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CYP3A4 Gene Is a Novel Biomarker for Predicting a Poor Prognosis in Hepatocellular Carcinoma

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Abstract. *Background/Aim: Project HOPE (High-tech Omics-based Patient Evaluation) began in 2014 using integrated gene expression profiling (GEP) of cancer tissues as well as diathesis of each patient who underwent operation at our Institution. The aim of this study was to identify novel genes displaying altered gene expression related to the survival and early recurrence after hepatectomy for hepatocellular carcinoma (HCC) using the results of integrated GEP analysis. Materials and Methods: The present study included 92 patients. Genes with aberrant expression were selected by the difference of expression levels with ≥10-fold change between tumor and non-tumor tissues. Results. GEP analysis showed that down-regulation was frequently observed in the PRSS8 (64%), CYP3A4 (61%) and EPCAM (57%) genes. Multivariate analysis revealed tumor stage ≥II (p=0.008) and down-regulation of the CYP3A4 gene (p=0.036) as independent predictor for overall survival. Furthermore, multivariate analysis identified maximum tumor diameter ≥74mm (p=0.008), presence of intrahepatic-metastasis (p=0.020), and down-regulation of CYP3A4 gene (p=0.019) as independent predictors for early recurrence. Conclusion: CYP3A4 was identified as a novel tumor suppressor gene related to a poor prognosis in HCC.*

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Key Words: Hepatocellular carcinoma, *CYP3A4* gene, down-regulation, overall survival, early recurrence.

Hepatocellular carcinoma (HCC) is the fifth leading cause of cancer-related death worldwide (1). Etiological factors of HCC include HBV, HCV, excess alcohol consumption, metabolic diseases, and specific carcinogen exposure. Therefore, the process of liver carcinogenesis is heterogeneous, and HCCs usually develop in the setting of chronic inflammation of the liver associated with a genomic mutation. The mechanism of liver carcinogenesis involves a unique combination of somatic alterations including genetic, epigenetic, transcriptomic and metabolic changes that form its unique molecular fingerprint (2). Thus, elucidating the molecular mechanisms and developing novel biomarkers are important for the early detection of HCC and improved outcomes (3, 4).

Recently, results of whole-genome sequencing analyses have shown that mutations in *TP53, CTNNB1, AXIN1, ARID1A, ARID2* and *BRD7* occur in 60% of patients with HCC (5-8). However, many microarray studies of HCC have shown quite different results, as each study focused on a somewhat different point (9-11).

Project HOPE (High-tech Omics-based Patient Evaluation) began from 2014 using integrated gene expression profiling (GEP) of each cancer tissue as well as diathesis of each patient, who receive operations at Shizuoka Cancer Center Hospital (12). The aim of this study was to identify novel genes displaying altered gene expression related to survival and early recurrence after hepatectomy for HCC using the results of the GEP analysis.

Materials and Methods

Subjects. Surgically-resected tumor specimens were obtained from 92 consecutive patients who underwent curative resection for HCC at the Division of Hepato-Biliary-Pancreatic Surgery of Shizuoka Cancer Center Hospital between January 2014 and October 2016 and had enrolled in Project HOPE. All pathological slides of specimens from those patients were reviewed. Clinical and pathological data were collected from our prospectively recorded database. The tumor stage was assessed based on the seventh edition of the Union for International Cancer Control (UICC) classification (13). Early recurrence was defined as recurrence within six months after hepatectomy.

Ethical approval for all experimental protocols and study was obtained from the institutional review board at the Shizuoka Cancer Center (Authorization Number: 25-33). Written informed consent was obtained from all patients enrolled in the study. All experiments using clinical samples were carried out in accordance with the approved guidelines.

Clinical samples. Tumor tissue samples with sizes corresponding to weights of ≥0.1 g were dissected from resected specimens, along with samples of surrounding normal tissue. The areas from which tumor samples were dissected were visually assessed as containing ≥50% tumor content. For the RNA analysis, tissue samples were submerged in RNAlater solution (Thermo Fisher Scientific, Waltham, MA, USA), minced, and stored at 4˚C before RNA extraction.

