High expression of Janus kinase 2 in background normal liver tissue of resected hepatocellular carcinoma is associated with worse prognosis

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Abstract. When assessing hepatocellular carcinoma (HCC), it is important to examine prognostic factors in the background normal liver tissue and consider malignant aspects of the primary lesion. Candidate genes were extracted from the background normal liver samples via multiarray analysis. Control samples, termed supernormal (SN) liver, were obtained from 11 cases of metastatic liver cancer. Corresponding normal (CN) liver tissue was surgically obtained from a typical HCC patient with chronic hepatitis C background for comparison. Expression profile and methylation array demonstrated that Janus kinase 2 (*JAK2*) gene expression was increased by 2.378-fold in the CN tissue. Methylation array reported a lower value for CN (0.125) than SN tissues (0.748). We then investigated *JAK2* expression by real-time quantitative reverse transcription-polymerase chain reaction in 100 consecutive resected HCC cases. The average expression level of *JAK2* (normalized to *GAPDH*) was significantly lower in CN (9.24±6.43, n=100) than in SN (35.21±21.38, n=11) tissues (P<0.001). As such a result was contrary to our expectation, the case used for array analysis seemed to be a rare incidence. One hundred HCC cases were subsequently divided into two groups based on *JAK2* expression in the adjacent normal tissue: one consisting of the upper 70% of cases (n=70) and the other of the remaining 30% (n=30). Higher *JAK2* expression in the adjacent tissue demonstrated significant correlation with worse survival (P=0.022). Furthermore, multivariate analysis identified higher *JAK2* expression in the background normal liver tissue of HCC as an independent prognostic factor (P=0.032). Our findings suggest that higher *JAK2* expression in the background normal liver tissue of HCC may be a good prognostic biomarker for resected HCC.

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Introduction

Hepatocellular carcinoma (HCC) represents the fifth most common malignancy and the third most common cause of cancer-related death worldwide (1). Hepatic resection is the main treatment option for HCC patients (2-4). However, even after curative resection, 80% of these patients develop intrahepatic recurrence, and 50% die within 5 years (5). Intrahepatic metastasis (IM) refers to HCC foci developing from tumor cells that spread into the remnant liver via the portal vein before or during hepatic resection. Multicentric occurrence (MO) refers to new HCC foci developing due to the presence of chronic active hepatitis, cirrhosis, or other HCC-relevant risk factors after resection (6-9). Previous studies have indicated that the clinical progression and outcomes of these two recurrence types differ significantly (8-10). Therefore, identification of such differences is important for both the determination of therapeutic strategies and prognosis prediction.

Several studies have utilized genetic background analysis of both recurrent and primary tumors to differentiate between IM and MO (11,12). The progression and outcome of truly relapsed HCC are distinct from those of a second primary tumor, thus clonal analysis of initial and recurrent HCC is of clinical significance. We previously reported clonal analysis data from the investigation of recurrent HCC genotypes by detecting mutations of the mitochondrial genome (13) or examining patterns of promoter hypermethylation in several tumor-suppressor genes in tumor cells (14). Our findings from the above-mentioned studies suggested the distinction between MO and IM.

Although early HCC may be cured by surgical resection, the major issue of this fatal disease is its tendency to develop MO (11). Many researchers have attempted to predict the prognosis of HCC patients by evaluating only risks associated with the resected tumor tissue. However, in consideration of the unique MO pattern in HCC, a mere focus on the tumor tissue would be insufficient. We hypothesized that molecular

changes in the background normal liver tissue surrounding HCC may also influence patient prognosis. Thus, the present study was designed to identify a unique biological marker, especially from those present in the background normal liver tissue of HCC, with outcome predictive values.

Materials and methods

Sample collection and DNA preparation for microarray analyses. Control samples, termed supernormal (SN) liver, were obtained from the normal tissues of 11 patients with metastatic liver cancer who underwent liver resection at our institution. Their primary diseases were colorectal cancer (n=5), gastrointestinal stromal tumor (n=2), and one each of gastric cancer, esophageal cancer, cervical cancer and tongue cancer. For comparison, non-neoplastic liver tissue was obtained from a typical HCC case that resulted from chronic hepatitis C [the corresponding normal (CN) sample] at the time of primary liver resection. This patient was a 58-year-old man with liver cirrhosis who had undergone liver resection but experienced recurrences 3 years after the primary lesion resection. DNA and RNA were extracted from the SN and CN tissues after appropriate pathological confirmation that the CN sample did not contain any cancerous regions. Expression profiling and methylation array were performed to compare the SN and CN samples and identify genes with differential expression and methylation rate.

