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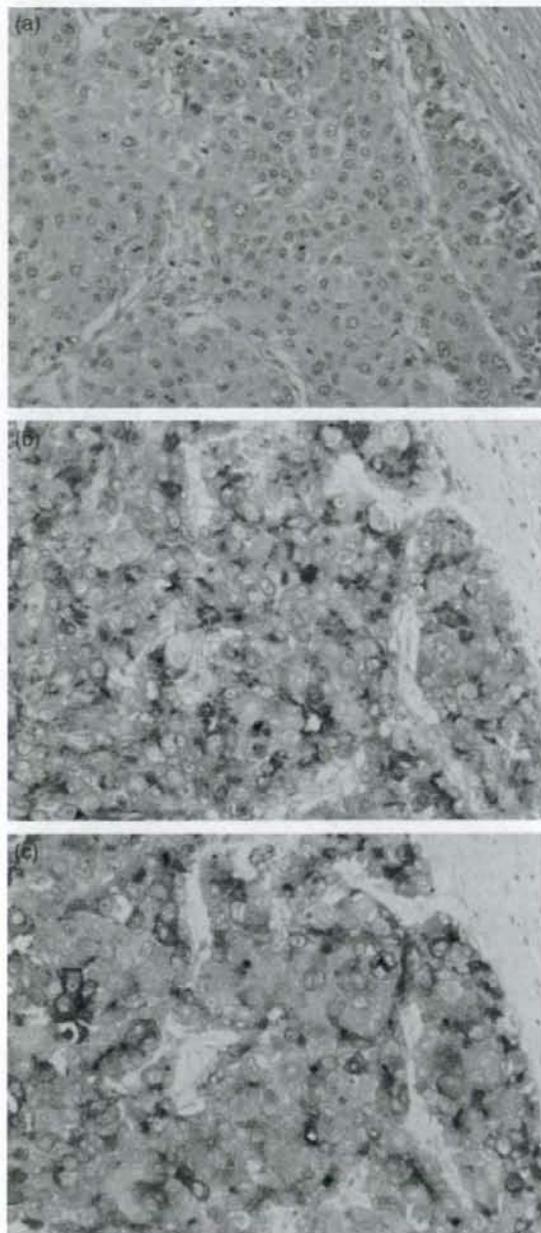
Fig. 3. Glypican 3 (GPC3) and α-fetoprotein (AFP) expression in hepatocellular carcinoma (HCC). (a) HCC (hematoxylin–eosin stain). Immunohistochemical staining for (b) GPC3 and (c) AFP.

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 $\alpha v\beta 3$.[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.

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

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.

Acknowledgments

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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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