

The Pro-Tumor Biological Function of IL-36 α Plays an Important Role in the Tumor Microenvironment of HCC

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Purpose: To investigate the role of IL-36 in the tumorigenesis of hepatocellular carcinoma (HCC). IL-36 composed of a natural antagonist (IL-36Ra) and three agonists (IL-36 α , - β , - γ) that stimulate inflammation by binding to a common receptor consisting of IL-36R and IL-1RAcP. HCC is a common malignancy associated with high morbidity and mortality, often diagnosed at later stages. Although the exact role of IL-36 α in HCC remains controversial, it is hypothesized that it may play a significant role in the development and progression of this cancer.

Materials and Methods: In the current study, we measured both circulating and intrahepatic levels of IL-36 α from HCC patients and healthy controls, using ELISA. The association between IL-36 and the differentiation of HCC was determined. Furthermore, the role IL-36 in both HCC and non-HCC cell lines was evaluated in vitro.

Results: Circulating and intra-hepatic IL-36 α was inversely correlated with differentiation of HCC, suggesting that IL-36 α contribute to protection during the development of HCC. Based on bioinformatics, miR-27b-3p is closely related to downstream IL-36 α . Thus, we determined miR-27b-3p expression in HCC tissues, showing upregulated miR-27b-3p was inversely correlated with IL-36 α in HCC, perhaps via CXCL1 in HCC cells. It was confirmed that IL-36 α inhibited HCC proliferation, viability and migration in vitro, consistent with reduced the expression of cytokines IL-1 β , IL-18, implying that IL-36 α inhibited the possible involvement of pyroptosis.

Conclusion: Our data suggests that IL-36 α may be a potential therapeutic target and a prediction biomarker for the management of HCC.

Keywords: hepatocellular carcinoma, interleukin-36 α , prognosis, biomarker

Introduction

Hepatocellular cancer (HCC) is the fourth most common malignancy with unacceptably high mortality and morbidity,¹ due to lack of a sensitive and a reliable biomarker for early diagnosis. Consequently, a large number of HCC patients are diagnosed at advanced stages with distant metastases, and receive only palliative care.¹ The 5-year survival rate has been reported to be as low as 9%,^{1,2} primarily due to high postoperative recurrence rates and resistance to chemotherapy.³ Alpha-fetoprotein (AFP), a routinely used biomarker for HCC, is not sensitive enough for early diagnosis.⁴ Thus, it is critically important to explore novel, reliable biomarkers for early detection and for predicting prognosis.

IL-36 is a member of the IL-1 family and is produced by a wide range of cells, including leukocytes, epithelial cells, and fibroblasts.⁵ The crystal structure of IL-36 α , IL-36 β , IL-36 γ , and IL-36Ra has confirmed that, like IL-1 β , these molecules share an evolutionarily conserved 12-stranded β -sheet structure.⁶ Successful activation of IL-36 α is initiated by binding to the IL-36R receptor and receptor accessory protein IL-1RAcP from the heterodimeric receptor complex.⁷

This generates an intracellular signaling cascade, leading to the activation of NF- κ B and mitogen-activated protein kinases that induce inflammatory responses.⁸ To explain the mechanism of IL-36 better, we have drawn a figure for clarification (Figure 1). The activity of IL-36 α was determined from its induction of CXCL1 mRNA transcripts by neutrophil chemokine, and CXCL1 was positively correlated with IL-36 α .⁹ Although IL-36 α has been reported to be involved in inflammatory diseases, the precise role of IL-36 α in the pathogenesis of HCC remains to be explored.

IL-36 α expression may contribute to the activation of adaptive T cell-mediated immunity.¹⁰ However, the underlying mechanism of IL-36 α in HCC remains to be elucidated. It has been suggested that miR-27b-3p, consisting of 19 to 25 base pairs, is involved in the oncogenesis of multiple cancers¹¹ and inhibits IL-36 α via CXCL1 in HCC based on bioinformatics database search. The upregulation of miR-27b-3p has been observed in HCC and cell lines.¹² The role of IL-36 α and the pyroptosis pathway during the development of HCC is unclear. Pyroptosis is an inflammatory cell death accompanied by the activation of inflammasomes and maturation of pro-inflammatory cytokines IL-1 β and IL-18.¹³

The objective of this study was to determine the correlation between IL-36 α and HCC differentiation and survival period, and its potential use as a diagnostic biomarker for HCC. The correlation between IL-36 α and AFP production was also examined. In addition, the study evaluated the inhibitory effects of IL-36 α on HCC proliferation, viability, and migration in vitro, as well as its potential role in inducing pyroptosis through the expression of cytokines IL-1 β and IL-18.¹⁴ These findings could potentially lead to the development of novel diagnostic and therapeutic targets for the management of HCC.

