Molecular background of α-fetoprotein in liver cancer cells as revealed by global RNA expression analysis

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α-Fetoprotein (AFP) is considered to be a diagnostic and prognostic biomarker in hepatocellular carcinoma (HCC). However, the role of AFP in the development of HCC is presently obscure. We hypothesized that a certain set of genes is expressed in a manner coordinate with AFP, and that these genes essentially contribute to the malignant characteristics of AFP-producing HCC. To address this hypothesis, we carried out global mRNA expression analysis of 21 liver cancer cell lines that produce varying levels of AFP. We identified 213 genes whose mRNA expression levels were significantly correlated with that of AFP ($P < 0.0001$). These included liver-specific transcription factors for AFP and other albumin family genes. Eighteen HCC-associated genes and 11 genes associated with malignancies other than HCC showed significant correlations with AFP production levels. Genes involved in lipid catabolism, blood coagulation, iron metabolism, angiogenesis, and the Wnt and mitogen-activated protein kinase pathways were also identified. Text data mining revealed that participation in the transcription factor network could explain the connection between 78 of the identified genes. Glypican 3, which is a component of the Wnt pathway and contributes to HCC development, had the fifth highest correlation coefficient with AFP. Reactivity to specific antibodies confirmed the significant correlation between AFP and glypican 3 expression in HCC tissues. These observations suggest that AFP-producing liver cancer cells may have a unique molecular background consisting of cancer-associated genes. From this genome-wide association study, novel aspects of the molecular background of AFP were revealed, and thus may lead to the identification of novel biomarker candidates. (*Cancer Sci* 2008; 99: 2402–2409)

Hepatocellular carcinoma (HCC) is one of the most common and aggressive malignancies worldwide and is the third leading cause of cancer death.[1] It is a major health problem with high prevalence in Asia and Africa,[2,3] and recent studies indicated that the incidence of HCC has increased substantially in the USA and UK over the last three decades.[4,5] The prognosis for HCC patients remains dismal at present, and novel diagnostic modalities as well as improvement of the therapeutic strategies currently in use are required to improve the clinical outcome for HCC patients.

Altered α-fetoprotein (AFP) level is a hallmark of HCC development;[6] a considerable proportion of HCC patients have elevated plasma AFP, and diagnostic value of AFP was suggested in the patients with liver cirrhosis.[7] Plasma AFP is a useful prognostic indicator, as the median survival rate of HCC patients with markedly elevated AFP is significantly shorter than that of patients with normal or moderately elevated AFP.[8] Preoperative AFP levels are predictive of HCC recurrence,[9,10] and may therefore be used in deciding therapeutic options for HCC patients after surgery. The lens culinaris agglutinin-reactive fraction of AFP, in particular, has been shown to be significantly associated with portal vein invasion and poor clinical outcomes.[11] AFP has been shown to function as a superoxide dismutase[12] and as an apoptotic factor,[13–15] and to directly promote proliferation in cultured cells.[16–20] Nevertheless, the molecular background of HCC associated with increased AFP levels in HCC patients and the mechanisms underlying the association of AFP with the onset of HCC and poor prognosis are presently unclear.

We hypothesized that a certain set of genes are expressed in a manner coordinate with AFP, and that these genes are responsible for the greater tumor size, portal vein thrombosis, and lack of histological differentiation that are observed in HCC tumors with higher AFP expression.[8] We have previously reported that the expression levels of 11 proteins correlate highly with that of AFP.[21] In the present study, we generated gene expression profiles of 21 liver cancer cell lines using DNA microarrays and investigated the genes whose expression level correlated significantly with AFP mRNA levels. The functional properties of the identified genes and their association with each other at the transcription level were examined using a text data mining program. The correlation or otherwise of AFP expression with the product of each identified gene was validated in HCC tissues using specific antibodies.

Materials and Methods

Cell lines. The following 21 cell lines were used: HuH-7, JHH-7, JHH-5, HepG2, HT17, HuH-1, Hep3B, Li-7, PLC/PRL/5, KIM-1, KYN-2, HLE, HLF, JHH-4, JHH-6, SK-Hep-1, KYN-3, PH5-CH, PH5-T, RBE, and SSP-25. Details of these cell lines are summarized in our previous proteomics report.[21]

Clinical specimens. HCC tissues were obtained from 23 HCC patients at the time of surgery, fixed in formalin, and embedded in paraffin. The project was approved by the institute's ethical committee and written informed consent for the use of the tissues for research purposes was obtained from the donors.

