


# Overexpression of interleukin-35 in intrahepatic cholangiocarcinoma is a prognostic indicator after curative resection

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Interleukin-35 (IL-35) is implicated in tumorigenesis, but its exact impact on intrahepatic cholangiocarcinoma (ICC) is not clear. The aim of the present study was to explore the specific effect of IL-35 on patient prognosis. Additionally, we formulated an effective prognostic nomogram for ICC patients after curative resection. Immunohistochemistry was applied to explore IL-35 expression as well as IL-35 receptor (IL-35R) in 102 ICC patients. Results showed that IL-35 was highly expressed in ICC tumor tissues and was positively associated with lymph node metastasis (LNM), TNM stage and vascular invasion and was an independent prognostic factor for patients' overall survival (OS) and recurrence-free survival (RFS). High expression of IL-35R (gp130 and IL-12R $\beta$ 2) was also observed in ICC cancer tissues, but only gp130 was an independent prognostic factor for OS and RFS and was indispensable in IL-35-mediated ICC clinical prognosis. The nomogram comprising carcinoembryonic antigen, LNM, IL-35 and gp130 expression achieved better predictive accuracy compared with TNM stage for OS. Our data support that high IL-35 expression correlates with ICC aggressiveness and emerges as a valuable biomarker for evaluating ICC progression and prognosis in clinical work.

## KEYWORDS

gp130, IL-12R $\beta$ 2, IL-35, intrahepatic cholangiocarcinoma, nomogram

**Abbreviations:** CEA, carcinoembryonic antigen; CI, confidence interval; C-index, concordance index; DCA, decision curve analysis; EBV3, Epstein-Barr virus (EBV)-induced gene 3; HR, hazard ratio; ICC, intrahepatic cholangiocarcinoma; IHC, immunohistochemistry; IL-35, interleukin-35; IL-35R, IL-35 receptor; LNM, lymph node metastasis; OS, overall survival; RFS, recurrence-free survival; TMA, tissue microarray.

Zhang, Gan, Jing, and Zheng contributed equally to this work and should be considered as co-first authors.

## 1 | INTRODUCTION

Intrahepatic cholangiocarcinoma is the second most common liver malignancy arising from the intrahepatic biliary tract,<sup>1</sup> and accounts for 10% to 15% of primary liver cancer.<sup>2</sup> As an

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uncommon disease, in recent years, the incidence of ICC is drastically increasing worldwide,<sup>3</sup> but there is hardly any progress in the treatment of ICC for OS rate which is still around 22%-40% after surgical resection.<sup>4,5</sup> To make matters worse, such a dismal situation manifests a major challenge as most ICC patients are not sensitive to conventional chemotherapy and radiotherapy.<sup>6,7</sup> In addition, early identification of ICC seems more difficult because of the early lymphatic metastasis and limited effective diagnoses.<sup>8</sup> Therefore, new and specific prognostic biomarkers or scoring systems to stratify the prognosis in ICC patients are urgently needed.

In recent years, much research has confirmed that the tumor microenvironment serves an important role in tumor development.<sup>9</sup> Tumor cells use multiple mechanisms to establish an immunosuppressive network for dampening antitumor immunity, predominately by the production of inhibitory immune molecules and induction of regulatory cells.<sup>10</sup> IL-35 is a newly identified cytokine, which belongs to the IL-12 family and is predominantly produced by regulatory T cells (Treg).<sup>11,12</sup> IL-35 is a heterodimeric cytokine composed of the P35 subunit of IL-12 and the EBI3 subunit.<sup>13,14</sup> IL-12R $\beta$ 2 and gp130 are reported as two subunits of IL-35R<sup>15</sup> and are critical for transmitting signals of IL-35. Downstream signals of IL-35 can go through a homodimer gp130:IL-12R $\beta$ 2 or gp130:gp130 homodimer and/or IL-12R $\beta$ 2:IL-12R $\beta$ 2 homodimer. After binding with IL-35R, IL-35 signals are initiated by the activation of members of the STAT family, then conducted into the nucleus and transcription of target genes is initiated.<sup>15,16</sup> Similar to IL-10 and transforming growth factor- $\beta$ , IL-35 has been suggested to show potent immunosuppressive effects to repress the functionality of effector T cells.<sup>11-13</sup> Recently, gene-expression analysis was used to discover that IL-35 has a broader tissue distribution.<sup>17</sup> Likewise, accumulating evidence notes that IL-35 is upregulated in several tumors, such as colorectal cancer,<sup>18</sup> pancreatic cancer<sup>19</sup> and hepatocellular carcinoma.<sup>20</sup> High expression of IL-35 contributes to promote tumor cell proliferation, inhibits apoptosis<sup>21</sup> and encourages tumor metastasis.<sup>19</sup> Furthermore, elevated levels of P35 and EBI3 expression are detected in poorly differentiated colorectal patients<sup>18</sup> and are associated with unfavorable prognosis in pancreatic patients.<sup>19</sup> Therefore, further studies are required to concentrate on the specific immune function that IL-35 exerts in diversified tumors. To the best of our knowledge, clinical correlation and prognostic significance of IL-35 in ICC patients is currently lacking.

Nomograms are commonly used statistical tools to estimate prognosis of individuals and provide more individualized outcome predictions based on integrating diverse biological and clinical variables. As a result of the obvious superiority compared with a traditional staging system, nomograms have been applied to several tumors to establish models for predicting prognostic factors.<sup>22</sup> Herein, we attempt to establish a nomogram integrating clinicopathological variables and IL-35 expression for evaluating the predictive effect of IL-35 in ICC patients.

## 2 | MATERIALS AND METHODS

### 2.1 | Tissue samples and clinical data collection

A total of 102 ICC patients who underwent curative resection were enrolled and retrospectively analyzed in the present study at Liver Cancer Institute, Zhongshan Hospital, Fudan University. We have followed the principles outlined in the Declaration of Helsinki for all human experimental investigations. Ethical approval was obtained from the Zhongshan Hospital Research Ethics Committee, and informed consent was obtained from each patient. Inclusion criteria for this study were as follows: (i) patients with no preoperative anti-cancer treatment; (ii) a definite pathological diagnosis of ICC; (iii) complete removal of tumor tissues; and (iv) complete clinicopathological data and follow-up information. Conventional clinicopathological variables such as vascular invasion, lymphatic metastasis, and TNM stage were recorded in our study. Child-Pugh scoring system was adopted for assessing liver function.<sup>23</sup> Clinical classification of tumors was estimated in accordance with the American Joint Committee on Cancer (AJCC, Seventh Edition) TNM classification. World Health Organization classification was used to estimate histological grade of tumors, and ICC tissue specimens were classified as well (G1), moderately (G2), or poorly differentiated (G3).<sup>24</sup> The interval between surgery and death was defined as OS and the interval between surgery and time of recurrence was defined as RFS.

### 2.2 | Tissue microarray construction and immunohistochemistry

Tissue microarrays were constructed according to a previous research report.<sup>25</sup> Two different tissue microarray blocks extracted from each case (3-mm-diameter each) were used for TMA construction.