RNA isolation. Total RNA was extracted from approximately 10 mg of minced tissue samples using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) as described previously (14). RNA samples with RNA integrity number ≥6.0 was used for microarray analysis.

Gene expression profiling (GEP) analysis. RNA samples with RNA integrity number ≥6.0 were used for the microarray analysis. Microarray analysis was performed as described previously (14). Briefly, total RNA (100 ng) was fluorescence-labeled and hybridized to the SurePrint G3 Human Gene Expression 8×60 K v2 Microarray (Agilent Technologies, Santa Clara, CA, USA). Microarray analysis was performed in accordance with the MIAME guidelines (15). Data analysis was performed using GeneSpring GX software (Agilent Technologies) and Microsoft Excel. Raw signal intensity values were log transformed and normalized to the 75th percentile. The fold change between tumor and non-tumor tissues from the same patient was calculated using the normalized intensity values. Probes expressed at raw signal values <10 in both tumor and non-tumor tissues were excluded from further analysis.

Reverse transcription polymerase chain reaction (RT-PCR) for mRNA analysis. Quantitative mRNA levels were determined using real-time RT-PCR with the Applied Biosystems 7900 HT Sequence Detection System (Applied Biosystems), a TaqMan Gene Expression assay for human CYP3A4 (assay ID Hs02514989; Applied Biosystems), and a Eukaryotic 18S rRNA Endogenous Control (Applied Biosystems) as an endogenous control. cDNA was generated using 100 ng of the total RNA and a High-capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-PCR was carried out in a total volume of 20 μl using 100 ng of cDNA, TaqMan Fast Advanced Master Mix (Applied Biosystems), and the respective TaqMan reagents for target genes. The conditions for amplification were 95˚C for 20 s followed by 40 cycles at 95˚C for 1 s and 60˚C for 20 s. Samples were analyzed in triplicate as biological replicates. The levels of CYP3A4 mRNA were defined from the cycle threshold (Ct). Ct were normalized with reference to the level of 18S rRNA in each sample using the comparative Ct method, and ΔCt was defined as the difference in threshold cycles for CYP3A4 mRNA and 18S rRNA (16).

Table I. *Patients demographics (n=92).*

Age (years)[#]	71 (42-87)
Gender (male/female)	73/19
Etiology of liver disease (viral/non-viral)	46/46
HBsAg-positive (%)	15 (16)
Anti-HCV Ab-positive (%)	31 (34)
AFP (ng/mL)[#]	12.0 (1.3-198,061)
DCP (mAU/mL)[#]	519 (13-446,000)
Size (mm)[#]	43 (9-180)
Tumor number (solitary/multiple)	72/20
Microscopic portal invasion (present)	23 (25)
Microscopic venous invasion (present)	22 (24)
Microsatellite lesions (present)	18 (20)
UICC stage (I/II+III)	46/46
Follow up duration (months)[#]	19.4 (1.2-33.7)

HBsAg, Hepatitis B surface antigen; HCV, hepatitis C virus; Ab, antibody; AFP, alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin. The values in parentheses are percentages unless otherwise indicated. [#]The value indicates the median (range).

Immunohistochemical (IHC) analysis. All resected specimens were fixed in 10% formalin, dehydrated and embedded in paraffin. Paraffin sections of 3-μm thickness containing representative histology of the tumor were used for the IHC analysis. IHC was performed using the Bond III automated stainer and BOND Polymer Refine Detection kit (Leica Biosystems). The sections were pretreated with epitope retrieval BOND1 for 20 min at 100˚C and then reacted with anti-CYP450 3A4 rabbit polyclonal antibody at 1:100 dilution (ab3572, Abcam). After reaction with diaminobenzidine chromogen, the sections were counterstained with hematoxylin, and the stained sections were independently evaluated by two investigators (R.A. and Y.K).