Material and Methods

Patients and Tissue

The study collected HCC liver tissues and serum samples from Gansu Provincial People's Hospital. Serum samples were obtained from HCC (n=15), HBV (n=15), cirrhosis (n=15), and healthy control (routine healthy checkup) (n=15) patients. Blood (3 mL) was collected from HCC patients and healthy controls in the early morning prior to breakfast, and serum was collected by centrifugation (1000 g/15 min) and kept at -80°C until used. None of the HCC patients received pre-operative chemotherapy prior to surgery. Hepatic tissues were collected from HCC (n=9) and non-tumor tissue (n=3). Surgical specimens of cancerous tissues and paired adjacent non-tumor tissue were obtained from three HCC cases. The study has been approved by the Ethics Committee of Gansu Provincial People's Hospital. The patients consent has been obtained. The study complied with the Declaration of Helsinki. Demographic information including patients' sex, age, liver cirrhosis, hematoxylin-eosin staining of pathological sections, AFP, and CEA level of serum were collected. The sample included 29 men and 31 women, with a mean age of 52 years (range 30–78 years, median 59 years for women, median 49.5 years for men). All tissue samples were stored at -80°C until tested.

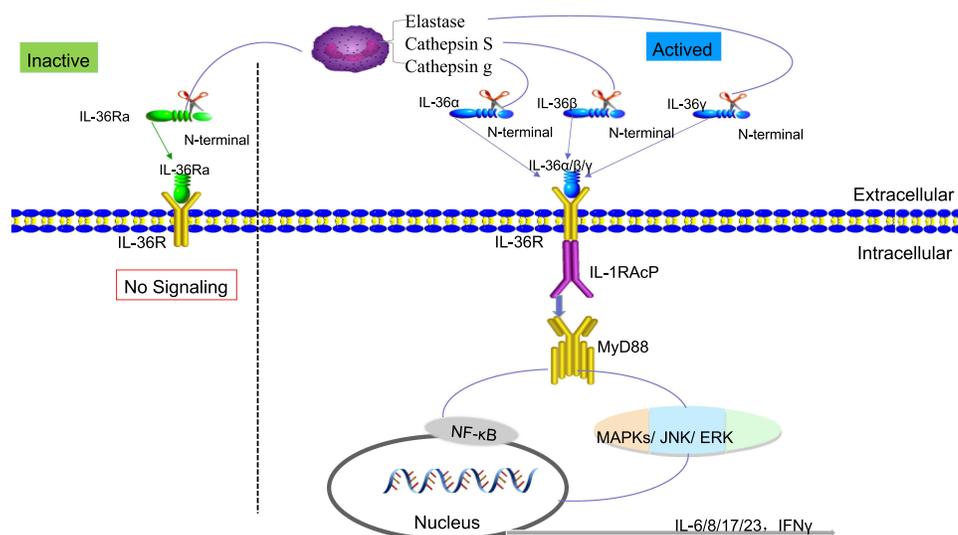


Figure 1 The activating process of IL-36 α .

Cell Lines

HepG2 (a low-differentiated human HCC cell line) and Lo2 (a normal human liver cell line) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Huh7 (a human high-differentiated HCC cell line) was obtained from The Second People's Hospital of Lanzhou University (Gansu, China) (ref14). Lo2 and Huh7 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin, whereas HepG2 cells were cultured in DMEM supplemented with 1% penicillin-streptomycin. The cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C incubator.

Bioinformatic Analysis

TARGETSCAN is a widely used database for predicting miRNA targets in vertebrates. It has undergone updates and improvements over time, including the incorporation of new algorithms to increase the accuracy of match predictions. In a search using gene symbols, the results can be displayed for different transcripts of a gene. When searching for the mRNA CXCL1, the results will show the predicted miRNA target(s) for the specific transcript of interest. The TARGETSCAN database can be accessed at https://www.targetscan.org/vert_80/.

The Encyclopedia of RNA Interactomes (ENCORI) is an open-source platform that facilitates the study of miRNA-mRNA, RNA-RNA, RBP-ncRNA, and RBP-mRNA interactions using degradome-seq and RNA-RNA interactome data. In our current study, we utilized ENCORI to predict the miRNA (miR-27b-3p) that regulates CXCL1 and to verify its correlation with CXCL1 expression. ENCORI is available at <http://starbase.sysu.edu.cn/index.php>.

