Evaluation of Jagged2 and Gli1 expression and their correlation with prognosis in human hepatocellular carcinoma

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Abstract. Jagged2 is closely associated with numerous congenital diseases and has an important role in multiple malignancies. It has been identified that Jagged2 is a sonic hedgehog-regulated factor. However, its expression and correlation with Gli1 in hepatocellular carcinoma (HCC) remain unknown. A total of 58 samples of surgically resected paired HCC and normal tumor-adjacent tissues were collected, and the Jagged2 and Gli1 expression was detected in the samples using immunohistochemical staining. The correlation between Jagged2 and Gli1 protein expression, and their correlation with the clinicopathological features of HCC were analyzed. The protein expression of Jagged2 and Gli1 were significantly upregulated in HCC tissues compared with the normal tumor-adjacent tissues ($P<0.001$, respectively), and Jagged2 expression was positively correlated with Gli1 protein in HCC tissues ($r=0.643$, $P<0.001$). Jagged2 and Gli1 protein were expressed at significantly higher levels in patients with intrahepatic metastasis, high histological grade and advanced tumor-node-metastasis stage (TNM) stage ($P<0.05$, respectively). With the Cox proportional hazard regression mode, the independent factors predictive of poor long-term HCC survival following radical liver resection included high expression of Jagged2, advanced TNM stage and high histological grade ($P<0.05$). In HCC, high expression of Jagged2 was closely correlated with poor clinicopathological features, and it may therefore be a potential prognosis predictor for patients with HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related mortality worldwide, with up to 75,0000 novel cases reported annually (1). Despite the fact that the major risk factors, including viral hepatitis (B and C) and/or alcohol abuse, have been identified, the therapeutic options remain limited due to incomplete understanding of the cellular and molecular mechanisms involved in the pathogenesis of HCC (2). Therefore, further investigation of the mechanisms involved in the genesis and development of HCC is important for identifying novel therapeutic approaches to improve the clinical outcomes for patients with HCC.

Notch signaling is a classical signaling pathway, which has an important role in regulating cell fate decisions, proliferation and apoptosis of multiple cell lineages (3). Notch signaling comprises several transmembrane receptors and their ligands, transcription factors, as well as negative and positive modifiers. Notch receptors (Notch 1-4) and two groups of ligands, Jagged (Jagged1 and Jagged2) and Delta-like (DLL1, DLL3, DLL4), have been identified in mammals (4). Notch signaling has been identified to be associated with numerous types of tumor, including human HCC, as evidenced by its contribution to the formation of liver tumors in mice (5). One Notch signaling ligands, Jagged2, is expressed widely in human organs, including the heart, skeletal muscle and pancreas (6). It has been demonstrated that a missense mutation in the *Jagged2* gene leads to mouse syndactylism with digit malformation (7). Mice with null mutations of *Jagged2* also exhibit a number of defects, including cleft palate, syndactyly and thymic abnormalities (6). Additionally, Jagged2 also appears to have an important role in various diseases, including malignant tumors. Jagged2 was reported to mediate lung adenocarcinoma epithelial-mesenchymal transition (EMT) and metastasis in mice (8). In hypoxic conditions, Jagged2 promoted breast cancer metastasis and self-renewal of cancer stem-like cells (9). Notch3 and Jagged2 contribute to gastric cancer development and glandular differentiation (10). Notably, the overexpression of Jagged2 has been identified in >90% of pancreatic cancer cell lines (11). However, the role of Jagged2 in the genesis and development of HCC remains elusive.

