



# IL-10 promoter hypomethylation is associated with increased IL-10 expression and poor survival in hepatocellular carcinoma

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**Background:** Epigenetic alterations of tumor-associated genes contribute to the pathogenesis of virtually all cancer types. We evaluated the methylation status of the interleukin-10 (IL-10) gene promoter and assessed its association with IL-10 mRNA expression and clinical prognosis in hepatocellular carcinoma (HCC) patients.

**Methods:** Methylation-specific polymerase chain reaction (MSP) and real-time polymerase chain reaction (PCR) were used to define the methylation index (MI) of the IL-10 gene and quantify IL-10 mRNA expression in 120 HCC samples and paired non-tumor tissues.

**Results:** Mean MI was 0.47 in HCC specimens and 0.59 in non-tumor controls, and was associated with metastasis classification and serum  $\alpha$ -fetoprotein (AFP) levels. IL-10 mRNA levels [mean  $-\Delta\Delta Ct$  of 1.678 in HCC cases with hypomethylation ( $\Delta MI \leq 0$ ) and  $-0.18$  in HCC cases with hypermethylation ( $\Delta MI > 0$ )] also correlated with metastasis classification and serum AFP. An association was detected between IL-10 mRNA and its gene's MI in HCC. Also, an association was found between IL-10 hypomethylation, but not IL-10 mRNA expression and reduced postoperative HCC survival.

**Conclusions:** These results indicate that IL-10 promoter hypomethylation is associated with increased IL-10 mRNA levels and indicative of poor survival in HCC.

**Keywords:** Interleukin-10 (IL-10); hepatocellular carcinoma (HCC); hypomethylation

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## Introduction

Liver cancer is the fifth most common cancer, the second most common cause of death from cancer worldwide, and is associated with a high recurrence rate. Reports from 2012 estimated 782,000 new cases and 745,000 liver cancer-related deaths, with an annual incidence of 5.6% (1).

Hepatocellular carcinoma (HCC) is the most common form of primary liver cancer (~90% of all cases) and is still associated with a poor survival rate after resection surgery. Many questions remain to be answered regarding HCC pathogenesis, which severely hampers our ability to develop novel and effective therapies for this dreadful disease (2).

The tumor microenvironment, basically comprises tumor, stromal, and immune cells, and is strongly influenced by the local balance of pro-inflammatory and anti-inflammatory cytokines (3). Inflammation plays a critical role in the genesis of HCC (4). Interleukin-10 (IL-10) is a cytokine mainly produced by B and T lymphocytes and macrophages that plays an essential role in limiting pro-inflammatory immune responses by inhibiting the effector function of Th1 cells (5). Deregulation of IL-10 expression is associated with allergic, autoimmune, and infectious disorders, along with cancer (6). Decreased expression of IL-10 typically leads to increased pro-inflammatory cytokine secretion and hinders anti-tumor immunity, thereby favoring tumor growth. As an anti-inflammatory and immunosuppressive cytokine, the IL-10 family of cytokines has vital functions closely related to immune tolerance in the tumor microenvironment, and its deficit can contribute to immune escape of neoplastic cells (7). Several studies have reported elevated IL-10 levels in HCC patients (8). Chan *et al.* found that hepatic injury in unresectable HCC patients could lead to higher serum IL-10 levels due to cirrhotic processes, rather than tumor load, and suggested the potential prognostic value of circulating IL-10 for these patients (9). Xue *et al.*, on the other hand, proposed that the presence of a large number of IL-10<sup>+</sup> B cells, observed in resected HCC samples, was responsible for restraining CD4<sup>+</sup> cytotoxic T cell activity (10). Chau *et al.* found much higher serum levels of IL-10 in HCC patients than in healthy subjects and suggested that pre-surgical serum IL-10 levels are related to postoperative survival (11). A study by Hsia *et al.*, meanwhile, proposed that serum IL-10 could serve as an HCC biomarker to help identify HCC cases with low levels of  $\alpha$ -fetoprotein (AFP) (12).