Western blotting. Cellular proteins were extracted from the cell lines using a urea lysis buffer (6 mol/L urea, 2 mol/L thiourea, 3% CHAPS, 1% Triton X-100), and 30 μg protein was separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis with an e-PAGE system (ATTO, Tokyo, Japan) as described previously.[21] Immunoblot analysis was carried out using primary antibodies against AFP (1:200 dilution, clone ZSA06; Zymed Laboratories, South San Francisco, CA, USA) and β-actin (1:1000 dilution, clone AC-15; Sigma, St Louis, MO, USA), peroxidase-conjugated secondary antibody (1:1000 dilution; GE Healthcare, Uppsala, Sweden), and enhanced chemiluminescence (GE Healthcare). The enhanced chemiluminescence signal was monitored using Fuji LAS-1000 (Fuji Film, Tokyo, Japan) and measured with ImageQuant TL (GE Healthcare).

Gene expression analysis. For gene expression analysis, total RNA was prepared from the 21 cell lines using an RNeasy mini

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kit (Qiagen, Hilden, Germany). The integrity of the purified RNA was confirmed using 2100 Bioanalyzer and an RNA 6000 nano LabChip kit (Agilent Technologies, Santa Clara, CA, USA). The DNA microarray used was a Human Genome U133 plus 2.0 array (Affymetrix, Santa Clara, CA, USA). Target cRNA was prepared from 1 μg of the purified RNA with a one-cycle cDNA synthesis kit and 3′-amplification reagents for *in vitro* transcription amplification and biotin-labeling (Affymetrix). Hybridization to the microarrays, washing, staining with the antibody amplification procedure, and scanning were carried out according to the manufacturers' instructions. The scanned image data were processed using the GeneChip Operating Software (version 1.4; Affymetrix). The signal expression value of each probe set was calculated and normalized by setting the signal value mean for each experiment to 100 so that minor differences between the experiments were adjusted. Among the 54 675 probes on the DNA microarray, we first selected 12 091 probes based on MicroArray Quality Control Project database analysis,[22] in which the intraplatform and interplatform reproducibility was examined for each gene using individual RNA samples and quantitative reverse transcription–polymerase chain reaction (RT-PCR). The 12 901 probes corresponded to 12 901 unique genes assigned to NCBI Entrez Gene ID numbers. The data were log-transformed (base 2) to produce a closer to normal distribution for statistical analysis.

To measure the similarity of gene expression profiles between AFP and other genes, we used Pearson's correlation coefficient r_{ij} as follows:

$$r_{ij} = \frac{\sum_{k=1}^{m}\left(p_{ik}-\overline{p}_i\right)\left(p_{jk}-\overline{p}_j\right)}{\sqrt{\sum_{k=1}^{m}\left(p_{ik}-\overline{p}_i\right)^2\sum_{k=1}^{m}\left(p_{jk}-\overline{p}_j\right)^2}},$$

where m is the number of observations, p_i is the AFP expression profile, p_j is the expression profile of the gene in question, and P_i is the arithmetic mean of p_i over m observations. We used the z-transforms of the observed correlation coefficients, calculated as follows:

$$z_{ij} = \frac{1}{2}\ln\frac{1+|r_{ij}|}{1-|r_{ij}|}.$$

The Z-statistic approximately follows the standard normal distribution:[23]

$$Z = \frac{z_{ij}}{\sqrt{1/(m-3)}}$$

We tested the observed correlation coefficients statistically under the null hypothesis that H0: $r_{ij} = 0$, with the significance level set at $\alpha/2$, that is, we rejected the null hypothesis if $Z > Z_{\alpha/2}$. The significance level was set at $\alpha = 0.0001$ in consideration for multiple tests.

Quantitative RT-PCR. cDNA was generated from mRNA using the SuperScript III kit (Invitrogen, Carlsbad, CA, USA). Quantitative amplification was carried out using the 7500 Real-time PCR system (Applied Biosystems) and was monitored with TaqMan Gene Expression Assays using premade primers, human glyceraldehydes-3-phosphate dehydrogenase (GAPDH), and TaqMan Universal PCR Master Mix according to the manufacturer's instructions (Applied Biosystems). All experiments were carried out in triplicate. The following 16 cancer-associated genes were examined: *AFP*, glypican 3 (*GPC3*), thrombopoetin (*THPO*), S100 calcium binding protein P (*S100P*), meprin Aα (*MEP1A*), prospero-related homeobox 1 (*PROX1*), frequently rearranged in advanced T-cell lymphomas 2 (*FRAT2*), carcinoembryonic antigen-related cell adhesion molecule 1 (*CEACAM1*), frequently rearranged in advanced T-cell lymphomas (*FRAT1*), v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (*ERBB3*), α2-HS-glucoprotein (*AHSG*), v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*), suppression of tumorigenicity (*ST7*), visinin-like-1 (*VSNL1*), regucalcin (*RGN*), and secretagogin EF-hand calcium binding protein (*SCGN*).

