Profiling cytochrome P450 family 4 gene expression in human hepatocellular carcinoma

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Abstract. Cytochrome P450 family 4 (CYP4) enzymes are known as microsomal omega (ω)-hydroxylases that metabolize fatty acids, eicosanoids, vitamin D and carcinogens. Thus, CYP4 enzymes may influence tumor development and progression. The aim of the present study was to evaluate the CYP4 expression profile in hepatocellular carcinoma (HCC) and its clinical relevance. The present study obtained CYP4 mRNA expression data for 377 HCC cases from The Cancer Genome Atlas cohort and performed Kaplan-Meier survival, Gene Ontology functional enrichment, and gene set enrichment analysis (GSEA). In addition, the level of CYP4F2 protein expression was evaluated in matched pairs of HCC and non-tumor tissue samples and the results were correlated with the clinicopathological characteristics of HCC (n=113). HCC survival analyses indicated better overall survival in patients with high CYP4F2, CYP4F12 and CYP4V2 mRNA expression levels; the results for histological grade and Tumor-Node-Metastasis stage supported these results. GSEA revealed high levels of CYP4F2, CYP4F12 and CYP4V2 mRNA expression to be negatively correlated with the expression of cell cycle-associated genes. CYP4F2 protein expression was higher in non-neoplastic liver tissue than in HCC tissue and positively correlated with favorable pathological tumor stage (I vs. II-IV; P=0.022) and was a good independent prognostic factor for overall survival (P=0.004). These results demonstrate that the expression levels of the genes CYP4F2, CYP4F12 and CYPV2 are favorable prognostic factors in HCC and suggest the potential predictive diagnostic and prognostic roles of CYP4F2, CYP4F12 and CYPV2 gene expression in HCC.

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Introduction

Cytochrome P450 enzymes (P450s) constitute a multi-gene family of constitutive and inducible heme-containing key oxidative enzymes (1,2). These enzymes not only have roles in endogenous functions but also participate in the metabolism of a wide variety of carcinogens and anti-cancer drugs. Thus, cytochrome P450s are considered to play important roles in tumor biology. Specifically, cytochrome P450 family 4 (CYP4) enzymes typically function as microsomal omega (ω)-hydroxylases that metabolize fatty acids, eicosanoids, and vitamin D and are important for chemical defense (3). Six CYP4 subfamilies exist in mammals: CYP4A, CYP4B, CYP4F, CYP4 V, CYP4X, and CYP4Z (4). The CYP4A, CYP4B, and CYP4F subfamilies have been shown to metabolize fatty acids of different chain lengths, whereas the fatty acid chain length specificity of CYP4AV, CYP4X, and CYP4Z is currently unknown (5). Subfamilies with large functional divergence likely perform different functions. Depending on the type of CYP4 gene, gene expression levels differ significantly among tissues, and their functions are unique (6). Therefore, CYP4 gene expression data may be utilized to obtain a better understanding of putative gene functions (7). Several studies have revealed marked mRNA upregulation of genes encoding CYP4 enzymes in some cancers, such as thyroid, breast, colon, and ovarian cancers (2,8). In addition, increased CYP4 gene expression has important clinical implications in various carcinomas (9). For example, CYP4 enzymes are expressed and play various metabolic roles in the liver. As expression of CYP4 family genes is increased in other cancers, CYP4 gene expression is also expected to be elevated in hepatocellular carcinoma (HCC); moreover, its elevation may be associated with prognosis, as in other carcinomas. Therefore, altered levels of CYP4 gene expression might be related to hepatocarcinogenesis.

In the present study, we investigated CYP4 mRNA expression levels and related clinical outcomes in HCC using The Cancer Genome Atlas (TCGA) cohort. In addition, we analyzed the underlying mechanism of the clinical outcomes

in these patients using gene set enrichment analysis (GSEA) as well as Database for Annotation, Visualization and Integrated Discovery (DAVID) and Cytoscape hierarchical analyses. Differential CYP4F2 and CYP4F3 protein expression between matched pairs of HCC and non-tumor tissue samples was evaluated. Our data may provide a useful strategy for identifying therapeutic targets in HCC.

Materials and methods

CYP4 mRNA expression analysis

Patients and data. mRNA sequence profiling (illuminahiseq_rnaseqv2-RSEM_genes_normalized) and clinical data of HCC patients were obtained from FIREHOSE (gdac.broadinstitute.org/). The methods of biospecimen procurement, RNA isolation and RNA sequencing have been previously described by The Cancer Genome Atlas Research Network (10). The TCGA RNA-seq data were cross-referenced with the clinical information of the patients. Patients with missing clinical/expression values were excluded from further analyses. In total, 377 samples were included in this study. The clinical characteristics of the patients in the cohort are presented in Table I. The mRNA-seq data were normalized using the Rank Normalize module in GenePattern (broadinstitute.org/cancer/software/genepattern).

GSEA. GSEA was performed to determine the biological significance of Kyoto Encyclopedia of Genes and Genomes (KEGG) canonical pathways. Enrichment analysis was performed for 20,502 genes. Phenotype labels, which were defined as CYP4 high 10% or low 10% according to CYP4 gene mRNA expression, were determined. $P<0.05$ indicated statistical significance. After performing GSEA, Enrichment Map Visualization was performed using Cytoscape (v3.5.1) to show the networks between the GSEA results. cBioPortal (www.cbioportal.org/) was also employed to analyze gene alterations and networks of CYP4 genes in HCC.

Survival analysis. Cutoff Finder (molpath.charite.de/cutoff) was utilized to determinate cutoff values for HCC mRNA expression. The CYP4 gene mRNA-seq data were uploaded from tab-separated files in which the rows represented patients and the columns represented variables (molpath.charite.de/cutoff/load.jsp). In Cutoff Finder, the cutoff value determination for survival significance was based on the fit of a mixture model that is conveniently applicable to molecular variables with bimodal-shaped distributions and is optimized based on the hypothesis that the variables are distributed according to a mixture of two Gaussian distributions. The cumulative event (death) rate was calculated using the Kaplan-Meier method, and the time to the first event was considered the outcome variable. The cutoff point optimization, briefly defined as the point with the most significant split, was determined by the significance of correlation with the survival variable. Additionally, the hazard ratio (HR) was calculated (11). The difference in overall survival between the poor and better survival groups, which were defined by the computed cutoff point for CYP4 expression, was depicted using Kaplan-Meier curves with calculated P-values (log-rank test, $P<0.05$).

