2N were calculated between two groups. Repetition of this permutation 10 000 times provided data matrix that was nearly the same as normal distribution. For each gene, the *P*-value was calculated from the original S2N ratio with reference to the distribution of permuted data matrix. We determined the optimal *P*-value and the informative gene set.

Pathway analysis

We further analysed the significant genes for resistance by the Ingenuity Pathways Analysis (Ingenuity Systems, Mountain View, CA, USA; http://www.ingenuity.com). The Ingenuity Pathway Knowledge Base (IPKB) is a database of earlier published findings on mammalian biology. Canonical pathways analysis identifies the pathways that were statistically significant from the submitted data matrix from the canonical pathways of IPKB. The *P*-value of each canonical pathway is calculated using Fischer's exact test determining the probability that the association between the genes in the data set and the canonical pathway is because of chance alone.

Network analysis was conducted as described earlier (Calvano *et al*, 2005). In brief, the submitted genes that were mapped to the corresponding gene objects in the IPKB were called 'focus genes'. The focus genes were used to generate biological networks. The Ingenuity software queries the IPKB for interactions between focus genes and then generates a set of networks. The *P*-value of each network is calculated according to the fit of the user's set of significant genes. The score of a network is displayed as a negative log of the *P*-value, indicating the probability that a collection of genes equal to or greater than the number in a network could be achieved by chance alone.

RT-PCR analysis

Complementary DNA was generated from 1 μg RNA with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA) as described earlier (Damdinsuren *et al*, 2006). Quantitative real-time PCR (qRT-PCR) assays were carried out using the Light Cycler (Roche Diagnostics, Mannheim, Germany), as described

earlier (Ogawa *et al*, 2004). Gene expression was measured in duplicate. The conditions set for qRT-PCR for TACSTD1, TCF3, AXIN2, MYC, CCND1 and β-actin were one cycle of denaturing at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 35 s, and final extension at 72°C for 10 min (or annealing at 58°C for β-actin). The primer sequences were as follows: TACSTD1 forward primer, 5′-TCCAGAAAGAAGAGA ATGGCA-3′; TACSTD1 reverse primer, 5′-AAAGATGTCTTCGT CCCACG-3′; TCF3 forward primer, 5′-ATCTGTGTCCCATGTCCC AG-3′; TCF3 reverse primer, 5′-CCAGGGTAGGAGACTTGCAG-3′; AXIN2 forward primer, 5′-GGTGTTTGAGGAGATCTGGG-3′; AXIN2 reverse primer, 5′-TGCTCACAGCCAAGACAGTT-3′; MYC forward primer, 5′-AAGAGGACTTGTTGCGGAAA-3′; MYC reverse primer, 5′-CTCAGCCAAGGTTGTGAGGT-3′; CCND1 forward primer, 5′-AAGGCCTGAACCTGAGGAG-3′; CCND1 reverse primer, 5′-CTTGACTCCAGCAGGGCTT-3′; β-actin forward primer, 5′-GA AAATCTGGCACCACACCTT-3′; and β-actin reverse primer, 5′-G TTGAAGGTAGTTTCGTGGAT-3′.

Immunohistochemical staining

For immunohistochemical staining of Ep-CAM expression, we used the method described earlier (Kondo *et al*, 1999) with minor modifications. Briefly, formalin-fixed, paraffin-embedded 4-μm thick sections were deparaffinised, then treated with an antigen retrieval procedure and incubated in methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase. The sections were incubated with normal protein block serum solution, and the biotin-blocking solution (Wako) was used as recommended by the manufacturer. Then, the sections were incubated overnight at 4°C with anti-Ep-CAM antibody as the primary antibody. After washing in phosphate-buffered saline (PBS), the sections were incubated with a biotin-conjugated secondary antibody (horse anti-mouse antibody for Ep-CAM) and with peroxidase-conjugated streptavidin. The peroxidase reaction was then developed with 0.02% 3, 30-diaminobenzidine tetrachloride (Wako) solution with 0.03% hydrogen peroxide. Finally, the sections were counterstained with Meyer's haematoxylin. For negative controls, sections were treated the same way except that they were incubated with Tris-buffered saline instead of the primary antibody.

Ep-CAM expression was assessed by two investigators (TN and NM) independently without knowledge of the corresponding clinicopathological data. Antigen expression was defined as the presence of specific staining on the surface membrane of tumour cells. Ep-CAM expression was evaluated by calculating the total immunostaining score, representing the product of the proportion score and the intensity score, as described earlier (Gastl *et al*, 2000). In brief, the proportion score described the estimated fraction of positively stained tumour cells (0, none; 1, $<10\%$; 2, 10–50%; 3, 50–80% and 4, $\geqslant 80\%$). The intensity of Ep-CAM expression was compared with staining of normal bile duct epithelium present in each section of positive control. The intensity score represented the estimated staining intensity (0, no staining; 1, weak; 2, moderate and 3, strong). The total score ranged from 0 to 12. Ep-CAM-positive cases represented those with a total score >4.

Western blot analysis

The cells were washed with PBS and collected with a rubber scraper. After centrifugation, the cell pellets were resuspended in RIPA buffer (25 mM Tris (pH 7.5), 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% sodium dodecyl sulphate, 1 mM phenylmethylsulphonyl fluoride and 500 KIE ml^{-1} 'Trasylol' proteinase inhibitor (Bayer LeverKusen, Germany)) with phosphatase inhibitor (Sigma). The extracts were centrifuged and the supernatant fraction was collected. Western blot analysis was carried out as described earlier (Kondo *et al*, 2005).

Luciferase reporter assay

The reporter assay kit was purchased from SA Biosciences (Frederick, MD, USA) to evaluate TCF/LEF transcriptional activity and is used according to the instructions provided by the manufacturer. In brief, 2×10^4 cells per well were added in triplicate to a 96-well microplate, and 24 h later, cells were transiently transfected with the transcription factor-responsive reporter or negative control by the Lipofectamine 2000 reagent (Invitrogen). Culture media were changed 16 h after transfection, and the transfected cells were treated with various concentrations of BIO (0–5 nM). After 24 h treatment, luciferase activity was measured with the Dual-Luciferase Assay System (Promega) using microplate luminometer, microlumat LB96P (Berthold Technologies, Calmbacher, Germany). The Firefly luciferase activity, indicating TCF-dependent transcription, was normalised to the *Renilla* luciferase activity as an internal control to obtain the relative luciferase activity.

Growth-inhibitory assays with 5-FU and IFN-α

The growth inhibitory assay was assessed by the 3-(4-, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) assay as described earlier (Eguchi *et al*, 2000). The tested concentrations of 5-FU were 0.05, 0.5 and 5 μg ml^{-1}, and those of IFN-α were 50, 500 and 5000 U ml^{-1}. The cells were incubated in a medium containing variable concentrations of 5-FU and IFN-α with DMSO or 5 nM BIO for 48 h. The proportion of cells incubated without drugs was defined as 100% viability.

DNA synthesis-inhibition assay

DNA synthesis inhibition was assessed by bromodeoxyuridine (BrdU) incorporation rate using the Cell Proliferation enzyme-linked immunosorbent assay (ELISA)-Chemiluminescent kit (Roche Applied Science, Indianapolis, IN, USA) according to the protocol provided by the manufacturer. In brief, HuH7 cells (1×10^4 per well) were seeded in triplicate into 96-well microplate. After treatment with control, 5-FU alone (5 μg ml^{-1}), IFN-α alone (5000 U ml^{-1}) and combination of 5-FU and IFN-α, with or without BIO (5 nM), the plates were incubated at 37°C under 5% CO_2 for 24 h. Then cells were labelled for 2 h with BrdU. Chemiluminescent signals were detected on the microplate luminometer (microlumat LB96P, Berthold Technologies).

Cell cycle analysis

Flow cytometric analysis was carried out as described earlier (Eguchi *et al*, 2000). In brief, cells were washed with PBS and then fixed in 70% cold ethanol. Propidium iodide (Sigma) and RNase (Sigma) were added for 30 min at 37°C. Samples were filtered, and data were acquired with a FACSort (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Analysis of the cell cycle was carried out using ModFIT software (Becton Dickinson Immunocytometry Systems).

Statistical analysis

Clinicopathological indicators were compared using χ^2-test, whereas continuous variables were compared using the Student's *t*-test. Survival curves were computed using the Kaplan–Meier method, and differences between survival curves were compared using the log-rank test. To evaluate the risk associated with the prognostic variables, the Cox model with determination of the hazard ratio was applied; a 95% confidence interval was adopted. All statistical analyses were calculated using the SPSS software

(version 11.0.1 J, SPSS Inc., Chicago, IL, USA), and P-value <0.05 was considered statistically significant.

RESULTS

Patients' characteristics

The characteristics of the 30 HCC patients are shown in Table 1. A total of 10 patients were IFNAR2-negative and 20 patients were IFNAR2-positive. In 20 cases with positive IFNAR2, 10 patients were classified as responders; the remaining 10 patients were classified as non-responders. All patients with negative IFNAR2 were non-responders. We have earlier reported that IFNAR2 expression correlated significantly with the response to IFN-α/5-FU therapy (Ota *et al*, 2005; Nagano *et al*, 2007a). A larger proportion of responders were infected with HCV than non-responders. But all other analysed parameters were comparable among these groups and there were no significant differences in these parameters.

Table 1 Clinicopathological characteristics of responders and non-responders

	IFNAR2-positive		IFNAR2-negative	
	Responders ($n=10$)	Non-responders ($n=10$)	Non-responders ($n=10$)	*P*-value
Age (year)				NS
<60	6	7	5	
⩾60	4	3	5	
Sex				NS
Male	9	9	9	
Female	1	1	1	
HBV infection				NS
Present	6	8	7	
Absent	4	2	3	
HCV infection				0.0180
Present	7	1	3	
Absent	3	9	7	
Child-pugh score				NS
A	7	7	5	
B, C	3	3	5	
Liver cirrhosis				NS
Present	4	7	3	
Absent	6	3	7	
α-fetoprotein (ng ml^{-1})				NS
<300	5	1	3	
⩾300	5	9	7	
Tumour size (cm)				NS
<5	2	3	2	
⩾5	8	7	8	
Histological grade				NS
Moderately differentiated	1	0	0	
Poorly differentiated	9	8	9	
Undifferentiated	0	2	1	
IFNAR2 expression				<0.0001
0	0	0	10	
1	8	10	0	
2	2	0	0	

HBV = hepatitis B virus; HCV = hepatitis C virus; IFNAR2 = type I interferon receptor 2.

Microarray analysis and pathway analysis

Genes with significant P-values ($P<0.001$) were defined by the random permutation test. These differentially expressed 161 genes were selected as informative gene set and are listed in Table 2. The status of gene expression was defined as expression in non-responders compared with responders. Of the total, 98 genes were relatively upregulated in the responder group and 63 genes were relatively downregulated.

Then we carried out the canonical pathway analysis of the 161 genes using the software Ingenuity. Eight canonical pathways were identified as pathways that significantly influenced the resistance of IFN-α/5-FU combination therapy in 161 informative genes (Table 3). We also simultaneously carried out network analysis of the same informative genes set. A total of 14 networks were identified, and these networks were ranked by the score on a P-value calculation, which ranged from 2 to 55. Then, we selected one network with the highest score. The network with the highest score consisted of 35 molecules in 25 focus molecules and 11 interconnecting molecules (Figure 1A). This network included AXIN2, TCF3, RARA, CREBBP and TACSTD1, which were all associated with Wnt/β-catenin signalling identified by the canonical pathway analysis. In recent reports, Wnt/β-catenin signalling has been shown to mediate radiation resistance and chemotherapy resistance of various malignancies. In the Wnt/β-catenin signalling-related genes, TACSTD1 was most highly upregulated in the non-responders at the level of transcription.