Statistical analyses. The categorical variables were compared using the chi-squared test or Fisher's exact test, as appropriate. Continuous variables were compared using the Mann-Whitney *U*-test. The cumulative overall survival (OS) curve was analyzed using the Kaplan-Meier method and compared using the log-rank test. A Cox proportional hazards model was used for the univariate and multivariate analyses, and all factors found to be significant predictors of the OS ($p<0.10$) in the univariate analysis were entered into the multivariate analysis. The multivariate analysis was performed via the logistic regression method using a backward stepwise selection model. All statistical analyses were performed using the SPSS 24.0 software package (SPSS, Inc., Chicago, IL, USA), and p-values of <0.05 in 2-tailed tests were considered to be significant.

Results

Patient characteristics. The patient characteristics are shown in Table I. The rate of patients with hepatitis B surface antigen was

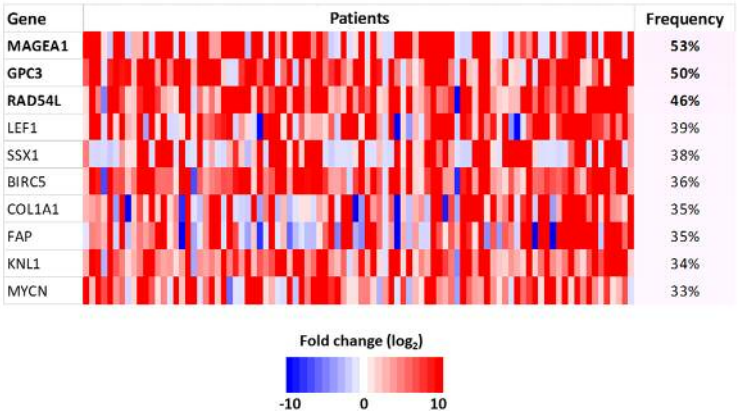

Figure 1. *Results of GEP using a microarray analysis. Genes that were frequently up-regulated in tumor tissue compared to non-tumor tissue.*

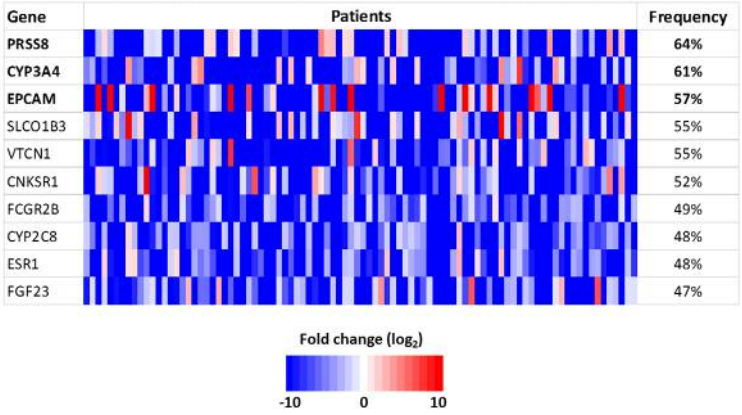

Figure 2. *Results of GEP using a microarray analysis. Genes that were frequently down-regulated in tumor tissue compared to non-tumor tissue.*

17%, and the rate of patients with anti-hepatitis C virus antibody was 34%. The median follow-up duration was 19.4 months.

GEP analyses. We first identified genes showing aberrant expression in HCC. To extract genes, we narrowed down to 820 cancer-related genes (SCC-820) (14) in order to focus on genes with oncogenic characteristics. Genes with aberrant expression were selected by the difference of expression levels with ≥10-fold change between tumor and non-tumor tissues. As a result, the top three up-regulated genes in tumor tissue were found to be *MAGEA1* in 49 patients (53%), *GPC3* in 46 patients (50%) and *RAD54L* in 42 patients (46%) among SCC-820 genes (Figure 1). The top three down-regulated genes in tumor tissue were found to be PRSS8 in 59 patients (64%), *CYP3A4* in 56 patients (61%) and *EPCAM* in 52 patients (57%) (Figure 2). We used these six genes as candidate novel biomarkers for predicting the prognosis in patients with HCC.