Monoclonal rabbit antihuman antibodies P35, EBI3, gp130 and IL-12R $\beta$ 2 (1:100 dilution) were purchased from Abcam (Cambridge, UK). IHC was used to detect the expression of P35, EBI3, gp130 and IL-12R $\beta$ 2 in tissues of ICC, and surgical produces were carried out as described.<sup>26</sup> Two experienced observers were responsible for sliced evaluation in a double blind method. Expression of IL-35, EBI3 gp130 and IL-12R $\beta$ 2 was evaluated by a semiquantitative scoring system.<sup>27</sup> The final score was made by positively stained tumor cells (0, no positive tumor cells; 1, <10%; 2, 10%-35%; 3, 35%-75%; 4, >75%) multiplied by staining intensity (1, no staining; 2, weak; 3, moderate; 4, strong). Eventually, total scores  $\geq 8$  were defined as high expression and those <8 were defined as low expression. Representative images of positive and negative controls for IHC are shown in Figure S1.

### 2.3 | Cell lines and cell culture

Human cholangiocarcinoma cell lines RBE, HUCCT-1, HCCC9810 and normal bile duct cell HIBEpic (HBP) were obtained from the

Chinese Academy of Sciences (Beijing, China). Cells were cultured in RPMI-1640 with 10% FBS and 1% penicillin-streptomycin (Hyclone, Logan, UT, USA), cultured in a 37°C incubator with 5% CO<sub>2</sub>.

## 2.4 | Western blot

Protein (30 µg) was separated by 10% SDS-PAGE electrophoresis, then transferred to PVDF (Millipore, Temecula, CA, USA), and the membranes were blocked in 5% BSA for 1 hour, then incubated with primary antibodies (P35, EB13, gp130 and IL-12Rβ2 (1:1000 dilution; Abcam) at 4°C overnight. β-Actin (Kangcheng, Shanghai, China) was used as an internal control. After 12 hours, the membranes were incubated with secondary antibody (Kangcheng) for 1 hour at room temperature. Finally, the protein bands were detected by enhanced chemiluminescent (ECL) substrate and processed by Image, Lab software (Bio-Rad, Mississauga, ON, Canada).

## 2.5 | Statistical analysis

Statistical analyses were carried out with SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Association between IL-35 and clinicopathological characteristics was analyzed using chi-squared test ( $\chi^2$ -test) or Fisher's exact test. Survival curves were evaluated by Kaplan-Meier analysis and compared by log-rank test. Cox proportional hazards regression model was adopted for univariate and multivariate analysis. Variables significant on univariate analysis (defined as  $P < .05$ ) were chosen as explanatory variables for the multivariate model.  $P < .05$  was considered statistically significant. The rms package in R project (R version 3.0.2) was used to establish a nomogram based on the results of multivariate analysis.<sup>28</sup> Predictive accuracy of the nomogram was measured by C-index and calibration curve. The larger the C-index, the more accurate the prognostic prediction.<sup>29</sup> DCA, a novel evaluation method, assessed the clinical usefulness of the nomograms for the prediction of prognosis.<sup>30</sup> This method used a different weight to the true- and false-positive results for incorporating the clinical outcomes of the nomogram. This weight was varied for reflecting divergence in patient preferences or differences in the risks of a procedure. These preferences were expressed in terms of a threshold probability. The following formula was used to calculate the net benefit of DCA.

$$\text{Netbenefit} = \frac{\text{true} - \text{positives}}{n} - \frac{\text{false} - \text{positives}}{n} \times \frac{\text{pt}}{(1 - \text{pt})}$$
, where  $n$  is total patients, and  $\text{pt}$  is threshold probability of OS. A high net benefit calculated with favorable probability was the optimal nomogram model. This method and the interpretation of final results are described in detail in these two studies.<sup>30,31</sup>

## 3 | RESULTS

### 3.1 | Patient clinicopathological profiles

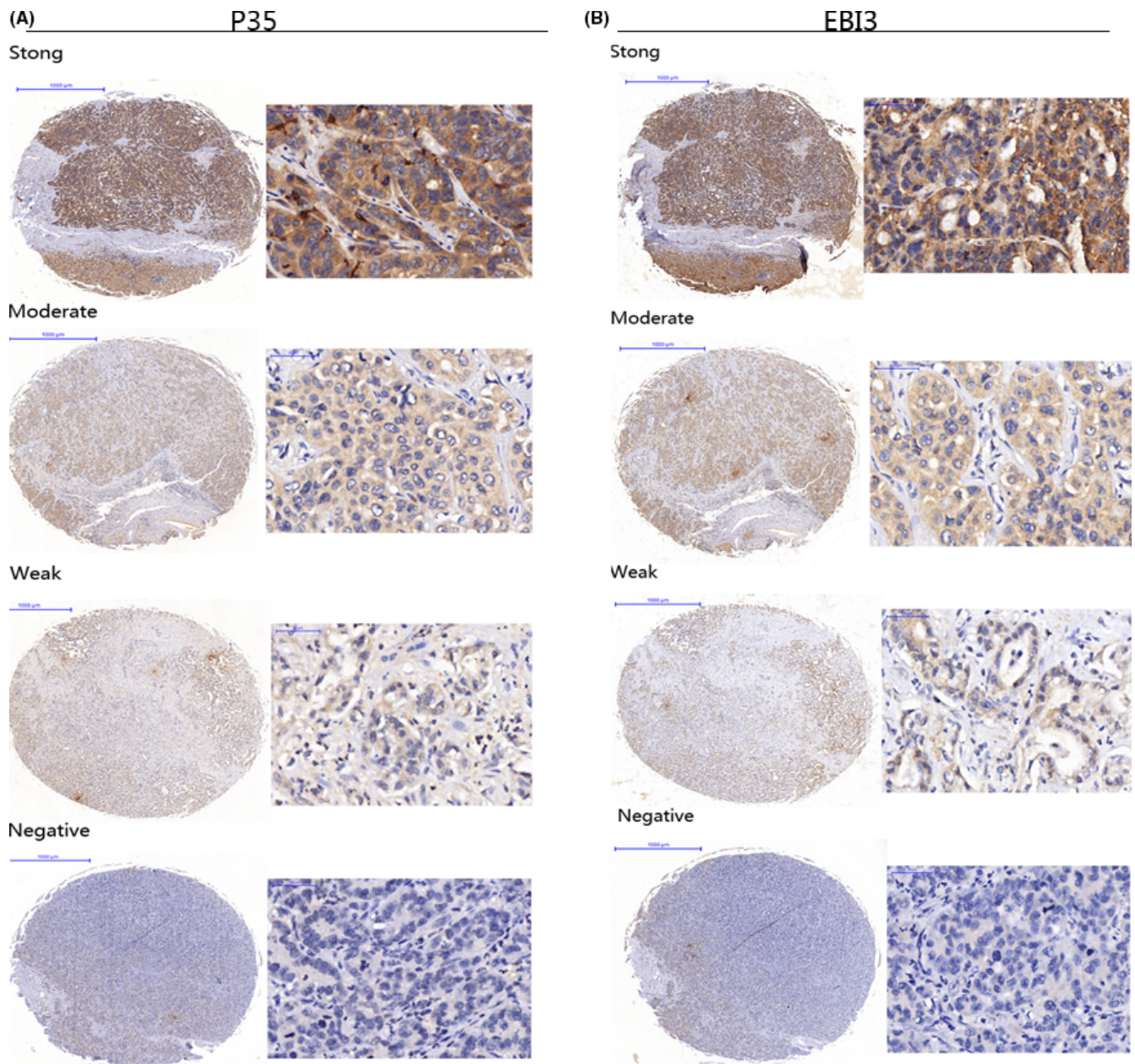
A total of 102 patients were included in the present study (64 males and 38 females) and average age of the patients was  $61.4 \pm 9.7$  years. Mean tumor size was  $(5.4 \pm 2.7)$  cm, and 61 patients (59.9%) were noted with tumor size  $\geq 5$  cm. Twenty-three (22.6%) patients had

