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ORIGINAL ARTICLE

Decreased expression of interleukin-36α correlates with poor prognosis in hepatocellular carcinoma

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Abstract Interleukin-36α (IL-36α) has been found to have a prominent role in the pathogenesis of inflammatory disorders; however, little is known about the role of IL-36α in cancer. In this study, we investigated the expression, prognostic value, and the underlying antitumor mechanism of IL-36α in hepatocellular carcinoma (HCC). From immunohistochemistry analysis, IL-36α expression was lower in poorly differentiated HCC cells. In clinicopathological analysis, low IL-36α expression significantly correlated with tumor size, histological differentiation, tumor stage, and vascular invasion, and low intratumoral IL-36α expression had significantly worse overall survival rates and shorter disease-free survival rates. Moreover, intratumoral IL-36α expression was an independent risk factor for overall survival. Consecutive sections were used to detect $CD3^+$, $CD8^+$, and $CD4^+$ tumor-infiltrating lymphocytes (TILs), and we found that high-IL-36α-expressing tumor tissues exhibited a significantly higher proportion of intratumoral $CD3^+$ and $CD8^+$ TILs, but not $CD4^+$ TILs. Our in vitro model confirmed that supernatant from IL-36α-overexpressing human HCC cells had an increased capacity to recruit $CD3^+$ and $CD8^+$ T cells. Consistently, mouse HCC cells engineered to overexpress IL-36α demonstrated markedly delayed growth in vivo, as well as higher levels of intratumoral $CD3^+$ and $CD8^+$ TILs, compared with control mice. In vitro chemotaxis analysis also showed that mouse HCC cells overexpressing IL-36α could recruit more number of $CD3^+$ and $CD8^+$ T cells. These results show that IL-36α expression may play a pivotal role in determining the prognosis of patients with HCC, which we attribute to the activation of adaptive T cell immunity, especially $CD8^+$ T cell immune response.

Keywords Hepatocellular carcinoma · Interleukin-36α · Prognosis · Tumor-infiltrating lymphocytes

Introduction

Interleukin-36α (IL-36α, formerly named IL-1F6) is a newly identified interleukin-1 (IL-1) family member. IL-36α is expressed primarily in the skin and other epithelial tissues and plays a prominent role in immune and inflammatory reactions [1, 2]. The majority of IL-36α research has primarily focused on its role in inflammatory disorders [1, 3–7]; for example, IL-36α is predominantly expressed by keratinocytes in psoriatic skin and can enhance inflammatory cell infiltration, as well as cytokine and chemokine expression [4]. These studies indicate that IL-36α plays a proinflammatory role in these diseases, similar to other IL-1 family members [8]. Despite this focus on inflammatory diseases, the role of IL-36α in the pathogenesis of cancer remains unclear.

Hepatocellular cancer (HCC) has an extremely poor prognosis, with a 5-year survival rate as low as 9 % [9]. This poor long-term survival rate is primarily associated with a high postoperative recurrence rate [10], which

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has been related to the HCC microenvironment [11], and immune response regulation at the local tumor site [12]. Indeed, it has been reported that cancer cells within the tumor secrete several types of cytokines into the tumor microenvironment, which can affect tumor progression and alter the antitumor immune response [13–16]. For example, Akiba et al. [16] found that high expression of intratumoral interleukin-8 correlated with a high frequency of invasion and metastasis in HCC patients. In contrast, Ikeguchi et al. [14] demonstrated that interleukin-2 produced by HCC cells conferred a favorable prognosis in HCC patients, because this factor activated tumor-infiltrating $CD8^+$ T cells to induce antitumor immunity. These results suggest that different cytokines produced from HCC cells might regulate the immunomicroenvironment through different pathways to affect the prognosis of HCC patients.