TACSTD1 expression by RT-PCR and correlation with microarray data

Next, we examined the correlation between the expression data of gene expression and qRT-PCR of TACSTD1 to verify the microarray expression data. qRT-PCR analysis was carried out on 13 HCC tissue samples with positive expression of IFNAR2. Individual mRNA levels were normalised to β-actin and expressed relative to those in a mixture of seven normal livers. In the 13 IFNAR2-positive samples, TACSTD1 expression correlated significantly with the microarray data (Figure 1B). The Pearson correlation coefficient (P-value) for TACSTD1 was 0.668 ($P=0.0107$). We then analysed TACSTD1 expression according to the clinical response to IFN-α/5-FU combination therapy. TACSTD1 expression was higher in several non-responders with IFNAR2-positive HCC or IFNAR2-negative HCC, compared with responders with IFNAR2-positive HCC (Figure 1C). Using a cut-off value of 10 for TACSTD1 expression ratio, it was possible to exclude some non-responders from patients with IFNAR2-positive HCC.

Immunohistochemical staining for Ep-CAM

We examined the Ep-CAM expression in 30 HCC patients who underwent palliative reduction surgery. In tumour lesions, Ep-CAM staining was specifically observed on the plasma membrane of cancer cells. In Figure 1D (left), strong Ep-CAM expression was noted in 80% of cancerous tissue in the representative case of non-responders with IFNAR2-negative HCC. On the other hand, no Ep-CAM expression was evident in the representative case of IFNAR2-positive responders (Figure 1D, right). Among the 30 patients examined, Ep-CAM expression was observed in six (20%). It is important that Ep-CAM expression was associated with resistance to IFN-α/5-FU therapy, and no Ep-CAM expression was noted in the responders (Table 4). However, the difference in the expression

Table 2 List of informative 161 genes defining responders and non-responders

Rank	Status	Gene symbol	Gene name	Ref Seq ID
1	Down	—	dj329I24.3 (member of mcm2/3/5 family)	—
2	Down	CREBBP	CREB-binding protein (Rubinstein-Taybi syndrome)	NM_004380
3	Down	C20orf116	Chromosome 20 open reading frame 116	NM_023935
4	Down	—	Ensembl genscan prediction	—
5	Down	SLC25A40	Solute carrier family 25, member 40	NM_018843
6	Up	ZNF598	Zinc-finger protein 598	NM_178167
7	Down	RCOR1	REST corepressor 1	NM_015156
8	Up	TACSTD1	Tumour-associated calcium signal transducer 1	NM_002354
9	Down	ZNF397	Zinc-finger protein 397	NM_032347
10	Down	RTP3	Receptor (chemosensory) transporter protein 3	NM_031440
11	Up	AARS2	Alanyl-tRNA synthetase 2, mitochondrial (putative)	NM_020745
12	Down	RARA	Retinoic acid receptor, alpha	NM_000964
13	Up	—	DNA segment on chromosome 4 (unique) 234 expressed sequence	NM_014392
14	Up	CASP7	Caspase 7, apoptosis-related cysteine peptidase	NM_033339
15	Up	AZIN1	Antizyme inhibitor 1	NM_148174
16	Down	—	Ensembl prediction	—
17	Down	—	Ensembl genscan prediction	—
18	Up	PABPN1	Poly(A)-binding protein, nuclear 1	—
19	Down	NDUFS7	NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20 kDa (NADH-coenzyme Q reductase)	NM_024407
20	Down	C9orf142	Chromosome 9 open reading frame 142	NM_183241
21	Down	—	Ensembl genscan prediction	—
22	Up	SNX21	Sorting nexin family member 21	NM_001042632
23	Down	C12orf47	Chromosome 12 open reading frame 47	XR_017874
24	Down	CCRL1	Chemokine (C–C motif) receptor-like 1	NM_178445
25	Down	GLG1	Golgi apparatus protein 1	NM_012201
26	Down	MRPS21	Mitochondrial ribosomal protein S21	NM_018997
27	Up	ABCA2	ATP-binding cassette, sub-family A (ABC1), member 2	NM_001606
28	Up	AXIN2	Axin 2 (conductin, axil)	NM_004655
29	Down	—	Ensembl genscan prediction	—
30	Down	NOXA1	NADPH oxidase activator 1	NM_006647
31	Down	COX4I1	Cytochrome c oxidase subunit IV isoform 1	NM_001861
32	Up	PCSK7	Proprotein convertase subtilisin/kexin type 7	XM_001128785
33	Up	FABP3	Fatty acid-binding protein 3, muscle and heart (mammary-derived growth inhibitor)	NM_004102
34	Down	C20orf111	Chromosome 20 open reading frame 111	NM_016470
35	Down	CAST	Calpastatin	NM_173061
36	Down	C12orf47	Chromosome 12 open reading frame 47	XR_017874
37	Up	—	Hypothetical protein xp_032244	—
38	Down	—	Ensembl genscan prediction	—
39	Up	PCDHA1	Protocadherin alpha 1	NM_018900
40	Down	MATK	Megakaryocyte-associated tyrosine kinase	NM_002378
41	Up	—	Hypothetical protein xp_051475	—
42	Up	UBE2Q1	Ubiquitin-conjugating enzyme E2Q (putative) 1	NM_017582
43	Up	GPATCH4	G patch domain containing 4	NM_182679
44	Down	PARP2	Poly (ADP-ribose) polymerase family, member 2	NM_005484
45	Down	HAL	Histidine ammonia-lyase	NM_002108
46	Up	ASCC3	Activating signal cointegrator 1 complex subunit 3	NM_006828
47	Down	KRTAP9-8	Keratin-associated protein 9–8	NM_031963
48	Up	MAGED4B	Melanoma antigen family D, 4B	NM_030801
49	Down	—	Hypothetical LOC339123	NM_001005920
50	Down	SPHK1	Sphingosine kinase 1	NM_021972
51	Up	—	Partial ighv ig h-chain v-region clone a81	—
52	Up	CCDC109A	Coiled-coil domain containing 109A	NM_138357
53	Up	GPR139	G protein-coupled receptor 139	NM_001002911
54	Up	C1orf78	Chromosome 1 open reading frame 78	NM_018166
55	Down	LRRC50	Leucine rich repeat containing 50	—
56	Down	FAM125B	Family with sequence similarity 125, member B	NM_033446
57	Down	IFT52	Intraflagellar transport 52 homolog (Chlamydomonas)	NM_016004
58	Down	C3orf36	Chromosome 3 open reading frame 36	NM_025041
59	Up	GUCA1B	Guanylate cyclase activator 1B (retina)	NM_002098
60	Down	EDF1	Endothelial differentiation-related factor 1	NM_003792
61	Down	CCDC69	Coiled-coil domain containing 69	NM_015621
62	Down	NDUFS6	NADH dehydrogenase (ubiquinone) Fe-S protein 6, 13 kDa (NADH-coenzyme Q reductase)	NM_004553
63	Up	CD93	CD93 molecule	NM_012072
64	Down	—	Ensembl genscan prediction	—
65	Down	ENO2	Enolase 2 (gamma, neuronal)	NM_001975
66	Down	CDCP2	CUB domain-containing protein 2	—
67	Down	—	Ensembl genscan prediction	—
68	Up	—	Similar to helicase-like protein nhl	—
69	Down	FOXN3	Forkhead box N3	NM_005197
70	Down	DEF8	Differentially expressed in FDCP 8 homolog (mouse)	NM_207514
71	Up	—	Hypothetical protein xp_034013	—

Table 2 (*Continued*)

Rank	Status	Gene symbol	Gene name	Ref Seq ID
72	Down	TNS3	Tensin 3	NM_022748
73	Up	FAM40A	Family with sequence similarity 40, member A	NM_033088
74	Down	PRDM7	PR domain containing 7	NM_052996
75	Up	—	Hypothetical protein xp_039419	—
76	Up	NNMT	Nicotinamide N-methyltransferase	NM_006169
77	Up	RPL9	Ribosomal protein L9	—
78	Up	ITM2C	integral membrane protein 2C	NM_030926
79	Up	—	Ensembl genscan prediction	—
80	Up	BEX4	BEX family member 4	XM_936467
81	Up	CSNK1G1	Casein kinase 1, gamma 1	NM_022048
82	Up	EEF2K	Eukaryotic elongation factor-2 kinase	NM_013302
83	Down	DNAJC8	DnaJ (Hsp40) homolog, subfamily C, member 8	—
84	Up	GP5	Glycoprotein V (platelet)	NM_004488
85	Down	DPH3	DPH3, KTI11 homolog (*S. cerevisiae*)	NM_001047434
86	Down	—	Ensembl genscan prediction	—
87	Up	RPS21	Ribosomal protein S21	—
88	Down	—	Kiaa1658 protein	—
89	Down	ADCYAP1R1	Adenylate cyclase activating polypeptide 1 (pituitary) receptor type 1	NM_001118
90	Down	C5orf25	Chromosome 5 open reading frame 25	XR_015120
91	Down	—	PRO0132 protein	NR_002763
92	Down	FGF3	Fibroblast growth factor 3 (murine mammary tumour virus integration site (v-int-2) oncogene homolog)	NM_005247
93	Up	FABP7	Fatty acid-binding protein 7, brain	NM_001446
94	Down	HSD11B1	Hydroxysteroid (11-beta) dehydrogenase 1	NM_181755
95	Up	—	Chondroitin sulphate glucuronyltransferase	NM_019015
96	Down	OPTN	Optineurin	NM_001008213
97	Up	—	Erythroid differentiation-related factor 2	—
98	Down	—	Truncated alpha ig h-chain of disease patient har	—
99	Down	WT1	Wilms tumour 1	NM_024425
100	Down	C8G	Complement component 8, gamma polypeptide	NM_000606
101	Down	—	pro1454	—
102	Down	CADM1	Cell adhesion molecule 1	NM_001098517
103	Down	GH1	Growth hormone 1	—
104	Down	DNPEP	Aspartyl aminopeptidase	NM_012100
105	Up	—	Actin-like gene	—
106	Down	—	Ensembl genscan prediction	—
107	Down	—	Ensembl genscan prediction	—
108	Up	INTS4	Integrator complex subunit 4	NM_033547
109	Down	SRA1	Steroid receptor RNA activator 1	NM_001035235
110	Down	—	Ensembl genscan prediction	—
111	Up	RPS25	Ribosomal protein S25	NM_001028
112	Down	—	KIAA1450 protein	NM_020840
113	Up	SH2D3C	SH2 domain containing 3C	NM_170600
114	Down	NDUFA12	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12	NM_018838
115	Down	—	NEFA-interacting nuclear protein NIP30	NM_024946
116	Down	TDRD1	tudor domain containing 1	NM_198795
117	Down	—	14a9ct dna sequence	—
118	Up	FBXW11	F-box and WD repeat domain containing 11	NM_012300
119	Down	CBFA2T3	Core-binding factor, runt domain, alpha subunit 2; translocated to, 3	NM_005187
120	Down	TCF3	Transcription factor 3 (E2A immunoglobulin enhancer-binding factors E12/E47)	NM_003200
121	Down	LASS5	LAG1 homolog, ceramide synthase 5	NM_147190
122	Down	—	Ensembl genscan prediction	—
123	Up	ACTR1B	ARP1 actin-related protein 1 homolog B, centractin beta (yeast)	NM_005735
124	Down	—	Hypothetical protein mgc5566	—
125	Up	RPS4X	Ribosomal protein S4, X-linked	XR_019325
126	Down	CDK6	Cyclin-dependent kinase 6	NM_001259
127	Up	AVIL	Advillin	—
128	Down	—	Hypothetical protein xp_043732	—
129	Down	C1orf136	Chromosome 1 open reading frame 136	—
130	Down	—	Hypothetical protein xp_043783	—
131	Down	—	Ews-fli-1	—
132	Up	CDC42BPG	CDC42-binding protein kinase gamma (DMPK-like)	NM_017525
133	Down	—	Ensembl genscan prediction	—
134	Down	GDAP1L1	Ganglioside-induced differentiation-associated protein 1-like 1	NM_024034
135	Up	C12orf4	Chromosome 12 open reading frame 4	NM_020374
136	Up	KIAA0415	KIAA0415	NM_014855
137	Down	PDLIM2	PDZ and LIM domain 2 (mystique)	NM_198042
138	Down	KHK	Ketohexokinase (fructokinase)	NM_006488
139	Down	SLC36A1	Solute carrier family 36 (proton/amino acid symporter), member 1	NM_078483
140	Up	—	Hypothetical protein xp_050311	—
141	Up	TBRG4	Transforming growth factor β regulator 4	NM_199122
142	Down	—	Rearranged vk3 of Hodgkin cell line	—