Little is known about the tissue-specific regulation of IL-10 expression in HCC and other cancers. Gene methylation is a prominent epigenetic mechanism that regulates the expression of tumor-related genes in virtually all types of cancer. Previous studies suggested that promoter methylation in cytokine genes contributes to cancer pathogenesis by shaping inflammatory responses in the tumor microenvironment (13). Epigenetic analysis of the promoter of the IL-10 gene in B cells highlighted the importance of methylation and acetylation polymorphisms in inflammatory diseases such as periodontitis (14). Along these lines, hypomethylation of a proximal CpGs site within the IL-10 promoter was correlated with abnormal IL-10 mRNA and serum levels in rheumatoid arthritis

patients (15). Another study showed that the methylation status of the IL-10 gene is reduced in patients with systemic lupus erythematosus, and suggested that detection of hypomethylated IL-10 promoter may be a clinical predictor in autoimmune diseases (16). On the other hand, IL-10 promoter methylation was found to be lower in breast cancer specimens compared with healthy breast tissues, leading the study authors to propose that hypomethylation of the IL-10 gene is involved in breast carcinogenesis (17).

Our previous studies analyzed promoter methylation patterns and highlighted the contribution of specific epigenetic events to HCC pathogenesis and progression (18). In the present study, we assessed the methylation status of the IL-10 promoter in matched HCC and non-tumor tissues to test the hypothesis that elevated IL-10 production correlates with hypomethylation of the IL-10 promoter in HCC. Our results indicate that IL-10 gene methylation could play a role in determining IL-10 mRNA expression and might be a useful prognostic indicator of outcome in HCC patients.

## Methods

### *Patients and tissue samples*

Matched tumor and surrounding non-tumor tissues were obtained from patients with a histologically confirmed diagnosis of HCC (n=120) that underwent surgical resection between 2007 and 2016 at Changzhou Cancer Hospital in China. The samples were frozen in liquid nitrogen right after surgical resection. Patients in this study were 96 men and 24 women, aged 39 to 85 years old [mean  $\pm$  standard deviation (SD): 55.32 $\pm$ 13.25 years]. We defined HCC classification by the 8th edition of AJCC for HCC [2017] (19). The study proposal and all ethical proceedings were approved by the committee on Human Experimentation of Changzhou Cancer Hospital. Written informed consent using a standardized questionnaire was obtained from all participants.

### *IL-10 promoter methylation analysis*

Genomic DNA was isolated from frozen samples using the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. DNA bisulfite modification was carried out with 1  $\mu$ g of DNA by NaOH (3 M), sodium bisulfite (2.5 M) and hydroquinone (1 M) (18). We detected the IL-10 gene promoter methylation by

methylation-specific polymerase chain reaction (MSP) assay. polymerase chain reaction (PCR) amplification with specific primers was performed to distinguish methylated from unmethylated DNA: methylated IL-10, forward primer 5'-GGGATTATAGGTATTTGTTATTATGT-3', reverse primer 5'-AAAAAATCTACCTCCCTTATCAA-3'. Unmethylated IL-10, forward primer 5'-GGGATTATAGGTATTTGTTATTACGT-3', reverse primer 5'-AAAAAATCTACCTCCCTTATCGAA-3' (20). PCR was carried out with 2  $\mu$ L modified DNA samples in a total reaction volume of 50  $\mu$ L in an Mx3000P thermal cycler (Stratagene, CA, USA) under the following conditions: 95 °C for 35 s, 50 °C for 60 s, and 72 °C for 45 s for 40 cycles, followed by a final 10 min extension. Unmethylated DNA products were 188 bp, and methylated DNA products were 187 bp. MSP products were separated by 2% agarose gel electrophoresis and visualized by Bio-Rad Gel Doc XR+ (Bio-Rad Laboratories, Inc., CA, USA). Gel intensity profile analyses were performed using Quantity One analysis software (Bio-Rad Laboratories, Inc.). Quantitative band comparison between samples was made in the same gel. We calculated the promoter methylation index (MI) of IL-10 gene using the following formula:  $100 \times \frac{\text{methylated reaction}}{\text{unmethylated reaction} + \text{methylated reaction}}$ .  $\Delta$ MI was defined as  $MI_{\text{HCC}} - MI_{\text{Non-tumor}}$  (21).