Microarray procedure. Total RNA from the 11 SN samples were mixed to eliminate individual background, then labeled with cyanine-3 dye using a Quick Amp Labeling kit (Agilent Technologies, Mississauga, ON, Canada), and hybridized to Agilent Whole Human Genome (4x44K) microarrays for 17 h in a rotating SciGene model 700 oven (SciGene, Sunnyvale, CA, USA). The arrays were scanned using an Agilent Technologies DNA Microarray Scanner (Agilent Technologies). Data were feature-extracted using Feature Extraction Software 10.5.1.1 and statistically analyzed using the default settings on GeneSpring GX 11.0.1 software (both from Agilent Technologies) (15). Methylation array was performed according to the Illumina Infinium HumanMethylation27 BeadChip (Illumina, San Diego, CA, USA) protocol, requiring 500 ng to 1 μg of bisulfite-converted DNA (16). Analysis of methylation array data was performed as previously described (17,18).

HCC cases for real-time quantitative reverse transcription-polymerase chain reaction analysis. Primary HCC and surrounding non-cancerous tissues were collected from 100 patients who underwent liver resection at Nagoya University Hospital between 1994 and 2002. The patient age ranged from 21 to 78 years (mean, 62.2±9.8 years), and the male-to-female ratio was 86:14. The median follow-up duration was 65.43 months (range, 0.3-208.9 months). Patient characteristics are summarized in Table I. All tissue samples were histologically confirmed as HCC. This study was approved by our institutional review board, and all patients provided written informed consent. Patient prognosis was predicted for all cases according to gene expression results by reverse transcription-polymerase chain reaction (RT-qPCR).

Table I. HCC patient characteristics (n=100).

Patient characteristics	Values
Age (years), mean ± SD (range)	62.2±9.8 (21-78)
Gender (n)	
Male	86
Female	14
Viral infection (n)	
HBV	18
HCV	75
Non-HBV/HCV	7
Child-Pugh classification (n)	
A	87
B	8
Liver damage classification (n)	
A	62
B	28
Albumin (mg/dl), mean ± SD	3.91±0.38
Total bilirubin (mg/dl), mean ± SD	0.82±0.33
PT (%), mean ± SD	75.9±12.8
AFP (ng/ml), mean ± SD	3,069±13,841.8
Tumor size (cm), mean ± SD (range)	4.47±3.08 (0.8-15)
Tumor multiplicity (n)	
Solitary	66
Multiple	30
ICG-R15, mean ± SD	12.49±9.82
Japanese stage (n)	
I	9
I	47
III	21
IV	15

HCC, hepatocellular carcinoma; n, number; SD, standard deviation; HBV, hepatitis B virus; HCV, hepatitis C virus; PT, prothrombin time; AFP, α-fetoprotein; ICG-R15, retention rate of indocyanine green 15 min after administration.

DNA preparation and RNA isolation from tissues. Surgically obtained tissue samples were immediately frozen in liquid nitrogen and stored at -80˚C until analysis. Genomic DNA was purified from the tissue samples by proteinase K digestion, followed by phenol/chloroform extraction. Total RNA was extracted from the SN and CN samples using a Qiagen miRNeasy Mini kit (Qiagen, Toronto, ON, Canada). RNA quality was confirmed with an RNA integrity number of ≥8 measured by an Agilent 2100 Bioanalyzer (Agilent Technologies).

RT-qPCR. The absolute quantification method was used to determine the input copy number by relating the PCR signal to a standard curve owing to its advantages in studies with a large number of samples. PCR was performed using a SYBR-Green PCR Core Reagent kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) under the following conditions: 50˚C

Table II. Expression profile and methylation array.