Gene Expression Profiling Interactive Analysis (GEPIA) is a web-based tool used for interactive online gene expression analysis. It is based on data from 9736 tumors and 8587 normal samples obtained from the TCGA and the GTEx databases.¹⁵ In our current study, we used GEPIA to explore the correlation between the receptor gene IL-36R and overall survival in HCC. Dot plots and body maps were also generated using this tool. GEPIA is available at <http://gepia.cancer-pku.cn/>.

Reagents

Recombinant human IL-36 α (LC12JU2903) was purchased from Sino Biological, Beijing, China. The CCK-8 kit, including human IL-36 α /IL1F6 (IL36A) Kit, human IL-18, and IL-1 β , was purchased from Solarbio, Beijing, China. RPMI 1640 and DMEM were obtained from HyClone Laboratories, Logan, UT, USA. Heat-inactivated fetal bovine serum was purchased from Gibco, Grand Island, NY, USA, and penicillin-streptomycin was obtained from Solarbio, Beijing, China.

ELISA

IL-36 α levels in HCC tissues, serum, and cell supernatant (without intervention) were measured using the human IL-36 α /IL1F6 (IL36A) Kit. The levels of IL-18 and IL-1 β in the supernatants from IL-36 α -treated HCC cells in vitro were determined using the human IL-18 Kit. All experiments were performed in triplicate.

Cell Growth (CCK-8)

HepG2, Lo2, and Huh7 cells were seeded in 96-well plates at a density of 1.5×10^3 cells per well in 200 μ L of the respective culture media mentioned previously. These cells were cultured with or without IL-36 α (500 ng/mL) for 24 hours in triplicate. Cell proliferation was determined, using the CCK-8 kit according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA).¹⁶

Based on preliminary experiments, HepG2 cells were selected for IL-36 α intervention, as their survival rate was the lowest among Lo2, Huh7, and HepG2 cells. HepG2 cells were treated with varying concentrations of IL-36 α (62.5, 125, 250, 500, 750 ng/mL) for 0, 24, 48, or 72 hours. The optimal time and concentration of exogenous IL-36 α for HepG2 cells were determined.

Wound Scratch

HepG2 and Lo2 cells were treated with or without IL-36 α (500 ng/mL) for 12 h until they reached 90% confluency. The monolayer cells were then scraped on the plate using a sterile pipette to create a gap, ensuring that no cells were present

in the gap and that it was straight, as previously described.¹⁷ The scratch area was imaged under an inverted microscope at 0, 24, and 48 h after IL-36 α treatment. The distance of cell migration (wound healing), was measured using ImagePro Plus 9 software, as previously described.¹⁸

Statistics

The statistical analysis was performed, using SPSS software (version 25.0). The results are presented as means \pm standard deviation of at least three independent experiments. The statistical significance of differences between groups was analyzed by one-way analysis of variance (ANOVA) followed by Student's *t*-test. The statistical significance was considered at $P < 0.05$. The significance levels are represented by asterisks as follows: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Results

Circulating Expression of IL-36 α , AFP, CEA from HCC Patients and HC

The levels of circulating IL-36 α were significantly higher in liver disease patients, in the order of CHB, cirrhosis, and HCC, compared to healthy controls (HC) (Figure 2A). In contrast, the levels of AFP gradually increased from CHB, cirrhosis to HCC patients, compared to HC (Figure 2B). A similar pattern was observed for CEA levels in the serum (Figure 2C).

IL-36 α Expression of HCC and Non-Tumor Tissues

The histopathological differentiation of HCC and non-cancer tissues is presented in Figure 3A, showing non-cancer, high, moderate, and poorly differentiated HCC. Constitutive expression of IL-36 α was observed in non-cancer tissues, while IL-36 α expression was significantly elevated in HCC tissues (Figure 3B). Interestingly, IL-36 α expression was highest in well-differentiated HCC tissues and gradually and significantly reduced in moderately and poorly differentiated HCC tissues ($P < 0.05$) (Figure 3B).

Bioinformatic Tools for microRNA-27b-3p Target Prediction

In the search for CXCL1 target miRNAs on TARGETSCAN, 766 potential matches were found according to the transcript. Among them, miR-27b-3p showed a perfect match to the seed region, ie nucleotides 2 to 7, which have perfect Watson-Crick pairing with the 3' UTR of CXCL1 (Figure 4A). To investigate whether miR-27b-3p can regulate CXCL1, we used the ENCORI database (Figure 4B) and found an inverse correlation between miR-27b-3p and CXCL1 expression, as calculated from 370 HCC samples in the database (Figure 4C).