Statistical analysis. Statistical analyses were performed using Prism v5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS v24 (IBM Corp., Armonk, NY, USA). Distributions between two groups were compared using t-tests (or the Kolmogorov-Smirnov test if the expected frequency within any cell was <5) for continuous variables and the χ^2 test (or Fisher's exact test if the expected frequency within any cell was <5) for categorical variables. Distributions of the characteristics among three or more groups were compared using analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

Assessment of CYP4F2 protein expression in matched pairs of HCC and non-tumor tissue samples

Tissue samples. We retrospectively screened 113 cases of HCC between 1999 and 2014 at Chungnam National University Hospital in Daejeon, South Korea. All formalin-fixed paraffin-embedded (FFPE) tissue samples were isolated from HCC patients who underwent segmentectomy or lobectomy. The two most representative viable tumor areas and one non-neoplastic area were selected and marked on hematoxylin and eosin (H&E)-stained slides. Tissue microarrays (TMAs) were created by punching tissue columns (3.0 mm in diameter) from the original paraffin blocks and inserting the columns into new recipient paraffin blocks (each containing 30 holes to receive the tissue columns). All clinical data were obtained from the National Biobank of Korea at Chungnam National University Hospital. The use of FFPE tissue for immunohistochemical analysis waived the prerequisite for informed consent for a retrospective comparison study using these tissues. In addition, all experimental procedures in this study were performed in accordance with relevant guidelines and regulations approved by the Institutional Review Board of Chungnam National University Hospital. The present study was approved by the Institutional Review Board of Chungnam National University Hospital (CNUH 2018-02-017).

Immunohistochemical staining analysis. Immunohistochemical staining of the tissue sections from the TMA paraffin blocks was performed as previously described (12). A primary rabbit polyclonal antibody against human CYP4F2 (ab111741, diluted 1:100; Abcam, Cambridge, UK) was used; the reactions were incubated at room temperature for 1 h. A modified Allred *et al* (13) method was applied to evaluate both the intensity of the immunohistochemical staining and the proportion of stained neoplastic or non-neoplastic hepatocytes on each slide. The proportion scores ranged from 0 to 5 (0, 0; 1, >0 to 1/100; 2, >1/100 to 1/10; 3, >1/10 to 1/3; 4, >1/3 to 2/3; and 5, >2/3 to 1), and the intensity scores ranged from 0 to 3 (0, negative; 1, weak; 2, moderate; and 3, strong). To generate the total immunohistochemical score, the intensity score and proportional score were multiplied for each specimen (range, 0-15). The results were examined separately and scored by KHK and IOS, who were blinded to the patient details. Discrepancies in scores were discussed to obtain a consensus.

Statistical analyses. Relationships between CYP4F2 expression and clinicopathological parameters were evaluated using Pearson's chi-square test and the Mann-Whitney U test.

Table I. Clinicopathologic information of the HCC patients.

Feature	Total n (%)
No.	377 (100.0)
Sex	377 (100.0)
Female	122
Male	255
Age, years	377 (100.0)
≤60	180
>60	196
NA	1
TNM stage	377 (100.0)
Stage I	175
Stage II	87
Stage III	86
Stage IV	5
NA	24
Histological grade	377 (100.0)
Grade 1	55
Grade 2	180
Grade 3	124
Grade 4	13
NA	5
Vital status	377 (100.0)
Alive	245
Dead	132
Child-Pugh classification	377 (100.0)
A	223
B	21
C	1
NA	132
Fibrosis Ishak score	377 (100.0)
0-no fibrosis	76
1,2-portal fibrosis	31
3,4-fibrous septa	30
5-nodular formation and incomplete cirrhosis	9
6-established cirrhosis	72
NA	159
Thrombocytopenia[a]	377 (100.0)
Yes	76
No	234
NA	67
Albumin level, g/dl	377 (100.0)
>3.5	217
≤3.5	86
NA	74
AFP (ng/ml)	377 (100.0)
≤20	152
>20	132
NA	93
History of hepatocellular carcinoma risk	
Hepatitis B	105
Hepatitis C	51
Hepatitis B + C	7
Alcohol consumption	118
Non-alcoholic fatty liver disease	18

[a]Thrombocytopenia was defined as a platelet nadir <150x10^9/l. HCC, hepatocellular carcinoma; TNM, Tumor-Node-Metastasis; AFP, α-fetoprotein.

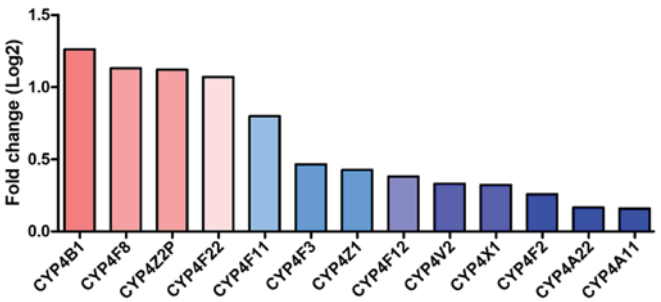

Figure 1. mRNA expression of CYP4 family genes in HCC. Relative increase and decrease in CYP4 family mRNA expression in HCC compared with normal control tissue. HCC, hepatocellular carcinoma; CYP4, cytochrome P450.

Differences in CYP4F2 expression levels between paired HCC tissue and non-tumor tissue sections were assessed using the Wilcoxon signed-rank test. One-way analysis of variance with a Newman-Keuls post hoc test was performed to analyze three or more groups. $P<0.05$ was considered to indicate a statistically significant difference. SPSS v24 software was used for analyses (IBM Corp.).

Results

CYP4 mRNA expression in HCC. CYP4 mRNA expression levels in HCC were examined, and the results are shown in Fig. 1. Interestingly, although the mRNA expression levels of CYP4B1, CYP4F8, CYP4Z2P and CYP4F22 were similar among normal controls, greatly decreased levels compared to normal controls were found for other CYP4 family genes, including CYP4A11, CYP4A22, CYP4F2, CYP4X1 and CYP4V2. The gene alteration in CYP4 did not reveal a significant difference. In addition, mRNA expression levels of CYP4F2, CYP4F3, CYP4F11 and CYP4V2 were significantly decreased in patients with higher histological and TNM stages (Figs. 2 and 3).

Effect of CYP4 mRNA expression on HCC patient survival. To determine the prognostic significance of CYP4 gene expression in patients with HCC, we examined correlations between CYP4 gene expression and overall survival. Initially, Kaplan-Meier curves were used to plot overall survival with