A, Expression profile

Gene symbol	Fold-change CN vs. SN	Regulation CN vs. SN	Raw CN	Raw SN	Flags CN	Flags SN
JAK2	2.378	Upregulated	220.579	76.974	Detected	Detected

B, Methylation array

Chromosome	Gene symbol	SN value	CN value
9	*JAK2*	0.748964	0.125348

SN, supernormal; CN, corresponding normal; *JAK2*, Janus kinase 2.

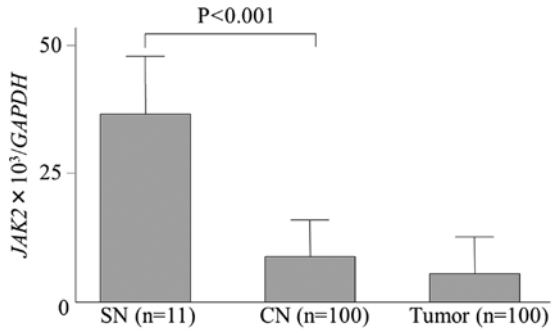

Figure 1. Comparison of Janus kinase 2 (*JAK2*) mRNA expression levels. Expression levels were determined by RT-qPCR. Total *JAK2* expression was significantly lower in the corresponding normal (CN) tissues than that in the supernormal (SN) samples [median, *JAK2* x10^3/GAPDH ratio, SN (35.21±21.38) vs. CN (9.24±6.43), P<0.001). Additionally, total *JAK2* expression was lower in the tumor tissues (5.21±5.71) than that in the CN tissues but the difference was not statistically significant.

for 2 min, 95˚C for 10 min, and 45 cycles of 95˚C for 15 sec and 60˚C for 30 sec. SYBR-Green signal was detected in real-time using an ABI Prism 7000 Sequence Detector (Perkin-Elmer Applied Biosystems). The PCR primers used were for a 93-bp fragment of Janus kinase 2 (*JAK2*) (sense, 5'-ACCTCCAGCCGTGCTTGA-3' in exon 11; antisense, 5'-TGATTACCTGCTTTCTTCAGTTTACTAATG-3' in exon 12). *GAPDH* expression was quantified in each sample for standardization purposes. All RT-qPCR experiments were performed at least three times, including negative controls without a template. *JAK2* expression was determined as the value of *JAK2* x10^3/*GAPDH* in each sample.

Statistical analysis. Continuous variables are expressed as median (range) and were compared using the Mann-Whitney U-test. Categorical variables were compared using the χ^2 or Fisher's exact test, as appropriate. Overall survival rates were estimated by the Kaplan-Meier method and compared using the log-rank test. All statistical analyses were performed using JMP Pro software version 11.0.0 (SAS Institute Inc., Cary, NC, USA). The level of statistical significance was set at P<0.05.

Results

Expression profiling and methylation array analysis. To identify novel tumor-related genes in the background normal liver tissue, we focused on genes with an increased expression in CN tissues compared with the corresponding SN tissue expression. Array analysis revealed that *JAK2*, which is involved in the JAK/STAT pathway, was strongly upregulated by 2.378-fold ($\log_2$ ratio) in the CN tissues (Table IIA).

Methylation array analysis showed continuous β values of 0.125 for CN vs. 0.748 for SN tissues, indicating a high degree of demethylation in the CN sample of the hepatitis C virus (HCV)-positive case (Table IIB). The result was of particular interest as it may imply that a possible mechanism of *JAK2* upregulation was demethylation of its promoter region in the HCV normal tissue.

RT-qPCR analysis of SN and background normal tissues from 100 HCC cases. However, when 100 HCC cases were analyzed, the overall *JAK2* expression (relative to *GAPDH*) was significantly lower in the adjacent normal than in the SN tissues (median ± SD of *JAK2* x10^3/*GAPDH* ratio, 9.24±6.43 vs. 35.21±21.38; P<0.001) (Fig. 1). In addition, *JAK2* expression in the tumor tissue was 5.21±5.71. Thus, the HCC case examined by array analysis was thought to be a rare incidence of upregulated and demethylated *JAK2*. As a result of the unexpected findings, we decided not to investigate the methylation status of *JAK2* in the CN tissues and instead focus on the clinical relevance of *JAK2* expression by evaluating its relationship with HCC prognosis. First, as we investigated *JAK2* expression in the tumor tissue, the HCC cases were divided into two groups: one with higher JAK2 tumor tissue expression than the average value (n=28) and the other with lower-than-average *JAK2* tumor expression (n=72). No significant differences in overall survival were observed between the two groups (Fig. 2A). Subsequently, the ratio of *JAK2* expression in the tumor tissue and that in the normal sample (HCC/CN) was calculated for each case to evaluate the risk of non-cancerous tissue. The group with a higher than the median HCC/CN ratio (n=50) tended to have longer overall survival than the one with lower than the median ratio