### IL-10 mRNA analysis

Total RNA was extracted from HCC and adjacent non-tumor tissues using TRIzol reagent (SBS, China). cDNA was generated using a first-strand cDNA synthesis kit (Invitrogen, China) from 2  $\mu$ g of total RNA in a final reaction volume of 20  $\mu$ L (1 $\times$  PCR buffer, 1 mmol/L dNTPs, 20 units AMV reverse transcriptase, 20 units RNA guard ribonuclease inhibitor, and 2.5 mmol/L random primers). Real-time PCR assays were performed to specifically quantitate the level of IL-10 mRNA transcripts in tissues. The forward primer of IL-10 mRNA for reverse transcription PCR (RT-PCR) reaction was 5'-TCAGGGTGGCGACTCTAT-3', and the reverse primer was 5'-TGGGCTTCTTTCTAAATCGTTC-3' (15). We carried out quantitative PCR (qPCR) in a total volume of 20  $\mu$ L SYBR Green master mix using the Mx3000P qPCR System. Reaction mixtures for IL-10 were subjected to the following amplification conditions: 35 s at 95 °C, 40 s at 50 °C, and 45 s at 72 °C (40 cycles).  $\beta$ -actin mRNA served as an internal control for qPCR by comparing its average computed tomography (CT) values with those of

IL-10 mRNA.

### Statistical analysis

Statistical analysis was performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). Categorical variables were compared using the chi-squared test and Pearson's correlation coefficient. Survival curves were based on Kaplan-Meier estimates. All P values presented were two-sided, and  $P < 0.05$  was regarded as statistically significant.

## Results

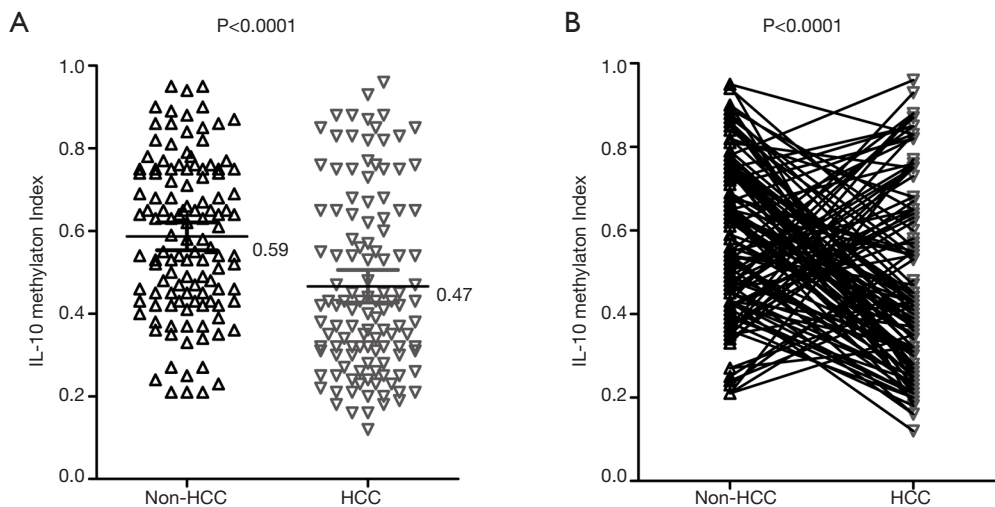
### IL-10 gene methylation status in HCC

The methylation status of the promoter region of the IL-10 gene was analyzed in matched HCC and non-tumor samples. The mean MI was 0.59 (95% CI, 0.55–0.62) in non-tumor samples, and 0.47 (95% CI, 0.43–0.51) in HCC specimens ( $P < 0.0001$ ) (Figure 1). Interestingly, relative to matched non-HCC specimens, 80 HCC cases (66.7%) showed decreased IL-10 promoter methylation ( $\Delta$ MI  $\leq 0$ ), while hypermethylation ( $\Delta$ MI  $> 0$ ) was seen in 40 cases (33.3%). Thus, while hypomethylation occurs in most cases, methylation of the IL-10 promoter appears to follow a biphasic distribution in our study population.

### Correlation between IL-10 gene methylation status and clinicopathological features of HCC

HCC clinicopathological parameters and corresponding IL-10 promoter methylation status change ( $\Delta$ MI) are summarized in Table 1. Clinicopathological parameters included gender, age, hepatitis B virus (HBV) history, tumor differentiation, cirrhosis, tobacco, alcohol, lymph node status, metastasis classification (M0 vs. M1), and AFP level. HCC patients were classified according to  $\Delta$ MI into hypomethylation ( $\Delta$ MI  $\leq 0$ ) and hypermethylation ( $\Delta$ MI  $> 0$ ) groups. While no correlation was observed between most clinicopathological variables and  $\Delta$ MI, significant associations were detected for  $\Delta$ MI  $\leq 0$  and both metastasis classification (i.e., M1  $>$  M0) and serum AFP levels ( $P = 0.001$  and  $P = 0.005$ , respectively).