Table 2 (*Continued*)

Rank	Status	Gene symbol	Gene name	Ref Seq ID
143	Up	CD59	CD59 molecule, complement regulatory protein	NM_203330
144	Down	PEX26	Peroxisome biogenesis factor 26	—
145	Up	VEGFC	Vascular endothelial growth factor C	NM_005429
146	Down	DTX2	Deltex homolog 2 (*Drosophila*)	XM_941785
147	Up	ELAVL3	ELAV (embryonic lethal, abnormal vision, *Drosophila*)-like 3 (Hu antigen C)	NM_032281
148	Up	BSDC1	BSD domain containing 1	NM_018045
149	Down	FUBP3	Far upstream element (FUSE)-binding protein 3	XM_001128545
150	Down	CCDC48	Coiled-coil domain containing 48	NM_024768
151	Down	EPHA6	EPH receptor A6	NM_001080448
152	Down	ST8SIA1	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1	NM_003034
153	Down	MKKS	McKusick-Kaufman syndrome	NM_018848
154	Down	MGA	MAX gene associated	NM_001080541
155	Up	—	Hypothetical protein xp_040140	—
156	Down	—	Hypothetical protein xp_043452	—
157	Up	MMP20	Matrix metallopeptidase 20 (enamelysin)	NM_004771
158	Up	SLC23A2	Solute carrier family 23 (nucleobase transporters), member 2	NM_005116
159	Up	GABARAPL1	GABA(A) receptor-associated protein like 1	NM_031412
160	Down	---	Ensembl genscan prediction	—
161	Up	PKLR	Pyruvate kinase, liver and RBC	NM_000298

Ranking was according to absolute value of signal-to-noise ratio. Status was defined as expression in non-responders compared with responders.

Table 3 List of significant pathways from 161 informative genes by canonical pathway analysis

Pathway	P-value
Ubiquinone biosynthesis	0.0004
Oxidative phosphorylation	0.0074
Mitochondrial dysfunction	0.0095
FXR/RXR activation	0.0162
Wnt/β-catenin signalling	0.0170
Complement system	0.0191
Histidine metabolism	0.0263
Sphingolipid metabolism	0.0389

rate was not significant probably because of the small sample size ($P = 0.0528$). In non-tumour lesions, Ep-CAM staining was observed in a few scattered cells and proliferating bile duct epithelium showed positive expression.

Analysis of the degree of Ep-CAM expression in tumour lesions showed five (16.7%) samples negative for Ep-CAM expression (score 0), 19 (63.3%) with weak expression (score 1–4), four (13.3%) stained moderately (score 6–8) and two (6.7%) samples exhibited strong Ep-CAM expression (score 9–12). These results suggest that Ep-CAM expression in advanced HCC could be a potentially useful marker for resistance to IFN-α/5-FU combination therapy.

Ep-CAM expression and activation of Wnt/β-catenin signalling by BIO

We analysed the protein expression level of Ep-CAM in hepatoma cell lines. Western blot analysis using an anti-Ep-CAM antibody confirmed the positive expression of Ep-CAM in three of the six cell lines (HuH7, HepG2 and Hep3B), whereas PLC/PRF/5, HLE and HLF were negative (Figure 2A). We earlier reported strong IFNAR2 expression in PLC/PRF/5 cells and weak IFNAR2 expression in HuH7 cells (Eguchi *et al*, 2000). We transfected a TCF/LEF reporter into PLC/PRF/5, HuH7 and HepG2 cells to evaluate TCF/LEF transcriptional activity, representing the activity of Wnt/β-catenin signalling pathway. We found that the luciferase activities were high in both Ep-CAM-positive HuH7 cells and HepG2 cells, whereas very low in Ep-CAM-negative PLC/PRF/5 cells (Figure 2B). And, HepG2 cell line was reported to have mutation and activated β-catenin (de La Coste *et al*, 1998). For these reasons, we used the cell line HuH7 to investigate how Wnt/β-catenin signalling affected on the growth-inhibitory effect of IFN-α/5-FU. In the next step, we examined whether Wnt/β-catenin signalling can be activated in HuH7 cells when treated with various concentrations of specific GSK-3 inhibitor, BIO. HuH7 cells treated with BIO showed a substantial, dose-dependent increase in TCF/LEF reporter activity. Consequently, treatment with BIO at 0.5, 1 and 5 nM induced 8.6-, 29.1-, and 48.6-fold increases in relative luciferase activity compared with HuH7 cells treated by DMSO, respectively (Figure 2C). To examine the effects of BIO on the expression of Wnt/β-catenin signalling targeted genes, qRT–PCR analysis of five targeted genes (TACSTD1, AXIN2, MYC, TCF3 and CCND1) was carried out in HuH7 cells after 24 h treatment with BIO (5 nM). The concentration of BIO was selected on the basis of the results of luciferase reporter assay. Treatment with BIO increased the mRNA expression of targeted genes from 1.3-fold to 7.6-fold compared with cells treated with DMSO (Figure 2D). In western blot analysis, the expression levels of Ep-CAM and c-MYC increased in a BIO dose-dependent manner in HuH7 cells, but not in PLC/PRF/5 cells (Figure 2E).

Growth inhibition assay and reduction of growth-inhibitory effect of 5-FU and/or IFN-α treatment

Next, we investigated the role of activation of Wnt/β-catenin signalling in the reduction of the growth-inhibitory effect of IFN-α/5-FU. The growth of HCC cells (PLC/PRF/5 and HuH7 cell lines) was suppressed by 5-FU and IFN-α in a dose-dependent manner. Concurrent addition of BIO and IFN-α/5-FU to the cell cultures significantly reduced the growth-inhibitory effects of IFN-α/5-FU in HuH7 cells. In HuH7 cells, the growth inhibitory effects of IFN-α/5-FU without BIO were 22.3 ± 2.8% at 0.5 $\mu g\,ml^{-1}$ 5-FU and 500 $U\,ml^{-1}$ IFN-α, and 44.6 ± 0.9% at 5 $\mu g\,ml^{-1}$ and 5000 $U\,ml^{-1}$. Concurrent addition of BIO decreased the growth inhibitory effect to 8.6 ± 3.9% ($P = 0.0012$; 0.5 $\mu g\,ml^{-1}$ of 5-FU and 500 $U\,ml^{-1}$ for IFN-α) and 29.0 ± 2.0% ($P < 0.0001$, 5 $\mu g\,ml^{-1}$ for 5-FU and 5000 $U\,ml^{-1}$ for IFN-α) of control cells. In contrast, no change in the growth-inhibitory effect was found in PLC/PRF/5 cell line (Figure 3A). We also investigated the effects of BIO when combined with 5-FU alone or IFN-α alone in HuH7 cells. The

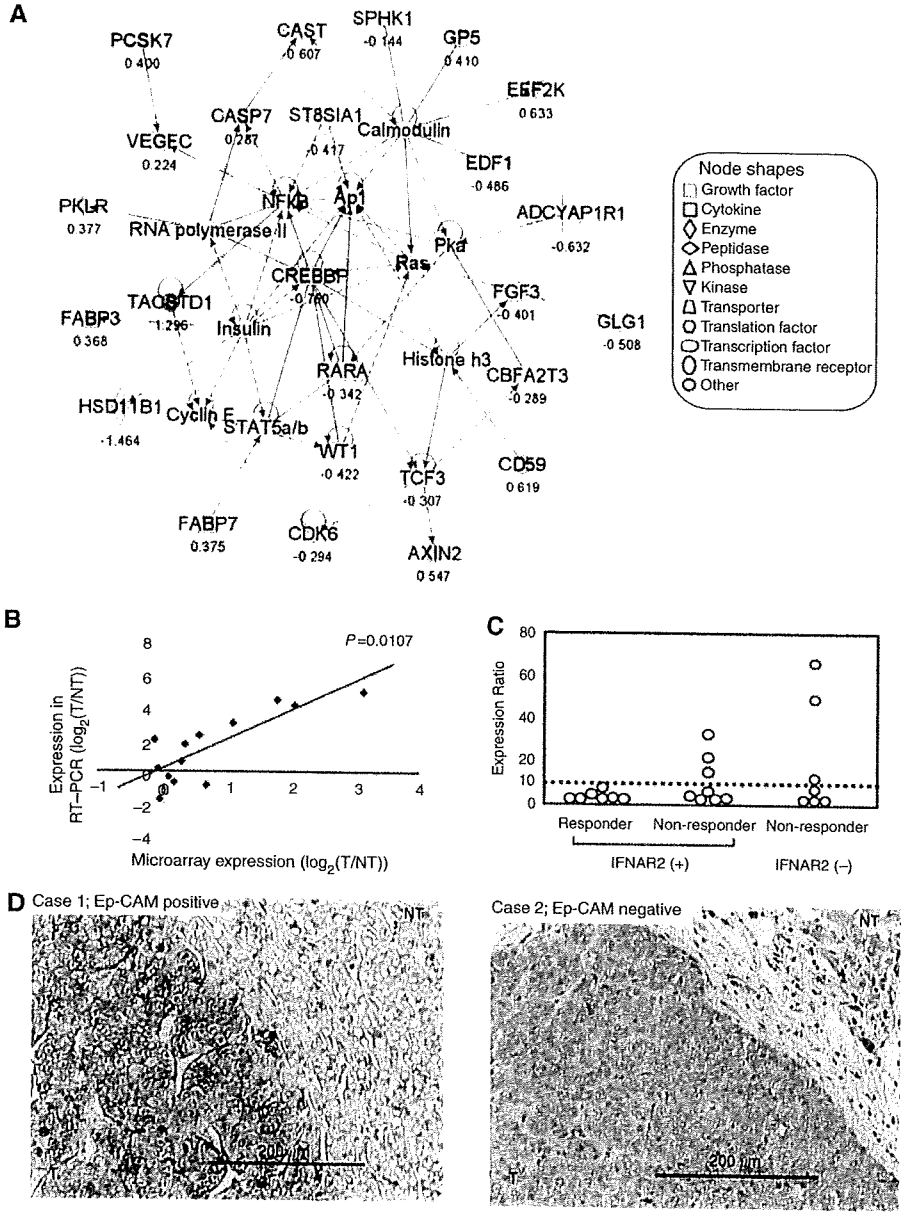

Figure 1 (**A**) Gene network of genes related to resistance to IFN-α/5-FU combination therapy. This network with the highest score consisted of 35 molecules in 19 focus molecules (red or green colour) and 16 interconnecting molecules (not coloured). The network included AXIN2, TCF3, RARA, CREBBP and TACSTD1, which are all associated with Wnt/β-catenin signalling. Each value of gene expression correlated directly with the intensity of the node colour. Red: upregulation in non-responders, green: downregulation in non-responders. The ratio of expression of each gene (non-responders/responders) is indicated below each node. (**B**) The expression levels determined by quantitative RT–PCR analysis correlated significantly with the microarray data. The Pearson correlation coefficient (*P*-value) for TACSTD1 were 0.668 ($P=0.0107$) (**C**) Among non-responders with IFNAR2-positive HCC or IFNAR2-negative HCC, the TACSTD1 expression ratio was higher in several cases than that in responders with IFNAR2-positive HCC. (**D**) Immunohistochemical staining for Ep-CAM in representative cases. Left panel: the majority of tumour cells showing staining for Ep-CAM on the plasma membrane. Right panel: tumour cells were negative for Ep-CAM. T, tumour lesion; NT, non-tumour lesion. (Magnification, × 200). The colour reproduction of this figure is available on the html full text version of the paper.