Sonic hedgehog (Shh) signaling has a key role in the regulation of embryogenesis, adult tissue homeostasis and carcinogenesis (12,13). It is activated by Shh ligand binding

to the Patched-1 receptor, which relieves the repression of transducer protein Smoothened and subsequently triggers activation of the Gli family of transcription factors, including Gli1. The activated Hedgehog signaling regulates cell proliferation, survival, angiogenesis and EMT in HCC (14). A transcriptome analysis identified Jagged2 as a sonic hedgehog-regulated factor, where inhibition of Shh signaling by a dominant-negative version of Gli3 ($Gli3^{Rep}$) resulted in the loss of Jagged2 expression. Conversely, constitutive activation of the Shh pathway, using $Gli3^{Act}$ resulted in the upregulation of Jagged2 (13,15). Nevertheless, the correlation between Gli1 and Jagged2 in human HCC remains poorly understood.

In the present study, the Jagged2 expression and its relation with Gli1 protein in human HCC was investigated, and then the correlation between Jagged2 expression and the clinicopathological features of HCC were analyzed, detailing its potential as a prognostic marker for HCC patients.

Materials and methods

Patients and tissue specimens. A total of 58 patients were recruited, including 15 females and 43 males (mean age, 51.7 years; range, 24-78). The patients had not been treated with preoperative chemotherapy or interventional embolization. All of the patients received curative liver resection in the Department of Hepatobiliary Surgery, First Affiliated Hospital of Medical College of Xi'an Jiaotong University, (Xi'an, China) between December 2009 and June 2010. All of the procedures were approved by the Xi'an Jiaotong University Ethics Committee and informed consent forms were signed by each patient. A total of 58 HCC tissues and matched normal tumor-adjacent tissues (>2 cm distance from the margin of the resection) were collected with an area of 0.5x0.5 cm and stored immediately in paraformaldehyde for immunohistochemistry. All of the patients were followed-up, either via visits or telephone contact, with a median follow-up time of 23 months (range, 3-36 months) after liver resection. The data of clinical features was obtained from the medical records of each patient. The maximum diameter of the tumor, intrahepatic metastasis, histological Edmonson classification and tumor-node-metastasis (TNM) stage were gathered from the pathological records and confirmed by two experienced pathologists who were blinded to the clinical data and the results of immunohistochemical staining.

Immunohistochemical analysis. Horseradish peroxidase staining was applied for immunohistochemical analysis. All of the paraformaldehyde-fixed paraffin sections were incubated at 60˚C for ≥4 h. Next, the sections were dewaxed in dimethylbenzene and rehydrated in alcohol of diminishing concentrations. All of the sections were then placed into citrate buffer and boiled for 15 min for antigen retrieval. A total of 3.0% hydrogen peroxide was utilized for eliminating the endogenous peroxidase. The sections were then blocked by 10% goat serum at 37˚C for 30 min and incubated at 4˚C overnight with primary antibody directed against Jagged2 (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; sc-5604) or Gli1 (1:100; Santa Cruz Biotechnology Inc.; sc-20687). The biotinylated goat anti-rabbit secondary antibody (Beijing Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China) was used to detect the primary antibody at 37˚C for 45 min. Next, the horseradish peroxidase-streptavidin conjugate was used to react with biotinylated secondary antibody at 37˚C for 30 min. The sections were reacted with diaminobenzidine and counterstained with hematoxylin. Finally, the sections were dehydrated in increasing concentrations of alcohol, transparentized by dimethylbenzene and coverslipped onto glass slides.

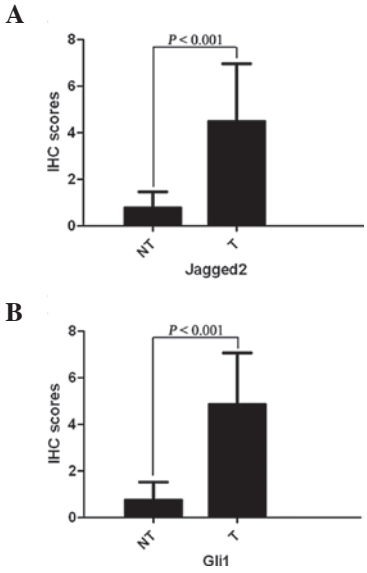

Figure 1. Expression level of Jagged2 and Gli1 in T and NT tissues. Expression level of (A) Jagged2 and (B) Gli1 in HCC tissues was significantly higher than those in normal NT tissues. HCC, hepatocellular carcinoma; T, HCC tissues; NT, normal tumor-adjacent tissues.