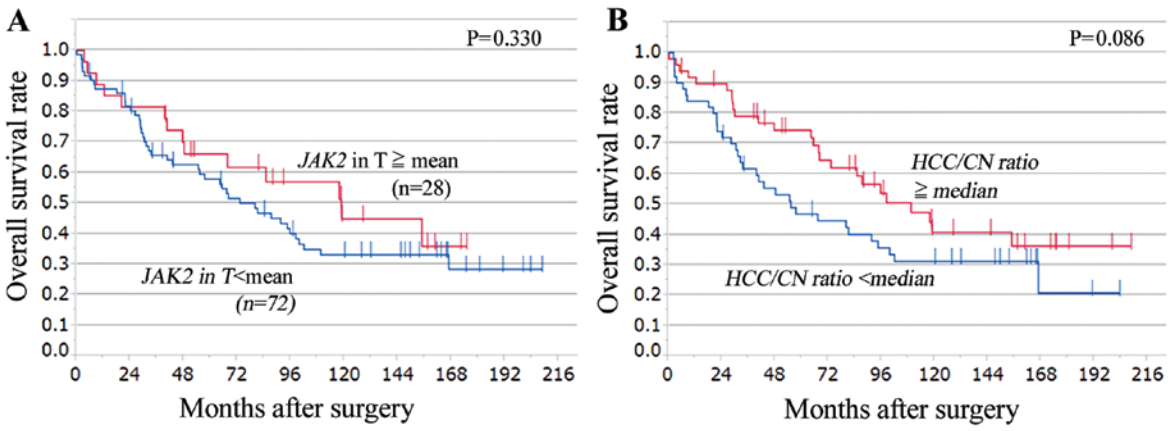

Figure 2. Overall survival rates of hepatocellular carcinoma (HCC) patients stratified by Janus kinase 2 (*JAK2*) expression in tumor tissues. (A) When HCC cases (n=100) were stratified based on whether *JAK2* tumor tissue (T) expression was higher (n=28) or lower (n=72) than the average value, no significant differences in overall survival were observed (log-rank, P=0.33). (B) We then subcategorized *JAK2* tumor tissue expression by that of the adjacent normal tissue to obtain an HCC/corresponding normal (CN) ratio. Patients with a greater HCC/CN ratio than the median tended to have better overall survival than those with a lower ratio; however, the difference was not statistically significant (P=0.086).