Next, we investigated the possible association between IL-10 gene methylation status and post-surgery survival in HCC patients (Figure 2). We found that there was a significantly shorter survival time (27 months) in HCC patients with IL-10 gene hypomethylation (MI  $\leq 0.5$ ),



**Figure 1** IL-10 gene promoter methylation index. (A) Mean methylation index of the IL-10 gene in paired HCC and non-tumor tissues; (B) IL-10 gene hypomethylation was detected in 66.7% of HCC cases. Statistical analyses were done using the paired *t*-test. IL-10, interleukin-10; HCC, hepatocellular carcinoma.

compared to HCC patients with hypermethylated IL-10 (MI > 0.5), for which mean survival was 38 months (log-rank  $P=0.0011$ , HR: 0.401, 95% CI: 0.23–0.69). Meanwhile, a median cumulative survival of 28 months was calculated for HCC patients with  $\Delta MI \leq 0$ , compared with 38 months for those with  $\Delta MI > 0$  (log-rank  $P=0.0002$ , HR: 0.347, 95% CI: 0.20–0.60). Our results suggest that IL-10 hypomethylation may serve as a clinical marker of poor prognosis in HCC.

### IL-10 mRNA expression in HCC

Relative IL-10 mRNA expression was assessed by real-time PCR in the 120 HCC samples and matched non-tumor tissues. We found that IL-10 mRNA levels were significantly higher in tumor samples (mean  $-\Delta\Delta Ct = -4.06$ ; 95% CI,  $-4.31$  to  $-3.80$ ) compared with non-tumor specimens (mean  $-\Delta\Delta Ct = -5.12$ ; 95% CI,  $-5.46$  to  $-4.77$ ) ( $P < 0.0001$ ; *Figure 3*). Specifically, IL-10 mRNA expression was elevated ( $-\Delta\Delta Ct > 0$ ) in 69 HCC cases (57.5%) and reduced ( $-\Delta\Delta Ct \leq 0$ ) in 51 (42.5%).

### Correlation between IL-10 mRNA expression and clinicopathologic features of HCC

The relationship between IL-10 mRNA expression in the 120 matched HCC samples and clinical factors were examined next (*Table 2*). There was a significant, positive association between IL-10 mRNA levels and both

metastasis classification (M1 > M0) and serum AFP ( $P < 0.002$  in both cases). The association between IL-10 mRNA expression and patient survival was assessed by the Kaplan-Meier method and the log-rank test, but no correlation was detected (*Figure 4*).

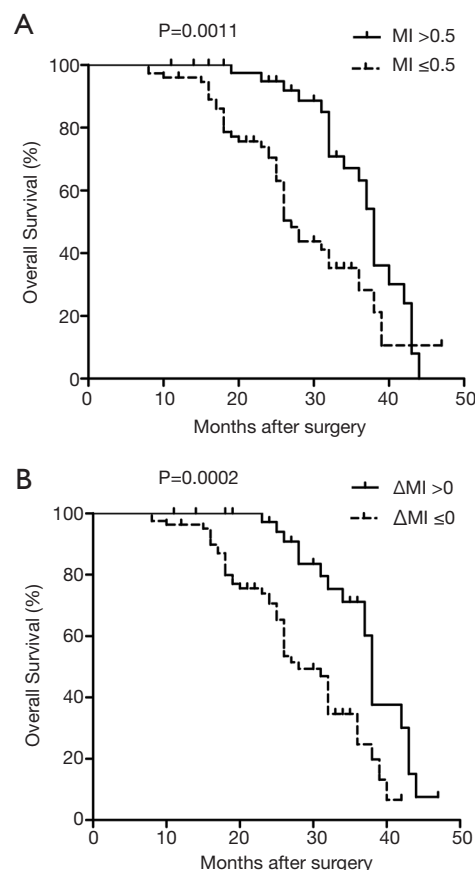
### Elevated IL-10 mRNA is associated with IL-10 promoter hypomethylation in HCC