Prognostic factors for overall survival. In the univariate analysis, a maximum tumor diameter ≥74 mm (p=0.037), UICC stage ≥II (p=0.005) and down-regulation of the CYP3A4 gene (p=0.041, Figure 3A) were significant predictors for the OS. The multivariate analysis to identify novel biomarkers revealed that UICC stage ≥II (hazard ratio [HR]=40.0, 95% confidence interval [CI] 2.65-500 p=0.008) and down-regulation of the *CYP3A4* gene (HR=21.7 95% CI=1.23-333 p=0.036) were independent predictors for the OS (Table II).

Predictors for early recurrence. Although there was no significant difference in the recurrence-free survival (RFS) according to the expression status of the *CYP3A4* gene, the slope of the survival curve in the patients with down-regulation of the *CYP3A4* gene markedly decreased within 6 months postoperatively compared with the survival curve in the patients with not down-regulation of the *CYP3A4* gene (Figure 4, p=0.221). We therefore investigated the

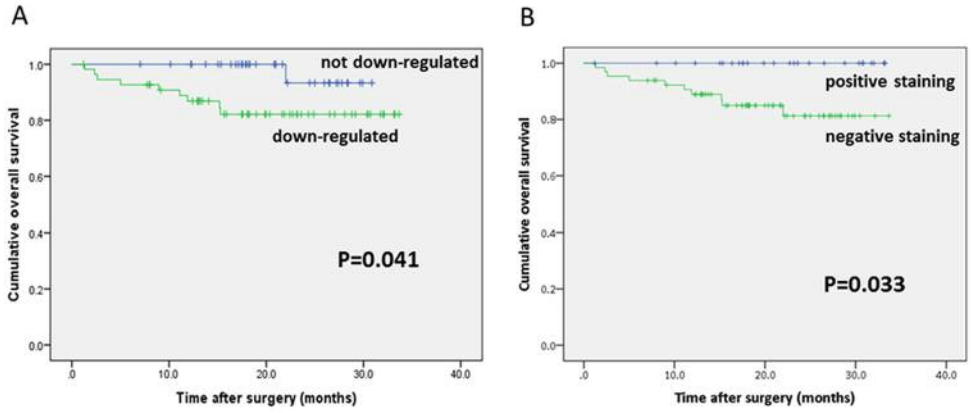

Figure 3. *Survival curves of patients who underwent hepatectomy using the Kaplan-Meier method. A. Overall survival curve according to the CYP3A4 gene expression in a microarray analysis. B. Overall survival curve according to the IHC staining status of CYP3A4 protein.*

associations between the expression of the *CYP3A4* gene and early recurrence. In the univariate analysis, des-gamma-carboxy prothrombin <62 (p=0.018), a maximum tumor diameter ≥74 mm (p=0.001), the presence of microscopic portal invasion (p=0.006), the presence of microscopic venous invasion (p=0.024), the presence of intrahepatic-metastasis (p=0.029), UICC stage ≥II (p=0.022) and the down-regulation of the *CYP3A4* gene (p=0.004) were significant predictors for early recurrence. A multivariate analysis identified a maximum tumor diameter >74 mm (odds ratio [OR] 6.10, 95% CI=1.60-23.26, p=0.008), the presence of intrahepatic-metastasis (OR=6.02, 95%CI=1.32-27.78 p=0.020), and the decreased expression of the *CYP3A4* gene (OR=15.87, 95%CI=1.59-166.67 p=0.019) as independent predictors for early recurrence (Table III).

