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Original Article

Prognostic signifcance of IL35 expression in human hepatocellular carcinoma

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Abstract: Object: This study was performed to explain the underlying role of IL35 on the prognosis of patients with HCC. Methods: The expression of IL35 at the protein level was detected with immunohistochemistry (IHC). Chi-square test was performed to analyze the relationship between IL35 expression and clinical parameters of HCC patients. The correlation between expression level of IL35 and the prognosis of patients with HCC was evaluated by Kaplan-Meier method and Cox regression method. Results: The positive rate of IL35 expression in HCC samples was about 62.7%, which was significantly higher than that in paired normal specimens (12%). The analyses suggested that there was no correlation between IL35 expression and age, gender, and tumor size ($P>0.05$), but a tight relationship was found between IL35 expression and metastasis, AFP, HBV infection, Child-Pugh ($P<0.05$). Susequent Kaplan-Meier analysis result indicated that positive expression of IL35 induced low survival rate of HCC patients, and the Cox regression analysis suggested that IL35 might be a biomarker for prognosis of patients with HCC. Conclusion: Generally, results of this study demonstrated that expression of IL35 shared a close association with the prognosis of patients with HCC. Therefore, IL35 could be considered as a novel index for prognosis of patients with HCC.

Keywords: Hepatocellular carcinoma, immunohistochemistry (IHC), interleukin 35 (IL35), prognosis

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer-related death worldwide [1]. According to the International Agency for Research on Cancer, more of an estimated 750,000 new liver cancer cases and 695,900 cancer deaths occurred worldwide every year [2]. The observed 5-year survival rate of HCC was 44%, and the observed HCC early recurrence and early hepatic decompensation rates were 21% and 10% respectively [3]. This mainly stems from a lack of early diagnosis, the deteriorated condition of the cirrhotic liver from which most HCC cases develop, and the high resistance of HCC to chemotherapy [4, 5]. Therefore more accurate and sensitive diagnostic methods are still needed to benefit HCC patients.

Interleukin-35 (IL-35) belongs to the IL-12 family of heterodimeric cytokines, These cytokines form via combinations of p19, p28, p35, p40, and Ebi3 subunits, and in addition to IL-35 (p35/Ebi3), also include IL-12 (p35/p40), IL-23 (p19/p40), and IL-27 (p28/Ebi3) [6]. Members of the IL-12 family exhibit mostly immunosuppressive effector (IL-27 and IL-35) functions or are mostly pro-inflammatory (IL-12 and IL-23). IL-35 itself is formed by a bond between p35 and Ebi3 [7]. IL-35 was first reported to be produced exclusively by T regulatory cells (Tregs) [8, 9]; after that, other studies demonstrated that IL-35 could also be produced by B cells [10-12] and tolerogenic dendritic cells (DC) [13]. Some non-immune cell types, such as several kinds of cancer cells, were also shown to express IL-35 [14, 15].

Several studies have shown that IL-35 played important roles in tumor immunology [16]: IL-35 could promote tumor growth via the enhancement of myeloid cell accumulation, tumor angiogenesis, and suppression of tumor immunity [15]; research identified a previously unrecognized role for IL-35-producing CD1d (hi) CD5 (+) B cells in the pathogenesis of pancreatic

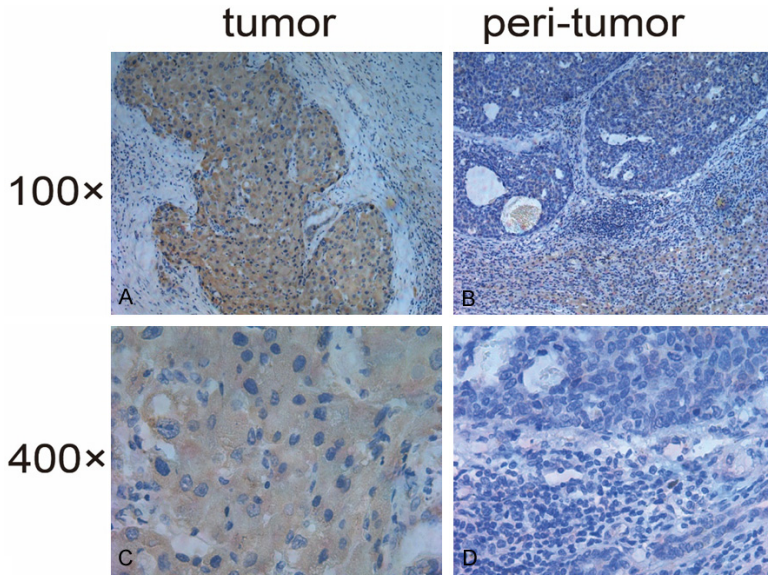

Figure 1. IHC staining of HCC tissue and peri-tumor tissue.