Methylation of genes within their promoter region is a common mechanism of transcriptional silencing. Accordingly, we found that IL-10 mRNA expression was lower (mean  $-\Delta\Delta Ct = -0.18$ ; 95% CI,  $-1.04$  to  $0.68$ ) in HCC cases with IL-10 promoter hypermethylation ( $\Delta MI > 0$ ), than in those showing hypomethylation of this gene ( $\Delta MI \leq 0$ ; mean  $-\Delta\Delta Ct = 1.68$ ; 95% CI,  $1.13$ – $2.22$ ) ( $P=0.0002$ ; *Figure 5*). Thus, IL-10 promoter hypomethylation and hypermethylation correlate, respectively, with increased and decreased IL-10 mRNA levels in HCC specimens ( $R^2=0.095$ ,  $P=0.001$  and  $R^2=0.063$ ,  $P=0.006$ , respectively). In contrast, no association was detected between IL-10 gene methylation status and IL-10 mRNA expression in non-tumor tissues ( $R^2=0.004$ ,  $P=0.467$ ). These results indicate that differential methylation of the IL-10 gene promoter could play an important role in determining IL-10 production in HCC. The results of the Cox regression analysis for survival factors in HCC are summarized in *Table 3*. Multivariate analysis revealed IL-10 MI and mRNA were associated with

**Table 1** Correlation between clinicopathologic variables and IL-10 methylation index in HCC

Variable	Cases (120)	$\Delta MI \leq 0$ (N=80)	$\Delta MI > 0$ (N=40)	P*
Gender				0.146
Male	96	67	29	
Female	24	13	11	
Age (y)				0.320
$\geq 50$	85	59	26	
$< 50$	35	21	14	
HBV history				0.760
Yes	92	62	30	
No	28	18	10	
Differentiation				0.429
G <sub>1</sub>	48	30	18	
G <sub>2-3</sub>	72	50	22	
Cirrhosis				0.781
Yes	82	54	28	
No	38	26	12	
Tobacco				0.135
Yes	76	49	37	
No	44	31	13	
Alcohol				0.358
Yes	71	45	26	
No	49	35	14	
Lymph node status				0.417
N0	42	26	16	
N1	78	54	24	
Metastasis classification				0.001
M0	53	27	26	
M1	67	53	14	
AFP ( $\mu\text{g/L}$ )				0.005
$\geq 30$	82	63	19	
$< 30$	38	17	21	

\*, statistical significance determined by Pearson's Chi-squared test. IL-10, interleukin-10; HCC, hepatocellular carcinoma; MI, methylation index; HBV, hepatitis B virus; AFP,  $\alpha$ -fetoprotein.