**TABLE 1** Correlations between IL-35 and clinical parameters in 102 ICC patients

Parameter	Total	IL35 expression		P-value
		High	Low	
Age (y)				
≤61	53	36	17	.779
>61	49	32	17	
Gender				
Female	38	28	10	.247
Male	64	40	24	
Liver cirrhosis				
Yes	17	12	5	.707
No	85	56	29	
Tumor encapsulation				
Complete	12	7	5	.514
None	90	61	29	
Tumor differentiation				
Well to moderate	44	29	15	.888
Poor	58	39	19	
Tumor number				
Single	79	53	26	.867
Multiple	23	15	8	
Tumor size (cm)				
<5	41	27	14	.886
≥5	61	41	20	
Vascular invasion				
Yes	27	13	14	<b>.017</b>
No	75	55	20	
Lymph node metastasis				
Yes	43	34	9	<b>.023</b>
No	59	34	25	
TNM stage				
I + II	39	20	19	<b>.010</b>
III + IV	63	48	15	
Child-Pugh score (A vs B)				
A	91	60	31	.748 <sup>a</sup>
B	11	8	3	
CEA (ng/mL)				
≥5	38	24	14	.562
<5	64	44	20	
gp130				
High	70	59	11	<b>.000</b>
Low	32	9	23	
IL-12Rβ2				
High	70	55	15	<b>.000</b>
Low	32	13	19	

<sup>a</sup>Fisher's exact test.

CEA, carcinoembryonic antigen; ICC, intrahepatic cholangiocarcinoma; IL-35, interleukin-35. The bold values were considered to be significant differences.



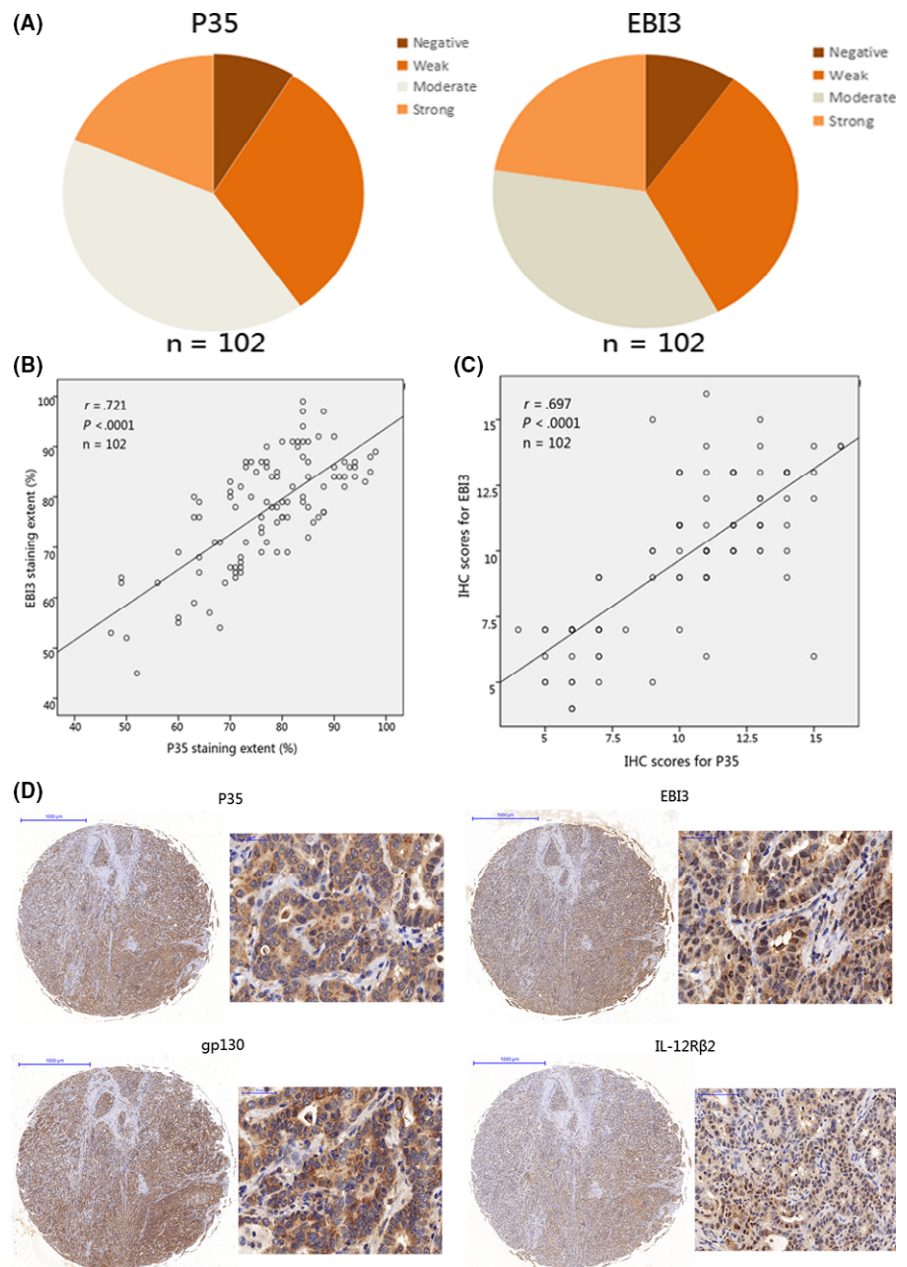
**FIGURE 1** Interleukin (IL)-35 expression in intrahepatic cholangiocarcinoma (ICC) tissues. Immunohistochemistry was used for analysis of the two subunit expression levels of IL-35: P35 and Epstein-Barr virus (EBV)-induced gene 3 (EBI3). (A,B) Representative images are shown for strong, moderate, weak and negative expression of P35 and EBI3 in ICC tumor tissues. (Magnification  $\times 20$ ,  $\times 400$ ; scale bars, 1000  $\mu\text{m}$ , 50  $\mu\text{m}$ )

multiple tumor nodules, 43 (42.1%) patients had LNM, and 27 (26.4%) patients had vascular invasion. Fifty-eight of 102 (56.9%) had poor differentiation. Based on the standard for TNM stage, 39 patients (38.2%) were in stage I + II, and 63 patients (61.8%) were in stage III + IV. Detailed characteristics of patients are presented in Table 1.