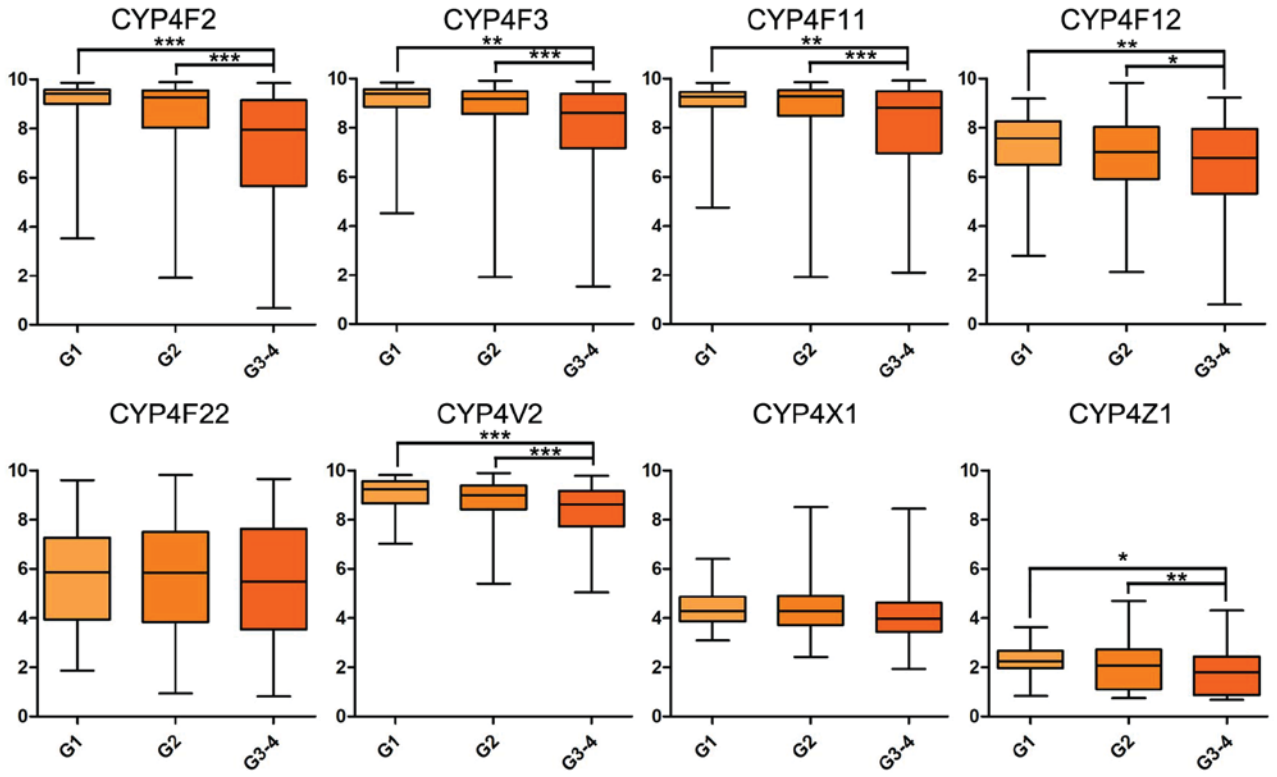

Figure 2. mRNA expression of CYP4 family genes by histological grade (G1-4). mRNA expression data for CYP4 family genes in hepatocellular carcinoma were obtained from FIREHOSE. *P<0.05, **P<0.01 and ***P<0.001, as indicated. CYP4, cytochrome P450.

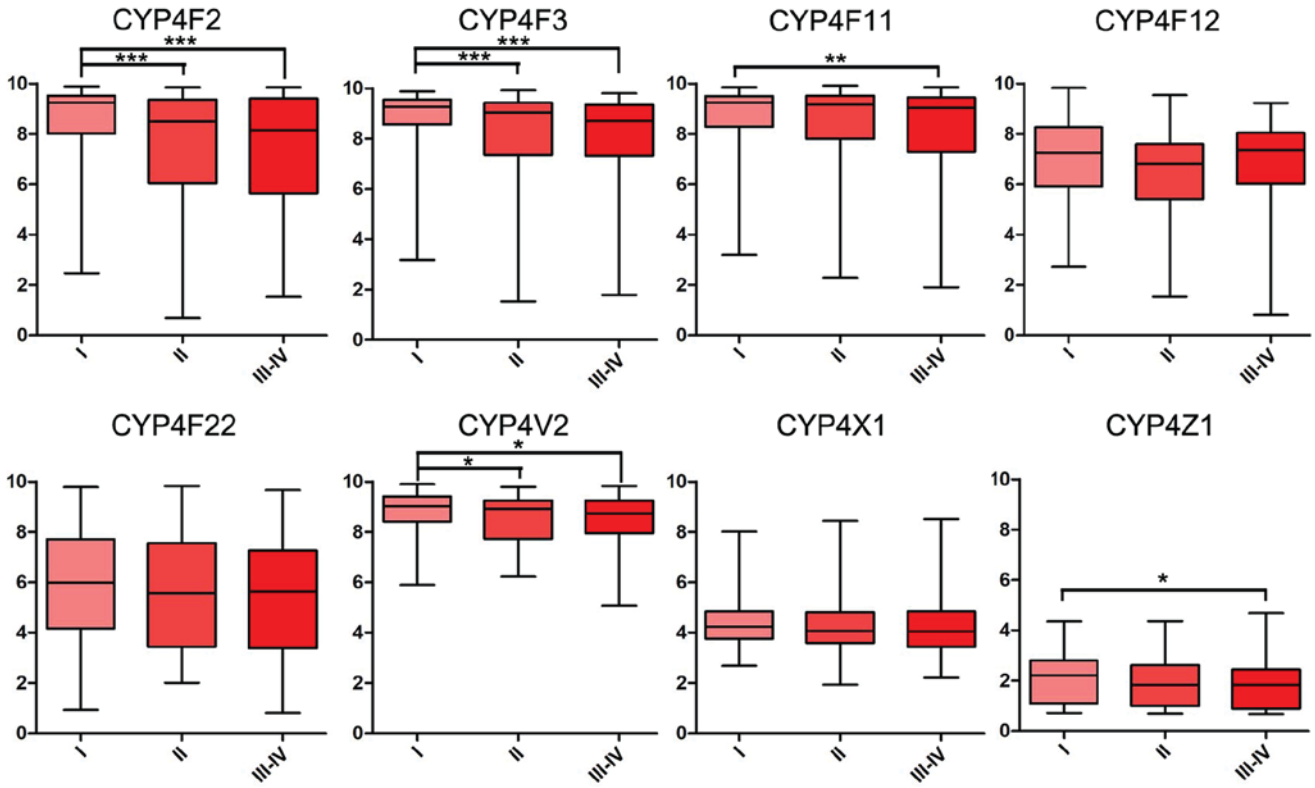

Figure 3. mRNA expression of CYP4 family genes by Tumor-Node-Metastasis stage. mRNA expression data for CYP4 family genes in hepatocellular carcinoma were obtained from FIREHOSE. *P<0.05, **P<0.01 and ***P<0.001, as indicated. CYP4, cytochrome P450.

mRNA expression using Cutoff Finder (molpath.charite.de/cutoff) (Fig. 4). High expression levels of CYP4F2, CYP4F12 and CYP4V2 were significantly associated with a better prognosis [HR: CYP4F2, 0.67 (95% CI, 0.47-0.95);