A comparison of the clinicopathological factors according to the CYP3A4 gene expression. The frequency of well-differentiated HCC in the patients with down-regulation of the *CYP3A4* gene was significantly lower than in the patients with normal expression of the *CYP3A4* gene (10.7% *vs.* 30.6%, p=0.017), but there were no significant differences between the two groups in other clinicopathological factors.

Association between the expression of the CYP3A4 gene in a microarray analysis and RT-PCR of tumor tissue. To verify the results of GEP, we evaluated the association between the expression of the *CYP3A4* gene in the microarray analysis and those in RT-PCR of the tumor tissue using Spearman's correlation coefficient, significantly high correlations were found (p<0.001, Figure 5A).

Association between IHC of CYP3A4 protein and the expression of the CYP3A4 gene in a microarray analysis of tumor tissue. In the IHC analysis, *CYP3A4* protein was stained in the cytoplasm of tumor cells (Figure 6A). Figure 6B shows negative staining of tumor cells in the IHC analysis. In an analysis of the association between IHC of CYP3A4 protein and the expression of the *CYP3A4* gene in a microarray analysis of tumor tissue using Spearman's correlation coefficient, significant correlations were found between these two factors (Figure 5B, p<0.001).

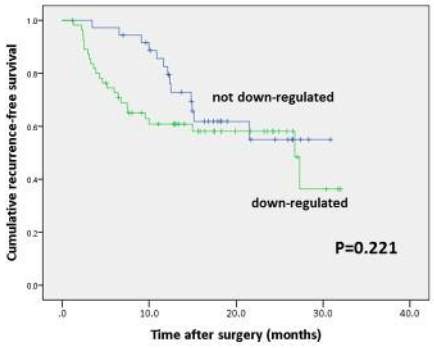

Figure 4. *Recurrence-free survival curve according to the CYP3A4 gene expression in a microarray analysis. There were no significant differences in the recurrence-free survival according to the expression status of CYP3A4 gene. The slope of the survival curve in the patients with down-regulation of the CYP3A4 gene markedly decreased within 6 months postoperatively compared to the survival curve in the patients with not down-regulation of the CYP3A4 gene.*

The optimal cut-off value of staining for CYP3A4 protein for dividing patients into two groups based on the greatest difference in overall survival (OS) was 30% when using the minimum p-value approach. The staining for CYP3A4 protein were classified according to the percentage of positive cells: staining in >30% of tumor cells was regarded as positive and

Table II. *Prognostic factors for overall survival by univariate and multivariate analysis*.

	Number of patients	2-years survival (%)	Univariate analysis *p*-Value	Multivariate analysis	
				Hazard ratio (95% Confidence interval)	*p*-Value
AFP			0.822		
<7	37	87.2			
≥7	55	88.1			
DCP			0.096		
<62	26	79.7			
≥62	66	89.9			
Etiology of liver disease			0.852		
Viral	46	88.6			
Non-viral	46	85.8			
Histologic differentiation			0.470		
Well	17	93.8			
Others	75	85.5			
Size			0.037		
<74 mm	64	92.6			
≥74 mm	28	81.4			
Tumor number			0.415		
Solitary	72	87.9			
Multiple	20	84.2			
Microscopic portal invasion			0.205		
Absent	69	89.2			
Present	23	80.1			
Microscopic venous invasion			0.208		
Absent	70	89.2			
Present	22	80.4			
Microsatellite lesions			0.371		
Absent	74	89.3			
Present	18	70.6			
Tumor stage			0.005		0.008
I	46	95.7		1	
II+III	46	78.4		40.0 (2.65-500)	
MAGEA1 gene			0.327		
Up-regulated	49	84.6			
Not up-regulated	43	89.0			
GPC3 gene			0.437		
Up-regulated	46	85.3			
Not up-regulated	46	88.6			
RAD54L gene			0.713		
Up-regulated	42	90.2			
Not up-regulated	50	84.0			
PRSS8 gene			0.860		
Not-down-regulated	33	87.1			
Down-regulated	59	86.3			
CYP3A4 gene			0.041		0.036
Down-regulated	56	82.2		21.7 (1.23-333)	
Not-down-regulated	36	93.3		1	
EPCAM gene			0.882		
Down-regulated	52	85.8			
Not-down-regulated	40	89.0			