Linkage Between IL-36 α and Prognosis

The Expression of the Only Receptor IL-36R via GEPIA

To investigate the prognostic value of IL-36 α in HCC, we used the GEPIA database. Since IL-36 α was not available in the database, we used IL-36R as a substitute to explore any potential relationship between IL-36 α and cancer in general (Figure 5A). We further focused on HCC and found that IL-36R was highly expressed in normal (peri-tumor) tissue but lowly expressed in HCC based on TPM maps (Figure 5B). We generated a survival plot using the Cox proportional

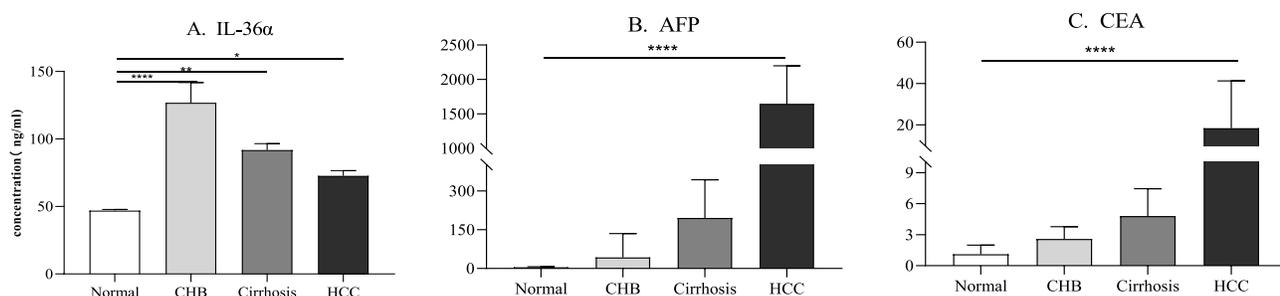
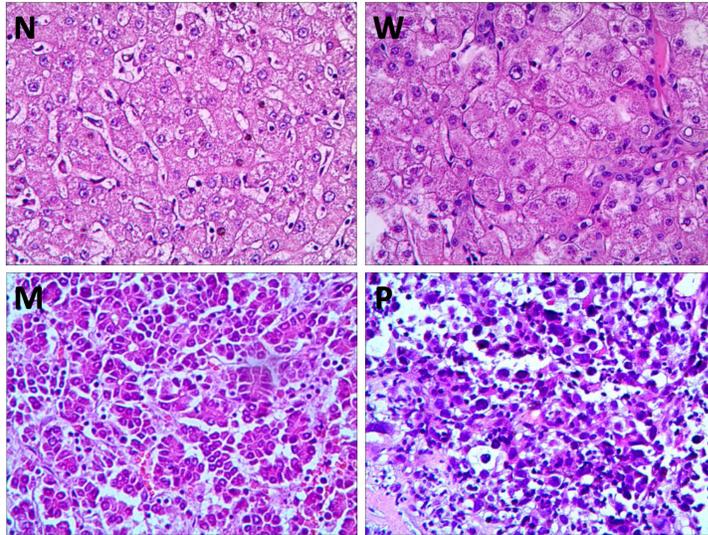


Figure 2 The expression of circulating (A) IL-36 α , (B) AFP and (C) CEA transcribed. The X-axis represents different groups. The Y axis represents concentrations of these molecules (ng/mL). **** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$.

A



B

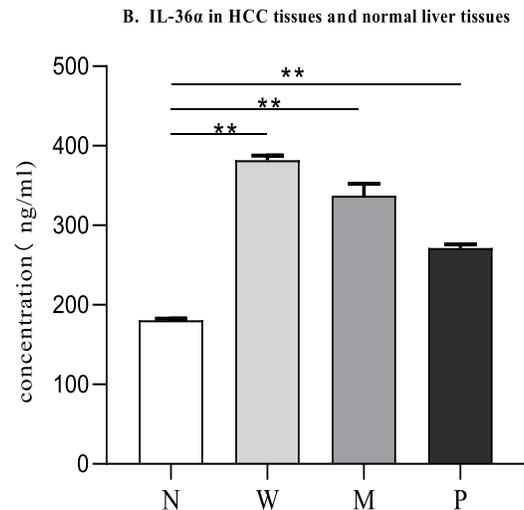
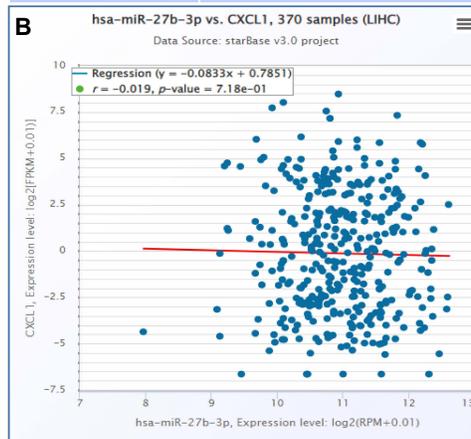