Text data mining. To find the transcriptional regulation network of the identified genes, we carried out text data mining using MetaCore (GeneGo, Saint Joseph, MI, USA; http://www.genego.com). Dijkstra's shortest path algorithms were first calculated using a prefilter based on tissue type (fetal or non-fetal liver).(24) Genes involved in transcriptional regulation were then extracted from the networks. Finally, genes not involved in the networks were excluded and a transcriptional regulation network of AFP-related genes was obtained.

Immunohistochemical study. Immunohistochemical staining for AFP and GPC3 was carried out using an automated immunohistochemical stainer according to the manufacturer's protocol (Envision; Dako Cytomation, Glostrup, Denmark). Serial sections of formalin-fixed, paraffin-embedded tissues (4 μm thick) were placed on silane-coated slides. Sections with the maximum tumor diameter were selected for immunohistochemical evaluation. A polyclonal antibody against AFP (rabbit, 1:100 dilution; Dako Cytomation) and an antibody against GPC3 (clone 1G12, 1:2000 dilution; BioMosaics, Burlington, VT, USA) were used. All sections were evaluated by H.O. and T.K. without knowledge of any clinical or pathological information; cases for which consensus was not reached were re-evaluated using a dual-headed microscope. AFP and GPC3 expression in the sections was scored as follows: negative, no membranous or cytoplasmic expression in the cancer cells; positive 1+, membranous and/or cytoplasmic expression observed in less than 50% of cancer cells; 2+, membranous and/or cytoplasmic expression in >50% of cancer cells.

Results

Expression of AFP in the liver cancer cell lines. We examined the AFP expression levels in 21 liver cancer cell lines using western blotting, DNA microarrays, and quantitative RT-PCR (Fig. 1a). AFP measured by DNA microarrays and quantitative RT-PCR showed consistent expression at the mRNA level in the 21 cell lines (r3 = 0.763). AFP expression at the protein level was concordant with that at the mRNA level as measured by DNA microarrays (r1 = 0.613) and quantitative RT-PCR (r2 = 0.546) (Fig. 1b). To examine the genes that had expression patterns similar to AFP, we used DNA microarray data because western blotting and quantitative RT-PCR do not generate gene expression data in a genome-wide manner. The presence of a significant correlation between western blotting data, DNA microarray data, and quantitative RT-PCR data suggested that the use of microarray data for measuring gene expression is quite acceptable.

Genes associated with AFP expression. From an initial DNA microarray data set consisting of 12 091 genes, we used MicroArray Quality Control criteria to select the 213 genes whose expression level significantly correlated with that of AFP ($P < \alpha = 0.0001$) (Supporting Table S1). The correlation coefficient value of the selected genes was at least 0.724980 (Supporting Table S1). The selected genes included *hepatocyte nuclear factor 4, alpha (HNF4A)*, *transcription factor 1 (TCF1)* and *forkhead box A3 (FOXA3)*, the transcription factors for AFP (Table 1; Supporting Table S2). Expression of the albumin family of genes, which is regulated by the same transcription factors as AFP, such as transthyretin, albumin, and vitamin D binding protein, was also associated with AFP expression (Table 1; Supporting Table 2). We found that the expression of 18 liver cancer-associated genes and 11 genes reported to be associated with malignancies other than liver cancer correlated highly with AFP expression