All of the stained sections were observed by two independent experienced pathologists in a blinded manner. Each stained section obtained a final score based on the intensity and percentage of positive cells following semi-quantitative assessment. The staining intensity was grouped into four grades: 0, negative staining; 1, weakly positive staining; 2, moderately positive staining; and 3, strongly positive staining. The stained sections with different percentages of positive cells were scored appropriately: 0 (<5%), 1 (5-25%), 2 (26-50%), 3 (51-75%) and 4 (>75%). These two scores were the mean of ten different high magnification (x400) fields and were multiplied to calculate the final score of each stained section. The sections with a total score of >1 were defined as exhibiting positive staining for the above proteins (16).

Statistical analysis. Each quantitative value was expressed as the mean ± standard deviation or the median. Comparison of Jagged2 or Gli1 protein expression between HCC tissue and normal tumor-adjacent tissue was calculated by a paired-samples t-test. Spearman's rank correlation coefficient test was used to analyze the correlation between Jagged2 and Gli1 protein expression. The differences in Jagged2 or Gli1 expression in the HCC tissues with different clinical features were compared by the Mann-Whitney U-test. The Kaplan-Meier method was used to obtain the survival curves of different clinical characteristics and the differences between these survival curves were analyzed

Table I. Correlation between the clinicopathological characteristics and protein expression of Jagged2 and Gli1 in the 58 hepatocellular carcinoma patients.

Characteristic	U-value (Jagged2)	P-value (Jagged2)	U-value (Gli1)	P-value (Gli1)
Age (<50/≥50)	341.0	0.214	393.0	0.679
Sex (male/female)	306.5	0.770	302.0	0.705
AFP (<400/≥400 ug/dl)	222.0	0.457	194.0	0.183
HBsAg (positive/negative)	247.5	0.389	256.0	0.479
Cirrhosis (yes/no)	246.0	0.554	260.0	0.749
Tumor size (<5/≥5 cm)	350.5	0.263	382.5	0.583
Intrahepatic metastasis (yes/no)	126.0	0.007	149.5	0.024
Hepatic capsule invasion (yes/no)	298.5	0.503	273.5	0.255
Portal vein tumor thrombus (yes/no)	185.5	0.748	177.0	0.618
TNM stage (I-II/III-IV)	220.5	0.001	230.0	0.007
Edmonson classification (I-II/III-IV)	209.0	0.004	256.5	0.034

AFP, α-fetoprotein.

Table II. Univariate analysis of Kaplan-Meier method.

Variable	χ^2-value	P-value
Jagged2 expression (low/high)	17.728	0.000
Gli1 expression (low/high)	4.612	0.032
AFP (<400/≥400 ug/dl)	4.585	0.032
Tumor size (<5/≥5 cm)	4.491	0.034
Intrahepatic metastasis (yes/no)	13.837	0.000
TNM stage (I-II/III-IV)	20.419	0.000
Edmonson classification (I-II/III-IV)	12.014	0.001

AFP, α-fetoprotein; TNM, tumor-node-metastasis.

Table III. Cox proportional hazard regression mode.