Table 4 Immunohistochemical analysis of Ep-CAM expression

	Ep-CAM expression		
	Negative	**Positive**	***P*-value**
Responders	10	0	0.0528
Non-responders	14	6	

combination of BIO and 5-FU alone and BIO and IFN-α exhibited reduced anti-proliferative effects (data not shown).

Activation of Wnt/β-catenin signalling interferes with the inhibitory effect of IFN-α/5-FU on DNA synthesis

To investigate whether activation of Wnt/β-catenin signalling is involved in the reduction of the growth inhibitory effects of

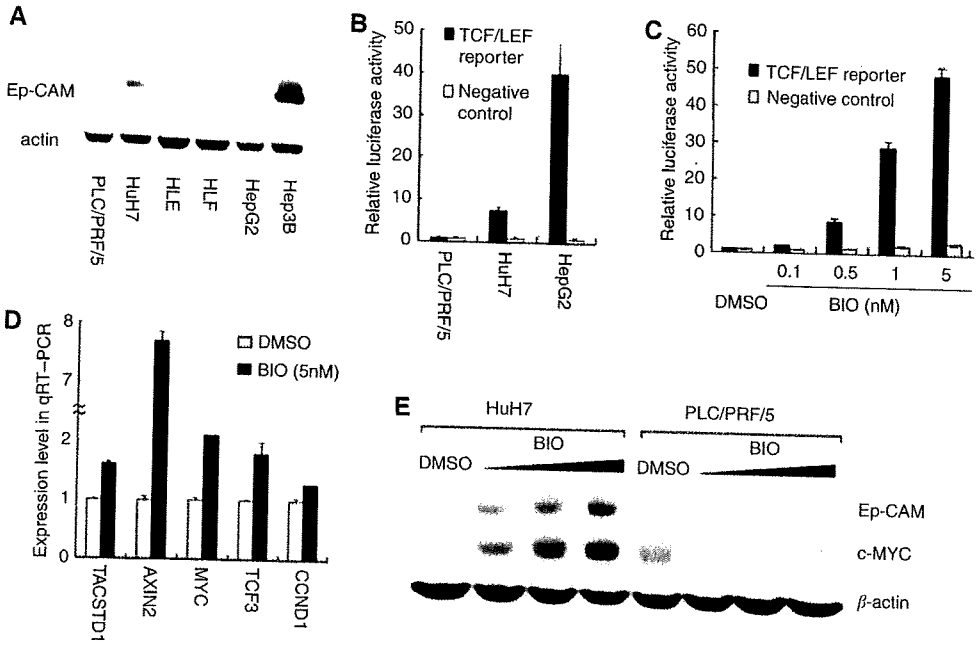

Figure 2 Changes in Ep-CAM expression and TCF/LEF transcription activity after treatment with BIO. (**A**) Western blot analysis of Ep-CAM in human hepatoma cell lines. Expression of Ep-CAM was positive in HuH7, HepG2 and Hep3B cell lines, but not in PLC/PRF/5, HLE and HLF. (**B**) Luciferase reporter assay of PLC/PRF/5, HuH7 and HepG2 cells. Relative luciferase activities were high in both Ep-CAM-positive HuH7 cells and HepG2 cells, whereas very low in Ep-CAM-negative PLC/PRF/5 cells. The assay was conducted in triplicate and results are shown as mean ± s.d. (**C**) Luciferase reporter assay of HuH7 cells treated with various concentrations of BIO for 24 h. Treatment with 5 nM induced 48.6-fold increase in relative luciferase activity compared with DMSO. The assay was conducted in triplicate and results are shown as mean ± s.d. (**D**) qRT–PCR analysis of HuH7 cells treated with BIO for 24 h. BIO increased the expression levels of TACSTD1, AXIN2, MYC, TCF3 and CCND1 compared with DMSO. Data are mean ± s.d. values of gene expression measured in duplicate. (**E**) Western blot analysis of HCC cell lines treated with BIO for 48 h. The expression of Ep-CAM and c-MYC increased in a BIO dose-dependent manner in HuH7 cells, but not in PLC/PRF/5 cells.

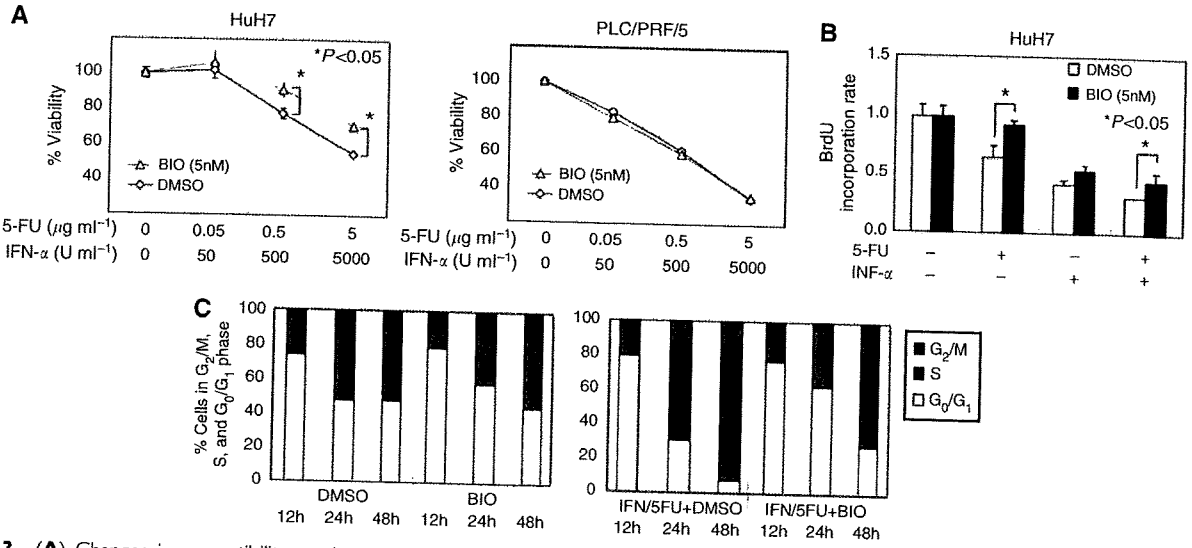

Figure 3 (**A**) Changes in susceptibility to the combination of 5-FU and IFN-α was measured by MTT assay. All cells were incubated with various concentrations of 5-FU and IFN-α and with BIO (5 nM) or DMSO. When BIO was combined with IFN-α/5-FU, it significantly reduced the growth inhibitory effects of IFN-α/5-FU in HuH7 cells, but not PLC/PRF/5 cells. The viability of cells incubated without drugs was defined as 100% and data are shown as mean ± s.d. (**B**) DNA synthesis-inhibition assay of HuH7 cell was assessed by BrdU incorporation rate. Cells were incubated with 5-FU and/or IFN-α and with BIO (5 nM) or DMSO. In both cell lines, the addition of BIO with 5-FU alone and IFN-α/5-FU significantly reduced the inhibitory effects of IFN-α/5-FU on DNA synthesis. Data was measured in triplicate and are shown as mean ± s.d. (**C**) Results of flow cytometric analysis of HuH7 cells treated with BIO and/or IFN-α/5-FU combination. Data represent percentages of cells in G_2/M, S and G_0/G_1 phases of the cell cycle. Concurrent use of BIO with IFN-α/5-FU delayed the accumulation of S-phase fraction.

IFN-α/5-FU, we evaluated the effects of BIO and IFN-α/5-FU on DNA synthesis using a BrdU-based cell proliferation ELISA. In HuH7 cells, the BrdU incorporation rates (representing DNA synthesis) in cultures treated with 5-FU alone and IFN-α/5-FU were 0.649 ± 0.052 and 0.312 ± 0.004, respectively. Activation of Wnt/β-catenin signalling by BIO resulted in a significant interference with the inhibitory effect of IFN-α/5-FU on DNA synthesis; the BrdU incorporation rates in cells cultured with BIO and 5-FU alone and with BIO and IFN-α/5-FU were significantly increased to 0.928 ± 0.020 ($P = 0.002$) and 0.458 ± 0.037 ($P = 0.007$) (Figure 3B).

Cell cycle assay

Finally, we used flow cytometric analysis to examine changes in cell cycle progression in cultures treated with BIO and IFN-α/5-FU. In cultures refed with serum plus 5-FU and IFN-α, the distribution of cells at different cell cycles was similar to that of cells treated with DMSO at 12 h. Thereafter, HuH7 cell lines treated with 5-FU and IFN-α showed accumulation of cells in S-phase and a gradual increase in S-phase fraction from 24 to 48 h. Addition of BIO and IFN-α/5-FU to the cell cultures delayed the accumulation of S-phase fraction. Marked accumulation of cells in S-phase (24 h; 69.4% and 48 h; 92.9%) was noted in cultures of cells treated with IFN-α/5-FU, whereas the percentage of cells in S-phase in cultures of BIO and IFN-α/5-FU decreased to 34.9% and 62.9% at the respective time points (Figure 3C).

DISCUSSION

Gene expression profiling analyses represent a high-throughput approach to dissect the biology underlining resistance to anticancer drugs in malignancies. We earlier identified a 63-gene set that could predict the response to IFN-α/5-FU combination therapy using a small-scale PCR array system of a total of 2666 genes (Kurokawa *et al*, 2004a). In this study, we used advanced technology with human whole genes analysis covering 30,336 human probes compared with the PCR array system. This comprehensive analysis allowed us to identify the biological actions of IFN-α/5-FU combination therapy. Furthermore, creating biological networks from comprehensive gene expression profiling could be useful for discovering certain targeted molecules and pathways. In fact, we reported recently genome-wide expression profiling of 100 HCC tissues using this network analysis, Ingenuity Pathway Analysis and identified novel targeted molecules related to specific signalling pathways (Kittaka *et al*, 2008).

In this study, gene expression profiling and pathway analysis identified Wnt/β-catenin signalling as a significant canonical pathway. The Wnt/β-catenin-signalling pathway plays an important role in the development of various malignancies, as well as cell proliferation and differentiation in several adult stem cells (Barker and Clevers, 2006; Klaus and Birchmeier, 2008). It has been also shown that anti-cancer drugs or irradiation often kill tumour cells, yet putative cancer stem/progenitor cells are resistant to these agents (Jamieson *et al*, 2004; Woodward *et al*, 2007; Klaus and Birchmeier, 2008). Cancer stem/progenitor cells provide an attractive explanation for chemotherapy-induced tumour remission as well as relapse. Analysis of the molecular and signalling mechanism of resistance of cancer stem/progenitor cells should be important for the development of new therapeutic strategies. Recent studies showed that the Wnt/β-catenin pathway plays a role in radiation and/or chemotherapy resistance of various malignancies such as leukaemia, head and neck tumours, prostate cancer and HCC (Jamieson *et al*, 2004; Ohigashi *et al*, 2005; Chang *et al*, 2008; Yang *et al*, 2008). In this study, we also showed that activation of Wnt/β-catenin signalling by a specific GSK-3 inhibitor in hepatoma cell lines decreased the susceptibility to IFN-α/5-FU through a reduction in their DNA synthesis inhibitory effects and regulation of cell cycle progression. We have already reported the mechanisms of the anti-proliferative effects of IFN-α/5-FU combination therapy, including regulation of cell cycle progression by increasing S-phase fraction (Eguchi *et al*, 2000), induction of apoptosis through IFNAR2, by downregulating Bcl-xl and by Fas/FasL pathway (Kondo *et al*, 2005; Damdinsuren *et al*, 2007; Nakamura *et al*, 2007; Nagano *et al*, 2007a), modulation of the immune response by inducing the TRAIL/TRAIL-receptor pathway (Yamamoto *et al*, 2004) and inhibition of tumour angiogenesis (Wada *et al*, 2007). In addition to the above mechanisms related to their anti-proliferative effects, this study showed that activation of Wnt/β-catenin signalling resulted in reduction of the inhibitory effects of IFN-α/5-FU on DNA synthesis, by decreasing the accumulation of cells in S-phase. With regard to the apoptotic effect of the combination therapy, it is reported that Wnt/β-catenin signalling is closely linked to JAK–STAT signalling (Yamashina *et al*, 2006), and regulates STAT3 expression, thus enhancing cell growth and anti-apoptotic activity of various cancer cells (Kusaba *et al*, 2007). We earlier reported that IFN-α/5-FU combination therapy increased the frequency of apoptosis in PLC/PRF/5 cells, but only minimally in HuH7 cells (<1%) (Eguchi *et al*, 2000). We also analysed the influence of activation of Wnt/β-catenin signalling on the apoptotic effects of IFN-α/5-FU combination therapy, but no significant change was observed in HuH7 cells probably because of the low rate of apoptosis. Further studies are needed to examine the molecular mechanisms of Wnt/β-catenin signalling-related enhancement of resistance to IFN-α/5-FU combination therapy.