AFP, Alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin; MAGEA1, melanoma-associated antigen 1; GPC3, glypican 3; RAD54L, RAD54-like (*S. cerevisiae*); PRSS8, protease, serine, 8; CYP3A4, cytochrome P450, family 3, subfamily A, polypeptide 4; EPCAM, epithelial cell adhesion molecule.

in <30% of cells as negative. The cumulative OS rate in patients with negative staining of CYP3A4 protein was significantly poorer than in patients with positive staining of CYP3A4 (Figure 3B, p=0.033).

Discussion

In the present study, we performed an integrated analysis of GEP for patients with HCC, and identified genes that were

Table III. *Univariate and multivariate analysis of factors predicting early recurrence.*

	Univariate analysis			Multivariate analysis	
	Early recurrence + (n=15)	Early recurrence – (n=77)	*p*-Value	Odds ratio (95% Confidence interval)	*p*-Value
AFP					
<7	9 (60%)	28 (36%)	0.088		
≥7	6 (40%)	49 (64%)			
DCP					
<62	8 (53%)	18 (23%)	0.018		
≥62	7 (47%)	59 (77%)			
Etiology of liver disease					
Viral	9 (60%)	37 (48%)	0.147		
Non-viral	6 (40%)	40 (52%)			
Histologic differentiation					
Well	1 (7%)	16 (21%)	0.258		
Others	14 (93%)	61 (79%)			
Tumor diameter					
<74 mm	5 (33%)	59 (77%)	0.001	1	0.008
≥74 mm	10 (67%)	18 (23%)		6.10 (1.60-23.26)	
Tumor number					
Solitary	9 (60%)	63 (82%)	0.085		
Multiple	6 (40%)	14 (18%)			
Microscopic portal invasion					
Absent	7 (47%)	62 (80%)	0.006		
Present	8 (53%)	15 (20%)			
Microscopic venous invasion					
Absent	8 (53%)	62 (80%)	0.024		
Present	7 (47%)	15 (20%)			
Microsatellite lesions					
Absent	9 (60%)	65 (84%)	0.029	1	0.020
Present	6 (4%)	12 (16%)		6.02 (1.32-27.78)	
Tumor stage					
I	3 (20%)	43 (56%)	0.022		
II+III	12 (80%)	34 (44%)			
MAGEA1 gene					
Not up-regulated	7 (47%)	36 (47%)	0.995		
Up-regulated	8 (53%)	41 (53%)			
GPC3 gene					
Not up-regulated	7 (47%)	39 (51%)	0.778		
Up-regulated	8 (53%)	38 (49%)			
RAD54L gene					
Not up-regulated	8 (53%)	42 (54%)	0.931		
Up-regulated	7 (47%)	35 (46%)			
PRSS8 gene					
Not-down-regulated	3 (20%)	30 (39%)	0.241		
Down-regulated	12 (80%)	47 (61%)			
CYP3A4 gene					
Not-down-regulated	1 (7%)	35 (45%)	0.004	1	0.019
Down-regulated	14 (93%)	42 (55%)		15.87 (1.59-166.67)	
EPCAM gene					
Not-down-regulated	9 (60%)	31 (60%	0.158		
Down-regulated	6 (40%)	46 (60%)			

AFP, Alpha-fetoprotein; DCP, des-gamma-carboxy prothrombin; MAGEA1, melanoma-associated antigen 1; GPC3, glypican 3; RAD54L, RAD54-like (*S. cerevisiae*); PRSS8, protease, serine, 8; CYP3A4, cytochrome P450, family 3, subfamily A, polypeptide 4; EPCAM, epithelial cell adhesion molecule.