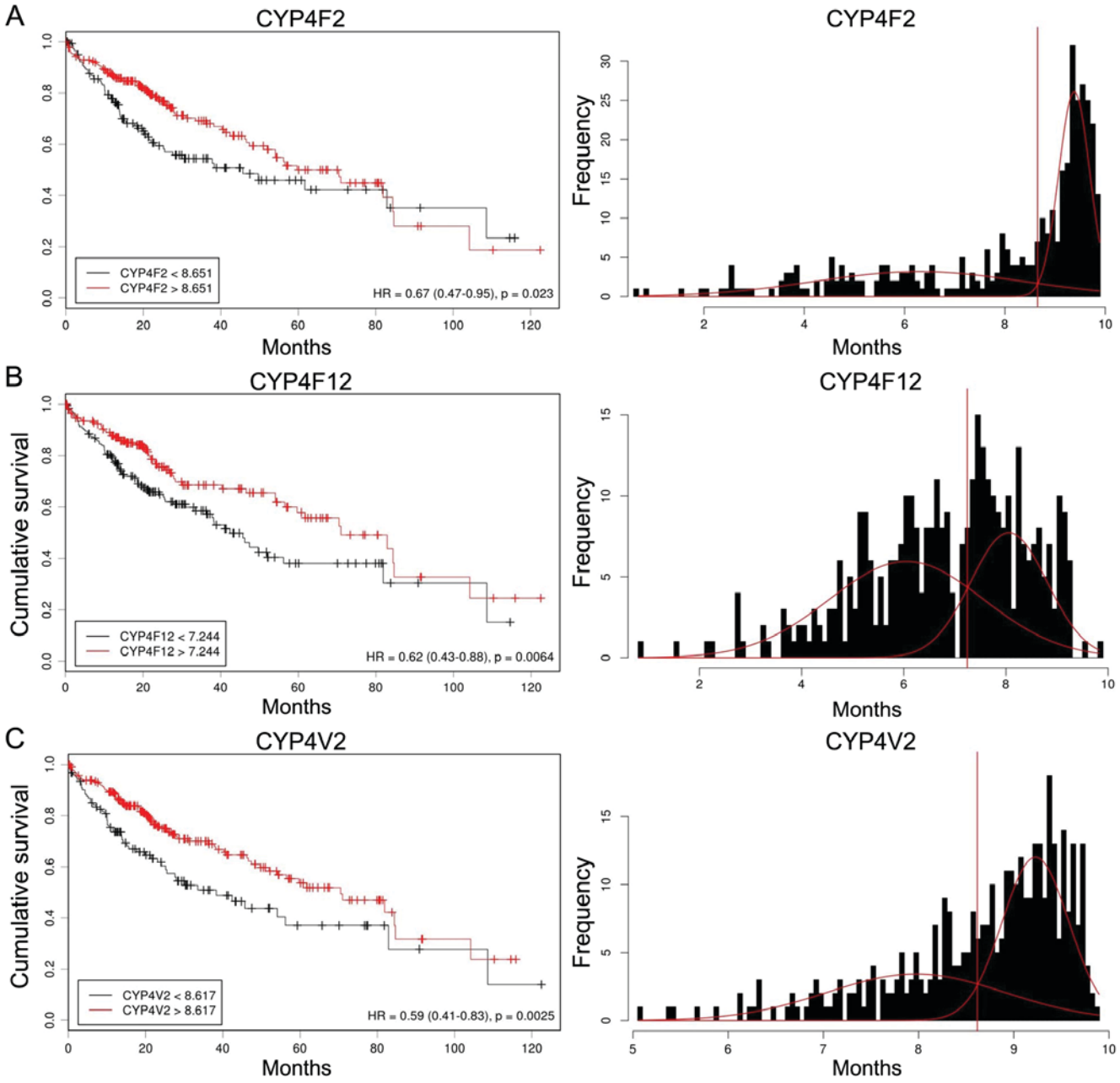

Figure 4. Survival analysis of CYP4 family genes in hepatocellular carcinoma. Kaplan-Meier analysis of the association between CYP4 family mRNA expression and overall survival. (A) High CYP4F2 expression was associated with a better prognosis; P=0.023 and hazard ratio=0.67. (B) High CYP4F12 expression was associated with a better prognosis; P=0.0064 and hazard ratio=0.62. (C) High CYP4V2 expression was associated with a better prognosis; P=0.0025 and hazard ratio=0.59. CYP4, cytochrome P450.

CYP4F12, 0.62 (95% CI, 0.43-0.88); CYP4V2, 0.59 (95% CI, 0.41-0.83)].

GSEA of CYP4 mRNA expression in HCC. GSEA was performed to identify significantly enriched pathways differing between high (top 10%) and low (bottom 10%) CYP4F2-, CYP4F12- and CYP4V2-expression groups based on pathways provided in curated gene set enrichment analysis and KEGG (Figs. 5 and 6). The networks of the GSEA results for CYP4F2, CYP4F12 and CYPV2 according to Cytoscape are shown in Fig. 7.

In the high CYPF2 group, significantly positively correlated pathways included metabolism-related pathways (retinol metabolism, drug metabolism, steroid biosynthesis, amino acid metabolism, and fatty acid metabolism) and the PPAR signaling pathway. Significantly negatively correlated pathways included the cell cycle, DNA replication, spliceosome, cancer-related pathways (bladder cancer, pathways in cancer, small cell lung cancer, pancreatic cancer and renal cell carcinoma) and the Notch signaling pathway.

Significantly positively correlated pathways in the high CYP4F12 group included metabolism-related pathways (retinol metabolism, drug metabolism, steroid biosynthesis, amino acid metabolism, and fatty acid metabolism) and the PPAR signaling pathway. Significantly negatively correlated pathways included cancer-related pathways (small cell lung cancer, pathways in cancer, renal cell carcinoma, pancreatic cancer, basal cell carcinoma, bladder cancer, colorectal cancer, melanoma and non-small cell cancer), Wnt signaling,