Table 1. Different expression levels of IL-35 in HCC

Tissue	Case NO	Expression		Positive rate	*P* value
		Positive	Negative		
HCC	150	114	36	76%	P<0.01
Normal	150	18	132	12%	P<0.01

cancer and underscored the potential significance of a B-cell/IL-35 axis as a therapeutic target [12]; research on lung adenocarcinoma suggested that plasma cells may be one of the major producers of IL-35 in lung cancer stroma, and IL-35 might play important roles in the progression of lung cancer [17]. Elevated levels of IL-35 could also be detected in lymphoma cells [18], and predicted poor outcomes in cases of leukemia, colorectal, and pancreatic cancer [19-21].

However, the prognostic significance of IL-35 in HCC remains unclear. In this study, we determined the expression of IL-35 and evaluated its prognostic significance in HCC patients. Our data indicated that IL-35 was remarkably increased in HCC compared with the peritumor tissues, and could be served as a promising biomarker of HCC prognosis.

Methods and materials

Patients and specimens

150 paraffin-embedded HCC tissues were used, of which 105 were in the primary cohort and 45 were in the validation cohort. HCC samples and paired peri-tumor specimens used in this study were obtained from 2009 to 2013, from 150 preoperatively untreated patients with histologically confirmed HCC, including 99 males and 51 females, aged from 21 to 64, with a mean age of 47 years, in Huashan Hospital. None of these selected patients experienced preoperative cancer treatment. This investigation lasted about 4 years, from April 2013 to May 2017. This retrospective study was approved by our institution's research ethics board. All of the patients involved in this study gave informed consent.

Immunohistochemistry and scoring

The expression level of IL-35 in 150 cases of HCC tissues and paired para-cancer tissue were tested with the immunohistochemistry (IHC) method. Specifically, the samples were fixed in 3% formaldehyde solution, embedded in paraffin and then cut into 4 μm-thick sections. Then the prepared sections were deparaffnized and rehydrated in a graded series of ethanol after baking at 65°C for 30 min. Following, 0.01 M citrate buffer (pH 6.0) was used to incubate with the sections at 100°C for 15 min, and cooled at room temperature for another 20 min. After that, the primary antibody rabbit anti IL-35 was added to the sections and the mixture was incubated at 4°C overnight. After that, biotin-labeled second antibody was added to each section, incubating for 15 min at room temperature, followed by washing with PBS twice, each for 5 min. Finally, staining signaling was determined using DAB by the avidin-biotin-peroxidase method. The sections were air-dried and reserved to use. Positive staining of IL-35 protein displayed mainly in the cytoplasm. Photographs of two representative fields were captured under high-power magnification (× 400), and identical settings were used for each photograph. The representative fields were defined as follows: first, we glance at × 100 magnification. If there were both high-expression and low-expression areas, we captured an