As stated above, little is known about the role of IL-36α in HCC. As such, in this study we investigated intratumoral IL-36α expression and analyzed its relationship with clinicopathologic factors and HCC prognosis for the first time. Furthermore, we investigated the potential mechanism by which IL-36α regulates the immunomicroenvironment in HCC in vitro and in vivo. Our studies provide novel insight into the role of IL-36α in HCC tumor progression. We believe this information may be helpful for developing effective cancer immunotherapy methods.

Materials and methods

Mice and cell lines

Female C57BL/6 mice (5–6 weeks old) were purchased from the Medical Experimental Animal Center of Guangdong Province. Mice were maintained in a specific pathogen-free environment, and all animal procedures were conducted in compliance with the guidelines of the laboratory animal ethics committee of Sun Yat-sen University. The mouse HCC cell line Hepa1-6 (syngeneic to C57L mice) and the human HCC cell line Sk-hep1 were purchased from the American Type Culture Collection (Manassas, VA, USA). The normal human liver cell line LO2 was obtained from the Committee of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were propagated in RPMI-1640 medium supplemented with 10 % heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA) and 1 % penicillin–streptomycin. Cells were grown in a humidified atmosphere of 5 % CO_2 at 37 °C.

Patients and tissue samples

Paraffin-embedded samples were obtained from 163 HCC patients who underwent surgery at Sun Yat-sen University Cancer Center between November 2003 and December 2004. Patients who received anticancer therapy prior to the operation or who had autoimmune diseases, HIV, and syphilis were excluded from this study. The 163 patients included 131 males and 32 females, with a median age of 47 years (range 15–70 years). Postoperative follow-up was conducted at our outpatient department as follows: The patients received clinical and laboratory examinations every 3 months for the first 2 years, every 6 months for the next 3 years, and annually for an additional 5 years or until death, whichever occurred first. Patients with confirmed recurrence received further treatment, such as a second surgery, local ablative therapy, percutaneous ethanol injection, and transcatheter arterial chemoembolization. The patients were followed up for 1–87 months, with a median follow-up of 36 months. Overall survival (OS) was defined as the period from the surgery to death or the last known follow-up. Disease-free survival (DFS) was defined as the period from the surgery to recurrence or the last follow-up if no recurrence was observed. The histological types were assigned according to the criteria proposed by Edmonson and Steiner [17]. Written informed consent was obtained from each patient, and the protocol was approved by the Ethics Committee of Sun Yat-sen University Cancer Center.

Immunohistochemical staining

Paraffin-embedded tissue blocks were sectioned at 2 μm and placed in the oven at 60 °C for 1 h, deparaffinized in xylene, and rehydrated in a graded series of ethanol to water. To unmask antigen epitopes, the slides were immersed in EDTA (1 mmol/l, pH 8.0), boiled for 15 min in a microwave oven, cooled for 30 min at room temperature (RT), and rinsed in phosphate-buffered saline (PBS, pH 7.4). Endogenous peroxidase activity was blocked with 0.3 % hydrogen peroxide solution for 15 min at RT. After rinsing three times with PBS, the slides were incubated with the primary antibodies overnight in a humidified chamber at 4 °C, including goat antihuman IL-36α polyclonal antibody (concentration at 2 μg/ml; R&D systems Inc., Minneapolis, USA), mouse antihuman CD3 monoclonal antibody (1:100 dilution; Zhongshan Golden Bridge Biotech., Beijing, China), rabbit antihuman CD8 monoclonal antibody (1:100 dilution; Zhongshan Golden Bridge Biotech.), and mouse antihuman CD4 monoclonal antibody (1:100 dilution; Zhongshan Golden Bridge Biotech., Beijing, China). After washing in PBS, the sections were incubated with horseradish peroxidase-conjugated antigoat (Zhongshan Golden Bridge Biotech.) or antimouse/rabbit secondary antibodies (Envision™ Detection Kit, GK500705, Gene Tech Co. Ltd., Shanghai, China) at RT for 30 min. The bound antibodies were then visualized with 3,3′-diaminobenzidine tetrahydrochloride, and the sections

were counterstained with hematoxylin. Finally, the slides were dehydrated, coverslipped and evaluated by microscopy. Negative control sections were prepared by replacing the primary antibody with PBS.