Activation of the Wnt/β-catenin signalling pathway is reported in various diseases including many malignancies (Moon *et al*, 2004; Reya and Clevers, 2005; Branda and Wands, 2006). The ideal method for detecting the signalling activity in human tissues remains controversial (Giles *et al*, 1980). A recent study identified Ep-CAM as a novel Wnt/β-catenin signalling target gene in HCC cell lines, which could also serve as a biomarker (Yamashita *et al*, 2007). Ep-CAM is a first tumour-associated antigen and encoded by the TACSTD1 gene (Herlyn *et al*, 1979; Litvinov *et al*, 1994). In liver neoplasia, Ep-CAM is expressed in almost all cholangiocarcinomas, whereas 14% of HCCs manifested the expression, which seems to be more pronounced in poorly differentiated HCCs (Breuhahn *et al*, 2006). Ep-CAM-positive HCC displayed a molecular signature with features of hepatic progenitor cells, including the presence of known stem/progenitor markers such as c-kit, cytokeratin 19. In earlier studies, we showed that the expression of IFNAR2 is the only significant predictor of clinical outcome of IFN-α/5-FU combination therapy (Ota *et al*, 2005; Nagano *et al*, 2007a). On the basis of the present results on 30 HCC tissue samples, Ep-CAM seems to be another predictor of IFN-α/5-FU combination therapy. Further studies are needed to validate this result using larger sample numbers to establish the precise clinical response to IFN-α/5-FU combination therapy.

In summary, we showed that activation of Wnt/β-catenin signalling enhanced the resistance to IFN-α/5-FU therapy by reducing the inhibitory effects of these drugs on DNA synthesis and regulation of cell cycle progression *in vitro*. Furthermore, the results identified Ep-CAM expression in HCC tissue specimen as a potential biological marker for resistance to IFN-α/5-FU combination therapy.

ACKNOWLEDGEMENTS

This work was supported by a grant-in-aid for cancer research from the Ministry of Culture and Science, the Ministry of Health, Labour and Welfare in Japan.

REFERENCES

Barker N, Clevers H (2006) Mining the Wnt pathway for cancer therapeutics. *Nat Rev Drug Discov* **5:** 997–1014

Baron S, Dianzani F (1994) The interferons: a biological system with therapeutic potential in viral infections. *Antiviral Res* **24:** 97–110

Branda M, Wands JR (2006) Signal transduction cascades and hepatitis B and C related hepatocellular carcinoma. *Hepatology* **43:** 891–902

Breuhahn K, Baeuerle PA, Peters M, Prang N, Tox U, Kohne-Volland R, Dries V, Schirmacher P, Leo E (2006) Expression of epithelial cellular adhesion molecule (Ep-CAM) in chronic (necro-)inflammatory liver diseases and hepatocellular carcinoma. *Hepatol Res* **34:** 50–56

Calvano SE, Xiao W, Richards DR, Felciano RM, Baker HV, Cho RJ, Chen RO, Brownstein BH, Cobb JP, Tschoeke SK, Miller-Graziano C, Moldawer LL, Mindrinos MN, Davis RW, Tompkins RG, Lowry SF (2005) A network-based analysis of systemic inflammation in humans. *Nature* **437:** 1032–1037

Chang HW, Roh JL, Jeong EJ, Lee SW, Kim SW, Choi SH, Park SK, Kim SY (2008) Wnt signaling controls radiosensitivity via cyclooxygenase-2-mediated Ku expression in head and neck cancer. *Int J Cancer* **122:** 100–107

Damdinsuren B, Nagano H, Kondo M, Natsag J, Hanada H, Nakamura M, Wada H, Kato H, Marubashi S, Miyamoto A, Takeda Y, Umeshita K, Dono K, Monden M (2006) TGF-beta1-induced cell growth arrest and partial differentiation is related to the suppression of Id1 in human hepatoma cells. *Oncol Rep* **15:** 401–408

Damdinsuren B, Nagano H, Wada H, Noda T, Natsag J, Marubashi S, Miyamoto A, Takeda Y, Umeshita K, Doki Y, Dono K, Monden M (2007) Interferon alpha receptors are important for antiproliferative effect of interferon-alpha against human hepatocellular carcinoma cells. *Hepatol Res* **37:** 77–83

de La Coste A, Romagnolo B, Billuart P, Renard CA, Buendia MA, Soubrane O, Fabre M, Chelly J, Beldjord C, Kahn A, Perret C (1998) Somatic mutations of the beta-catenin gene are frequent in mouse and human hepatocellular carcinomas. *Proc Natl Acad Sci USA* **95:** 8847–8851

Eguchi H, Nagano H, Yamamoto H, Miyamoto A, Kondo M, Dono K, Nakamori S, Umeshita K, Sakon M, Monden M (2000) Augmentation of antitumor activity of 5-fluorouracil by interferon alpha is associated with up-regulation of p27Kip1 in human hepatocellular carcinoma cells. *Clin Cancer Res* **6:** 2881–2890

Gastl G, Spizzo G, Obrist P, Dunser M, Mikuz G (2000) Ep-CAM overexpression in breast cancer as a predictor of survival. *Lancet* **356:** 1981–1982

Giles Jr RC, Tramontin R, Kadel WL, Whitaker K, Miksch D, Bryant DW, Fayer R (1980) Sarcocystosis in cattle in Kentucky. *J Am Vet Med Assoc* **176:** 543–548

Gutterman JU (1994) Cytokine therapeutics: lessons from interferon alpha. *Proc Natl Acad Sci USA* **91:** 1198–1205

Herlyn M, Steplewski Z, Herlyn D, Koprowski H (1979) Colorectal carcinoma-specific antigen: detection by means of monoclonal antibodies. *Proc Natl Acad Sci USA* **76:** 1438–1442

Jamieson CH, Ailles LE, Dylla SJ, Muijtjens M, Jones C, Zehnder JL, Gotlib J, Li K, Manz MG, Keating A, Sawyers CL, Weissman IL (2004) Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. *N Engl J Med* **351:** 657–667

Kittaka N, Takemasa I, Takeda Y, Marubashi S, Nagano H, Umeshita K, Dono K, Matsubara K, Matsuura N, Monden M (2008) Molecular mapping of human hepatocellular carcinoma provides deeper biological insight from genomic data. *Eur J Cancer* **44:** 885–897

Klaus A, Birchmeier W (2008) Wnt signalling and its impact on development and cancer. *Nat Rev Cancer* **8:** 387–398

Komori T, Takemasa I, Yamasaki M, Motoori M, Kato T, Kikkawa N, Kawaguchi N, Ikeda M, Yamamoto H, Sekimoto M, Matsubara K, Matsuura N, Monden M (2008) Gene expression of colorectal cancer: Preoperative genetic diagnosis using endoscopic biopsies. *Int J Oncol* **32:** 367–375

Kondo M, Nagano H, Wada H, Damdinsuren B, Yamamoto H, Hiraoka N, Eguchi H, Miyamoto A, Yamamoto T, Ota H, Nakamura M, Marubashi S, Dono K, Umeshita K, Nakamori S, Sakon M, Monden M (2005) Combination of IFN-alpha and 5-fluorouracil induces apoptosis through IFN-alpha/beta receptor in human hepatocellular carcinoma cells. *Clin Cancer Res* **11:** 1277–1286

Kondo M, Yamamoto H, Nagano H, Okami J, Ito Y, Shimizu J, Eguchi H, Miyamoto A, Dono K, Umeshita K, Matsuura N, Wakasa K, Nakamori S, Sakon M, Monden M (1999) Increased expression of COX-2 in nontumor liver tissue is associated with shorter disease-free survival in patients with hepatocellular carcinoma. *Clin Cancer Res* **5:** 4005–4012

Kurokawa Y, Matoba R, Nagano H, Sakon M, Takemasa I, Nakamori S, Dono K, Umeshita K, Ueno N, Ishii S, Kato K, Monden M (2004a) Molecular prediction of response to 5-fluorouracil and interferon-alpha combination chemotherapy in advanced hepatocellular carcinoma. *Clin Cancer Res* **10:** 6029–6038

Kurokawa Y, Matoba R, Takemasa I, Nagano H, Dono K, Nakamori S, Umeshita K, Sakon M, Ueno N, Oba S, Ishii S, Kato K, Monden M (2004b) Molecular-based prediction of early recurrence in hepatocellular carcinoma. *J Hepatol* **41:** 284–291

Kusaba M, Nakao K, Goto T, Nishimura D, Kawashimo H, Shibata H, Motoyoshi Y, Taura N, Ichikawa T, Hamasaki K, Eguchi K (2007) Abrogation of constitutive STAT3 activity sensitizes human hepatoma cells to TRAIL-mediated apoptosis. *J Hepatol* **47:** 546–555

Litvinov SV, Velders MP, Bakker HA, Fleuren GJ, Warnaar SO (1994) Ep-CAM: a human epithelial antigen is a homophilic cell-cell adhesion molecule. *J Cell Biol* **125:** 437–446

Miyamoto A, Umeshita K, Sakon M, Nagano H, Eguchi H, Kishimoto S, Dono K, Nakamori S, Gotoh M, Monden M (2000) Advanced hepatocellular carcinoma with distant metastases, successfully treated by a combination therapy of alpha-interferon and oral tegafur/uracil. *J Gastroenterol Hepatol* **15:** 1447–1451

Moon RT, Kohn AD, De Ferrari GV, Kaykas A (2004) WNT and beta-catenin signalling: diseases and therapies. *Nat Rev Genet* **5:** 691–701

Motoori M, Takemasa I, Doki Y, Saito S, Miyata H, Takiguchi S, Fujiwara Y, Yasuda T, Yano M, Kurokawa Y, Komori T, Yamasaki M, Ueno N, Oba S, Ishii S, Monden M, Kato K (2006) Prediction of peritoneal metastasis in advanced gastric cancer by gene expression profiling of the primary site. *Eur J Cancer* **42:** 1897–1903

Motoori M, Takemasa I, Yano M, Saito S, Miyata H, Takiguchi S, Fujiwara Y, Yasuda T, Doki Y, Kurokawa Y, Ueno N, Oba S, Ishii S, Monden M, Kato K (2005) Prediction of recurrence in advanced gastric cancer patients after curative resection by gene expression profiling. *Int J Cancer* **114:** 963–968

Nagano H, Miyamoto A, Wada H, Ota H, Marubashi S, Takeda Y, Dono K, Umeshita K, Sakon M, Monden M (2007a) Interferon-alpha and 5-fluorouracil combination therapy after palliative hepatic resection in patients with advanced hepatocellular carcinoma, portal venous tumor thrombus in the major trunk, and multiple nodules. *Cancer* **110:** 2493–2501