### 3.2 | Correlation between IL-35 expression and clinicopathological features

With no specific antibodies for IL-35, in the present study, we used perfect coexpression of two IL-35 subunits EBI3 and P35 as a

surrogate for IL-35 level. ICC samples were viewed as "IL-35 high" (both EBI3 and P35 IHC scores were  $\geq 8$ ). To explore the correlation between IL-35 and ICC, we carried out IHC analysis of coexpression of P35 and EBI3 in both tumor tissues and matched paratumor specimens. P35 and EBI3 showed perfect colocalization and were primarily localized in the cytoplasm of ICC tissues. In addition, P35 and EBI3 expression was also identified in some nuclei (Figure 1). Expression level of both P35 and EBI3 in tumor tissues was similar to paratumor tissues. P35 and EBI3 were rarely expressed in infiltrating lymphocytes and endothelial cells in ICC tissues (Figures S2,S3).



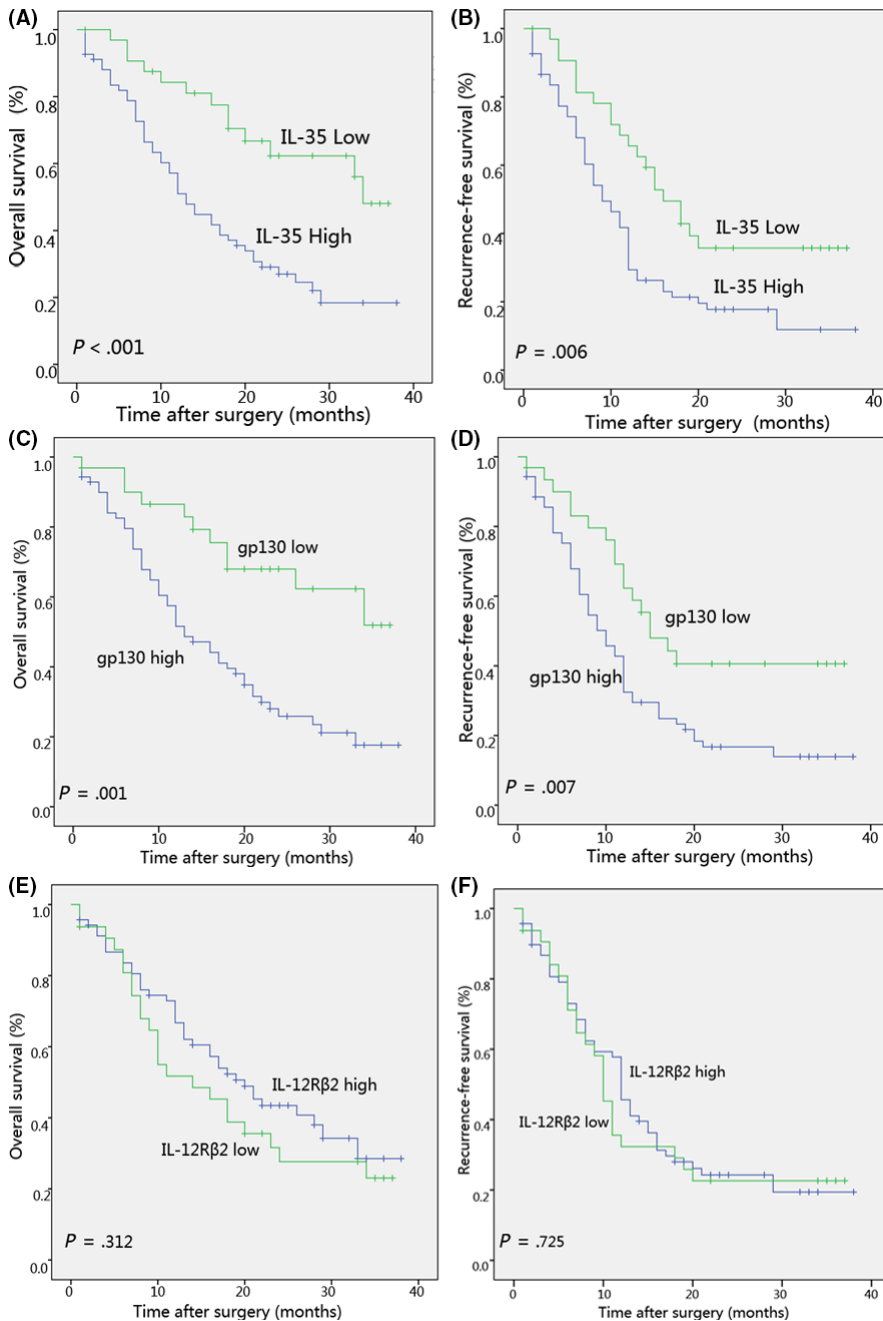
**FIGURE 2** Correlation analysis between P35 and EB13 expression in intrahepatic cholangiocarcinoma (ICC) tissues. (A) Distribution of immunohistochemistry (IHC) results of staining intensity of negative, weak, moderate, strong of P35 and Epstein-Barr virus (EBV)-induced gene 3 (EB13) expression. (B,C) Distribution of IHC results for P35 and EB13 expression. (B) Staining extent correlation analysis ( $r = .721$ ,  $P < .0001$ , Pearson's correlation analysis). (C) Final IHC scores correlation analysis ( $r = .697$ ,  $P < .0001$ , Spearman's correlation analysis). P35 expression was positively associated with EB13 level in ICC tissues. (D) Both gp130 and interleukin (IL)-12Rβ2 were highly expressed in ICC tumor tissues, and frequently coexpressed with P35 and EB13. (Magnification  $\times 20$ ,  $\times 400$ ; scale bars, 1000 μm, 50 μm)

Staining intensities, namely negative, weak, moderate, strong of P35 and EB13 were approximately 8.9%, 31.3%, 18.7%, 41.2% and 9.8%, 32.4%, 22.6%, 35.3%, respectively (Figure 2A). More importantly, extent of staining ( $r = .721$ ,  $P < .0001$ , Pearson's correlation analysis) and final IHC scores ( $r = .697$ ,  $P < .0001$ , Spearman's correlation analysis) of EB13 was noted to be positively correlated to P35 in ICC specimens (Figure 2B,C).

By using coexpression level of EB13 and P35 as a proxy for IL-35 level, the final high expression of IL-35 was assessed as 66.7% (68 out of 102) in ICC tumor tissues. Considering the importance of IL-35R in transmitting the signals of IL-35, expression of IL-35R (gp130 and IL-12Rβ2) was also detected in ICC tissues. As our results showed, both gp130 and IL-12Rβ2 were mainly expressed in the cytoplasm of ICC tissues, rarely expressed in infiltrating lymphocytes

and endothelial cells, and even frequently coexpressed with P35 and EB13, in accordance with recent research in pancreatic cancer<sup>19</sup> (Figures 2D, S2,S3).