Image quantification

Immunohistochemical staining of IL-36α protein expression was quantitatively estimated by Image Pro-Plus 6.0 (Media cybernetics Inc., Silver Spring, USA) as previously described [18, 19]. In short, six to ten random digital images (2,560 × 1,920 pixels) at 400× magnification were photographed using a Nikon Eclipse 80i microscope coupled to a camera and an image analysis system. Mean optical density (MOD), representing the concentration of positive staining as measured per positive pixel, was calculated as a quantitative measure of protein expression. All digital photographs were taken and measured using the same microscope light source intensity to eliminate variation.

$CD3^+$, $CD8^+$, and $CD4^+$ tumor-infiltrating lymphocytes (TILs) were investigated by manually counting positively stained cells in ten random fields at 400× high-power magnification. The density of stained TILs was evaluated by calculating the mean number of positively stained cells per high-power field [20].

Transduction of tumor cells

To construct a recombinant retroviral plasmid encoding IL-36α, human *IL-36α* gene was cloned by RT-PCR from RNA isolated from the normal liver cell line LO2, using the forward primer 5′-CCG CTC GAG GCC ACC ATG GAA AAA GCA TTG AAA ATT GAC ACA CCT CAG C-3′ and the reverse primer 5′-GCG TCG ACT TAA AAC AGC ATA GTT AAC CCA AAG TCA GTA GTG TTG GC-3′. The resulting PCR product was inserted into the retroviral vector pLNCX2 (Clontech Laboratories Inc., Palo Alto, CA, USA), which we called pLNCX2-IL-36α. Briefly, replication-incompetent retrovirus was obtained by transfecting GP2-293 cells (Clontech Laboratories Inc.) with the pLNCX2-IL-36α (or empty pLNCX2 vector control) and pVSV-G (Clontech Laboratories Inc.), a plasmid expressing the G glycoprotein of the vesicular stomatitis virus. Sk-hep1 cells were infected with pLNCX2-IL-36α (or the empty pLNCX2 vector control) retrovirus supernatant in the presence of 8 μg/ml polybrene (Sigma Chemical Co., St. Louis, MO, USA). After infection for 48 h, the cells were selected using 800 μg/ml G418 and screened for stable expression of IL-36α (Sk-hep1/IL-36α). G418-resistant Sk-hep1 cells infected with the empty pLNCX2 vector (Sk-hep1/pLNCX2) were used as controls. Hepa1-6 cells were similarly infected with pLNCX2-IL-36α or the control pLNCX2 retroviral supernatant and were selected using 600 μg/ml G418 to produce Hepa1-6/IL-36α and Hepa1-6/pLNCX2 cells, respectively. In addition, Hepa1-6 cells were also infected with recombinant lentiviruses overexpressing murine IL-36α (GenePharma, Shanghai, China) and were selected in the presence of 2 μg/ml puromycin to produce Hepa1-6/mIL-36α and Hepa1-6/mCon cells, respectively. Successful induction of IL-36α expression in all cell types was assessed by Western blotting.

Preparation of culture supernatants and chemotaxis assays

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (Guangzhou Blood Center) by Ficoll-Hypaque gradient centrifugation. Mice splenocytes from female C57BL/6 mice were isolated and filtered through cell strainers. $CD3^+$ and $CD8^+$ T cells were purified from human PBMCs or mice splenocytes by negative selection using immunomagnetic beads (Invitrogen Dynal AS, Oslo, Norway). Tumor culture supernatants were prepared by plating 3×10^5 tumor cells in 3 ml complete RPMI-1640 medium in six-well culture dishes for 72 h, and then the supernatant was collected, centrifuged, and stored in aliquots at −80 °C.