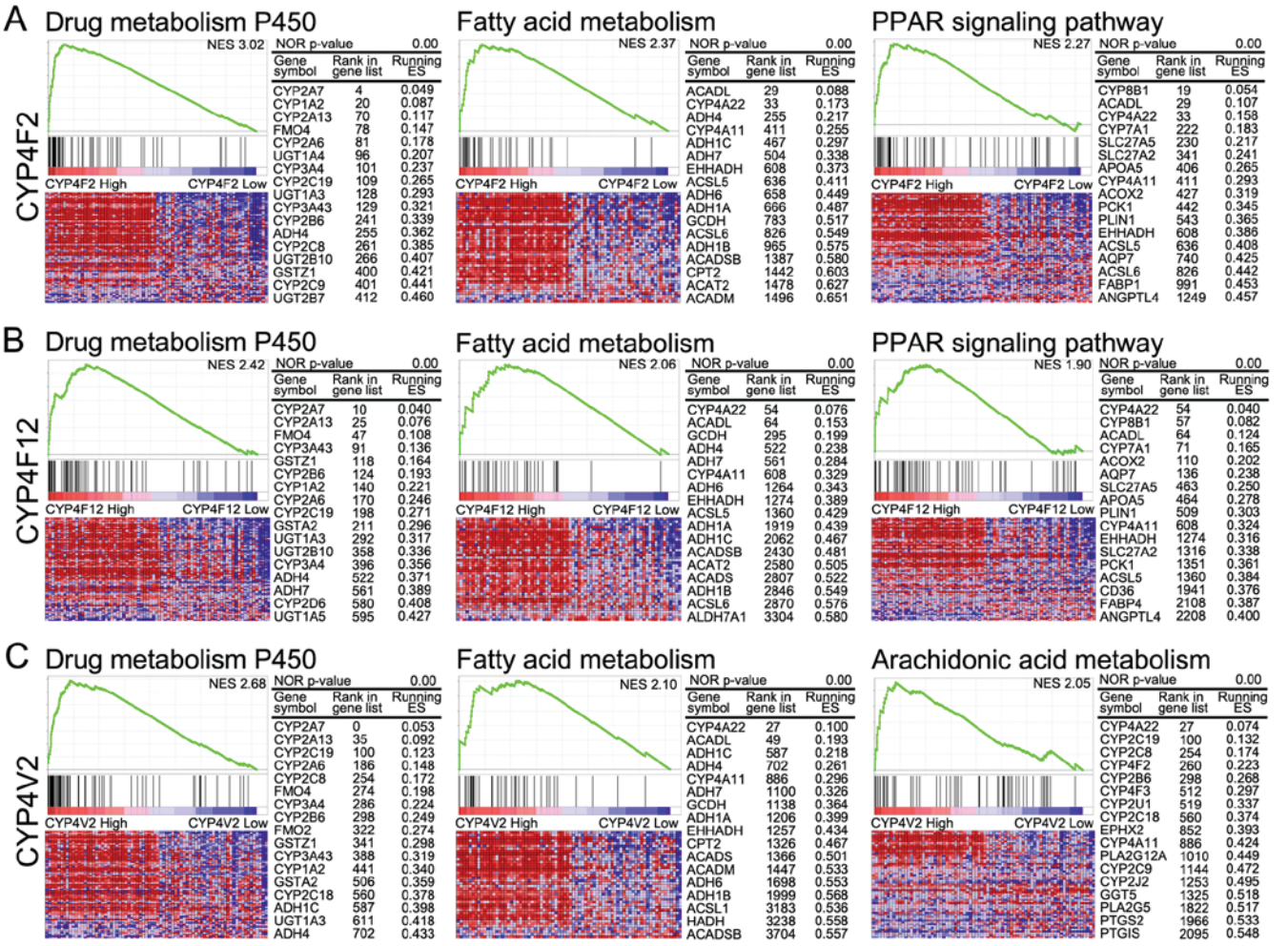

Figure 5. Gene set enrichment analysis plots representing positively correlated pathways with CYP4F2, CYP4F12 and CYP4V2. (A) CYP4F2. (B) CYP4F12 and (C) CYP4V2. P-values of CYP4F2, CYP4F12 and CYP4V2 are shown. CYP4, cytochrome P450; PPAR, peroxisome proliferator activated receptor.

Hedgehog signaling, TGF beta signaling, MAPK signaling, ERBB signaling and Notch signaling.

As with the high CYP4F12 group, significantly positively correlated pathways in the high CYP4V2 group, included metabolism-related pathways (retinol metabolism, drug metabolism, steroid biosynthesis, amino acid metabolism and fatty acids metabolism) and the PPAR signaling pathway. Significantly negatively correlated pathways in this group included cell cycle, spliceosome, DNA replication, homologous replication and cancer-related pathways (bladder cancer, pathways in cancer, small cell lung cancer and pancreatic cancer).

Association between the level of CYP4F2 protein expression and the clinicopathological features of 113 HCC cases. The 113 HCC cases were immunohistochemically evaluated for CYP4F2 expression in tumor cells and in non-neoplastic hepatocytes. Most non-neoplastic hepatocytes were strongly and diffusely positive for CYP4F2 expression and scored higher than did the HCC cells (P<0.001; Figs. 8 and 9).

The clinicopathological characteristics of the 113 HCC patients in association with CYP4F2 protein expression by immunohistochemical staining are presented in Table II (14,15). High CYP4F2 expression positively correlated with a favorable pathological TNM stage (stage I vs. stages II-IV) (P=0.022). Univariate and multivariate analyses using Cox's proportional hazard regression model were performed for age, sex, hepatitis B or C viral infection, liver cirrhosis, histologic grade, pathologic tumor stage and CYP4F2 expression in the 113 HCC cases (Tables III and IV). The univariate analyses showed decreased CYP4F2 expression to be a prognostic factor indicating shorter disease-free and overall survival (P=0.043 and P=0.008, respectively). The multivariate analysis revealed that CYP4F2 expression was an independent favorable prognostic factor for overall survival (P=0.004).

Discussion

CYP4 proteins are traditionally known as ω-hydroxylases responsible for endogenous fatty acid metabolism. Several previous studies have demonstrated that specific CYP4 subfamilies are selectively associated with certain cancers, such as thyroid, breast, colon, and ovarian cancers (2,8). However, variation in expression among CYP4 subfamily members in HCC is unknown. The present study defined the CYP4 gene expression profile in HCC and correlated the CYP4 mRNA expression levels of CYP4 subfamilies as well