To assess the correlation of IL-35 with ICC biology, associations between IL-35 and clinicopathological features were analyzed. As shown in Table 1, high expression of IL-35 was positively correlated with advanced TNM stage ( $P = .010$ ,  $\chi^2$ -test), regional LNM ( $P = .023$ ,  $\chi^2$ -test) and vascular invasion ( $P = .017$ ,  $\chi^2$ -test). However, no significant correlation was detected in terms of gender, age, liver cirrhosis, tumor size, Child-Pugh score, or differentiation. Significant positive correlation between IL-35 and IL-35R expression was found (gp130;  $P < .001$ ,  $\chi^2$ -test), (IL-12Rβ2;  $P < .001$ ,  $\chi^2$ -test). These findings suggested that the IL-35 ligand was usually accompanied by IL-35R and was consistent with the reported literature.<sup>15,19</sup>



**FIGURE 3** Significance of interleukin (IL)-35 and IL-35 receptor (IL-35R) expression in intrahepatic cholangiocarcinoma (ICC) patients. Kaplan-Meier analysis of overall survival (OS; left) and recurrence-free survival (RFS; right) of ICC patients, showing that both (A,B) IL-35 and (C,D) gp130 were independent prognostic factors for OS and RFS, but (E, F) IL-12Rβ2 was not

### 3.3 | Prognosis of IL-35 expression in ICC patients

Patients with high IL-35 expression were proven to have significantly poorer OS ( $P < .001$ ,  $\chi^2 = 12.495$ ) and shorter RFS ( $P = .006$ ,  $\chi^2 = 7.609$ ) than those with low IL-35 expression (Figure 3A,B). High expression of gp130 correlated with poor OS ( $P = .001$ ,  $\chi^2 = 11.764$ ) and short RFS ( $P = .007$ ,  $\chi^2 = 7.153$ ) (Figure 3C,D). However, IL-12Rβ2 was not an independent prognostic factor for both OS ( $P = .312$ ,  $\chi^2 = 1.020$ ) and RFS ( $P = .725$ ,  $\chi^2 = .124$ ) (Figure 3E,F). To obtain further evaluation on the role of IL-35 in ICC, we investigated the relationship between IL-35 and prognosis by Cox proportional hazards model. Univariate analysis showed that IL-35, gp130, tumor size, TNM stage, LNM, and CEA were independent

prognostic factors for OS. Independent prognostic factors for RFS were IL-35, gp30, TNM stage, and LNM (Table 2). Additionally, multivariate analyses indicated that IL-35, gp130, LNM, and CEA were independent prognostic factors for OS, and that LNM was an independent prognostic factor for RFS (Table 3). Regrettably, the significant differences of IL-35 and gp130 on RFS disappeared on multivariate analysis. IL-35-high and gp130-high patients were nearly twofold more likely to suffer from poorer OS (HR, 2.141; 95% CI, 1.008-4.544,  $P = .048$ ; HR, 2.234; 95% CI, 1.026-4.866,  $P = 0.043$ , respectively). The above findings prompted us to further examine the correlation between IL-35 and its receptor (gp130 and IL-12Rβ2) in ICC tissues. We therefore investigated the significance of combined IL-35 expression and IL-35R in patient prognosis. Patients

**TABLE 2** Univariate analyses of factors associated with OS and RFS

Variable	OS		RFS	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (y)	1.250 (0.758-2.601)	.382	1.218 (0.770-1.928)	.400
Gender	0.809 (0.488-1.342)	.413	0.810 (0.507-1.294)	.378
Liver cirrhosis	0.837 (0.436-1.607)	.594	0.785 (0.430-1.431)	.429
Tumor encapsulation	0.548 (0.219-1.366)	.197	0.642 (0.295-1.401)	.266
Tumor differentiation	1.241 (0.751-2.052)	.400	1.598 (0.994-2.568)	.053
Tumor number (multiple vs single)	1.533 (0.886-2.653)	.126	1.419 (0.841-2.349)	.189
Tumor size ( $\geq$ 5 cm vs <5 cm)	1.798 (1.054-3.068)	<b>.031</b>	1.404 (0.87-2.266)	.164
Vascular invasion	1.163 (0.673-2.011)	.588	1.387 (0.841-2.288)	.200
Lymph node metastasis	3.069 (1.840-5.120)	<b>.000</b>	1.969 (1.241-3.124)	<b>.004</b>
Child-Pugh score (A vs B)	1.394 (0.663-2.930)	.381	1.324 (0.658-2.665)	.431
TNM stage	3.008 (1.675-5.402)	<b>.000</b>	1.889 (1.152-3.097)	<b>.012</b>
CEA ( $\geq$ 5 ng/mL vs <5 ng/mL)	2.204 (1.337-3.632)	<b>.002</b>	1.561 (0.980-2.484)	.061
IL35 expression (high vs low)	2.868 (1.545-5.324)	<b>.001</b>	1.999 (1.191-3.356)	<b>.009</b>
gp130 expression (high vs low)	2.939 (1.528-5.655)	<b>.001</b>	2.023 (1.174-3.484)	<b>.011</b>
IL-12R $\beta$ 2 expression (high vs low)	1.299 (0.775-2.177)	.321	1.089 (0.668-1.776)	.733

CEA, carcinoembryonic antigen; 95% CI, 95% confidence interval; HR, hazard ratio; IL, interleukin; OS, overall survival, RFS, recurrence-free survival. The bold values were considered to be significant differences.

were divided into four groups: both IL-35 and gp130 were high; either IL-35 or gp130 was high; both IL-35 and gp130 were low; either IL-35 or gp130 was low. The poorest OS and shortest RFS were found in ICC patients with a high IL-35 level and high gp130 staining (Figure 4A). We continued carrying out subgroups to verify our results, showing again that raised IL-35 and gp130 expression implied poorer OS and shorter RFS (Figure 4B). Similarly, the role of IL-35 combined with IL-12R $\beta$ 2 was also explored as gp130. Intriguingly, no significant differences in OS and RFS were observed when IL-35 level was combined with IL-12R $\beta$ 2 expression (Figure 4C). Our subgroup analysis further confirmed this result (Figure 4D). Collectively, these outcomes supported that IL-35-related poor prognosis in ICC was associated with gp130 only, but not with IL-12R $\beta$ 2.

To further investigate whether IL-35 can discriminate between patients with different clinicopathological features, subgroup analyses were carried out. IL-35 was identified to stratify OS regarding LNM, tumor differentiation, and TNM stage (Figure 5). Additionally, IL-35 had the capability of stratifying RFS such as tumor with LNM, vascular invasion and some other clinicopathological features (Figure 6).