Chemotaxis assays were performed using polycarbonate filters of 5 μm pore size in 24-well transwell chambers (Corning Incorporated, New York, USA). Six hundred microliters of culture supernatants was added to the lower chamber; then 5×10^6 purified $CD3^+$ or $CD8^+$ T cells were added to the upper chamber in 100 μl of complete medium and incubated for 4 h at 37 °C. A 500-μL aliquot of the cells that migrated to the bottom chamber was collected and counted. Each experiment was performed in duplicate. Scores were calculated as percentage of migrated cells versus control.

In vivo tumor models and immunohistochemistry

Parental or transduced Hepa1-6 cells were harvested and injected subcutaneously into the posterior flank of mice (5×10^6 tumor cells in 100 μl of PBS). Tumor growth was monitored every 3 days, and the tumor volume was calculated using the formula ($1/2 \times \text{length} \times \text{width}^2$). Tumors were extracted, cut into 2 mm^3 cubes, fixed in 10 % formalin, and embedded in paraffin. Sections were cut at 2 μm thickness and mounted onto poly-L-lysine-coated microscope slides. $CD3^+$- and $CD8^+$-positive cells were detected using a rabbit antimouse CD3γ monoclonal antibody (1:500 dilution; Epitomics Inc., Burlingame, USA) or rabbit antimouse CD8A polyclonal antibody (1:300 dilution; Abnova Co., Walnut, CA, USA), respectively. $CD3^+$ and $CD8^+$ TILs were quantified as the mean number of positively stained cells per random high-power field (400× magnification) from ten fields per section, as described above.

Statistical analysis

All statistical analyses were performed using the SPSS software package (version 16.0, SPSS Inc., Chicago, IL, USA). Results are expressed as the mean ± SD or median ± range. The median values for each immunohistochemical variable were used as cutoffs for defining the subgroups, as described previously [21, 22]. The association between IL-36α expression and clinical variables was analyzed using the Pearson χ^2 test. OS and DFS curves were calculated according to the Kaplan–Meier method and were analyzed by the log-rank test. Univariate and multivariate analyses based on the Cox proportional hazards regression model were used to explore the effects of IL-36α expression and HCC clinicopathological variables on survival. The relationship between IL-36α and intratumoral T cells was tested by the Mann–Whitney *U* test. The Student's *t* test was used to assess differences in the chemotaxis assays, tumor volumes, and tumor weights. Tests were two-tailed, and p values <0.05 were judged to be statistically significant.

Results

IL-36α expression in HCC clinical samples and its relationship to clinicopathological variables

IL-36α expression was detected in 163 HCC surgical specimens using immunohistochemical staining (Fig. 1a–h). Positive IL-36α expression was found primarily in the cytoplasm of normal hepatocytes and well-differentiated HCC cells. However, in moderately and poorly differentiated HCC tissues, the expression level of IL-36α was much lower. According to our quantitative analysis, the median MOD of IL-36α for all samples was 0.0398 (range 0–0.1124), which was used as the cutoff for defining high or low IL-36α expression. The association between the IL-36α expression level and various clinicopathological features of the HCC patients is listed in Table 1. Low intratumoral IL-36α expression was significantly correlated with tumor size ($p = 0.001$), histological differentiation ($p = 0.04$), tumor stage ($p = 0.031$), and vascular invasion ($p = 0.002$). No correlation was found between IL-36α expression and age, gender, liver cirrhosis, number of lesions, HBsAg, tumor capsule, or AFP.

IL-36α expression and patient survival

To investigate the prognostic value of IL-36α in HCC patients, OS and DFS were estimated for patients with high and low IL-36α expression by Kaplan–Meier survival analysis. This analysis revealed that patients in low-IL-36α-expressing group had either worse OS (log-rank test, $p = 0.001$; Fig. 1i) or shorter DFS (log-rank test, $p = 0.002$; Fig. 1j) than the high-IL-36α-expressing group. To investigate the effect of IL-36α expression and other clinicopathological parameters on the prognosis of HCC patients, univariate and multivariate analyses were performed to identify the prognostic factors for OS. In univariate survival analysis, IL-36α expression level, histological differentiation, number of lesions, TNM stage, and vascular invasion were significant risk factors for OS (Table 2). Multivariate Cox regression analysis demonstrated that IL-36α expression was an independent prognostic factor for OS ($p = 0.003$; Table 2), as well as number of lesions ($p = 0.031$). Thus, IL-36α expression may be useful for predicting the OS of HCC patients.