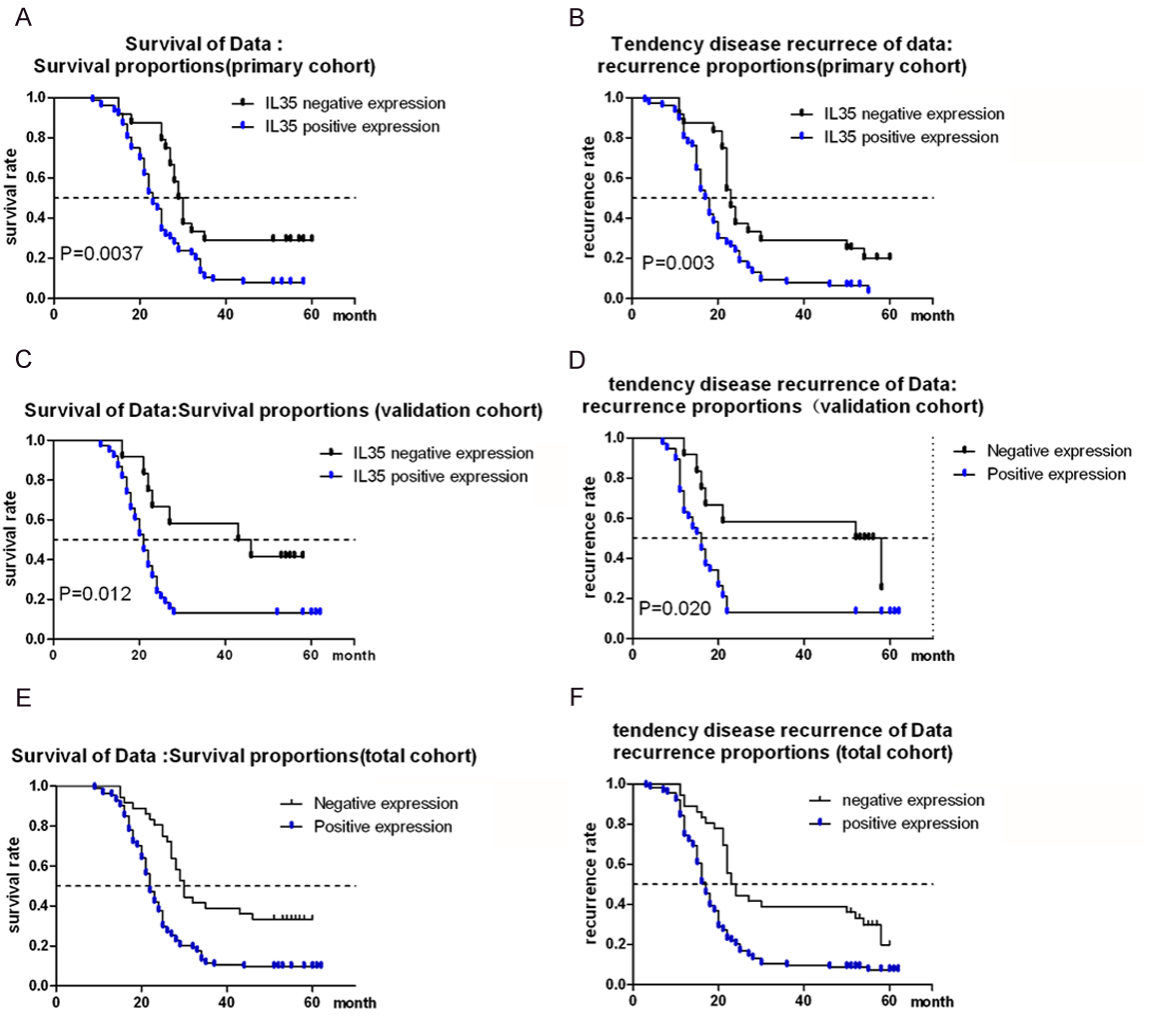

Figure 2. A. Correlation of survival time (OS) and IL-35 expression in primary cohort. B. Correlation of tendency to disease recurrence (TTR) and IL-35 expression in primary cohort. C. Correlation of survival time (OS) and IL-35 expression in validation cohort. D. Correlation of tendency to disease recurrence (TTR) and IL-35 expression in validation cohort. E. Correlation of survival time (OS) and IL-35 expression in total cohort. F. Correlation of tendency disease recurrence (TTR) and IL-35 expression in total cohort.

image at × 400 magnification, that contained both a high-expression and low-expression area. Image-Pro Plus v6.0 software was used to count and measure integrated optical density (IOD), and mean IOD was calculated from two photographs per patient. X-tile plots were created for assessment of IL-35 expression, and optimization of cutpoints was based on the outcome of IOD value [22]. Statistical significance was assessed using the cutoff score derived from 150 cases by a standard log-rank method, with *p* values obtained from a lookup table.

Follow-up

Patients were prospectively followed after surgery according to a formulated schedule. Follow-up information of all patients was updated every 3 months for the first 2 years, every 6 months for the next 3 years, and then every year thereafter. The follow-up time ranged from 9 months to 62 months (median, 28.3 months). At reference date (Nov 30, 2017), patients still alive were censored at their last consultation, and patients who died were censored at their death date. Overall survival time was calculated from the date of the initial surgical operation to death.