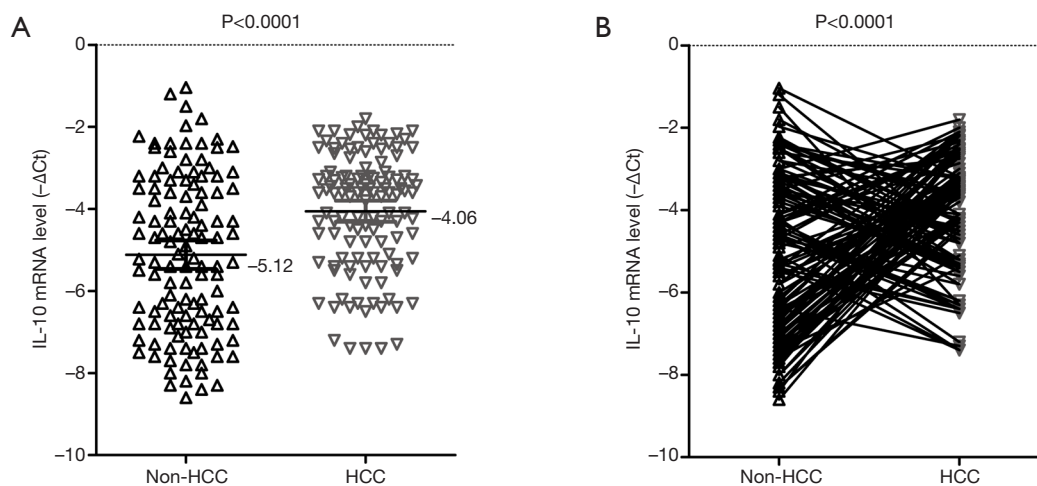


**Figure 2** IL-10 gene hypomethylation correlates with poor overall HCC survival. (A) Kaplan-Meier analysis of survival in HCC patients discriminated by IL-10 methylation index. Low IL-10 promoter methylation ( $MI < 0.5$ ) was significantly correlated with poor overall survival after surgery in HCC patients; (B) HCC patients with hypomethylated ( $\Delta MI \leq 0$ ) IL-10 showed poorer survival.  $\Delta MI = MI_{HCC} - MI_{non-tumor}$ . IL-10, interleukin-10; HCC, hepatocellular carcinoma; MI, methylation index.

overall survival (OS) ( $P=0.009$ ,  $P=0.014$ , respectively).

## Discussion

Chronic inflammatory liver disease and liver cirrhosis are the main risk factors for the development of HCC, the most common form of primary liver cancer, which is now the second leading cause of cancer-related deaths worldwide (1). Tumor metastasis is the primary cause of death in HCC patients. The tumor microenvironment plays critical roles in hepatocarcinogenesis and is a main determinant of metastatic progression. Inflammatory cytokines promote chronic inflammatory liver disease and, as cancer eventually



**Figure 3** IL-10 mRNA expression in matched HCC and non-tumor tissues. (A) IL-10 mRNA expression in paired tumor and non-tumor tissues. IL-10 mRNA expression was higher in HCC than in matched, non-tumor specimens ( $P < 0.0001$ ); (B) IL-10 mRNA levels increased from non-tumor tissues to HCC. Statistical analyses were done using the paired *t*-test. IL-10, interleukin-10; HCC, hepatocellular carcinoma.

develops, sustain tumor progression through a complex interplay with both tumor cells and carcinoma-associated immune cells (22,23).

IL-10 is an anti-inflammatory cytokine that is highly expressed in HCC. While IL-10 overexpression correlates with tumor progression and has a predominantly immunosuppressive role, it is not clear whether an association between IL-10 expression and the risk for developing HCC exists (24). Thus, addressing the molecular mechanisms regulating IL-10 expression should lead to a better understanding of the pathophysiological involvement of this cytokine in HCC. The present study shows that hypomethylation of the IL-10 gene promoter correlates with increased IL-10 mRNA expression in clinical HCC samples, and is a predictor of poor outcome. Moreover, the IL-10 MI is related to tumor metastasis classification; i.e., hypomethylation is more prevalent in HCC patients classified as M1 rather than M0 (metastasis-free), and is also inversely related to serum AFP levels, a common diagnostic and prognostic biomarker of HCC. In addition, we detected a significantly shorter survival (27 months) in HCC patients with hypomethylated IL-10 genes compared with patients with IL-10 hypermethylation (38 months). HCC patients with IL-10 gene hypomethylation also had a shorter median cumulative survival of 28 months compared with 38 months for those with hypermethylated IL-10. These results suggest that IL-10 hypomethylation might be a useful prognostic factor in HCC.

Our study found that IL-10 mRNA levels were overall higher in HCC samples than in paired non-tumor specimens, and also correlated with metastatic status and elevated serum AFP. However, a relationship between IL-10 mRNA levels and HCC outcome could not be established. Our previous research investigated IL-10 gene expression in clinical HCC samples and indicated its key role in the pathogenesis of this disease. While DNA methylation exerts widespread control of gene transcription, several other regulatory mechanisms intervene at the mRNA and protein level. Thus, further studies addressing the regulation of IL-10 at the mRNA and protein levels in a more extensive population sampling are warranted to clarify this discrepancy.