frequently up- or down-regulated in tumor tissue compared with non-tumor tissue using a microarray analysis. We analyzed the relationship between the expression of candidate genes and the prognosis and found that down-regulation of the *CYP3A4* gene was an independent predictor for the survival and early recurrence. To verify the results of the microarray analysis, we performed RT-PCR and IHC. Both RT-PCR and IHC correlated with the findings of the

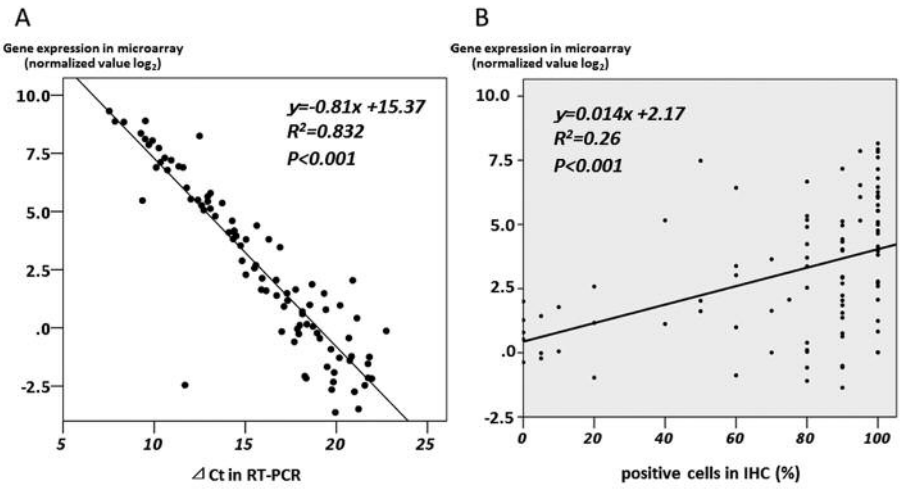

Figure 5. *A: Correlation analysis between the expression of the CYP3A4 gene in a microarray analysis and by RT-PCR of tumor tissue. B: Correlation analysis between the IHC staining of CYP3A4 protein and the expression of CYP3A4 gene in a microarray analysis of tumor tissue.*

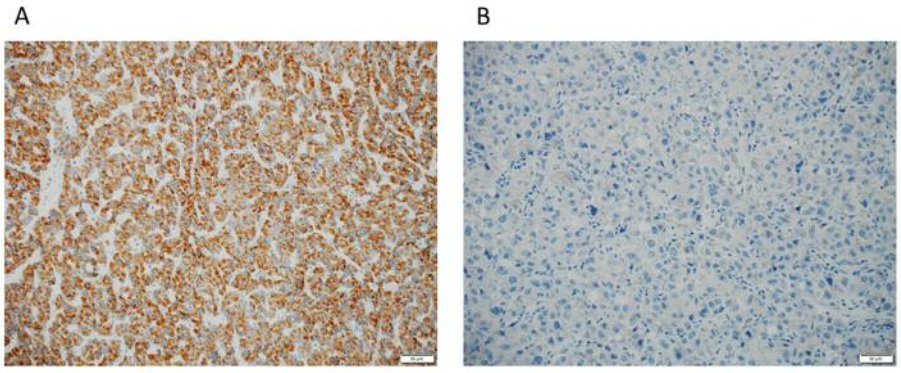

Figure 6. *A: IHC analysis of CYP3A4 in tumor tissue, that showed positive staining in the cytoplasm. B: IHC analysis of CYP3A4 in tumor tissue, which showed negative staining.*

microarray analysis, and we identified *CYP3A4* as a novel, potential clinically useful biomarker for the prognosis of HCC. Although many reports have performed comprehensive microarray analyses of the GEP for patients with HCC (10, 17-24), the frequency of down-regulation and the prognostic impact of the *CYP3A4* gene have not yet described in any reports of integrated microarray analyses. Most of these reports have instead focused on genes related to cancer pathways, and few have focused on the frequency of aberrant gene expression and their impact on the prognosis. The present study is the first report to describe the frequency of down-regulation of the *CYP3A4* gene and its prognostic impact for the OS and early recurrence.