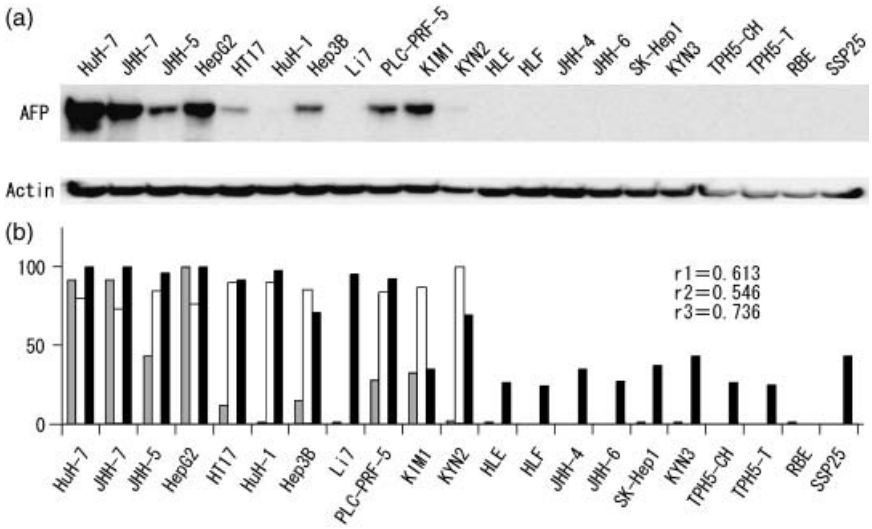

Fig. 1. α-Fetoprotein (AFP) expression in the liver cancer cell lines examined. (a) AFP expression using Western blotting. Actin served as positive control. (b) AFP expression at the protein level was concordant with that at the mRNA level as measured by DNA microarrays and quantitative reverse transcription–polymerase chain reaction (RT-PCR). r1, r2, and r3 are correlation coefficiency values; r1, western blotting versus DNA microarray; r2, western blotting versus quantitative RT-PCR; r3, quantitative RT-PCR versus DNA microarray.

Table 1. List of α-fetoprotein (AFP)-associated genes that were correlated with AFP and malignancies

Correlation coefficiency value	Gene symbol	Gene title
Transcription factors for AFP		
0.881559	HNF4A	Hepatocyte nuclear factor 4α
0.800777	TCF1	Hepatic nuclear factor 1 (HNF1)
0.748229	FOXA3	Forkhead box A3
Albumin family		
0.901225	ALB	Albumin
0.738336	GC	Vitamin D binding protein
0.917934	TTR	Transthyretin (prealbumin, amyloidosis type I)
Hepatocellular carcinoma-related genes		
0.931226	GPC3	Glypican 3
0.896721	ASGR2	Asialoglycoprotein receptor 2
0.885392	ASGR1	Asialoglycoprotein receptor 1
0.875061	AHSG	α-2-HS-glycoprotein
0.836128	THPO	Thrombopoietin
0.814368	HPN	Hepsin
0.804988	VTN	Vitronectin
0.799604	PROX1	Prospero-related homeobox 1
0.771268	CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)
0.75632	ERBB3	Erythroblastic leukemia viral oncogene homolog 3 (avian)
0.741689	REL	v-rel reticuloendotheliosis viral oncogene homolog (avian)
0.72498	FGFR3	Fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)
0.725946	GLUL	Glutamate-ammonia ligase (glutamine synthetase)
0.76969	GJB1	Gap junction protein β1, 32 kDa (connexin 32, Charcot-Marie-Tooth neuropathy, X-linked)
0.833362	PC	Pyruvate carboxylase
0.850565	VIL1	Villin 1
0.734071	ZG16	Zymogen granule protein 16
0.724989	PRAP1	Proline-rich acidic protein 1
Genes associated with cancer other than liver cancer		
0.790659	EVA1	Epithelial V-like antigen 1
0.745726	SHD	Src homology 2 domain containing transforming protein D
0.80636	MEP1A	Meprin Aα (PABA peptide hydrolase)
0.83576	S100P	S100 calcium binding protein P
0.790596	FRAT2	Frequently rearranged in advanced T-cell lymphomas 2
0.766911	FRAT1	Frequently rearranged in advanced T-cell lymphomas
0.730513	CHEK2	CHK2 checkpoint homolog (*Schizosaccharomyces pombe*)
0.777384	ST7	Suppression of tumorigenicity 7
0.75987	DMD	Dystrophin (muscular dystrophy, Duchenne and Becker types)
0.810027	VSNL1	Visinin-like 1
0.724989	PRAP1	Proline-rich acidic protein 1

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(Table 1; Supporting Table S2). As the aim of the present study was to find genes whose expression correlated significantly with AFP expression, and to discuss the possible mechanisms underlying the contribution of aberrant AFP expression to the HCC phenotypes, validation of the detected gene expression levels using other methods was critical. The expression of 16 selected genes, including *AFP*, was examined by quantitative RT-PCR, showing consistent expression levels for all genes except *S100P* and *BRAF* (Supporting Fig. S1).

Reported biological function of genes associated with AFP expression. The identified genes were grouped based on their function as reported previously: 11 genes are known to be involved in lipid metabolism, 14 in the blood coagulation pathway, six in iron metabolism, four in angiogenesis, and three encode complement factors (Table 2). Five of the genes identified are reported to be involved in signal transduction, including three in the Wnt signaling pathway and two in the mitogen-activated protein kinase pathway (Table 2).