Variable	B	SE	Wald	Exp (B)	P-value	95% CI
Gli1 expression (low/high)	-0.711	0.428	2.755	0.491	0.097	0.212-1.137
Jagged2 expression (low/high)	1.309	0.417	9.866	3.701	0.002	1.636-8.375
AFP (<400/≥400 ug/dl)	0.459	0.452	1.030	1.582	0.310	0.653-3.834
Tumor size (<5/≥5 cm)	-0.329	0.455	0.525	0.719	0.469	0.295-1.753
Intrahepatic metastasis (yes/no)	0.873	0.460	3.593	3.749	0.058	0.971-5.901
TNM stage (I-II/III-IV)	1.979	0.582	11.567	7.234	0.001	2.313-22.626
Edmonson classification (I-II/III-IV)	1.321	0.411	10.352	2.393	0.001	1.676-8.385

B, partial regression coefficient; SE, partial regression coefficient standard error; AFP, α-fetoprotein; TNM, tumor-node-metastasis; CI, confidence interval.

using a log-rank test. The Cox proportional hazard regression model was applied to determine the independent prognostic factors in multivariate survival analysis. SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used in this statistical analysis and P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of Jagged2 and Gli1 in HCC and matched normal tumor-adjacent tissues. Immunohistochemical staining demonstrated that Jagged2 protein expression in HCC tissues was found to be significantly higher than that in the matched

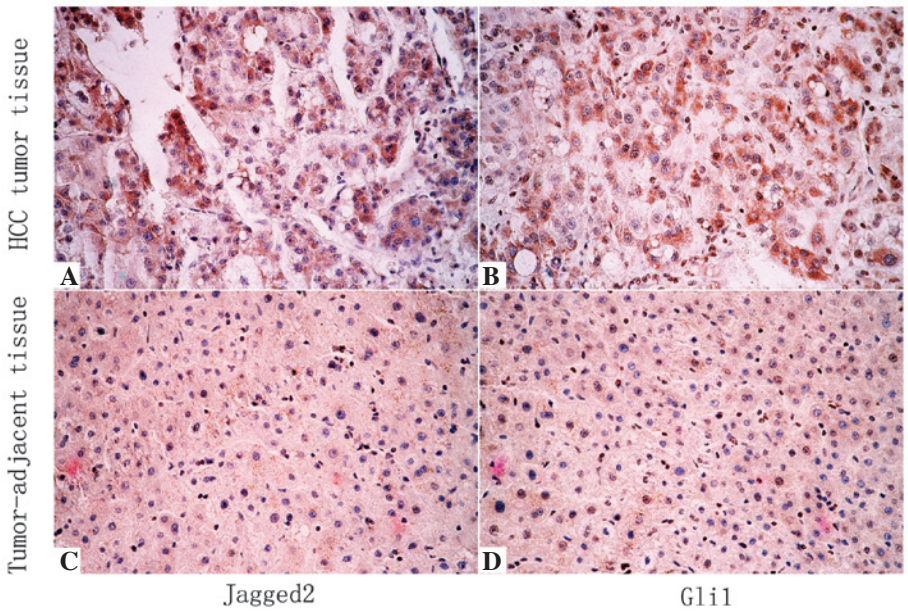

Figure 2. Immunohistochemical staining of Jagged2 and Gli1 in HCC tissues and normal tumor-adjacent tissues. In the cases of high Jagged2 protein expression (A), there was strong staining of Gli1 in the same HCC tissue section (B). By contrast, in the case of low Jagged2 protein expression (C), there was no detectable Gli1 protein expression in the same normal tumor-adjacent tissue section (D) (original magnification, x400; staining, streptavidin peroxidase). HCC, hepatocellular carcinoma.

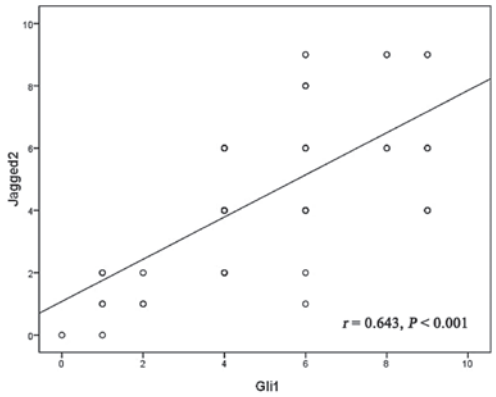

Figure 3. In the Spearman's rank correlation coefficient test, Jagged2 demonstrated a positive correlation with Gli1 protein expression in hepatocellular carcinoma tissues (r=0.643, P<0.001).