CYP3A4 is mainly expressed in the liver and intestine, and its enzymes are involved in the metabolism of about 50% of all drugs, participating in the metabolic activation and metabolism of several pre-carcinogens (25-27). Ba *et al*. reported that benzo[a]pyrene, which is metabolized by CYP3A4, promoted HCC metastasis and progression *in vitro* and in mouse models. They also indicated that the survival curves of benzo[a]pyrene-exposed HCC-bearing mice was significantly poor (28). Although similar results have not been obtained in human HCC tissue, the down-regulation of CYP3A4 might be associated with a lower metabolism of pre-carcinogens, which leads to higher exposure of pre-carcinogens. As a result, carcinogenesis easily occurs in patients with down-regulation of the *CYP3A4* gene.

Furthermore, the present study showed that tumor differentiation is more aggressive in patients with down-regulation of the *CYP3A4* gene than in those with normal expression. Many papers have reported an association between tumor differentiation and a poor prognosis, and this

finding might be related to the poor prognosis of patients with down-regulation of the *CYP3A4* gene (29). For these reasons, down regulation of the *CYP3A4* gene might be associated with aggressive tumor behaviors, thereby leading to a higher rate of early recurrence and a poorer OS. The lack of a significant difference in the RFS according to the *CYP3A4* gene expression despite these findings might be due to the follow-up duration being too short.

The present study revealed high correlation between the expression of the *CYP3A4* gene in microarray analyses and in RT-PCR, suggesting that the GEP using the microarray had been compiled accurately and the *CYP3A4* gene was a potential novel biomarker for the prognosis of HCC. However, microarray analyses and RT-PCR are difficult to perform in daily clinical practice. The present study therefore confirmed the expression of CYP3A4 protein using IHC. Evaluating the IHC status of CYP3A4 protein may be a useful novel biomarker for predicting the prognosis in daily clinical practice. Although an appropriate adjuvant chemotherapy regimen for HCC has not yet been established, such therapy may be beneficial for patients with negative staining of CYP3A4 protein, as these patients may have potential residual cancer. Improving the prognosis of HCC will require proper identification of patients who may benefit from adjuvant chemotherapy for HCC.

Regarding the mechanisms underlying the down-regulation of the *CYP3A4* gene, the expression of the *CYP3A4* gene was regulated by nuclear receptors, well-established xenobiotic sensors capable of binding to various structurally diverse chemicals, such as pregnane X receptor (PXR) (30) and constitutive androstane receptor (CAR) (30, 31). Promoter hyper-methylation has been reported to result in repression of CAR and PXR expression, which induces the down-regulation of drug-metabolizing enzymes, including the *CYP3A4* gene, in human pluripotent stem cell-derived hepatocyte-like cells (32). CpG methylation of the *CYP3A4* promoter was also reported to induce the down-regulation of the *CYP3A4* gene in human non-cancerous liver and human hepatoma cell lines (33, 34). Although the precise mechanisms underlying the down-regulation of the CYP3A4 gene were not elucidated in human HCC tissue, several mechanisms, such as promoter hyper-methylation of *CYP3A4*, CAR and PXR, might repress the expression of the *CYP3A4* gene.

There are several limitations associated with the present study. First, the follow-up duration of the present study was slightly short. Second, this study was conducted at a single center, and the number of patients was slightly small. Further prospective multi-institutional studies are, therefore, needed to validate the results of the present study objectively.

In conclusion, the down-regulation of the *CYP3A4* gene and protein were correlated with a poor prognosis in HCC.

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