Transcription network of the identified genes. We used a text data mining approach to examine the transcriptional network of the identified genes (Fig. 2; a higher resolution image of the network is shown in Supporting Fig. S2). This literature-based interpretation

Table 2. List of α-fetoprotein (AFP)-associated genes that were involved in normal regulatory pathways

Correlation	Gene symbol	Gene title
Lipid catabolism[11]		
0.953605	APOB	Apolipoprotein B
0.932435	APOA1	Apolipoprotein A-I
0.928801	FABP1	Fatty acid binding protein 1, liver
0.911320	APOC2	Apolipoprotein C-II
0.897877	APOC3	Apolipoprotein C-III
0.789613	DHCR24	24-Dehydrocholesterol reductase
0.785963	APOH	Apolipoprotein H
0.743957	LIPC	Lipase, hepatic
0.741831	SCARB1	Scavenger receptor class B
0.741049	HMGCS2	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 2
0.741007	PCSK9	Kexin type 9
Blood coagulation[14]		
0.931987	SERPIND1	Heparin cofactor
0.899619	PROZ	Vitamin K-dependent plasma glycoprotein
0.875732	F7	Coagulation factor VII
0.874013	F10	Coagulation factor X
0.854635	SERPINF1	α-2 antiplasmin
0.840493	FGL1	Fibrinogen-like 1
0.832607	LOC55908	Hepatocellular carcinoma-associated gene TD26
0.811034	KNG1	Kininogen 1
0.793770	SERPINF2	α-2 antiplasmin PEDF
0.776527	PROC	Inactivator of coagulation factors Va and VIIIa
0.773841	FGG	Fibrinogen gamma chain
0.764485	SERPINC1	Antithrombin
0.749890	F13B	Coagulation factor XIII, B polypeptide
0.746955	F5	Coagulation factor V
Iron metabolism[6]		
0.872017	LEAP-2	Liver-expressed antimicrobial peptide 2
0.860938	TF	Transferrin
0.825157	HPX	Hemopexin
0.783582	TFR2	Transferrin receptor 2
0.751094	HAMP	Hepcidin antimicrobial peptide
0.727492	SLC11A2	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2
Angiogenesis[4]		
0.763843	ANGPTL3	Angiopoietin-like 3
0.739551	HRG	Histidine-rich glycoprotein
0.724980	FGFR3	Fibroblast growth factor receptor 3
0.811034	KNG1	Kininogen 1
Complement[3]		
0.803829	C8A	Complement component 8, α polypeptide
0.766028	C2	Complement component 2
0.775684	C8B	Complement component 8, β polypeptide
Wnt pathway[3]		
0.931226	GPC3	Glypican 3
0.790596	FRAT2	Frequently rearranged in advanced T-cell lymphomas 2
0.766911	FRAT1	Frequently rearranged in advanced T-cell lymphomas
Mitogen-activated protein kinase pathway[2]		
0.832252	MAP3K13	Mitogen-activated protein kinase kinase kinase 13
0.797606	MAPK6	Mitogen-activated protein kinase 6