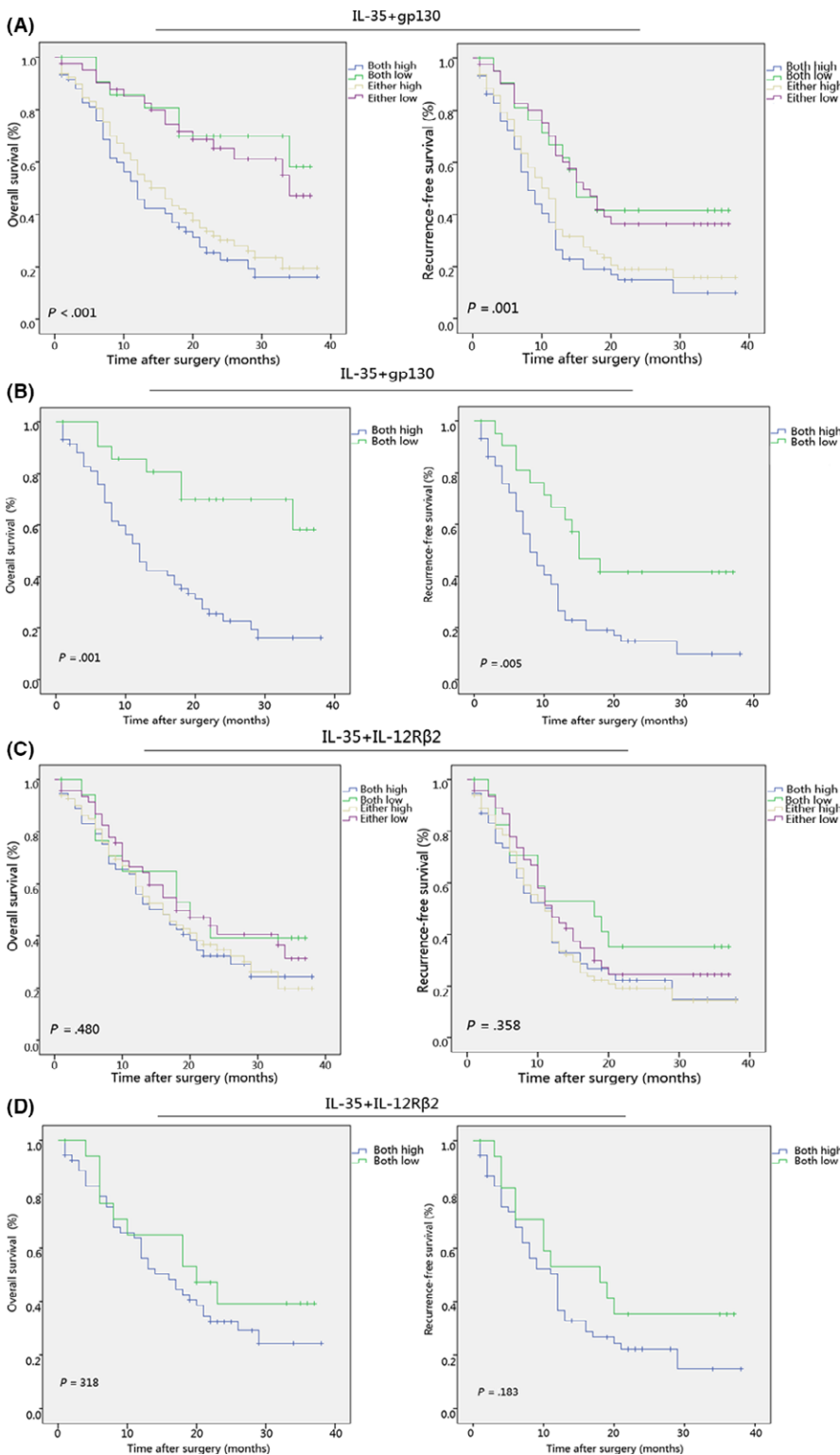
### 3.4 | Prognostic nomogram for OS

Finally, we integrated all significant independent prognostic factors according to multivariate analysis (Table 3) to create a prognostic nomogram (Figure 7A). C-index for OS prediction of the nomogram

integrating CEA, lymph node metastasis, IL-35 and gp130 expression was 0.72 (95% CI, 0.69-0.75) and the C-index for OS prediction of CEA, gp130, and IL-35 was 0.595 (95% CI, 0.592-0.598), 0.61 (95% CI, 0.607-0.613), and 0.616 (95% CI, 0.613-0.619), respectively. Calibration plot for the probabilities of OS at 1, 2, and 3 years after surgery fitted well between the prediction by nomogram and the actual observation. (Figure 7B-D). Nomogram was reported to be a more accurate model than the conventional staging systems in predicting prognosis of cancers.<sup>28,32,33</sup> In line with recent research, our nomogram showed a better predictive capability for prediction of OS compared to TNM stage, with a C-index of 0.72 (95% CI, 0.69-0.75) and 0.633 (95% CI, 0.630-0.636), respectively. On decision curve analysis, the nomogram showed superior net benefit with a wider range of threshold probability for predicting 1-, 2-, and 3-year OS relative to TNM stage (Figure 7E-G). This suggested that if ICC patients had a high expression of IL-35 and its receptor gp130, they should be classified as a high-risk population for predicting a poorer OS. Measures to detect the expression of IL-35 and gp130 early should be instituted.

### 3.5 | Expression of IL-35 and IL-35R in ICC cell lines

Western blot analysis showed that protein expression of P35, EB13, gp130 and IL-12R $\beta$ 2 was highly expressed in ICC cells compared with HBP cells (Figure S4). Our results suggested that the expression of IL-35 and IL-35R was upregulated in ICC cells.



**FIGURE 4** gp130 expression is essential for interleukin (IL)-35-associated poor prognosis of patients with intrahepatic cholangiocarcinoma (ICC). (A–D) Kaplan-Meier analysis of overall survival (OS; left) and recurrence-free survival (RFS; right) of ICC patients showing a high IL-35 level combined with high gp130 expression implied the poorest OS and RFS in ICC patients