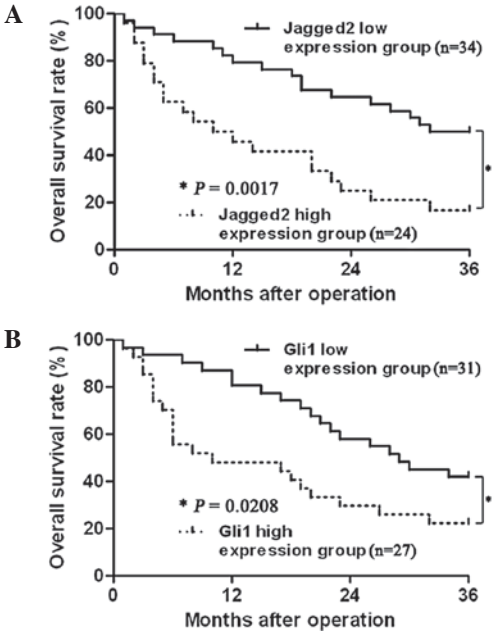

Figure 4. Kaplan-Meier overall survival curves of patients with hepatocellular carcinoma according to the level of Jagged2 and Gli1 protein. (A) The Jagged2 high expression group (n=24), Jagged2 expression level ≥ median value of Jagged2 expression level; low Jagged2 expression group (n=34), Jagged2 expression level < median value of Jagged2 expression level. (B) The high Gli1 expression group (n=27), Gli1 expression level ≥ median value of Gli1 expression level; low Gli1 expression group (n=31), Gli1 expression level < median value of Gli1 expression level.

normal tumor-adjacent tissues (4.47±2.48 vs. 0.78±0.68; P<0.001; Fig. 1A). The same was observed for Gli1 protein expression (4.84±2.24 vs. 0.74±0.79; P<0.001; Fig. 1B). The positive staining of Jagged2 was observed mainly in the cytoplasm, while Gli1 was localized in the cytoplasm and nuclei in HCC cells (Fig. 2A-B).

Correlation between Jagged2 and Gli1. A previous study indicated that there is a correlation between Jagged2 and Gli1 expression in various malignancies; however, this association has not been observed in HCC (15). Using the Spearman's rank correlation coefficient test, Jagged2 was demonstrated to be positively correlated with Gli1 protein expression (r=0.643, P<0.001; Fig. 3)

Correlation between Jagged2 or Gli1 protein expression in HCC tissues and clinicopathological features. The correlation between Jagged2 or Gli1 protein expression and clinicopathological parameters in 58 HCC patients was analyzed by the Mann-Whitney U-test, and the results are shown in Table I. Jagged2 protein expression was significantly correlated with intrahepatic metastasis (P=0.007), advanced TNM stage (P=0.001) and high Edmonson pathological classification

(P=0.004). Gli1 protein expression was also closely associated with intrahepatic metastasis (P=0.024), advanced TNM stage (P=0.007) and high Edmonson pathological classification (P=0.034).

Analysis of risk factors for patient survival. The Kaplan-Meier method was used in univariate analysis. Median values of Jagged2 and Gli1 were used as cut-off points to divide the patients into two groups: The low expression group and the high expression group. As demonstrated in Table II, Jagged2, Gli1, α-fetoprotein (AFP level), maximum tumor diameter, intrahepatic metastasis, TNM stage and Edmonson pathological classification were associated with patient 3-year overall survival (P<0.05, respectively). The survival curves of variables are demonstrated in Fig. 4. With further investigation, Cox proportional hazard regression analysis was conducted with the seven variables that were previously determined, to select the potential risk factors for determining prognosis. The results demonstrated that TNM stage, Edmonson pathological classification and Jagged2 expression were potential risk factors, which affected the prognosis of HCC patients (P<0.05, respectively; Table III).