Nagano H, Sakon M, Eguchi H, Kondo M, Yamamoto T, Ota H, Nakamura M, Wada H, Damdinsuren B, Marubashi S, Miyamoto A, Takeda Y, Dono K, Umeshit K, Nakamori S, Monden M (2007b) Hepatic resection followed by IFN-alpha and 5-FU for advanced hepatocellular carcinoma with tumor thrombus in the major portal branch. *Hepatogastroenterology* **54:** 172–179

Nakamura M, Nagano H, Sakon M, Yamamoto T, Ota H, Wada H, Damdinsuren B, Noda T, Marubashi S, Miyamoto A, Takeda Y, Umeshita K, Nakamori S, Dono K, Monden M (2007) Role of the Fas/FasL pathway in combination therapy with interferon-alpha and fluorouracil against hepatocellular carcinoma *in vitro*. *J Hepatol* **46:** 77–88

Obi S, Yoshida H, Toune R, Unuma T, Kanda M, Sato S, Tateishi R, Teratani T, Shiina S, Omata M (2006) Combination therapy of intraarterial 5-fluorouracil and systemic interferon-alpha for advanced hepatocellular carcinoma with portal venous invasion. *Cancer* **106:** 1990–1997

Ogawa M, Yamamoto H, Nagano H, Miyake Y, Sugita Y, Hata T, Kim BN, Ngan CY, Damdinsuren B, Ikenaga M, Ikeda M, Ohue M, Nakamori S, Sekimoto M, Sakon M, Matsuura N, Monden M (2004) Hepatic expression of ANG2 RNA in metastatic colorectal cancer. *Hepatology* **39:** 528–539

Ohigashi T, Mizuno R, Nakashima J, Marumo K, Murai M (2005) Inhibition of Wnt signaling downregulates Akt activity and induces chemosensitivity in PTEN-mutated prostate cancer cells. *Prostate* **62:** 61–68

Oken MM, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, Carbone PP (1982) Toxicity and response criteria of the Eastern Cooperative Oncology Group. *Am J Clin Oncol* **5:** 649–655

Ota H, Nagano H, Sakon M, Eguchi H, Kondo M, Yamamoto T, Nakamura M, Damdinsuren B, Wada H, Marubashi S, Miyamoto A, Dono K, Umeshita K, Nakamori S, Wakasa K, Monden M (2005) Treatment of hepatocellular carcinoma with major portal vein thrombosis by combined therapy with

subcutaneous interferon-alpha and intra-arterial 5-fluorouracil; role of type 1 interferon receptor expression. *Br J Cancer* **93:** 557–564

Patt YZ, Yoffe B, Charnsangavej C, Pazdur R, Fischer H, Cleary K, Roh M, Smith R, Noonan CA, Levin B (1993) Low serum alpha-fetoprotein level in patients with hepatocellular carcinoma as a predictor of response to 5-FU and interferon-alpha-2b. *Cancer* **72:** 2574–2582

Reya T, Clevers H (2005) Wnt signalling in stem cells and cancer. *Nature* **434:** 843–850

Rhodes DR, Chinnaiyan AM (2005) Integrative analysis of the cancer transcriptome. *Nat Genet* **37**(Suppl): S31–S37

Sakon M, Nagano H, Dono K, Nakamori S, Umeshita K, Yamada A, Kawata S, Imai Y, Iijima S, Monden M (2002) Combined intraarterial 5-fluorouracil and subcutaneous interferon-alpha therapy for advanced hepatocellular carcinoma with tumor thrombi in the major portal branches. *Cancer* **94:** 435–442

Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH (2004) Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med* **10:** 55–63

Wada H, Nagano H, Yamamoto H, Arai I, Ota H, Nakamura M, Damdinsuren B, Noda T, Marubashi S, Miyamoto A, Takeda Y, Umeshita K, Doki Y, Dono K, Nakamori S, Sakon M, Monden M (2007) Combination therapy of interferon-alpha and 5-fluorouracil inhibits tumor angiogenesis in human hepatocellular carcinoma cells by regulating vascular endothelial growth factor and angiopoietins. *Oncol Rep* **18:** 801–809

Woodward WA, Chen MS, Behbod F, Alfaro MP, Buchholz TA, Rosen JM (2007) WNT/beta-catenin mediates radiation resistance of mouse mammary progenitor cells. *Proc Natl Acad Sci USA* **104:** 618–623

Yamamoto T, Nagano H, Sakon M, Wada H, Eguchi H, Kondo M, Damdinsuren B, Ota H, Nakamura M, Wada H, Marubashi S, Miyamoto A, Dono K, Umeshita K, Nakamori S, Yagita H, Monden M (2004) Partial contribution of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/TRAIL receptor pathway to antitumor effects of interferon-alpha/5-fluorouracil against Hepatocellular Carcinoma. *Clin Cancer Res* **10:** 7884–7895

Yamashina K, Yamamoto H, Chayama K, Nakajima K, Kikuchi A (2006) Suppression of STAT3 activity by Duplin, which is a negative regulator of the Wnt signal. *J Biochem* **139:** 305–314

Yamashita T, Budhu A, Forgues M, Wang XW (2007) Activation of hepatic stem cell marker EpCAM by Wnt-beta-catenin signaling in hepatocellular carcinoma. *Cancer Res* **67:** 10831–10839

Yang W, Yan HX, Chen L, Liu Q, He YQ, Yu LX, Zhang SH, Huang DD, Tang L, Kong XN, Chen C, Liu SQ, Wu MC, Wang HY (2008) Wnt/beta-catenin signaling contributes to activation of normal and tumorigenic liver progenitor cells. *Cancer Res* **68:** 4287–4295

Yang XJ, Tan MH, Kim HL, Ditlev JA, Betten MW, Png CE, Kort EJ, Futami K, Furge KA, Takahashi M, Kanayama HO, Tan PH, Teh BS, Luan C, Wang K, Pins M, Tretiakova M, Anema J, Kahnoski R, Nicol T, Stadler W, Vogelzang NG, Amato R, Seligson D, Figlin R, Belldegrun A, Rogers CG, Teh BT (2005) A molecular classification of papillary renal cell carcinoma. *Cancer Res* **65:** 5628–5637

Zembutsu H, Ohnishi Y, Tsunoda T, Furukawa Y, Katagiri T, Ueyama Y, Tamaoki N, Nomura T, Kitahara O, Yanagawa R, Hirata K, Nakamura Y (2002) Genome-wide cDNA microarray screening to correlate gene expression profiles with sensitivity of 85 human cancer xenografts to anticancer drugs. *Cancer Res* **62:** 518–527

Ann Surg Oncol (2009) 16:2058–2064
DOI 10.1245/s10434-009-0476-7

Annals of
SURGICAL ONCOLOGY
OFFICIAL JOURNAL OF THE SOCIETY OF SURGICAL ONCOLOGY

ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Dickkopf-1 Expression as a Marker for Predicting Clinical Outcome in Esophageal Squamous Cell Carcinoma

Tomoki Makino, MD[1], Makoto Yamasaki, MD[1], Ichiro Takemasa, MD[1], Atsushi Takeno, MD[1], Yurika Nakamura, PhD[1], Hiroshi Miyata, MD[1], Shuji Takiguchi, MD[1], Yoshiyuki Fujiwara, MD[1], Nariaki Matsuura, MD[2], Masaki Mori, MD[1], and Yuichiro Doki, MD[1]

[1]Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan; [2]Department of Molecular Pathology, School of Allied Health Science, Faculty of Medicine, Osaka University, Suita, Osaka, Japan

ABSTRACT

Background and Objectives. Dickkopf-1 (DKK1) is the inhibitor of the canonical Wnt signaling pathway, however it is highly transactivated in various cancers, suggesting the presence of unknown mechanism. Its implication in human esophageal squamous cell carcinoma (ESCC) has not been sufficiently investigated.

Patients and Methods. We evaluated DKK1 protein expression in resected specimens from 170 patients with ESCC by immunohistochemistry. Tumors were categorized as positive or negative for DKK1. The relationships between DKK1 expression in ESCC and various clinicopathological parameters and prognosis (disease-free survival; DFS) were analyzed separately.

Results. Immunohistochemically, 72 (42.4%) tumors were DKK1 positive while no significant staining was observed in the normal squamous epithelium except for few basal cells. There was no significant relationship between DKK1 expression in ESCC and any of the clinicopathological parameters tested in this study. Patients with DKK1-positive tumors had poorer DFS than those with negative ESCC (5-year DFS; 31.5% versus 53.6%, $P = 0.0062$). Univariate analysis showed a significant relationship between pT [hazard ratio (HR) = 2.944, 95% confidence interval (CI) = 1.713–5.059, $P < 0.0001$], number of pN (HR = 2.836, 95% CI = 1.866–4.309, $P < 0.0001$), lymphatic invasion (HR = 2.892, 95% CI = 1.336–6.262, $P = 0.0070$), and DKK1 expression (HR = 1.763, 95% CI = 1.167–2.663, $P = 0.0071$) and DFS. Multivariate analysis including the above four parameters identified pT (HR = 2.053, 95% CI = 1.157–3.645, $P = 0.0140$), pN number (HR = 2.107, 95% CI = 1.362–3.260, $P = 0.0008$), and DKK1 expression (HR = 1.813, 95% CI = 1.195–2.751, $P = 0.0052$) as independent and significant prognostic factors for DFS.

Conclusion. Our data suggest the usefulness of DKK1 as a novel predictor of poor prognosis of patients with ESCC after curative resection and also as a therapeutic target for future tailored therapies against ESCC.

Esophageal squamous cell carcinoma (ESCC), the major histological form of esophageal cancer in East Asian countries, is one of the most lethal malignancies of the digestive tract and, in most cases, the initial diagnosis is made when malignancy is in the advanced stage.[1] In spite of recent improvements in multitreatment modalities, including surgical techniques, radiotherapy, and chemotherapy, the prognosis of patients with ESCC is still unsatisfactory.[2] Assessment of prognosis through clinicopathological features remains inadequate even when using the staging systems of tumor–node–metastasis (TNM) classification because of the considerable variability and heterogeneity within the same stage.[3] Therefore, there is a need to identify novel biological markers that allow a more accurate identification of high-risk population of recurrent disease and help in the design of appropriate treatment strategies for ESCC patients.

Dickkopf-1 (German for "big head, stubborn"), also known as DKK-1, is a secreted protein involved in embryonic development and known as a potent inhibitor of the Wnt signaling pathway, which enables appropriate

First Received: 9 December 2008;
Published Online: 30 April 2009

M. Yamasaki, MD
e-mail: myamasaki@gesurg.med.osaka-u.ac.jp

positioning and development of the embryonic brain and other organ structures.[4–6] Specifically, Wnt-1 protein binds to the frizzled receptor (Fz) and the low-density lipoprotein receptor-related protein-5/6 (LRP5/6), triggering signals important for proliferation via β-catenin.[7] DKK1 binds to LRP5/6 and blocks interaction with Wnt-1, resulting in β-catenin degradation and retardation of proliferation.[8] In contrast to other Wnt/β-catenin signaling antagonists, DKK1 is reported to be overexpressed in many malignant tissues including breast cancer, lung cancer, esophageal carcinomas, ovarian endometrioid adenocarcinomas, multiple myeloma, Wilms' tumor, hepatoblastoma, and hepatocellular carcinoma (HCC), indicating negative feedback of Wnt/β-catenin signaling or the presence of unknown mechanism for DKK1, other than its association with chemo- or hormone sensitivity.[9–15] On the other hand, in human colon tumors, DKK1 expression decreases and DKK1 acts as a tumor suppressor gene where the DKK1 promoter is selectively hypermethylated, resulting in epigenetic silencing as observed in leukemia.[16–18] Thus, the expression and role of DKK1 might vary according to cancer location. The direct mechanisms in each type of cancer are in fact under investigation at present. Notwithstanding the above previous studies, there is little or no information on the clinical significance of DKK1 in ESCC, especially prognosis of patients with ESCC.