Figure 3 The expression of IL-36 α in HCC and non-cancer tissues. **(A)** Results of HE staining at $\times 200$ magnification. **(B)** IL-36 α expression levels in non-cancer liver tissue (N), well-differentiated HCC (W), moderately differentiated HCC (M), and poorly differentiated HCC (P) measured by ELISA. The mean concentration of each group is represented by A. The horizontal axis represents the different groups, and the vertical axis represents the concentration of IL-36 α (ng/mL). The IL-36 α concentration in W, M, and P was significantly higher than that in N (* $P < 0.05$, ** $P < 0.01$).

A

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length
Position 16-22 of CXCL1 3' UTR	5' ...CCAGAAGGGAGGAGGAGCUCAC...		-0.20	89	-0.20	0.085
hsa-miR-27b-5p	3' CAAGUGGUUAGUCGAUUCGAGA					



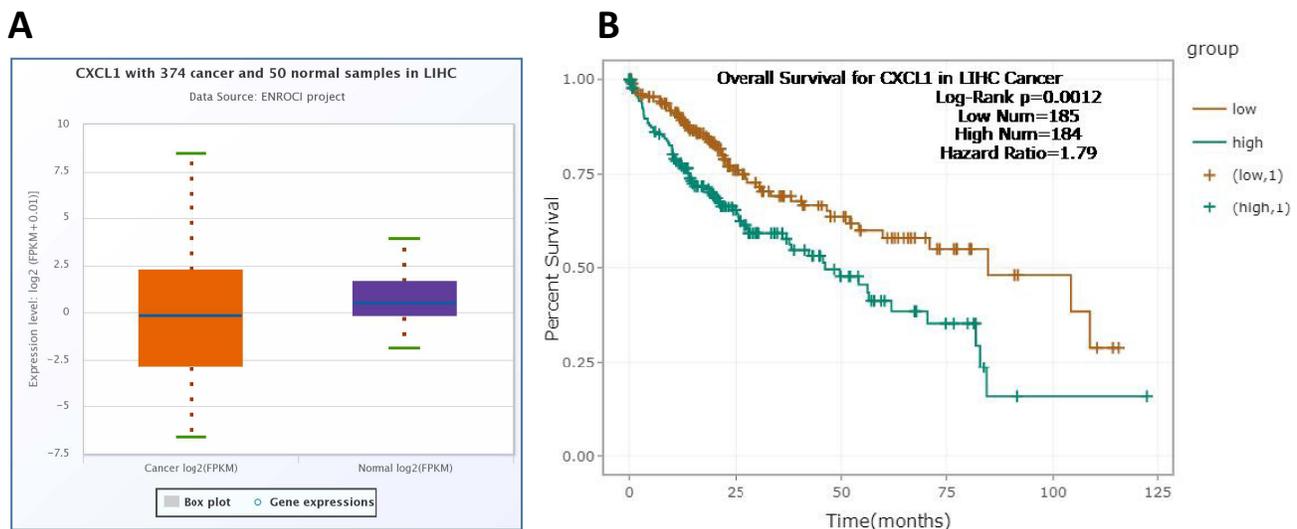
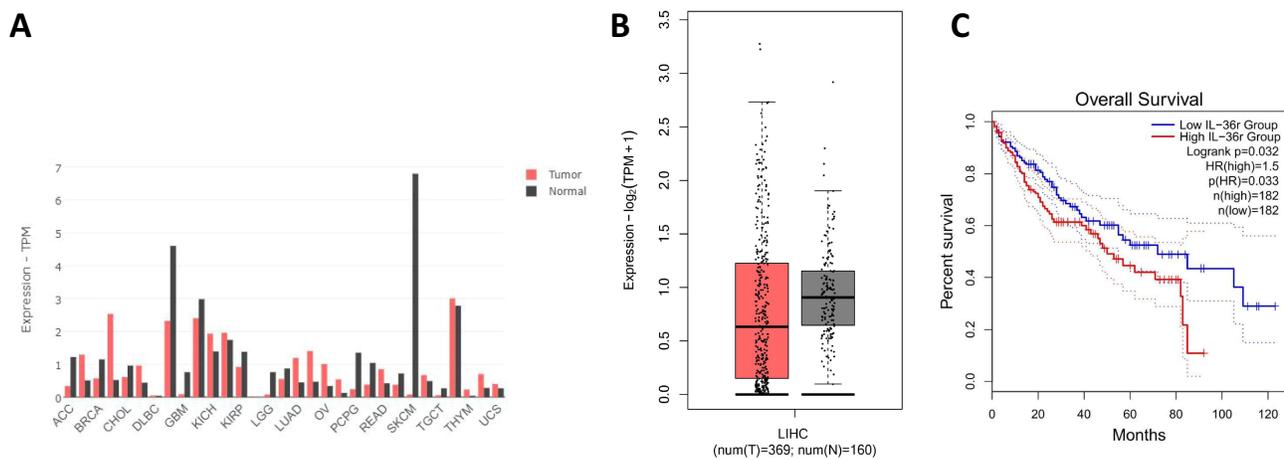
	CANCER	LIHC
CancerFullName		Liver Hepatocellular Carcinoma
SampleNum		370
Coefficient-R		-0.019
p-value		7.18e-1