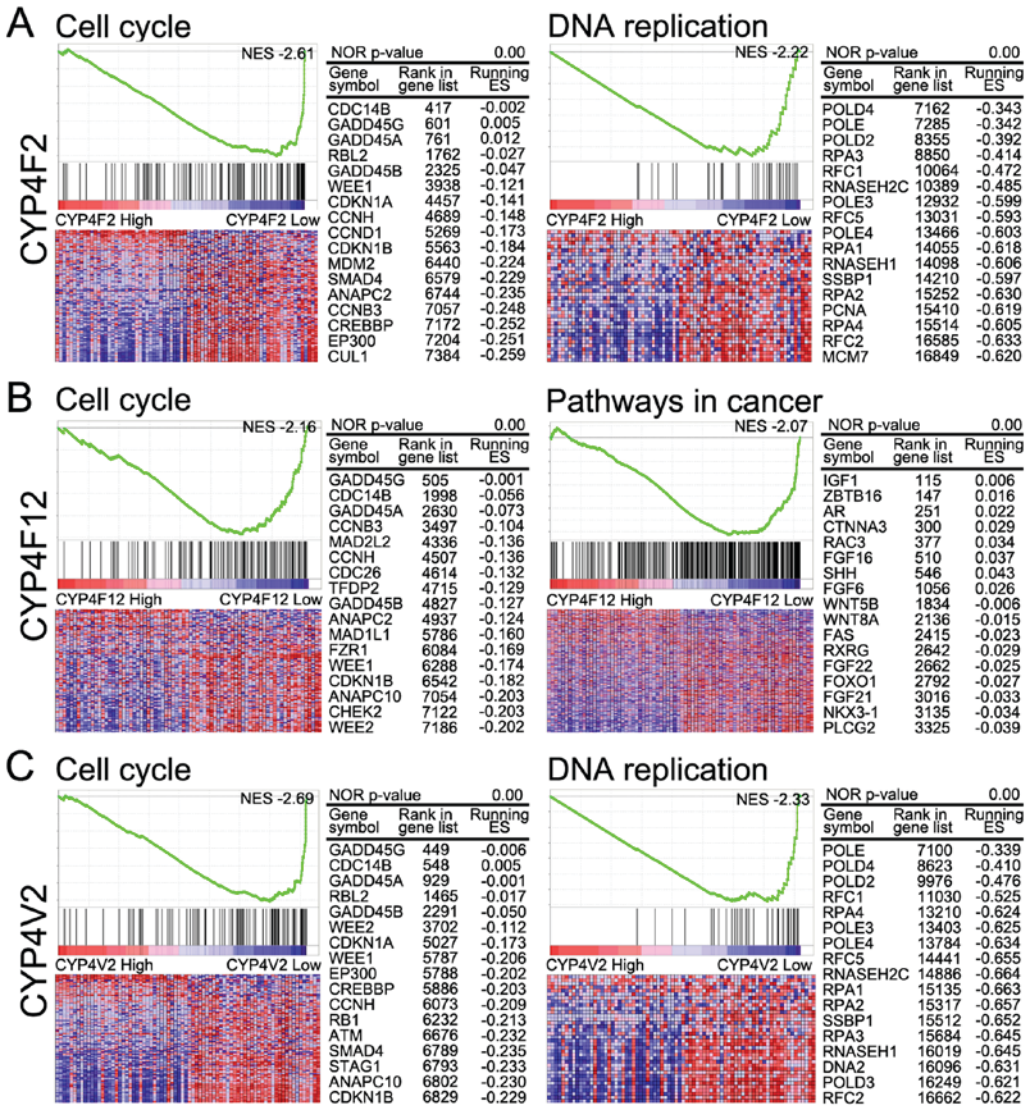

Figure 6. Gene set enrichment analysis plots representing negatively correlated pathways for CYP4F2, CYP4F12 and CYP4V2. (A) CYP4F2, (B) CYP4F12 and (C) CYP4V2. P-values of CYP4F2, CYP4F12 and CYP4V2 are shown. CYP4, cytochrome P450.

as the CYP4F2 protein expression level with clinicopathologic values, including prognostic factors. This study found that the mRNA expression levels of CYP4F2, CYP4F12, and CYP4V2 were significantly associated with good prognostic factors, including histologic grade, TNM stage, and overall survival. The protein level of CYP4F2 based on immunohistochemistry was higher in non-neoplastic hepatocytes than in HCC cells, and positive correlations were observed between low CYP4F2 protein expression and poor prognostic factors, including higher pathologic TNM stage and shorter overall and disease-free survival.

CYP4F subfamily enzymes are known for catalyzing ω-hydroxylation of long-chain fatty acids, leukotrienes, prostaglandins, vitamins with long alkyl side chains, and hydroxyeicosatetraenoic acid (HETE) (6,16). Such mammalian CYP4F gene amplification is associated with increased diversity in the metabolism of both endogenous and exogenous compounds (5,17,18). For example, CYP4F2 and -4F3 hydroxylate pro- and anti-inflammatory leukotrienes, whereas CYP4F11 metabolizes eicosanoids and drugs, and CYP4F8 and 4F12 metabolize prostaglandins, endoperoxides and arachidonic acid (5,19). In addition to these metabolic roles, CYP4F is well known as a biomarker of tumors, such as thyroid, ovarian, breast, and colon cancers (8). In particular, progression of HCV-infected liver disease to HCC tends to occur less frequently patients with lower CYP4F expression (20).

CYP4V2 is predicted to perform fatty acid metabolism and is associated with Bietti crystalline dystrophy, an autosomal recessive disorder that causes progressive night blindness and constriction of vision and is characterized by the presence of shiny yellow crystals with complex lipid deposits in the cornea and retina (21). CYP4V2 is expressed in human THP1 macrophages that exhibit fatty acid metabolism catalytic activity, and its expression is regulated by peroxisome proliferator activated receptor gamma (PPARγ) (22). CYP4V2 expression has also been correlated with lower tumor grades in breast cancer (1,2).

In the present study, CYP4F2, CYP4F12, and CYPV2 were found to be important indicators of cumulative survival differences based on histologic grade and TNM stage in patients with HCC. Consistently, the level of CYP4F2 protein expression