Statistical analysis

Data collected in this study was analyzed by SPSS18.0 software (SPSS Inc, USA). The correlation between IL-35 expression and clinical

Table 2. Correlation between IL-35 expression and clinical pathological features of HCC

		IL-35 protein expression			
		Positive	Negative	X^2	*P* value
Gender	Male	75	24	0.009	0.923
	Female	39	12		
Age (years)	≤55	47	17	0.402	0.526
	>55	67	19		
Liver cirrhosis	Yes	108	2	106.76	0
	No	6	34		
TNM stage	I-II	54	10	4.293	0.038
	III-IV	60	26		
Tumor sizes (cm)	≤4.5	77	22	0.505	0.478
	>4.5	37	14		
Metastasis	Yes	94	19	12.969	0
	No	20	17		
AFP (ug/L)	<400	21	20	18.995	0
	≥ 400	93	16		
HBV infection	Yes	105	6	80.928	0
	No	9	30		
HCV infection	Yes	64	16	1.504	0.22
	No	50	20		
Child-Pugh classification	A	60	16	86.655	0
	B	54	20		
Edmondson grade	III	46	14	82.927	0
	IV	68	22		
Surgical intervention	Radical resection	90	10	57.143	0
	Palliative resection	24	26		
Post-operative TACE	Yes	24	26	32.237	0
	No	90	10		

parameters of patients with HCC was evaluated by Chi-squared test. Then Kaplan-Meier and Cox regression analysis were conducted to evaluate the factors that could influence the prognosis of patients with HCC. Differences were considered significant if P<0.05.

Results

Expression of IL-35 in HCC tissues and paired peritumor (normal) tissues

We determined the expression of IL-35 in HCC patients with IHC (**Figure 1**). The result showed that among the 150 HCC samples, 114 (62.7%) specimens had positive expression, but among paired normal samples, only 18 (12%) specimens were positive (**Table 1**). The result illustrated that the expression of IL-35 protein in HCC tissues was significantly higher than that of paired normal tissues (P<0.05).

Correlation of IL-35 expression with prognosis of HCC patients

IHC analysis was performed to assess the IL-35 expression of 150 paraffin-embedded HCC tissues and paired peri-tumor tissues, in which 100 were in the primary cohort and 50 were in the validation cohort. The survival and recurrence of HCC patients was analyzed with Kaplan-Meier analysis. As shown in **Figure 2A**, HCC patients in the primary cohort with high IL-35 expression had much shorter OS times, (mean OS 23 vs 29.5 months, P = 0.0037). **Figure 2B** suggested that the cases with high expression of IL-35 had a higher tendency of disease recurrence (mean TTR 17.5 vs 23 months, P = 0.003). Likewise, **Figure 1C** showed that HCC patients in validation cohort with high IL-35 expression had much shorter OS times (mean OS 44.5 vs. 21 months, P =

Table 3. Univariate and multivariate analyses of clinical parameters and overall survival

Univariate analysis of clinical parameters			
	HR (hazard ratio, HR)	95% CI	P
Gender Male vs. female	0.577	0.385-0.865	0.008
Age (years) ≤55 vs. >55	0.657	0.459-0.94	0.022
Tumor size (cm) ≤4.5 vs. >4.5	0.562	0.371-0.85	0.006
Liver cirrhosis Yes vs. No	3.977	2.439-6.486	0
TNM stage I-II vs. III-IV	0.591	0.413-0.847	0.004
Metastasis Yes vs. No	8.284	4.395-15.616	0
AFP (ug/L) ≤400 vs. >400	6.258	3.626-10.802	0
HBV Yes vs. No	3.479	2.158-5.607	0
HCV Yes vs. No	1.862	1.279-2.711	0.001
Child-Pugh class Yes vs No	0.567	0.392-0.82	0.003
Edmondson class III vs. IV	1.227	0.854-1.762	0.269
Surgery Radical resection vs. palliative resection	0.526	0.354-0.782	0.002
Post-operative TACE Yes vs No	0.526	0.354-0.782	0.002
IL-35	9.706	4.871-19.34	0
Multivariate analysis of clinical parameters			
	HR (hazard ratio, HR)	95% CI	P
IL-35 Yes vs No	5.001	1.822-13.727	0.002
Tumor size (cm) ≤4.5 vs. >4.5	1.905	1.2-3.024	0.006
Metastasis Yes vs. No	3.707	1.474-9.325	0.005