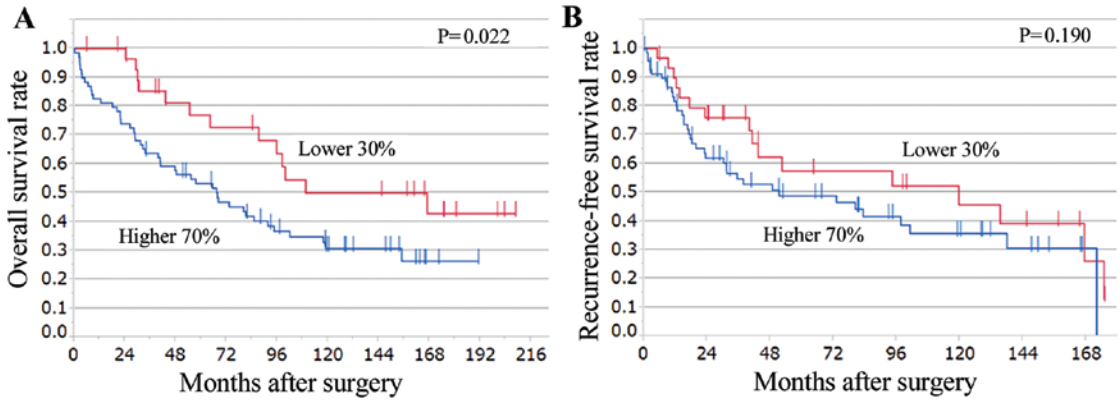

Figure 3. Overall and recurrence-free survival of hepatocellular carcinoma (HCC) patients stratified by Janus kinase 2 (*JAK2*) expression in the adjacent liver tissue. (A) HCC cases (n=100) were divided into two groups: the higher 70% group, consisting of the upper 70% of cases with higher corresponding normal tissue *JAK2* expression levels (n=70), and the lower 30% group containing the remaining 30% (n=30). A log-rank test demonstrated significant differences in overall survival rates (P=0.022) between the two groups. (B) Patients in the lower 30% group exhibited a trend towards better recurrence-free survival, but the difference was not statistically significant (P=0.19).

(n=50), but the differences were not statistically significant (P=0.086) (Fig. 2B). Based on these findings, *JAK2* expression in the background normal tissue of HCC, but not in the tumor tissue itself, appeared to relate to the overall survival of HCC patients. We then sorted these HCC cases according to *JAK2* expression and divided them into two groups: the higher 70% group, which consisted of the upper 70% of cases (n=70), and the lower 30% group containing the remaining 30% of cases (n=30). The higher 70% group showed significantly worse overall survival than the lower 30% group (P=0.022) (Fig. 3A). Additionally, the higher 70% group tended to have worse recurrence-free survival than the lower 30% group, but the differences were not statistically significant (Fig. 3B).

Correlation between upregulated JAK2 expression in background normal tissue and clinicopathological characteristics of HCC. *JAK2* expression was significantly different between well/moderately and poorly differentiated tissues (8.86 vs. 13.25; P=0.014) (Table III).

Univariate analysis revealed significant correlation of overall survival with liver cirrhosis (P=0.018), vascular invasion (P=0.001), α-fetoprotein levels of >20 ng/ml (P=0.038), and higher *JAK2* expression (>70 vs. <30%, P<0.017) (Table IV).

Furthermore, multivariate analysis confirmed significant correlation of overall survival with liver cirrhosis (P=0.01), vascular invasion (P=0.0003), and higher *JAK2* expression in the background normal liver tissue of the HCC cases (P=0.032) (Table IV).

Discussion

A major obstacle for HCC treatment is the high frequency of tumor recurrence even after curative resection and liver transplantation (19). In cases of small and well-differentiated tumors, the recurrence rate remains high (20). We previously reported that MO was more common than IM in HCC (13,14). Accordingly, the detection of metachronous multicentric recurrent carcinoma at an earlier stage and the instigation of

Table III. Clinicopathological findings of 100 HCC patients according to *JAK2* expression.

	Adjacent normal tissue		
Clinicopathological factor	N	Expression value	P-value
Age (years)			
≥65	52	9.15	0.518
<65	48	9.32	
Gender			
Male	84	9.11	0.316
Female	16	9.85	
HCV			
+	73	9.93	0.077
-	25	7.48	
HBV			
+	18	7.46	0.184
-	80	9.72	
Liver cirrhosis			
+	45	9.30	0.658
-	48	9.41	
Tumor size			
≥5 cm	35	10.36	0.434
<5 cm	59	8.64	
Tumor multiplicity			
Solitary	66	9.35	0.399
Multiple	30	9.19	
Septal formation			
+	74	8.92	0.165
-	26	10.12	
Growth form			
Expansive	79	9.19	0.123
Infiltrative	14	10.33	
Vascular invasion			
+	25	9.67	0.383
-	68	9.13	
Differentiation 1			
Well	16	8.26	0.873
Others	76	9.33	
Differentiation 2			
Well, moderately	86	8.86	**0.014**
Poorly	6	13.25	
AFP			
≥20 ng/ml	57	9.25	0.952
<20 ng/ml	39	9.17	
Japanese stage			
I, II	58	9.65	0.717
III, IV	37	8.73	

HCC, hepatocellular carcinoma; *JAK2*, Janus kinase 2; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, α-fetoprotein.

appropriate additional therapy may prolong survival in patients with MO (21). Furthermore, molecular elucidation of the risks for MO using CN liver tissue could provide useful information alongside evaluation of the cancer tissue itself.