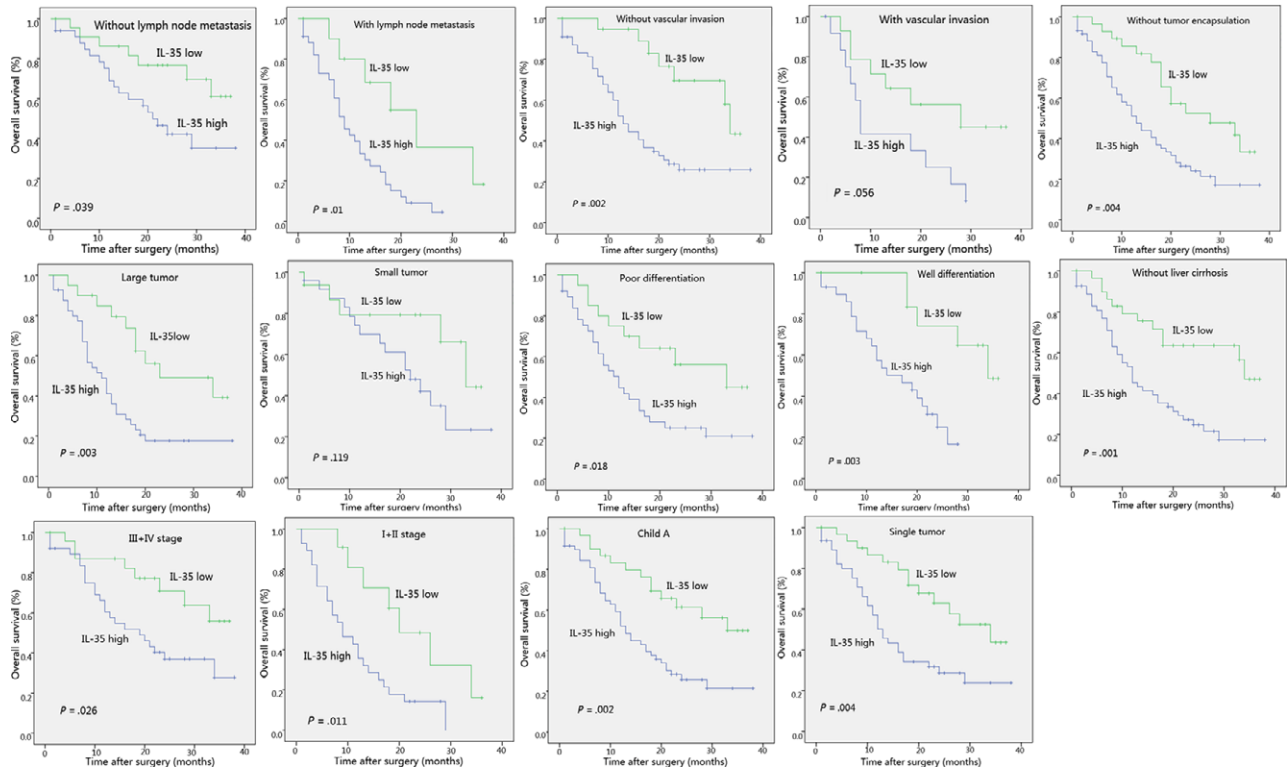
## 4 | DISCUSSION

Herein, we detected expression of IL-35 in 102 ICC patients and conducted a series of prognostic analyses. We first used coexpression of two IL-35 subunits EB13 and P35 to serve as a proxy to determine IL-35 level. As discovered, EB13 and P35 were highly coexpressed in ICC tumor tissues, which was consistent with previous findings.<sup>34–36</sup> In line

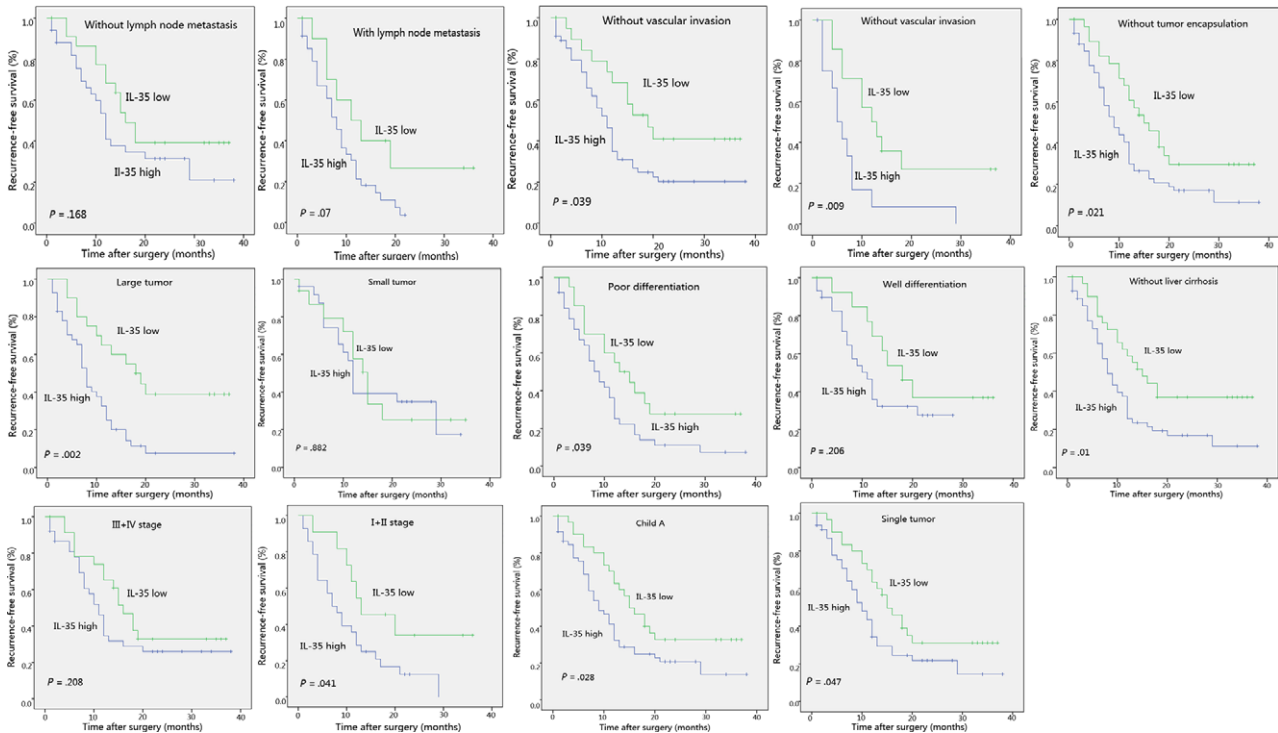
with the related research on pancreatic cancer,<sup>19</sup> raised expression of IL-35R (gp130 and IL-12R $\beta$ 2) was also observed in ICC tissues. Additionally, our western blot results in ICC cells supported these conclusions, namely that elevated expression of IL-35 and IL-35R was also detected in ICC cells compared with normal bile duct cells HBP.

Results of analyzing the relationship between IL-35 and clinicopathological features suggested that IL-35 overexpression was