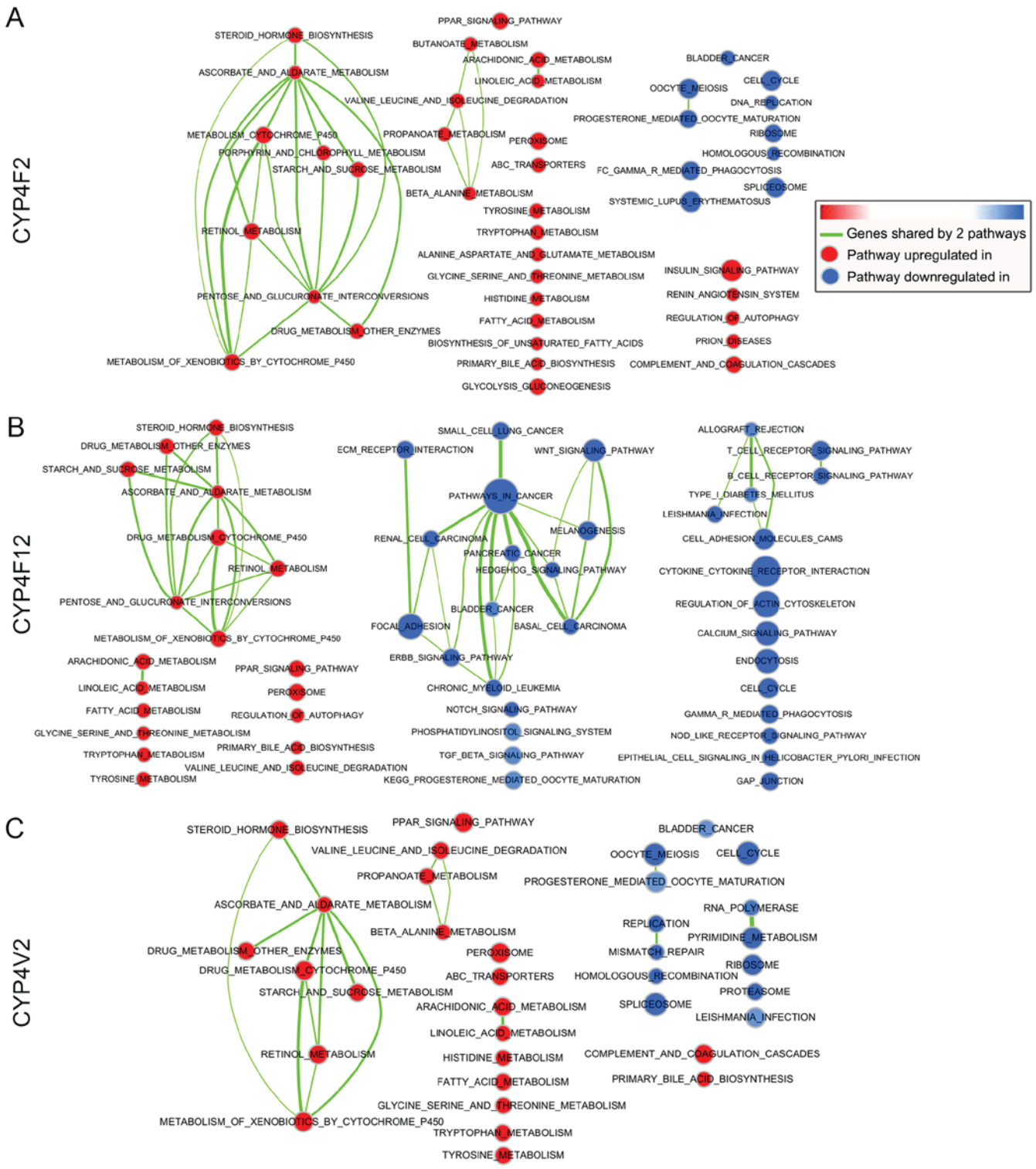

Figure 7. Enrichment map of CYP4F2, CYP4F12 and CYP4V2 between high 10% and low 10% groups. The map displays enriched gene sets between the high 10% and low 10% groups. Red nodes represent enrichment in upregulated pathways, and blue nodes represent enrichment in downregulated pathways; the color intensity is proportional to the enrichment significance. Clusters indicate functionally associated gene sets. (A) CYP4F2, (B) CYP4F12 and (C) CYP4V2. CYP4, cytochrome P450.

was lower in HCC cells than in matched non-neoplastic hepatocytes.

Indeed, the GSEA data support the results of improved survival in patients with high expression of CYP4F2, CYP4F12 and CYP4V2 genes. Correlations were observed between these patients and upregulation of specific metabolic pathways, such as drug metabolism pathways related to cytochrome P450, fatty acid metabolism and the PPAR signaling pathway, in HCC. A previous study showed that CYP4 is associated with PPAR signaling, which is related to cancer proliferation and metastasis; CYP4F is also significantly decreased in groups with a low incidence of breast cancer, though this is not related

Table II. Patient characteristics according to the immunohistochemical expression of CYP4F2 in hepatocellular carcinoma (n=113).

Characteristics	Total n (%)	CYP4F2 expression		P-value
		Low (%) [80 (100.0)]	High (%) [33 (100.0)]	
Age (years)	113 (100.0)	57.53±9.467	59.48±10.028	0.376
Gender				0.932
Male	85 (75.2)	60 (75.0)	25 (75.8)	
Female	28 (24.8)	20 (25.0)	8 (24.2)	
HBV or HCV				0.241
Negative	23 (20.4)	14 (17.5)	9 (27.3)	
Positive	90 (79.6)	66 (82.5)	24 (72.7)	
Liver cirrhosis				0.932
Negative	28 (24.8)	20 (25.0)	8 (24.2)	
Positive	85 (75.2)	60 (75.0)	25 (75.8)	
Histologic grade				0.321[a]
1 (well)	16 (14.2)	12 (15.0)	4 (12.1)	
2 (moderate)	76 (67.3)	55 (68.8)	21 (63.6)	
3 (poorly)	21 (18.6)	13 (16.3)	8 (24.2)	
4 (undifferentiated)	0 (0.0)	0 (0.0)	0 (0.0)	
Pathologic stage				0.022[b]
I	31 (27.4)	17 (21.3)	14 (42.4)	
II	70 (61.9)	51 (63.8)	19 (57.6)	
III	11 (9.7)	11 (13.8)	0 (0.0)	
IV	1 (0.9)	1 (1.3)	0 (0.0)	

[a]Grade 1/2 vs. Grade 3/4; [b]Stage I vs. Stage II-IV. HBV, hepatitis B viral infection; HCV, hepatitis C viral infection' CYP4F2, cytochrome P450 family 4 member 2.

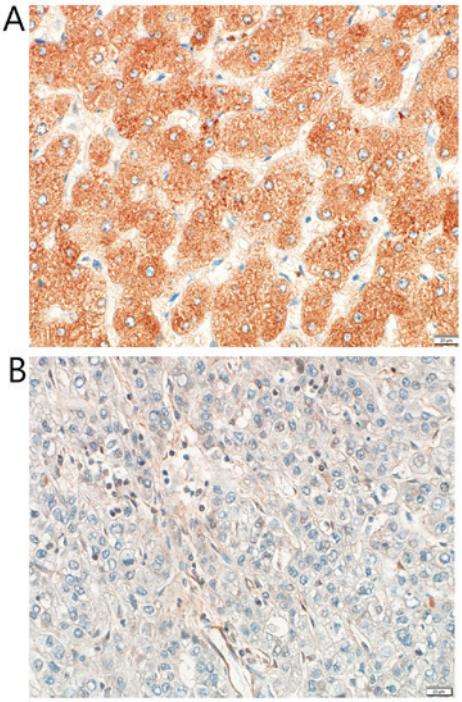

Figure 8. Representative photographs of cytochrome P450 family member 2 immunohistochemical staining of HCC samples. Paired HCC tissue and non-tumor tissue sections showing (A) non-tumor hepatocytes with strong positive staining and (B) HCC cells with faint staining (scale bar=20 μm). HCC, hepatocellular carcinoma.

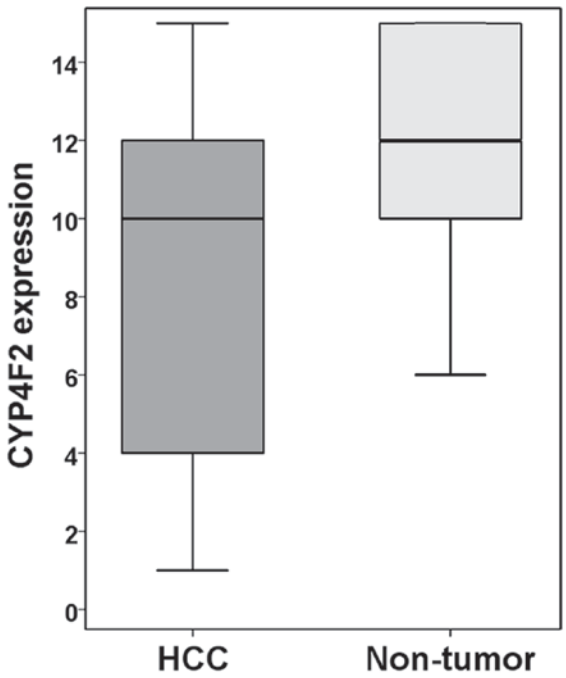

Figure 9. In total, 113 matched pairs of hepatocellular carcinoma and non-tumor tissue sections were assessed for CYP4F2 expression using the Wilcoxon signed-rank test (P<0.001). The line in the middle of the boxes represents the median. The box length indicates the interquartile range. The ends of the whiskers represent the maximum and minimum values. CYP4F2, cytochrome P450 family member 2; HCC, hepatocellular carcinoma.