In the present retrospective study, we first conducted immunohistochemical (IHC) analysis of DKK1 protein expression in 170 resected specimens of ESCC and then determined its association with prognosis of patients with ESCC.

MATERIALS AND METHODS

Patients and Treatments

The present study involved 170 patients with histopathologically confirmed primary thoracic esophageal cancer who underwent surgical resection at our hospital between 1998 and 2007. They included 18 female and 152 male patients, with age ranging from 38 to 82 years (median 63.3 years). Subtotal esophagectomy via right thoracotomy with two- or three-field lymphadenectomy was performed in all patients. Curative resection (R0) was achieved in 162 patients (95.3%), while the outcome in the remaining 8 (4.7%) patients was noncurative resection (R1, 2). None of the patients died of postoperative complications. Ninety-five patients with lymph node metastasis at initial diagnosis received neoadjuvant chemotherapy (NACT), which consisted of two courses of 5-fluorouracil (5-FU), cisplatin (CDDP), and adriamycin (ADM).[19–22] We provided adjuvant chemotherapy (docetaxel or CDDP plus 5-FU regimen) to 11 patients with larger numbers of pathologically positive lymph nodes.[23]

After surgery, the patients were surveyed every 3 months by physical examination and serum tumor markers [squamous cell carcinoma (SCC) antigen, carcinoembryonic antigen (CEA)], every 6 months by computed tomography (CT) scan and abdominal ultrasonography, and every year by endoscopy until tumor recurrence was evident. Patients with tumor recurrence received chemotherapy or chemoradiotherapy, as long as their systemic condition permitted. Mean overall survival was 32.0 months and mean disease-free survival was 27.8 months. Mean follow-up period after surgery was 44.7 months.

Immunohistochemical Analysis

DKK1 protein accumulation was examined by IHC staining of formalin-fixed and paraffin-embedded ESCC tissue sections. One representative slide with the deepest tumor invasion was selected from each patient and subjected to immunohistochemistry as follows. Briefly, after deparaffinization in xylene and dehydration in graded ethanol solutions, endogenous peroxidase activity was blocked by incubation with 30 mL/L hydrogen peroxide for 20 min. Then tissue sections were heated at 95°C for 40 min in citrate buffer (0.05 mol/L, pH 6.0) for antigen retrieval. After incubation with rabbit polyclonal primary antibody DKK1 (Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1:60) for 2 h at room air temperature, staining was performed by labeled streptavidin–biotin (LSAB) method. Negative controls of immunohistochemical reactions included omission of the primary antibody. Normal human placenta was used as a positive control. DKK1 staining for each ESCC sample was judged positive when more than 10% of the cancer cells in the section were immunoreactive to DKK1, and otherwise negative when 10% or less of the cells were positive. All slides were assessed independently by two pathologists and then by conference in case of disagreement. Both pathologists were blinded to the clinicopathological data.

Statistical Analysis

Correlations between DKK1 expression and various clinicopathological parameters were each evaluated by the χ^2 test and Fisher's exact probability test. The association between continuous parameters was evaluated by Mann–Whitney's *U*-test. Prognostic variables were assessed by log-lank test, and disease-free survival (DFS) was analyzed by the method of Kaplan and Meier. Cox's proportional hazard regression model with stepwise comparisons was used to analyze the independent prognostic factors. With

TABLE 1 Patient characteristics ($n = 170$)

Parameters	Number of patients (%)
Age (year)	63.3 [38–82][a]
Gender (male/female)	152(89.4)/18(10.6)
Histology (well/mod/poor)[b]	41(24.1)/85(50.0)/44(25.9)
Location (upper/middle/lower)[c]	18(10.6)/81(47.6)/71(41.8)
Neoadjuvant chemotherapy (yes/no)	95(55.9)/75(44.1)
pT (0/1/2/3/4)[d]	0(0)/24(14.1)/29(17.1)/ 103(60.6)/14(8.2)
pN (N0/N1/M1lym)	53(31.2)/117(68.8)/42(24.7)
Number of pN (0/1–3/4–7/≥ 8)	50(29.4)/64(37.7)/ 24(14.1)/32(18.8)
pStage (0/I/II/III/IV)	0(0)/13(7.6)/60(35.3)/ 55(32.4)/42(24.7)

[a] Average and range

[b] Well-, moderately, and poorly differentiated squamous cell carcinoma

[c] Middle, lower, and upper thoracic esophagus

[d] pN, pT, pStage (pathological classification) and M1lym (distant lymph node metastasis) according to TNM classification

respect to survival analysis, we adopted four lymph nodes as the cutoff value based on the guidelines of the Japanese Society for Esophageal Diseases (JSED).[24] These analyses were carried out using SPSS version 10 (SPSS, Inc., Chicago, IL) for Windows. A P value of less than 0.05 denoted the presence of statistical significance.

RESULTS

DKK1 Expression in ESCC

A total of 170 samples (Table 1) that contained both cancerous and noncancerous lesions were evaluated for DKK1 protein expression by immunohistochemical analysis. Of these, 72 (42.4%) showed positive DKK1 expression, mainly in the cytoplasm of tumor cells, with faint nuclear staining (Fig. 1a), while the remaining 98 (57.6%) were negative (Fig. 1b). The positive staining was almost homogeneous at single cancer nest and among different areas (surface, central, and deepest areas) of the cancer lesion. In contrast, none of the normal squamous epithelium showed significant level of immunohistochemical staining, although some basal cells showed faint nuclear immunostaining (Fig. 1c). The grading of immunostained sections was almost identical by the two pathologists, with interobserver variation of less than 10%.

Correlation Between DKK1 Expression and Clinicopathological Parameters

Table 2 lists the correlations between DKK1 expression and various clinicopathological parameters. DKK1-positive tumors tended to be associated with larger number of pathologically positive lymph nodes compared with DKK1-

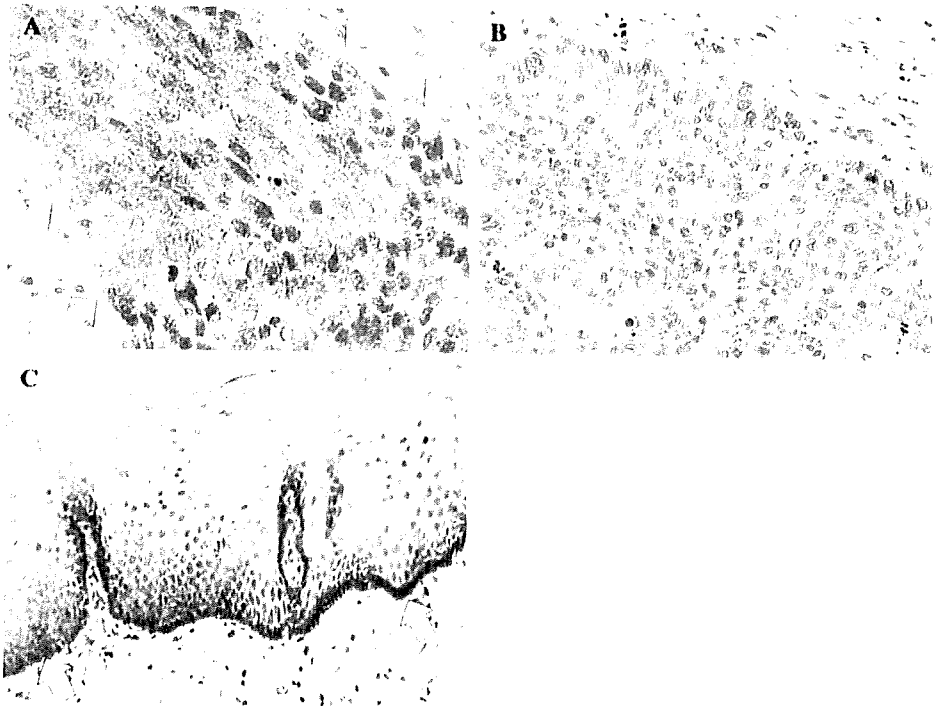

FIG. 1 DKK1 expression by immunohistochemical staining. **a** Representative DKK1-positive esophageal squamous cell carcinoma showing staining mainly in the cytoplasm of tumor cells (arrows) (magnification ×400). **b** Representative DKK1-negative esophageal squamous cell carcinoma showing almost no appreciable staining of tumor cells (magnification ×200). **c** Representative normal squamous epithelium negative for DKK1 expression except in a few basal cells (arrows) (magnification ×200)

negative tumors, although not statistically significantly so (3.4 versus 6.3, $P = 0.2199$). No significant correlations were observed with other parameters, including age, gender, tumor location, use of neoadjuvant chemotherapy, and pT and pStage (Table 2).

Correlation Between DKK1 Expression and Survival

Disease recurrence after curative resection was diagnosed in 83 (51.2%) of 162 patients with curative resection (R0) and the mean time to recurrence was 9.5 months. The total 5-year disease-free survival (5-year DFS) rate was 44.4%. Patients with DKK1-positive tumors showed poorer DFS than those with negative tumors (5-year DFS; 31.5% versus 53.6%, $P = 0.0062$) (Fig. 2a). Analysis of each subgroup showed that this trend was apparent in the advanced pT stage group (pT3/4) (5-year DFS; 19.1% versus 44.4%, $P = 0.0063$) and pN1 group (5-year DFS; 18.5% versus 46.7%, $P = 0.0021$), but not in early pT stage group (pT1/2) (5-year DFS; 63.4% versus 71.9%, $P = 0.5404$) or pN0 group (5-year DFS; 62.3% versus 66.7%, $P = 0.9673$). Similarly, there was a significant difference in prognosis of patients with DKK1-positive and DKK1-negative tumors, especially at pStage III (5-year DFS; 17.6% versus 55.1%, $P = 0.0122$), but not pStage I (5-year DFS; 100% versus 83.3%, P value not applicable), pStage II (5-year DFS; 37.7% versus 65.8%, $P = 0.1019$) or pStage IV (5-year DFS; 11.8% versus 27.8%, $P = 0.1140$) (Fig. 2b).

On univariate analysis, the relationships between pT (HR = 2.944, 95% CI = 1.713–5.059, $P < 0.0001$), number of pathologically positive lymph nodes (HR = 2.836, 95% CI = 1.866–4.309, $P < 0.0001$), lymphatic invasion (HR = 2.892, 95% CI = 1.336–6.262, $P = 0.0070$), and DKK1 expression (HR = 1.763, 95% CI = 1.167–2.663, $P = 0.0071$) and DFS were significant, but not between all other parameters tested (e.g., age, gender, histology, tumor location, and venous invasion) (Table 3). Neoadjuvant chemotherapy group, which mainly consisted of cN1 patients, tended to show unfavorable prognosis but without significance ($P = 0.0521$). Furthermore, the number of patients who received adjuvant chemotherapy ($n = 11$, 6.5%) was too small to evaluate its prognostic significance. Multivariate analysis using the above four parameters with statistical significance ($P < 0.05$) in univariate analysis identified that four or more pathological lymph node metastases ($pN \geq 4$) was the poorest prognostic factor (HR = 2.107, 95% CI = 1.362–3.260, $P = 0.0008$), followed by pathological tumor invasion (pT3,4) (HR = 2.053, 95% CI = 1.157–3.645, $P = 0.0140$) and positive DKK1 expression (HR = 1.813, 95% CI = 1.195–2.751, $P = 0.0052$) (Table 4).