Figure 4 Bioinformatic tools for microRNA-27b-3p target prediction. **(A)** Position 32–39 of CXCL1 3' UTR targeted by miR-27b-5p. 7mer-A1 indicates that the position of 2–7 bases is paired with the target gene, and the first base at the 5' end of the target gene is A. The Context++ score represents the sum of the contribution of 14 features for each of the four site types, with a more negative score indicating greater repression. The Context++ score percentile represents the percentage of sites for the miRNA with a less favorable Context++ score. **(B)** Scatter plot showing the negative correlation between miR-27b-3p and CXCL1 expression levels, with expression levels represented as $\log_2(\text{RPM} + 0.01)$. **(C)** Coefficient-R is an illustration of B, with a value less than -1 indicating a negative correlation.

hazard ratio and the 95% confidence interval information. The overall survival of HCC based on IL-36R expression is shown in Figure 5C, indicating an inverse correlation between IL-36R expression and HCC survival.

The Expression of the mRNA CXCL1 by Using ENCORI Database

Using ENCORI database, we compared the transcriptional levels CXCL1 in HCC (n=374) with normal liver samples (n=50) (Figure 6A), showing a correlation between CXCL1 and IL-36R at the transcriptional levels. The data generated from ENCORI, showing that constitutive expression of CXCL1 in peri-tumor, and such expression was significantly



suppressed in HCC. Then we further determined the correlation between overall survival and CXCL1 expression, showing CXCL1 expression was associated with prognosis of overall survival ($P < 0.0012$) (Figure 6B).

IL-36 α Inhibited Viability and Migration in Cells Lines

Constitutive levels of IL-36 α were detected in non-HCC cells in vitro. Huh7 or HepG2 HCC cells showed 1.4 or 1.7-fold higher expression of IL-36 α (* $P < 0.5$) compared to non-HCC cells, indicating upregulation of IL-36 α in HCC cells that depended on differentiation (Figure 7A). To investigate the effect of exogenous IL-36 α on HCC cells, HepG2 cells were treated with 750, 500, 250, 125, and 62.5 ng/mL IL-36 α for 24 h. Results revealed a dose-dependent inhibition of cell viability by IL-36 α (Figure 7B), with the highest inhibition observed at 500 ng/mL. Wound scratch assay was conducted to evaluate the effect of IL-36 α (500 ng/mL) on the migration ability of HepG2 cells at 24 or 48 h post-treatment