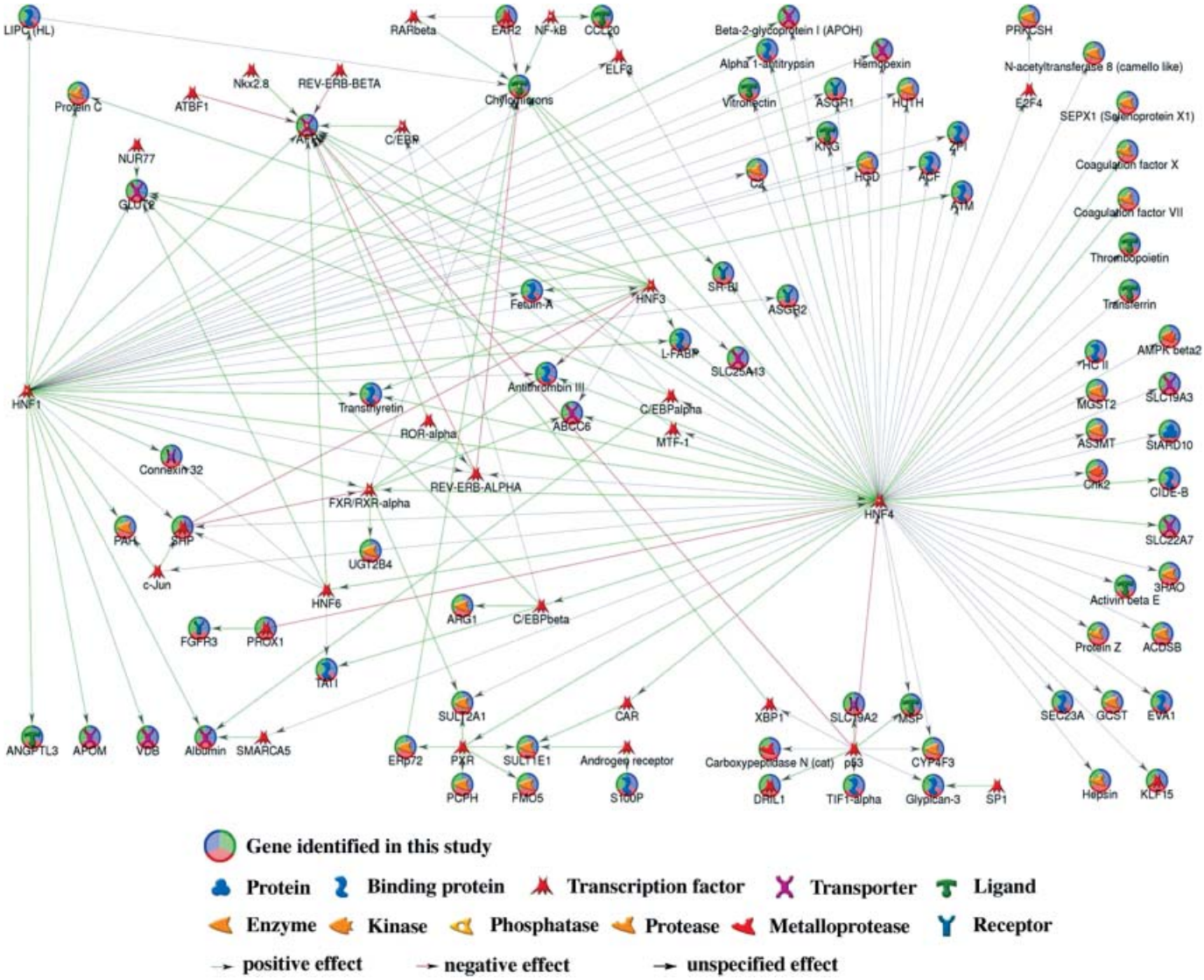

Fig. 2. The transcriptional network of the identified genes with known function.

of the data provided an overview of the genetic background of the correlation between AFP and the identified genes. In Supporting Table S2, the references by which the network was created are shown. Expression coordinate with that of AFP has been published for 78 of the 213 genes based on the known functional networks of 34 transcription factors. The most common transcription factors in this network were HNF4, HNF1, p53, and pregnane X receptor (PXR), which are responsible for the functional connection between 53, 29, eight, and five genes, respectively. AFP and GPC3 were found to be linked through their common participation in the p53 transcription factor network (Fig. 2; Supporting Table S2).

Association of AFP and GPC3 expression in HCC tissues. To confirm the molecular findings of the *in vitro* study, we examined the correlation between GPC3 and AFP expression in clinical samples. We found that the immunohistochemical expression level of GPC3 correlated significantly with that of AFP in HCC tissues (Fisher's exact test $P = 0.0065$ and Spearman correlation $P = 0.0003$) (Fig. 3; Supporting Table S3), as well as with AFP levels in the patients' sera (Jonckheere's trend test $P = 0.00001$). The degree of histological differentiation did not correlate with GPC3 levels in the tumor tissues.

Discussion

We identified 213 genes whose expression was concordant with that of AFP in 21 liver cancer cell lines. The coordinated expression of these genes may contribute to the malignant phenotypes of AFP-producing HCC. Furthermore, our study also showed that global expression studies, as opposed to functional studies on single genes, have the potential to reveal novel aspects of the molecular background of biomarkers of unknown function.

The expression of GPC3 in the liver cancer cell lines had the fifth highest correlation with that of AFP (Supporting Table S1). Text data mining revealed that AFP is functionally associated with GPC3 through their common participation in the p53 transcription factor network (Fig. 2); p53 is a negative regulatory factor for AFP[25] and a genome-wide p53-association study revealed the presence of a p53 binding motif in the *GPC3* gene sequence.[26] Clinicopathological observations have shown that higher AFP serum levels are associated with mutant p53 overexpression in HCC,[27,28] suggesting that loss of wild-type p53 function in HCC could increase AFP production and secretion into the serum. Recently, Morford *et al*. reported that GPC3 and AFP may share the transcription factors zinc fingers and homeoboxes