In the present study, a multi-combination array analysis, using SN liver tissue obtained from surgically resected specimens of metastatic liver cancer as a control, revealed that *JAK2* overexpression and promoter region demethylation were striking characteristics of one particular CN specimen. Constitutive *JAK2* activation is known to be largely responsible for the development of polycythemia vera and myeloproliferative neoplasms (22,23). JAK family members, including JAK1, JAK2, JAK3 and TYK2, have also been detected in the human liver by both immunohistochemistry and western blotting (24). JAK2 protein expression is relatively high in normal human liver tissue compared with other intestinal organs according to GeneCards (25). Although JAK2 phosphorylation is not detected in the normal liver tissue, its expression is significantly increased from the surrounding non-neoplastic liver to HCC (26). The activation of the JAK/STAT pathway in HCC was previously demonstrated by measuring the phosphorylation of JAK/STAT proteins (26). On the basis of the array analysis results and subsequent literature review, we hypothesized that *JAK2* overexpression in the background normal liver tissue adjacent to HCC could be a novel molecular candidate risk factor for HCC. Since there have been few studies demonstrating differential *JAK2* expression among HCC, CN and SN liver tissues, we proceeded to evaluate *JAK2* expression using surgically resected specimens from patients with HCC.

Contrary to our expectations, RT-qPCR revealed lower *JAK2* expression in CN than in SN. Indeed, only one of 100 CN specimens exhibited higher *JAK2* expression than the mean value of all SN samples. Thus, the HCC case chosen for multi-combination array analysis turned out to be a rare incidence of higher *JAK2* expression through promoter demethylation. However, subsequent evaluation of *JAK2* expression in the clinical specimens of HCC did produce some significant findings. First, we assessed the correlation between *JAK2* expression in tumor tissue and survival as JAK2 reportedly contributed to oncogenesis through activation of STAT3 in a previous study involving various human solid tumor cell lines (27). Additionally, high expression of phosphorylated *JAK2* in HCC tissue was found to associate with poor prognosis (28). However, in the present study, we found that *JAK2* in tumor tissue was not significantly correlated with survival in HCC patients. We also observed that a greater HCC/CN ratio tended to associate with better overall survival. Thus, we speculated that in HCC cases, the *JAK2* expression level in CN but not in cancerous tissues affected overall survival, which we subsequently demonstrated in this study. Lower *JAK2* expression levels in CN tissue may reflect liver conditions such as chronic inflammation, liver cirrhosis or viral infection. However, some studies showed that DNA binding activity of STAT3 was markedly increased in the remnant rat liver immediately after partial hepatectomy (27,28). Higher *JAK2* expression in SN tissue may also reflect the better regeneration potential of an intact liver (29). Since an intact liver generally exhibits high *JAK2* expression after liver resection, varying levels of *JAK2* expression can only be observed in patients with a damaged liver and thus suppressed *JAK2* expression. Nonetheless, the reason why higher *JAK2* expression persisted despite viral or inflammatory damage inherent to CN tissue

Table IV. Univariate and multivariate analyses of overall survival.

Clinicopathological factor	N	Univariate analysis			Multivariate analysis		
		HR	95% CI	P-value	HR	95% CI	P-value
Age (years)							
≥65	52	1					
<65	48	1.17	0.706-1.98	0.529			
Gender							
Male	84	1					
Female	16	1.1	0.543-2.04	0.77			
HCV							
+	73	1.88	0.997-3.96	0.051			
-	25	1					
HBV							
+	18	1					
-	80	1.7	0.827-4.12	0.157			
Liver cirrhosis							
+	45	1.88	1.11-3.20	**0.018**	2.09	1.18-3.71	**0.01**
-	48	1					
Tumor size							
≥5 cm	35	1.28	0.745-2.17	0.359			
<5 cm	59	1					
Tumor multiplicity							
Solitary	66	1					
Multiple	30	1.44	0.828-2.44	0.19			
Septal formation							
+	74	1					
-	26	1.08	0.574-1.93	0.786			
Growth form							
Expansive	79	1					
Infiltrative	14	1.38	0.633-2.69	0.39			
Vascular invasion							
+	25	2.63	1.49-4.51	**0.001**	3.1	1.70-5.51	**0.0003**
-	68	1					
Differentiation 1							
Well	16	1					
Others	76	1.98	0.921-5.17	0.082			
Differentiation 2							
Well, moderately	86	1					
Poorly	6	1.71	0.594-3.89	0.286			
AFP							
≥20 ng/ml	57	1.76	1.02-3.12	**0.038**	1.5	0.860-2.72	0.152
<20 ng/ml	39	1					
Japanese stage							
I, II	58	1					
III, IV	37	1.62	0.961-2.72	0.069			
JAK2 expression in CN							
Higher 70%	70	2.04	1.12-3.96	**0.017**	1.91	1.04-3.74	**0.032**
Lower 30%	30	1					