Association between the level of IL-36α expression and the number of TILs

As cytokine production by tumor cells affects the tumor immunomicroenvironment, we further investigated the relationship between IL-36α expression and TILs in tumor tissues from HCC patients. The number of $CD3^+$ and $CD8^+$ TILs appeared to positively correlate with the intratumoral IL-36α expression level (Fig. 2a). No obvious correlation was observed between $CD4^+$ TILs and IL-36α expression (Supplementary Fig. 1a). Comparative analysis further confirmed that intratumoral IL-36α expression positively correlated with the number of $CD3^+$ or $CD8^+$ TILs ($p = 0.011$ and 0.035, respectively; Fig. 2b), but not with $CD4^+$ TILs ($p = 0.408$; Supplementary Fig. 1b). From the survival analysis, we found that higher levels of $CD3^+$ or $CD8^+$ TILs correlated with a better OS in HCC patients (log-rank test, $CD3^+$, $p = 0.031$; $CD8^+$, $p = 0.001$; Fig. 2c, d). Thus, these results indicate that IL-36α might play an antitumor role through activating adaptive $CD8^+$ T cell immunity.

IL-36α-overexpressing HCC cell lines attract T lymphocytes

To explore the mechanism linking IL-36α expression to T cell immunity, we tested the ability of IL-36α-overexpressing HCC cell lines to recruit T lymphocytes in vitro. Sk-hep1 cells were transfected to overexpress IL-36α, which was confirmed by Western blotting (Fig. 3a). In the transwell chemotaxis assays, we found that Sk-hep1/IL-36α cells recruited a significantly higher portion of purified $CD3^+$ T cells ($p = 0.03$; Fig. 3b) or $CD8^+$ T cells ($p = 0.045$; Fig. 3c) compared with control Sk-hep1/pLNCX2 cells. These results further indicate that IL-36α could activate antitumor immunity via recruiting immunoeffector cells to tumor sites.