Discussion

As one of the ligands of Notch signaling, Jagged2 expression was found to be associated with a variety of tumor types. However, there is a controversy regarding the effect of Jagged2 on tumorigenesis and tumor development. One study reported that Jagged2 regulated the expression of cytokines, which promote antitumor immunity (17). However, another study rejected this and demonstrated that Jagged2 enhances cell growth, invasion and migration in two uveal melanoma cell lines (Mel285 and Mel290) (18). In the cutaneous melanoma cell line, *Jagged2* was the most markedly overexpressed gene in the highly invasive clone, with an RNA level ~15-fold higher than that in the less invasive cells (19). Furthermore, when Jagged2 was induced at the transcriptional level under hypoxic conditions, it was significantly correlated with angiogenic processes in breast cancer, renal cell carcinoma and epithelial tumor cells (20). However, to the best of our knowledge no study has been conducted regarding the association between HCC and Jagged2. In the present study, the results identified that Jagged2 expression in HCC tissues was notably higher than that in the matched normal tumor-adjacent tissues. In addition, the clinical pathological parameter analysis demonstrated that Jagged2 was significantly correlated with intrahepatic metastasis, advanced TNM stage and high Edmonson pathological classification, suggesting that Jagged2 may have an important role in tumorgenesis and tumor development. This observation is consistent with those of previous studies concerning the oncogenic effect of Jagged2 in several malignant tumor types (8-11). It was also identified that patients with low Jagged2 expression had an improved outcome compared with patients with high Jagged2 expression. Furthermore, Cox proportional hazard regression mode analysis suggested that Jagged2 was an independent factor for the prediction of poor long-term HCC survival following radical liver resection.

Hedgehog/Gli1 signaling pathway is involved in a variety of human cancer types and aberrant activation of this signaling has also been identified to be associated with HCC (21-23). Recent studies identified that the transcription factor Gli1 was associated with poor prognosis among the patients with HCC and the expression of the *Gli1* gene in tumor tissues was significantly correlated with disease-free survival and overall survival rates (24). Using immunohistochemical staining, the present study demonstrated that Gli1 expression in HCC tissues was higher than that in the matched tumor-adjacent tissues. Gli1 was significantly correlated with intrahepatic metastasis, advanced TNM stage and high Edmonson pathological classification, and the 3-year overall survival of the patients with Gli1 high expression was lower than that in those with low Gli1 expression. Additionally, the AFP level, maximum tumor diameter, intrahepatic metastasis, TNM stage and Edmonson pathological classification were associated with the 3-year overall survival. According to the clinical research, HCC patients with advanced TNM stage and high Edmonson pathological classification have a poor long-term survival.

Hedgehog signaling induced Jagged2 upregulation and tumor growth factor-β1 secretion to promote the motility and invasiveness of cancer cells (18). A previous transcriptome analysis identified Jagged2 as a sonic hedgehog-regulated factor (15). However, the accurate mechanisms involved in Hedgehog signaling-induced Jagged2 upregulation remain unclear. In the present study, it was demonstrated that Jagged2 protein expression was positively correlated with Gli1. This result indicated that Gli1, the key transcriptional factor in Shh signaling, may regulate Jagged2 expression in HCC.

In conclusion, the present study demonstrated that Jagged2 and Gli1 were overexpressed in HCC tissues and Jagged2 was positively correlated with Gli1 protein expression. High-expression of Jagged2 or Gli1 was associated with a poor 3-year overall survival in HCC patients and Jagged2 acted as an independent predictor of an unfavorable prognosis. Elucidating the regulation between Gli1 and Jagged2 expression, and the mechanisms involved in promoting HCC invasion and metastasis by Jagged2, requires further investigation.

Acknowledgements

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