HR, hazard ratio; CI, confidence interval; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, α-fetoprotein; *JAK2*, Janus kinase 2; CN, corresponding normal.

and the mechanism underlying its association with poor prognosis observed in this study remain unclear. Recent findings suggest that high JAK2 expression could reflect carcinogenesis potential in the background liver tissue and may indicate the existence of hormones or cytokines upstream of JAK2 that potentially may affect HCC outcomes (30,31).

In high-risk HCC cases, such as those with viral hepatitis or steatohepatitis, measurement of *JAK2* expression in liver biopsies or surgically resected specimens may enable the prediction of early disease recurrence via higher *JAK2* expression. Patients with high *JAK2* expression could then be offered a more intense follow-up program, such as frequent examination with ultrasonography or computed tomography. Further study is needed to elucidate how genes such as *JAK2* in adjacent normal liver tissue response to HCC development and recurrence. Such knowledge could provide excellent opportunity for novel approaches to prediction, prevention, and exploitation of molecular-targeted therapy for HCC.

In conclusion, our findings suggest that higher *JAK2* expression in the background normal liver tissue of HCC may be a good prognostic biomarker for resected HCC. Thus, its combination with other tumor prognostic factors may lead to a more accurate prediction of HCC prognosis.

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References

1. Parkin DM, Bray F, Ferlay J and Pisani P: Global cancer statistics, 2002. CA Cancer J Clin 55: 74-108, 2005.
2. Rahbari NN, Mehrabi A, Mollberg NM, *et al*: Hepatocellular carcinoma: current management and perspectives for the future. Ann Surg 253: 453-469, 2011.
3. Kobayashi A, Kawasaki S, Miyagawa S, *et al*: Results of 404 hepatic resections including 80 repeat hepatectomies for hepatocellular carcinoma. Hepatogastroenterology 53: 736-741, 2006.
4. Chen MS, Li JQ, Zheng Y, *et al*: A prospective randomized trial comparing percutaneous local ablative therapy and partial hepatectomy for small hepatocellular carcinoma. Ann Surg 243: 321-328, 2006.
5. Taura K, Ikai I, Hatano E, Fujii H, Uyama N and Shimahara Y: Implication of frequent local ablation therapy for intrahepatic recurrence in prolonged survival of patients with hepatocellular carcinoma undergoing hepatic resection: an analysis of 610 patients over 16 years old. Ann Surg 244: 265-273, 2006.
6. Chen PJ, Chen DS, Lai MY, *et al*: Clonal origin of recurrent hepatocellular carcinomas. Gastroenterology 96: 527-529, 1989.
7. Imamura H, Matsuyama Y, Tanaka E, *et al*: Risk factors contributing to early and late phase intrahepatic recurrence of hepatocellular carcinoma after hepatectomy. J Hepatol 38: 200-207, 2003.
8. Portolani N, Coniglio A, Ghidoni S, *et al*: Early and late recurrence after liver resection for hepatocellular carcinoma: prognostic and therapeutic implications. Ann Surg 243: 229-235, 2006.
9. Cucchetti A, Piscaglia F, Caturelli E, *et al*: Comparison of recurrence of hepatocellular carcinoma after resection in patients with cirrhosis to its occurrence in a surveilled cirrhotic population. Ann Surg Oncol 16: 413-422, 2009.
10. Matsuda M, Fujii H, Kono H and Matsumoto Y: Surgical treatment of recurrent hepatocellular carcinoma based on the mode of recurrence: repeat hepatic resection or ablation are good choices for patients with recurrent multicentric cancer. J Hepatobiliary Pancreat Surg 8: 353-359, 2001.