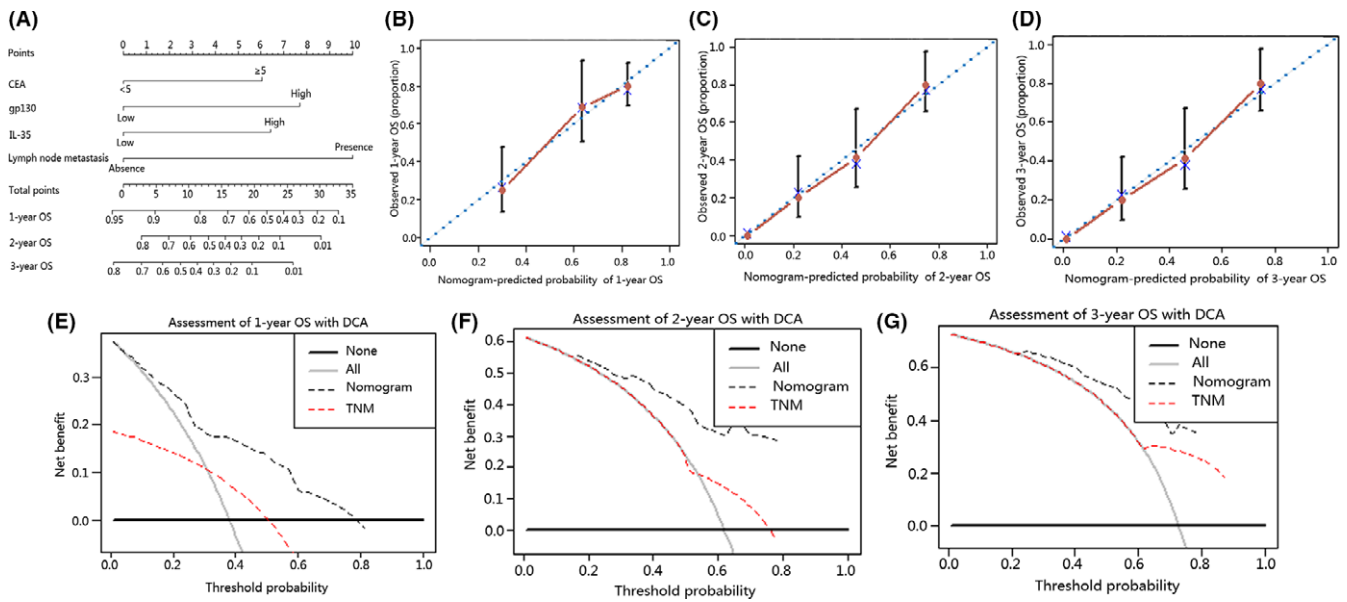




**FIGURE 5** Overall survival (OS) curves for the expression of interleukin (IL)-35 among intrahepatic cholangiocarcinoma subgroups. Subgroup analysis indicated that significant differences in OS were found between IL-35 high and IL-35 low patients after categorization by clinicopathological variables



**FIGURE 6** Recurrence-free survival (RFS) curves for the expression of interleukin (IL)-35 among intrahepatic cholangiocarcinoma subgroups. Subgroup analysis indicated that significant differences in RFS were found between IL-35 high and IL-35 low patients after categorization by clinicopathological variables



**FIGURE 7** Intrahepatic cholangiocarcinoma (ICC) prognostic nomogram, calibration curve and decision curve analysis (DCA). (A) Nomogram predicting overall survival (OS) in patients with ICC. (To use the nomogram, an individual patient's value is located on each variable axis, and a line is drawn upwards to determine the number of points received for each variable value. The sum of these numbers is located on the Total Points axis, and a line is drawn downwards to the survival axes to determine the likelihood of 1-, 2- and 3-y OS.) Calibration curve for predicting OS at (B) 1 y, (C) 2 y and (D) 3 y. Nomogram-predicted probability of overall survival is plotted on the x-axis and actual overall survival is plotted on the y-axis. DCA depict the clinical net benefit in pairwise comparisons across the different models. Nomogram is compared with the TNM stage in terms of (E) 1-y, (F) 2-y and (G) 3-y OS. Dashed lines indicate the net benefit of the predictive models across a range of threshold probabilities (black: nomogram; red: TNM stage). Horizontal solid black line represents the assumptions that no patient will experience the event, and the solid grey line represents the assumption that all patients will experience the event. On DCA, the nomogram showed superior net benefit compared with TNM stage across a wider range of threshold probabilities. CEA, carcinoembryonic antigen

Variable	OS		RFS	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Tumor size (≥5 cm vs <5 cm)	1.326 (0.752-2.339)	.329	NA	
Lymph node metastasis	2.440 (1.157-5.147)	<b>.019</b>	2.538 (1.353-4.761)	<b>.004</b>
TNM stage	1.173 (0.583-2.362)	.654	1.505 (0.799-2.835)	.206
CEA (≥5 ng/mL vs <5 ng/mL)	1.920 (1.142-3.226)	<b>.014</b>	NA	
IL-35 expression (high vs low)	2.141 (1.008 -4.544)	<b>.048</b>	1.570 (0.878-2.806)	.128
gp130 expression (high vs low)	2.234 (1.026-4.866)	<b>.043</b>	1.610 (0.891-2.909)	.115

CEA, carcinoembryonic antigen; 95% CI, 95% confidence interval; HR, hazard ratio; IL, interleukin; NA, not applicable; OS, overall survival, RFS, recurrence-free survival. The bold values were considered to be significant differences.

significantly associated with LNM, advanced TNM stage, and vascular invasion, thus suggesting a special function of IL-35 in ICC metastasis and poor prognosis. More importantly, the close correlation between IL-35 expression and IL-35R (gp130 and IL-12Rβ2) was uncovered in our research, which prompted us to suspect the significance of IL-35 combined with IL-35R in prediction of ICC outcome.

No matter whether in univariate or multivariate analysis, high IL-35 level and gp130 expression more likely implied poorer OS; intriguingly, no significant correlation was observed between IL-

**TABLE 3** Multivariate analyses of factors associated with OS and RFS

12Rβ2 and prognosis in ICC patients. IL-35 expression was strengthened as an independent prognostic factor only along with gp130 receptor. We reasonably speculated that IL-35 overexpression promoted ICC progression through an IL-35 ligand-gp130 receptor signaling pathway. With the desire to verify this hypothesis, we investigated the significance of combined IL-35 expression and IL-35R in patient prognosis. Our results confirmed that more remarkable disparity between different groups was observed when IL-35 was combined with gp130. High IL-35 expression and gp130 level

illustrated the poorest OS and shortest RFS, but no significant positive correlation was observed when IL-35 was combined with IL-12R $\beta$ 2, which was identical to a recent study.<sup>19</sup> This evidence was provocative in suggesting that although both subunits of IL-35R were expressed in ICC, only gp130 was indispensable in IL-35-mediated ICC clinical prognosis.

Research has shown that nomograms can establish a more effective model in the prediction of prognosis of cancers compared with the traditional staging system, and the serological marker CEA has been viewed as independent risk factor for the prognosis of ICC.<sup>28</sup> It is therefore logical to create a nomogram integrating CEA, lymph node metastasis, IL-35 and gp130 expression to verify whether the predictive performance of a nomogram is better than traditional staging systems in ICC. Fortunately, our nomogram provided better predictive accuracy in the prediction of OS relative to TNM stage. Our results were supportive in suggesting that immune molecules (IL-35, gp130) should be incorporated into the ICC prognostic system to improve discriminative ability.

Several limitations in the present study should be noted. First, the study was retrospective in nature and the patients were from a single institution in China. Second, as a result of the relatively small number of clinical patients included in this study, external validation was not carried out. Third, follow-up time was relatively short, as only 3 years of OS and RFS were included in our study.

In conclusion, despite the acknowledged limitations, our results suggest that high IL-35 level positively associates with ICC aggressiveness and can be viewed as a convenient novel prognostic factor for the prediction of survival and recurrence in ICC patients.

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## CONFLICT OF INTEREST

Authors declare no conflicts of interest for this article.

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#### SUPPORTING INFORMATION

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