TABLE 2 Correlation between DKK1 and various clinicopathological parameters

Parameters	DKK1 expression		
	Positive (%)	Negative (%)	P value
Age (years)			
<65	43 (45.3)	52 (54.7)	0.4359
≥65	29 (38.7)	46 (61.3)	
Gender			
Male	63 (41.4)	89 (58.6)	0.6151
Female	9 (50.0)	9 (50.0)	
Histopathology			
Well[a]	13 (31.7)	28 (68.3)	0.1467
Mod, poor	59 (45.7)	70 (54.3)	
Location			
Upper, middle[b]	44 (44.4)	55 (55.6)	0.5331
Lower	28 (39.4)	43 (60.6)	
Neoadjuvant chemotherapy			
Yes	40 (42.1)	55 (57.9)	>0.9999
No	32 (42.7)	43 (57.3)	
pT[c]			
T1	12 (50.0)	12 (50.0)	0.1748
T2	9 (31.0)	20 (69.0)	
T3	42 (40.8)	61 (59.2)	
T4	9 (64.3)	5 (35.7)	
Number of pN[d]	6.3	3.4	0.2199
pStage			
Stage I	7 (53.8)	6 (46.2)	0.3742
Stage II	21 (35.0)	39 (65.0)	
Stage III	27 (49.1)	28 (50.9)	
Stage IV	17 (40.5)	25 (59.5)	

[a] Well-, moderately, and poorly differentiated squamous cell carcinoma

[b] Middle, lower, and upper thoracic esophagus

[c] pT, pN, pStage (pathological classification) according to TNM classification

[d] Average

DISCUSSION

The present IHC study of DKK1 expression in 170 ESCC resected specimens showed positive cancer-cell DKK1 expression in 42.4% of the specimens with negative immunostaining in the normal squamous epithelium, apart from basal cells. DKK1 protein expression did not correlate with various clinicopathological parameters. With respect to prognosis, immunostaining of ESCC for DKK1 was a significant determinant of unfavorable prognosis, especially in patients with pStage III disease. In addition to DKK1-positive expression, multivariate analysis also identified pT and the numbers of pN as independent prognostic predictors for DFS. Therefore, immunostaining

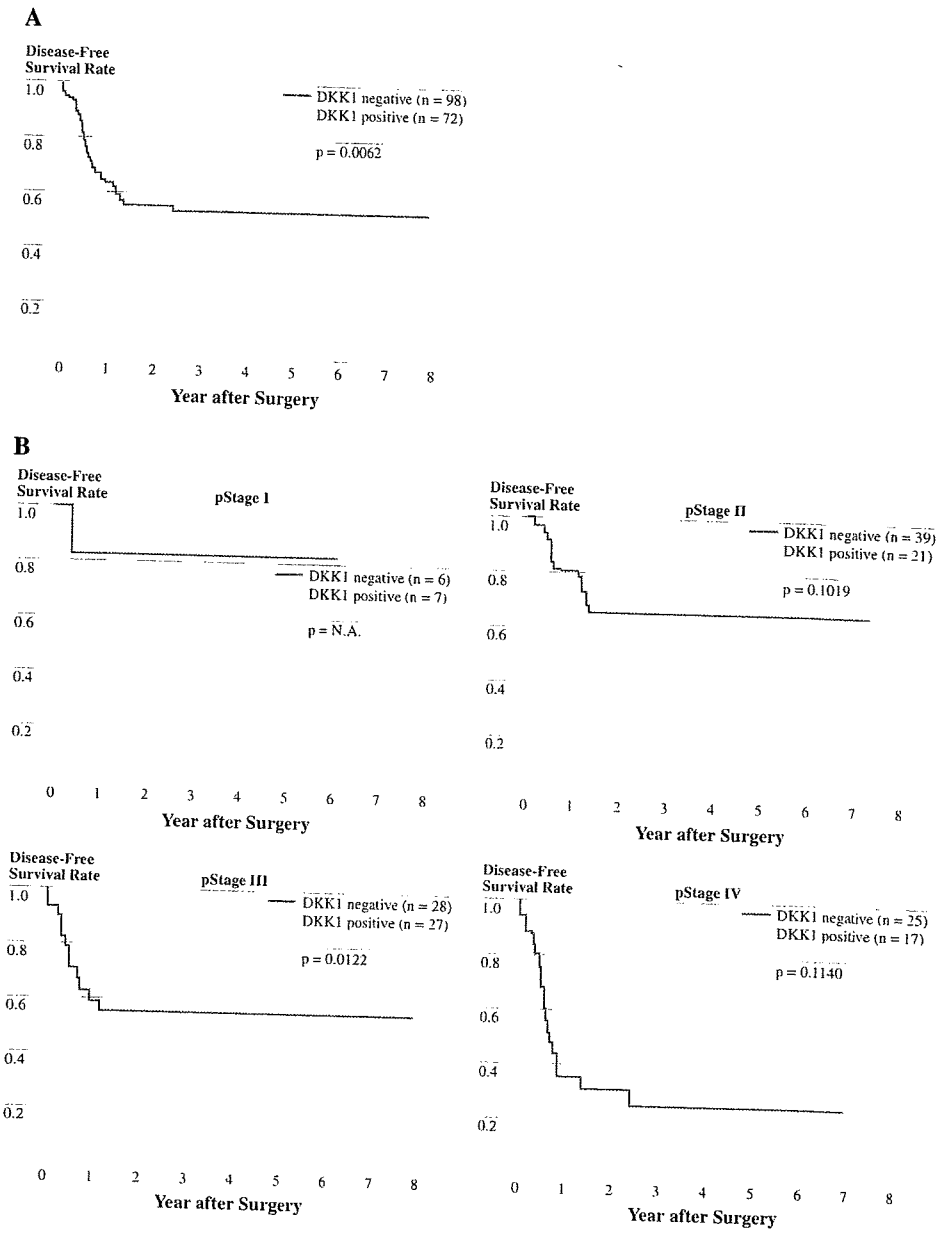

FIG. 2 Survival curves according to DKK1 expression. **a** Disease-free survival curve classified according to DKK1 expression for all patients plotted by Kaplan–Meier methods. **b** Disease-free survival curves according to DKK1 expression at each pathological stage. Differences between two groups were evaluated by log-rank test. Ordinate: disease-free survival rate, abscissa: time after surgery (years)

for DKK1 could be potentially considered a predictor of unfavorable prognosis of patients who undergo ESCC resection.

The human DKK-related gene family is composed of DKK-1, DKK-2, DKK-3, and DKK-4, together with a unique DKK-3-related protein termed Soggy (Sgy). hDKKs 1–4 contain two distinct cysteine-rich domains in which the positions of ten cysteine residues are highly conserved between family members.[25] Members of the human DKK-related family differ not only in their structures and expression patterns but also in their abilities to inhibit Wnt signaling, which is thought to regulate the proliferation and renewal of stem cells, whereas dysregulated activation of Wnt/β-catenin signaling has been implicated in carcinogenesis.[26] DKK1 is a 35-kDa protein that contains signal peptide sequence and functions as a negative regulator of the Wnt signaling through disruption of the complex formation of Wnt and its receptors, LRP5/6 and Fz receptor.[27] DKK1 is reported to play a crucial role in head formation in vertebrate development, although there is a big question mark on its role in cancer and whether it is similar to its known function in normal cells or embryogenesis.[4,8,25,28,29]

In human cancers, DKK1 is overexpressed in breast cancer, lung cancer, esophageal carcinomas, ovarian endometrioid adenocarcinomas, human hepatoblastomas, Wilms' tumors, HCC, and multiple myeloma.[9–14] However,

TABLE 3 Univariate survival analysis of disease-free survival by Cox's proportional hazard model

Parameters	Number of cases	hazard ratio	95% CI	*P*
Age (<65 years/≥65 years)	95/75	1.153	0.759–1.752	0.5056
Gender (male/female)	152/18	1.016	0.510–2.022	0.9646
Histology (mod-poor/well)[a]	127/43	1.677	0.974–2.889	0.0623
Location (lower/upper-middle)[b]	73/97	1.018	0.673–1.540	0.9333
Neoadjuvant chemotherapy (yes/no)	95/75	1.521	0.996–2.324	0.0521
pT (T3,4/T1,2)[c]	117/53	2.944	1.713–5.059	<0.0001
Number of pN (≥4/<4)	56/114	2.836	1.866–4.309	<0.0001
Lymphatic invasion (present/absent)	145/25	2.892	1.336–6.262	0.0070
Venous invasion (present/absent)	88/82	1.251	0.827–1.892	0.2882
DKK1 expression (positive/negative)	72/98	1.763	1.167–2.663	0.0071

CI confidence interval

[a] Well-, moderately, and poorly differentiated squamous cell carcinoma

[b] Middle, lower, and upper thoracic esophagus

[c] pT and pN based on TNM classification

TABLE 4 Multivariate analysis of disease-free survival by Cox's proportional hazard model

Parameters	Number of cases	HR	95% CI	*P*
pT (T3,4/T1,2)	117/53	2.053	1.157–3.645	0.0140
Number of pN (≥4/<4)	56/114	2.107	1.362–3.260	0.0008
Lymphatic invasion (present/absent)	145/25	2.055	0.925–4.565	0.0770
DKK1 expression (positive/negative)	72/98	1.813	1.195–2.751	0.0052

For abbreviations, see Table 3

Gonzalez-Sancho et al. reported the loss of DKK1 expression in colon cancer, suggesting that colon carcinogenesis involves the removal of the inhibitory effect of DKK1 on the Wnt/β-catenin pathway.[16] Only a few studies reported the clinical or prognostic significance of DKK1. Forget et al. found preferential expression of DKK1 in hormone-resistant breast tumors, which was associated with poor prognosis.[9] Yamabuki et al. examined the possible role of DKK1 as a serologic and prognostic biomarker for lung and esophageal carcinomas.[10] They evaluated DKK1 expression by IHC using tumor tissue microarrays and suggested its association with poor prognosis for ESCC, although they failed to identify it as an independent prognostic factor. They also showed experimentally that exogenous expression of DKK1 increased the migration/invasion activity of mammalian cells, suggesting a significant role for DKK1 in progression of human cancer. Considered together, the above results and our findings suggest that DKK1 expression seems to play an important role in the development and/or progression of certain types of human tumors including ESCC, although the link between DKK1 and Wnt signaling pathway in the context of cancer progression or carcinogenesis remains under investigation at present.

With respect to the results of survival analysis, our survival data by pathological stage based on TNM classification were comparable to those of previous reports on ESCC in Japan.[3] The 5-year DFS of pStage III DKK1-negative tumors determined in the present study was 55.1%, which was equivalent to 56.8% of DFS for pStage II tumors in the above study. On the other hand, the 5-year DFS of pStage III DKK1-positive tumors in our study was 17.6%, which was similar or rather worse than that of 20.4% of pStage IV tumors. Therefore, for prediction of prognosis, especially for patients with pStage III tumors, it might be useful to integrate DKK1 expression level with the pathological TNM classification although other stages, especially stage IV, had too small samples to detect a prognostic significance of DKK1 expression.[3] Furthermore DKK1 expression could be a valuable guide for treatment strategy of ESCC. Patients with pStage III, pT3/4 or pN1 DKK1-positive tumors, who are highly likely to show disease recurrence, should receive chemotherapy and be followed up closely; a second line taxan-based chemotherapy for nonresponders to NACT (or the same protocol as NACT for responders) could be an effective postoperative chemotherapy. It should be noted, however, that one cannot apply our results in ESCC to adenocarcinomas of the esophagus, which is the commonest histopathological type in Western countries, because this study involved only analysis of squamous cell carcinoma, which is common in East Asian countries.

In conclusion, DKK1 expression as determined immunohistochemically was significantly associated with poor prognosis of patients with ESCC. However, because Wnt signaling pathway alone could not provide an explanation for the prognostic significance of DKK1, further studies should evaluate the potential mechanisms of increased DKK1 expression and poor prognosis. Nevertheless, we hope the findings of this study open the door for exploration of efficacious treatment strategies and development of new therapeutic approaches, such as antibody therapy or functional inhibition of expression, for ESCC.