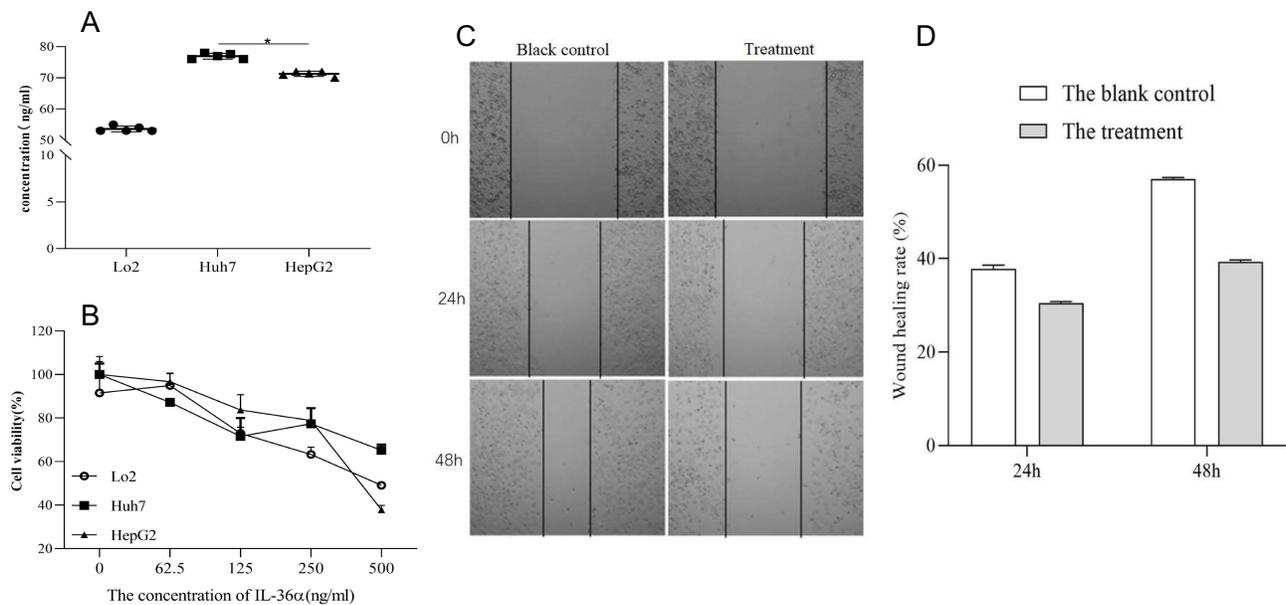


Figure 7 The expression of IL-36 α in different cell lines was investigated. **(A)** The expression levels of IL-36 α were measured in Lo2 (normal liver cell), Huh7 (well-differentiated HCC cell), and HepG2 (poor-differentiated HCC cell), using ELISA. Higher expression of IL-36 α was observed mainly in hepatocellular carcinoma cells. **(B)** The viability of the cell lines (Lo2, Huh7, and HepG2) was assessed after treatment with different concentrations of IL-36 α (750, 500, 250, 125, and 62.5 ng/mL) for 24 hours. The viability of cells decreased with increasing IL-36 α concentrations, with the greatest effect observed at 500 ng/mL. **(C and D)** The concentration of the treatment group was significantly lower than that of the blank control group. The scratch healing rate of lateral migration of HepG2 cells was assessed using a scratch healing experiment (* $P < 0.05$).

(Figure 7C), while DMEM only was used as control. Results showed that IL-36 α suppressed wound healing by 10% or 20% at 24 or 48 h post-treatment, respectively.

IL-36 α Suppressed IL-1 β and IL-18 in HepG2 Cells in vitro

IL-18 and IL-1 β have been reported to be produced by gastrointestinal cells during pyroptosis. Therefore, we measured the levels of IL-18 and IL-1 β in HepG2 cells in response to IL-36 α stimulation with 500 ng/mL for 24 h, using ELISA. We found that the expression of IL-18 and IL-1 β was suppressed by approximately 40% and 10%, respectively, in HepG2 cells in the presence of IL-36 α (500 ng/mL) for 24 h, compared to mock-treated cells (Figure 8).

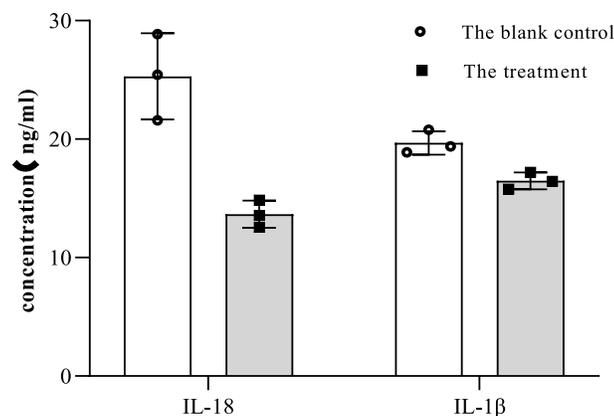


Figure 8 IL-36 α suppressed IL-1 β and IL-18 expression in HepG2 cells in vitro. To test this, we set up blank control groups and treatment groups to measure the expression of IL-18 and IL-1 β in HepG2 cells. The treatment group was treated with IL-36 α (500 ng/mL) for 24 hours, while the control group was only added DMEM.