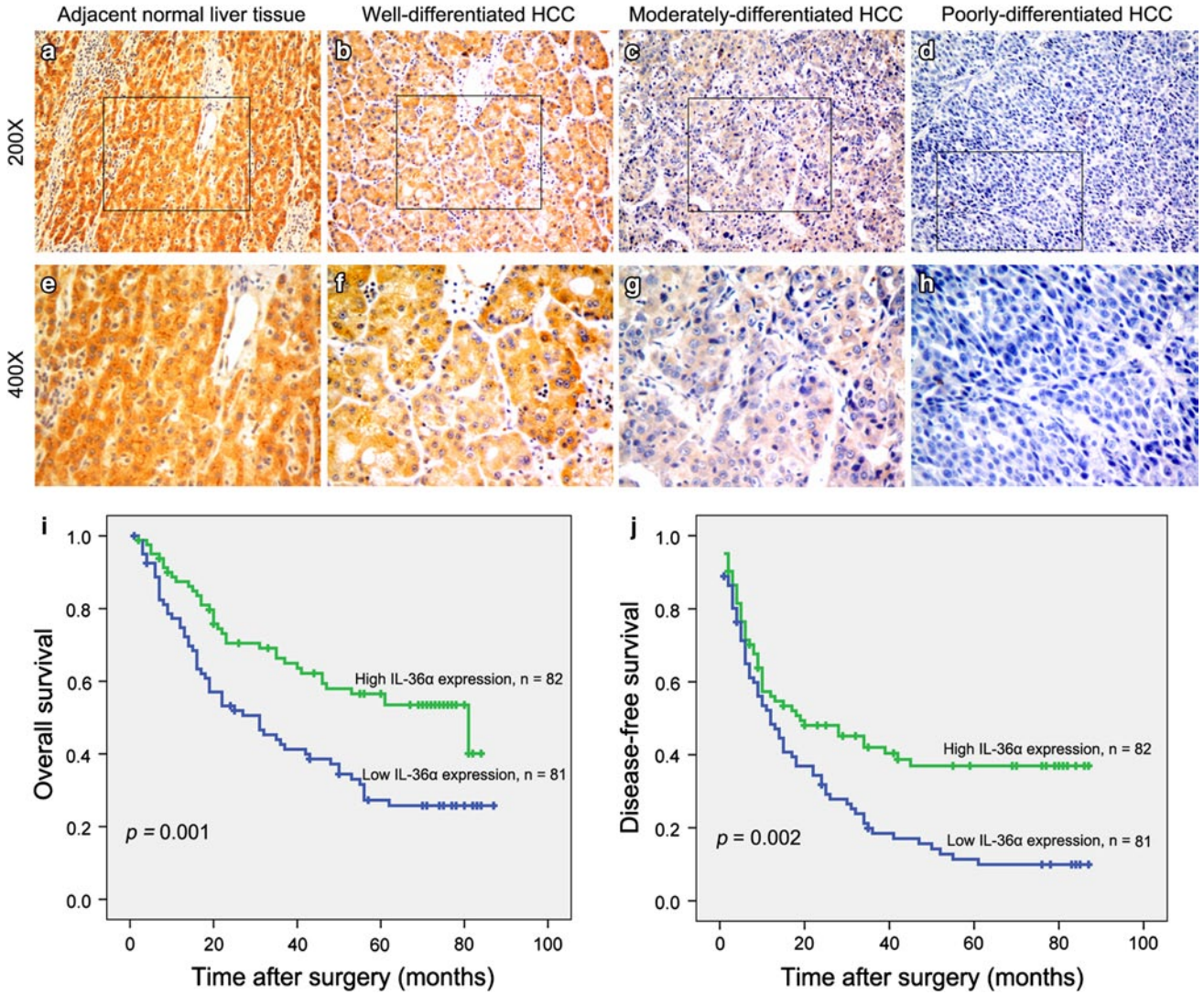

Fig. 1 IL-36α expression and its prognostic value in primary hepatocellular carcinoma tissues. Representative images of IL-36α staining are shown in normal liver tissue (**a**, **e**; MOD = 0.1127), well-differentiated HCC (**b**, **f**; MOD = 0.0581), moderately differentiated HCC (**c**, **g**; MOD = 0.0328), and poorly differentiated HCC (**d**, **h**; MOD = 0). The *top row* of images is shown at ×200 magnification, and the *boxed area* in the *bottom row* is shown at ×400 magnification. The OS (**i**) and DFS (**j**) rates of the patients in the low-IL-36α-expressing group were significantly lower than those of the patients in the high-IL-36α-expressing group

Overexpression of IL-36α in mouse HCC cell lines delays tumor growth in vivo

To further examine whether IL-36α could indeed affect tumor growth in vivo, we transduced IL-36α into the mouse HCC cell line Hepa1-6 using retroviral vectors; successful transduction of IL-36α was confirmed by Western blotting (Fig. 4a). There was no significant difference in the in vitro growth rate between tumor cells transfected with IL-36α and with the control vector (data not shown). Next, we subcutaneously inoculated Hepa1-6/IL-36α, Hepa1-6/pLNCX2, or Hepa1-6 parental tumor cells into syngeneic C57BL/6 mice and examined their tumor volumes. Hepa1-6/IL-36α tumors grew more slowly and were significantly smaller (19.5 ± 11.2 mm^3) at day 16 post-implantation, compared with either Hepa1-6/pLNCX2 (297 ± 114 mm^3) or parental Hepa1-6 (255 ± 76.8 mm^3) tumors (Hepa1-6 parental vs. Hepa1-6/IL-36α, $p < 0.001$; Hepa1-6/pLNCX2 vs. Hepa1-6/IL-36α, $p = 0.001$; Fig. 4b). Accordingly, the mean tumor weight of the IL-36α-overexpressing group was markedly lower than the other two groups (Fig. 4c). To exclude that the antitumor effects observed are related to the recognition of human IL-36α by the mouse immune system, Hepa1-6 cells stably expressed murine IL-36α were used for in vivo studies. Similarly to human IL-36α, murine IL-36α transduced into