0.012, **Figure 2C**), and a higher tendency of disease recurrence (mean TTR 16 vs 55 months, P = 0.020, **Figure 2D**). The association between IL-35 expression and OS/TTR was also analyzed in all 150 HCC patients, including both a primary cohort and validation cohort. The results showed that HCC patients with high IL-35 expression had much shorter OS times (mean OS 22 vs 30 months, **Figure 2E**, P< 0.0001) and a higher tendency of disease recurrence (mean TTR 17 vs. 23.5 months, **Figure 2F** P< 0.0001).

Correlation between IL-35 protein expression and clinical parameters of HCC patients

We further investigated the relationship between the expression of IL-35 and the clinical parameters of HCC patients. Chi-square result showed that there was a significant relationships between the expression level of IL-35 and certain clinical parameters, such as metastasis, AFP, HBV infection, Child-Pugh (P<0.05), but no tightly correlation was found between expression level of IL35 and other clinical parameters, likely age, gender, and tumor size (P>0.05) (**Table 2**).

Next we analyzed the correlation between clinical parameters and prognosis of patients with HCC by univariate and multivariate analyses. The data suggested that the clinical parameters such as tumor size (>4.5 cm), metastasis (positive), and HBV (positive) predicted poor prognosis of HCC patients (**Table 3**).

Discussion

HCC is one of the most prevalent cancers with high rate of metastasis and mortality [23]. Surgery and interventional therapy for HCC have made enormous strides in recent years; however, a dismal prognosis for HCC patients after surgical resection is still common [24]. Although several molecular biomarkers, such as Smad [25], OPN [26], CD133 [27], and β-catenin [28], have been reported to have clinical significance for predicting HCC prognosis, biomarkers for HCC diagnosis and prognostic prediction are still urgently needed.

The interleukin family is widely distributed in the tumor microenvironment, and types of

interlukin molecules play different roles in tumor progression and metastasis. As a newly discovered interleukin family member, IL-35 has been reported to play important roles in tumor immunology [16] and autoimmune disease [29]. Recent findings suggested that IL-35 could inhibit immune activity and enhance tumor metastasis through various signaling pathways: for example, pancreatic cancer cells could produce IL-35 to recruit monocytes via CCL5 and induce macrophage to promote angiogenesis by expression of several chemokines [30], However, another study suggested that the pro-tumorigenic effect of B cells was mediated by their expression of IL-35 through a mechanism involving IL-35-mediated stimulation of tumor cell proliferation, and the results identified a previously unrecognized role for IL-35-producing CD1d high CD5+ B cells in the pathogenesis of pancreatic cancer [12]. This suggested that IL-35 could promote progression and metastasis of tumor (especially pancreatic cancer) through multiple, alternative pathways, and it proved the complexity of interaction between tumor and the tumor microenvironment. Nonetheless, the role played by IL-35 in the progress and metastasis of HCC is still unknown.

Our results showed that IL-35 protein level was significantly upregulated in HCC tissues compared with normal tissues. HCC patients with high IL-35 expression had much shorter OS times; the cases with high expression of IL-35 had higher tendency of cancer recurrence. Further investigation suggested a significant relationship between the expression level of IL-35 and certain clinical parameters, such as metastasis, AFP, HBV infection, and Child-pugh, but no tight correlation was found between expression level of IL-35 and other clinical parameters, likely age, gender, and tumor size. Next, the correlation between clinical parameters and prognosis of patients with HCC was analyzed. Clinical parameters such as tumor size (larger than 4.5 cm), metastasis (positive), and HBV (positive) predicted poor prognosis of HCC patients.

Thus far, there are no similar study to research the relationship between the expression of IL-35 and the prognosis of HCC. But, multi-center trials with various tumor markers are still needed to confirm our conclusion, and the molecular mechanism of IL-35 promoting the progression and metastasis of HCC still needs to be clarified.

In conclusion, our research suggests that the expression of IL-35 was significantly higher in HCC tissues compared with paired normal tissues, and high expression of IL-35 could induce poor prognosis of HCC patients.

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Disclosure of conflict of interest

All authors contributed equally to this work.

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