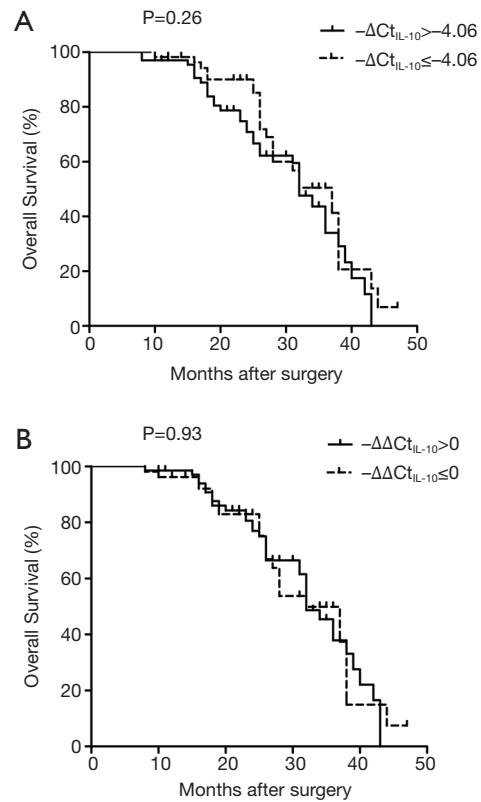
The importance of epigenetic regulation of oncogene, tumor suppressor gene, and cytokine expression has received substantial support in recent years, as aberrant DNA promoter-specific hypermethylation or hypomethylation was shown to contribute to the development of human cancers (25,26). In a previous study, we performed a large-scale promoter methylation analysis of tumor-related genes in HCC and normal surrounding tissues and found various types of abnormal methylation patterns in HCC. Specifically, we reported that Line-1 gene hypomethylation was the most common molecular abnormality in HCC, and this event was associated with the overexpression of CD133 mRNA (18).

Hypermethylation of CpG dinucleotides in gene

**Table 2** Correlation between clinicopathologic variables and IL-10 mRNA expression in HCC

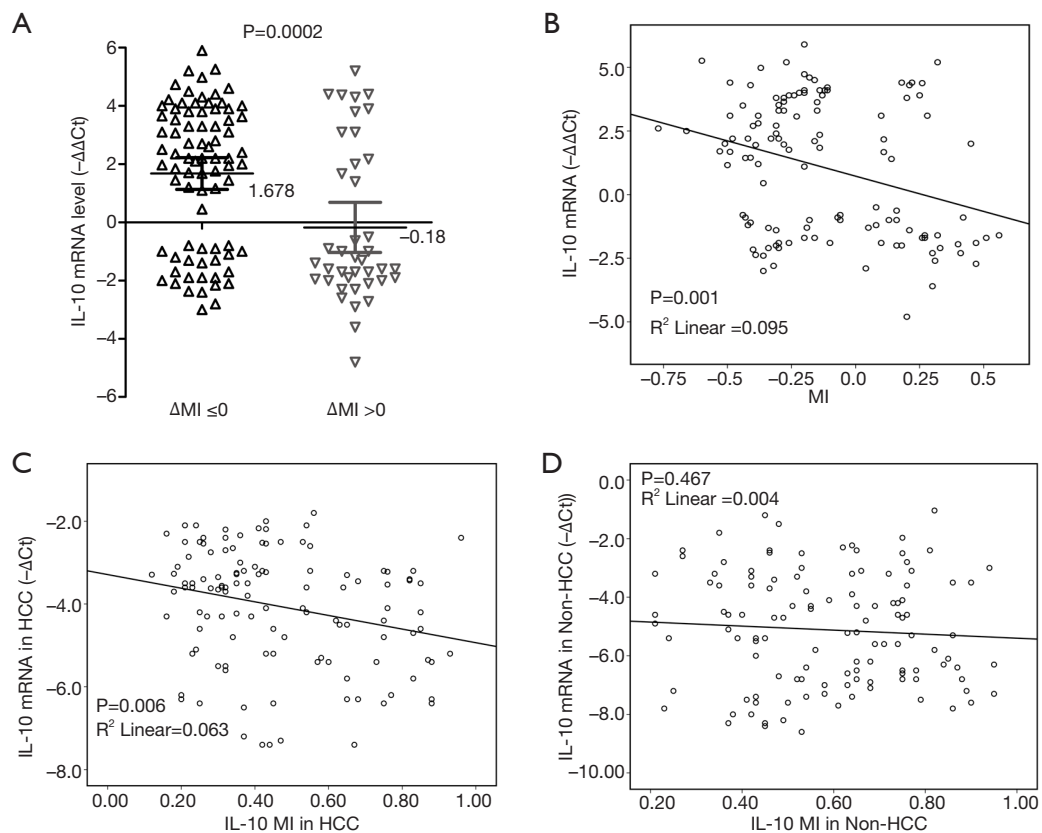
Variable	Cases (120)	$-\Delta\Delta Ct_{IL-10} \leq 0$ (N=51)	$-\Delta\Delta Ct_{IL-10} > 0$ (N=69)	P*
Gender				0.140
Male	96	44	52	
Female	24	7	17	
Age (y)				0.243
$\geq 50$	85	39	46	
$< 50$	35	12	23	
HBV history				0.407
Yes	92	41	51	
No	28	10	18	
Differentiation				0.327
G <sub>1</sub>	48	23	25	
G <sub>2-3</sub>	72	28	44	
Cirrhosis				0.193
Yes	82	37	45	
No	38	22	16	
Tobacco				0.156
Yes	76	36	40	
No	44	15	29	
Alcohol				0.659
Yes	71	29	42	
No	49	22	27	
Lymph node status				0.405
N0	42	20	22	
N1	78	31	47	
Metastasis classification				0.002
M0	53	31	22	
M1	67	20	47	
AFP ( $\mu\text{g/L}$ )				0.002
$\geq 30$	82	27	55	
$< 30$	38	24	14	