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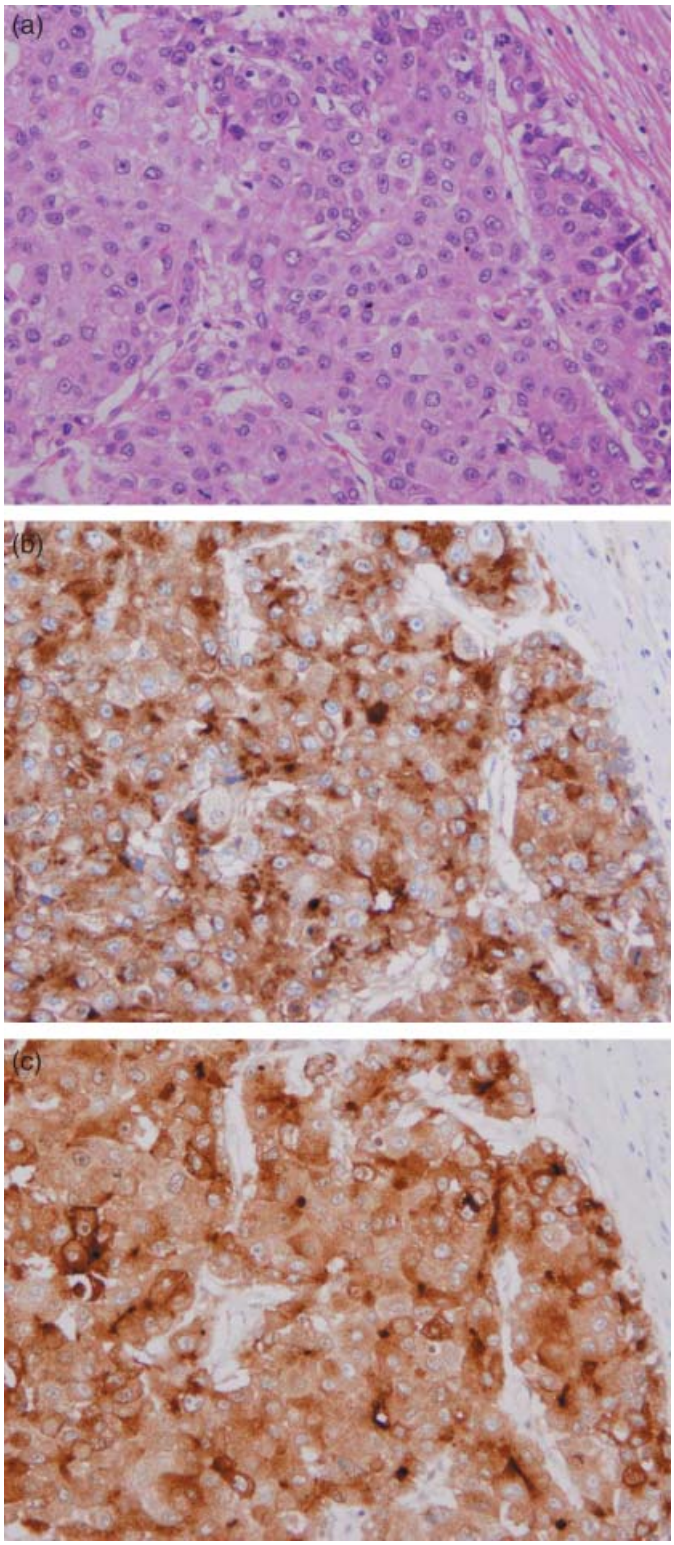

Fig. 3. Glypican 3 (GPC3) and α-fetoprotein (AFP) expression in hepatocellular carcinoma (HCC). (a) HCC (hematoxylin–eosin stain). Immunohistochemical staining for (b) GPC3 and (c) AFP.

2 (Zfh2) and AFP regulator 2 (Arf2) in the mouse.[29] We did not find these transcription factors in our text data mining study, probably because the results of their report were not included in the current version of the text database of the Metacore software. GPC3 is a member of the glypican family of glycosyl phosphatidylinositol-anchored cell-surface heparan sulfate proteoglycans. GPC3 is expressed in most HCC but is not detected in normal liver and benign hepatic lesions[30,31] and is thus considered as a diagnostic marker for HCC.[32] GPC3 promotes HCC progression by activating the Wnt pathway[33,34] and by inhibiting fibroblast growth factor 2 and bone morphogenetic protein 7.[35] GPC3 and its fragment have also been reported to be significantly elevated in the serum of a large proportion of HCC patients; their expression, however, did not correlate with that of AFP.[36,37] These findings were in contrast to a study in which plasma GPC3 was found to be a sensitive marker for AFP-producing gastric carcinoma,[32] as well as to our study, in which plasma AFP levels correlated with the GPC3 immunohistochemical expression levels in HCC tissues (Fig. 3; Supporting Table S3). Plasma AFP and GPC3 expression may be regulated differently depending on the tissue type from which the malignancy has arisen.

In addition to GPC3, we found liver cancer-associated genes whose expression correlated with that of AFP. Asialoglycoprotein receptors are candidate receptors for hepatitis B virus (HBV) attachment to hepatocytes[38] and are considered as a potential target for anti-HBV drugs.[39] *PROX1*[40] and *AHSG*[41] have been used as poor prognosis indicators for HCC patients. Furthermore, from the present study, the expression of *CEACAM1* and hepsin (*HPN*) were found to be inversely correlated with parameters denoting malignancy in HCC. The correlation of the aforementioned genes with AFP has not been reported previously.