Table III. Univariate analysis results of overall survival and disease-free survival in 113 patients with hepatocellular carcinoma.

	Overall survival		Disease-free survival	
Prognostic factor	HR (95% CI)	P[a]	HR (95% CI)	P[a]
CYP4F2 expression	0.893 (0.821-0.971)	0.008	0.948 (0.901-0.998)	0.043
Age at operation	1.019 (0.983-1.055)	0.303	0.992 (0.969-1.016)	0.511
Sex				
Male	1 (reference)		1 (reference)	
Female	0.631 (0.244-1.636)	0.344	1.002 (0.582-1.725)	0.996
HBV or HCV				
No	1 (reference)		1 (reference)	
Yes	0.825 (0.388-1.756)	0.618	1.171 (0.665-2.064)	0.584
Cirrhosis				
No	1 (reference)		1 (reference)	
Yes	1.950 (0.811-4.691)	0.136	2.143 (1.191-3.857)	0.011
Histologic grade		0.011		0.005
1 (well)	1 (reference)		1 (reference)	
2 (moderate)	1.117 (0.419-2.974)	0.825	1.429 (0.699-2.920)	0.328
3 (poorly)	3.408 (1.151-10.090)	0.027	3.204 (1.418-7.240)	0.005
4 (undifferentiated)	NA.	NA.	NA.	NA.
Pathologic stage		0.003		<0.001
I	1 (reference)		1 (reference)	
II	0.872 (0.408-1.866)	0.725	1.167 (0.688-1.981)	0.567
III	3.524 (1.356-9.157)	0.010	4.357 (2.001-9.490)	<0.001
IV	8.942 (1.088-73.469)	0.041	8.873 (1.128-69.803)	0.038

[a]Univariate Cox regression analysis. HR, hazard ratio; CI, confidence interval; HBV, hepatitis B viral infection; HCV, hepatitis C viral infection.

to HCC (23). Moreover, the CYP4F12 gene has been linked to malignancy-associated metabolic abnormalities in cholesterol (fatty acid metabolism) and primary bile acid metabolic homeostasis (24). Specifically, the CYP4F12 gene has been connected to genetic and epigenetic alterations in Sirt6, a member of the sirtuin family of NAD-dependent deacetylases, which are involved in HCC development and progression (24).

Furthermore, our study revealed that high expression of genes CYP4F2, CYP4F12, and CYP4V2 is related to downregulation of cell cycle pathways. Therefore, HCC with low expression of these CYP genes is associated with tumor proliferation. In addition, genes related to the cell cycle, DNA replication, cancer, and Wnt signaling pathways were significantly downregulated in patients with high expression levels of CYP4F2 and CYP4F12. These results indicate higher cancer cell survival in HCC patients with low expression of CYP4F2, CYP4F12, and CYP4V2 genes, and these factors may contribute to tumor progression and decreased survival. Moreover, high expression of these CYP genes was related to downregulation of several cancer-related pathways. For example, higher expression of CYP4F12 is related to aberrant Wnt signaling. In HCC, as in other types of tumors, aberrant activation of the canonical Wnt/beta-catenin signaling pathway is an important contributor to tumorigenesis (25).

Taken together, CYP4F2, CYP4F12, and CYP4V2 in HCC are involved in patient survival via components of various metabolic pathways. Our study suggests that the gene expression levels of CYP4F2, CYP4F12, and CYP4V2 may not only serve as diagnostic markers but may also function as prognostic factors for HCC. Further clinicopathologic studies are required to verify the roles of CYP4F2, CYP4F3 and CYP4V2 gene expression in HCC.

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Availability of data and materials

The datasets generated and analyzed during the current study are available in TCGA (cancergenome.nih.gov/) and Firebrowse (firebrowse.org/?cohort=LIHC&download_dialog=true; the 'illuminahiseq_rnaseqv2-RSEM_genes_normalized (MD5) dataset').

Table IV. Multivariate analysis results of overall survival and disease-free survival in 113 patients with hepatocellular carcinoma.

	Overall survival		Disease-free survival	
Prognostic factor	HR (95% CI)	P[a]	HR (95% CI)	P[a]
CYP4F2 expression	0.872 (0.794-0.958)	0.004	0.948 (0.896-1.003)	0.064
Age at operation	1.028 (0.987-1.071)	0.188	0.995 (0.970-1.019)	0.667
Sex				
Male	1 (reference)		1 (reference)	
Female	0.886 (0.314-2.504)	0.820	1.205 (0.664-2.189)	0.540
HBV or HCV				
No	1 (reference)		1 (reference)	
Yes	0.936 (0.395-2.216)	0.880	1.106 (0.601-2.037)	0.746
Cirrhosis				
No	1 (reference)		1 (reference)	
Yes	1.969 (0.747-5.196)	0.171	2.139 (1.144-3.999)	0.017
Histologic grade		0.009		
1 (well)	1 (reference)		1 (reference)	
2 (moderate)	1.145 (0.371-3.529)	0.814	1.297 (0.599-2.810)	0.509
3 (poorly)	3.856 (1.201-12.380)	0.023	2.940 (1.232-7.016)	0.015
4 (undifferentiated)	NA.	NA.	NA.	NA.
Pathologic stage		0.074		0.113
I	1 (reference)		1 (reference)	
II	0.783 (0.331-1.854)	0.578	1.112 (0.624-1.984)	0.719
III	2.808 (0.944-8.355)	0.064	2.734 (1.167-6.400)	0.021
IV	1.916 (0.210-17.457)	0.564	3.210 (0.379-27.193)	0.285

[a]Multivariate Cox regression analysis. HR, hazard ratio; CI, confidence interval; HBV, hepatitis B viral infection; HCV, hepatitis C viral infection.

Authors' contributions

KK, HSE and SYC designed the study. KK, HSE, SYC, BSL and IOS performed the study. KK conducted the pathological analysis. HSE and SYC drafted the original manuscript, and KK edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of Chungnam National University Hospital (CNUH 2018-02-017). The requirement for written informed consent was waived due to the retrospective nature of the study; however, consent was originally obtained at the time of data collection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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