\*, statistical significance determined by Pearson's chi-squared test. IL-10, interleukin-10; HCC, hepatocellular carcinoma; MI, methylation index; HBV, hepatitis B virus; AFP,  $\alpha$ -fetoprotein.



**Figure 4** IL-10 mRNA expression does not correlate with HCC survival. (A) Kaplan-Meier analysis of HCC survival as a function of IL-10 mRNA expression. No correlation between IL-10 mRNA levels ( $-\Delta Ct \leq -4.06$ ) and post-operative overall survival was detected; (B) no correlation between IL-10 mRNA levels ( $-\Delta\Delta Ct \leq 0$ ) and post-operative overall survival was detected.  $-\Delta Ct$ ,  $-(Ct_{HCC} - Ct_{\beta-actin})$  in tumor tissues;  $-\Delta\Delta Ct$ ,  $-(\Delta Ct_{HCC} - \Delta Ct_{non-tumor})$ . IL-10, interleukin-10; HCC, hepatocellular carcinoma.

promoter regions is typically associated with transcription silencing, while hypomethylation usually leads to activation of gene expression (27). Accordingly, our study found that hypomethylation of the IL-10 promoter prevailed in HCC patients (66.7%) and correlated with higher IL-10 mRNA levels, while IL-10 mRNA expression was decreased in the remaining HCC patients (33.3%) with hypermethylated IL-10. In future research, it will be of interest to investigate the molecular bases of the biphasic distribution of IL-10 methylation profiles observed in our study; based on the present data, we speculate that IL-10 demethylation



**Figure 5** Correlation between IL-10 promoter methylation status and IL-10 mRNA expression. (A,B) Association between IL-10 relative methylation index ( $\Delta$ MI) and IL-10 mRNA expression; (C) IL-10 mRNA levels were higher in HCC samples with IL-10 gene hypomethylation; (D) scatter plots summarizing IL-10 mRNA expression and MI in non-tumor tissues. IL-10, interleukin-10; HCC, hepatocellular carcinoma; MI, methylation index.

**Table 3** Results of cox regression analysis of survival in HCC

Factor	$\beta$	SE	Hazard ratio (95% CI)	P
AFP $\geq$ 30 $\mu$ g/L	0.021	0.036	1.021 (1.005, 1.084)	0.032
$\Delta$ MI $\leq$ 0	1.924	0.325	3.425 (2.126, 10.623)	0.009
$-\Delta\Delta C_{t_{IL-10}} > 0$	1.334	0.416	2.124 (1.162, 8.712)	0.014

HCC, hepatocellular carcinoma; SE, standard error; CI, confidence interval; AFP,  $\alpha$ -fetoprotein; MI, methylation index.

may represent a molecular switch indicative of metastatic progression in HCC (28).

In sum, the hypomethylation of the IL-10 gene promoter was significantly correlated with increased IL-10 mRNA expression in clinical HCC samples. But the results in our study have some limitations due to individual differences between tumor patients and data of recurrence cannot be obtained. A more thorough understanding of the mechanisms of epigenetic deregulation of the IL-10

gene and the role its transcript plays in the tumor microenvironment might lead to the development of novel targeted therapies for HCC.

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## Footnote

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2019.07.33>). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All of the patients gave written informed consent, and approval was given by the Ethics Committee of Changzhou Cancer Hospital of Soochow University (No. 2016006).

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