Discussion

In the current study, it was demonstrated that circulating IL-36 α levels were significantly upregulated in patients with CHB, cirrhosis, and HCC, compared to healthy controls. The hepato-protective role of IL-36 in CHB has been well documented,¹⁹ as IL-36 can be produced by both hepatocytes²⁰ and peripheral leukocytes.²¹ However, the protective role of IL-36 may be compromised in susceptible individuals, resulting in persistent liver chronic inflammation.²² Additionally, cirrhosis often causes persistent liver damage without effective intervention,²³ and the host immunity in cirrhotic patients is often compromised, contributing to incomplete protection from IL-36. The lower IL-36 levels in cirrhotic patients than in CHB patients may be related to the severity of liver damage, with more severe hepatic damage in cirrhosis than in CHB. Similarly, the lowest levels of IL-36 in HCC patients, where the liver is most severely damaged among these three liver diseases, could be explained by this phenomenon. These findings suggest that IL-36 α could be a novel biomarker for the detection of HCC.

In our study, we observed that IL-36 α expression was mainly detected in well-differentiated HCC *in vitro*, while it was weakly expressed in poorly differentiated HCC. We also found a correlation between the expression of mir-27b-3p and differentiation status, where higher levels of mir-27b-3p were associated with poorer differentiation. Therefore, the reduced expression of IL-36 α in HCC could be attributed to the upregulation of mir-27b-3p. Moreover, given the consistent upregulation of IL-36 α and CXCL1 in HCC cells, suggesting that mir-27b-3p may negatively regulate IL-36 α via CXCL1 in HCC cells.

On the other hand, IL-36R (IL-1R6), is initiated by binding to IL-36 ligands.²⁴ We performed bioinformatics analysis to investigate the overall survival of HCC based on IL-36R expression and found an inverse correlation between IL-36R expression and HCC survival. Given the secretory nature of the IL-36 ligands and the distribution patterns of the IL-36R, we assigned an important role for IL-36 α cytokines in binding with IL-36R. Using bioinformatics, we also found that down-regulated expression of CXCL1 was associated with a better prognosis of overall survival, suggesting that CXCL1 contributes to the activity of IL-36 α , which is consistent with other studies showing that IL-36 α is regulated by CXCL1 in neutrophil chemokine.²⁵ These findings suggest that IL-36 α may play a protective role during the development of HCC, and that up-regulated IL-36 α may serve as an early predictor of prognosis.

In our study, we observed that IL-36 α inhibited HCC proliferation, viability, and migration *in vitro*, which correlated with the reduced expression of IL-1 β and IL-18. Pyroptosis is an inflammatory cell death process that is accompanied by the activation of inflammasomes and the maturation of pro-inflammatory cytokines, including IL-1 β and IL-18.¹³ Although we do not have firm evidence, our data suggest that IL-36 α may inhibit IL-1 β and IL-18-mediated pyroptosis during the development of HCC, which will require further investigation.

The incidence of HCC is highest in China,²⁶ and the currently routinely used biomarker for its diagnosis is AFP, which is both economic and versatile.²⁷ However, the sensitivity and specificity of AFP in diagnosing HCC need improvement.²⁸ Our current data suggest that IL-36 α is rather sensitive and specific in HCC and may be used as a biomarker for early diagnosis and prediction of prognosis. Our speculation is supported by others, indicating that IL-36 plays a critical role in tumor treatment and prognosis.²⁹ Furthermore, our data suggest that IL-36 α may be a potential therapeutic target in the management of HCC, which is in line with the findings in colorectal cancer during the early stages of the disease.³⁰ Therefore, we believe that IL-36 α is a novel prognostic marker for HCC.

Finally, our data is relatively in small sample size, and future study will extend to large sample size from multi-centers, particularly in exploring pyroptosis during the development of HCC at molecular and cellular level. In the current study, we focused on investigating the role of a single gene, which may not fully represent the complexity of the potential pathways involved in the development of HCC studies. We plan to explore the involvement of additional genes using gene sets to better understand the underlying pathogenesis of HCC. Framework method for the analysis of qualitative data has been used by many researchers, which could boost the credit of the study. We will perform our study to clarify our hypothesis in future.

Conclusion

We conclude that IL-36 α is constitutively expressed and upregulated in HCC, and its expression is inversely correlated with mir-27b-3p, which is associated with HCC progression and prognosis. IL-36 α exerts a protective role by inhibiting HCC proliferation, viability, and migration. In addition, we found that reduced IL-36R and CXCL1 expression is associated with overall survival in HCC patients. Our data suggest that IL-36 α could be a novel prognostic marker and indicator in HCC.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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