11. Morimoto O, Nagano H, Sakon M, *et al*: Diagnosis of intrahepatic metastasis and multicentric carcinogenesis by microsatellite loss of heterozygosity in patients with multiple and recurrent hepatocellular carcinomas. J Hepatol 39: 215-221, 2003.
12. Nakata T, Seki N, Miwa S, *et al*: Identification of genes associated with multiple nodules in hepatocellular carcinoma using cDNA microarray: multicentric occurrence or intrahepatic metastasis? Hepatogastroenterology 55: 865-872, 2008.
13. Nomoto S, Yamashita K, Koshikawa K, Nakao A and Sidransky D: Mitochondrial D-loop mutations as clonal markers in multicentric hepatocellular carcinoma and plasma. Clin Cancer Res 8: 481-487, 2002.
14. Nomoto S, Kinoshita T, Kato K, *et al*: Hypermethylation of multiple genes as clonal markers in multicentric hepatocellular carcinoma. Br J Cancer 97: 1260-1265, 2007.
15. Stangegaard M: Gene expression analysis using agilent DNA microarrays. Methods Mol Biol 529: 133-145, 2009.
16. Bibikova M and Fan JB: GoldenGate assay for DNA methylation profiling. Methods Mol Biol 507: 149-163, 2009.
17. Okamura Y, Nomoto S, Hayashi M, *et al*: Identification of the bleomycin hydrolase gene as a methylated tumor suppressor gene in hepatocellular carcinoma using a novel triple-combination array method. Cancer Lett 312: 150-157, 2011.
18. Takai D and Jones PA: The CpG island searcher: a new WWW resource. In Silico Biol 3: 235-240, 2003.
19. Wang Z, Zhang G, Wu J and Jia M: Adjuvant therapy for hepatocellular carcinoma: current situation and prospect. Drug Discov Ther 7: 137-143, 2013.
20. Takenaka K, Adachi E, Nishizaki T, *et al*: Possible multicentric occurrence of hepatocellular carcinoma: a clinicopathological study. Hepatology 19: 889-894, 1994.
21. Kumada T, Nakano S, Takeda I, *et al*: Patterns of recurrence after initial treatment in patients with small hepatocellular carcinoma. Hepatology 25: 87-92, 1997.
22. Amato D and Wang C: Resolution of thrombocytopenia with JAK2 mutation in a patient with Gaucher disease. Blood 122: 4287-4288, 2013.
23. Kralovics R, Passamonti F, Buser AS, *et al*: A gain-of-function mutation of JAK2 in myeloproliferative disorders. N Engl J Med 352: 1779-1790, 2005.
24. Hellgren G, Jansson JO, Carlsson LM and Carlsson B: The growth hormone receptor associates with Jak1, Jak2 and Tyk2 in human liver. Growth Horm IGF Res 9: 212-218, 1999.
25. GeneCards. Weizmann Institute of Science, 2014. Available from http://www.genecards.org.
26. Calvisi DF, Ladu S, Gorden A, *et al*: Ubiquitous activation of Ras and Jak/Stat pathways in human HCC. Gastroenterology 130: 1117-1128, 2006.
27. Cressman DE, Diamond RH and Taub R: Rapid activation of the Stat3 transcription complex in liver regeneration. Hepatology 21: 1443-1449, 1995.
28. Trautwein C, Rakemann T, Niehof M, Rose-John S and Manns MP: Acute-phase response factor, increased binding, and target gene transcription during liver regeneration. Gastroenterology 110: 1854-1862, 1996.
29. Ono M, Chia DJ, Merino-Martinez R, Flores-Morales A, Unterman TG and Rotwein P: Signal transducer and activator of transcription (Stat) 5b-mediated inhibition of insulin-like growth factor binding protein-1 gene transcription: a mechanism for repression of gene expression by growth hormone. Mol Endocrinol 21: 1443-1457, 2007.
30. Yeh YT, Lee KT, Tsai CJ, Chen YJ and Wang SN: Prolactin promotes hepatocellular carcinoma through Janus kinase 2. World J Surg 36: 1128-1135, 2012.
31. Yoshikawa H, Matsubara K, Qian GS, *et al*: SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. Nat Genet 28: 29-35, 2001.