Angiogenic factors, such as angiopoietin-like 3 (ANGPTL3), fibroblast growth factor receptor 3 (FGFR3), histidine-rich glycoprotein (HRG) and pigment epithelium-derived factor (PEDF), were found to be associated with AFP expression in our study. ANGPTL3 induces blood vessel formation by stimulating endothelial cell adhesion and migration through the integrin αvβ3.[42] FGFR3 plays an important role in lymphatic vessel development,[40] whereas both HRG and PEDF inhibit angiogenesis.[43] The serum concentration of PEDF is reduced in chronic liver diseases and HCC.[44] Hypervascularity of HCC tumors is associated with poor prognosis for HCC patients.[45] Furthermore, a correlation between AFP production and angiogenesis has been observed in AFP-producing gastric carcinoma.[46,47] These observations and findings suggest that AFP may be involved in the molecular network of aberrant angiogenesis in HCC.

We found that AFP was associated with genes involved in iron metabolism (Table 1). Iron overload facilitates liver carcinogenesis by generating oxygen-reactive species and carcinogenic oxidative damage.[48] Aberrant iron metabolism may therefore also be involved in the development of malignant phenotypes in HCC with higher AFP expression.

Genes involved in blood coagulation, inflammation, and lipid metabolism were also identified as being associated with AFP. The products of some of these genes have been reported to be elevated in the plasma of liver cancer patients.[49,50] Our findings suggest that these proteins may share common expression mechanisms with AFP in liver cancer cells.

In the present study, the AFP transcription factor HNF4 was found to be upregulated in cell lines with higher AFP levels. Naiki *et al*. reported that transfection of HNF4α into liver cancer (HuH-7) cells resulted in the upregulation of 56 genes;[51] however, only six of these were identified in our study. These included apolipoprotein M, apolipoprotein C-III, acetyl-coenzyme A acetyltransferase 1, apolipoprotein A-I, nuclear receptor subfamily 0, and alpha-1 antitrypsin (Supporting Table S1). As multiple transcription factors act in synergy regulated by each other, transfection of single genes may not be able to fully reproduce the biological events that occur *in vivo*. However, such experiments are of value as they can evaluate the degree to which the constructed virtual gene network reflects reality.

In our previous study, we reported 11 proteins whose expression was highly correlated with AFP expression. We found that the genes corresponding to seven of them showed upregulation or downregulation consistent with that of AFP. These included ubiquitin-conjugating enzyme E2R2, lectin galactoside-binding (soluble 1), BH3-interacting domain death agonist, aldehyde dehydrogenase 1 family (member A1), isocitrate dehydrogenase 1 (NADP+, soluble), annexin A1, and vinculin. In contrast, four genes, namely glucose-6-phosphate dehydrogenase, solute carrier family 25, keratin 7, and ribosomal protein (large, P0), did not show regulation consistent with that of AFP in either study. This inconsistent finding may be due to differences between proteome and transcriptome features.

The genetic background of AFP-related genes is presently obscure. Zhang *et al*. reported that the CpG island methylator phenotype is associated with elevated serum AFP levels in HCC.[52] Further studies integrating genomic, transcriptomic, and proteomic data may lead to a more comprehensive understanding of the molecular background of AFP expression in relation to cancer phenotypes.

According to the produced gene network (Fig. 2), AFP-coordinate gene expression appears to be largely attributed to liver-enriched transcription factors such as HNF1 and HNF4. These transcription factors are highly expressed at the early stages of liver development, suggesting that the genes under their regulation are also likely to be involved in liver development. As AFP is also expressed in liver development, some of these genes may also represent novel biomarker candidates for HCC.

Biomarker discovery has to a large extent been achieved based on statistical analysis, whereas less emphasis was placed on the functional assessment of the biomarker candidates. The limited sensitivity and specificity of the existing biomarkers may be due to our lack of understanding of their molecular background and functional network. By investigating the genes and proteins associated with established biomarkers, we may be able to develop novel diagnostic strategies and reveal the molecular mechanisms underlying diseases, efforts that may also lead to novel drug target identification.

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Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1. The expression levels of α-fetoprotein (AFP) and 14 other selected genes as measured by quantitative reverse transcription–polymerase chain reaction.

Fig. S2. An enlarged image of the transcriptional network of the identified genes with known function.

Table S1. List of 213 genes whose expression level significantly correlated with that of α-fetoprotein as selected using MicroArray Quality Control

Table S2. List of transcription factors linked to the identified genes and relevant literature

Table S3. Glypican 3 immunohistochemical expression in relation to α-fetoprotein expression and histological differentiation

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