Table 1 Relationship between IL-36α expression and clinicopathological features in HCC patients

Variable	*n*	IL-36α expression		χ^2	*p* value
		Low-level group ($n = 81$)	High-level group ($n = 82$)		
Age (years)				0.491	0.483
<50	93	44	49		
≥50	70	37	33		
Gender				0.001	0.969
Male	131	65	66		
Female	32	16	16		
Tumor size (cm)				10.480	0.001*
<5	73	26	47		
≥5	90	55	35		
Histological differentiation				6.462	0.040*
Well (I)	30	10	20		
Moderate (II)	101	50	51		
Poor (III–IV)	32	21	11		
Liver cirrhosis				2.073	0.150
Yes	136	71	65		
No	27	10	17		
Number of lesions				1.037	0.308
1	136	70	66		
≥2	27	11	16		
HBsAg				3.123	0.077
Negative	17	5	12		
Positive	146	76	70		
AFP (ng/ml)				0.520	0.471
Negative (≤400)	92	48	44		
Positive (>400)	71	33	38		
TNM stage					
I	100	43	57	4.637	0.031*
II–III	63	38	25		
Tumor capsule					
Intact	79	40	39	0.054	0.816
Absent or not intact	84	41	43		
Vascular invasion					
Yes	21	17	4	9.422	0.002*
No	142	64	78		

* p value < 0.05

mouse HCC cells could also significantly inhibit the tumor growth in vivo (Supplementary Fig. 2a-c). These results indicate that human IL-36α is homologous to murine IL-36α and could activate mouse immune system.

Overexpression of IL-36α in mouse HCC cell lines enhances $CD3^+$ and $CD8^+$ T cell infiltration into tumor tissues in vivo

To investigate alterations in the host immune response induced by IL-36α overexpression within the tumor microenvironment, we next analyzed the number of $CD3^+$ and $CD8^+$ T cells in mouse tumor tissues using immunohistochemical staining. A dramatic increase in intratumoral $CD3^+$ and $CD8^+$ T cell infiltration was observed within Hepa1-6/IL-36α tumors, compared with either Hepa1-6/pLNCX2 or Hepa1-6 parental tumors (Fig. 4d, e). To further verify whether IL-36α-overexpressing mouse HCC cell line Hepa1-6 could recruit mouse $CD3^+$ or $CD8^+$ T cells in vitro, we isolated mouse splenocytes to perform the in vitro transwell assays and found that IL-36α-overexpressing mouse HCC cell line had chemotaxis roles

Table 2 Univariate and multivariate analyses of overall survival in HCC

Variable	Univariate analysis			Multivariate analysis		
	HR	95 % CI	*p* value	HR	95 % CI	*p* value
IL-36α	0.491	0.323–0.746	0.001*	0.521	0.339–0.801	0.003*
Age	1.179	0.785–1.771	0.428			
Gender	1.009	0.603–1.688	0.973			
Tumor size	1.471	0.973–2.225	0.067			
Histological differentiation	1.505	1.077–2.101	0.016*	1.395	0.970–2.006	0.072
Liver cirrhosis	0.926	0.532–1.611	0.786			
HBsAg	0.934	0.470–1.859	0.847			
AFP	0.853	0.562–1.295	0.456			
Number of lesions	1.714	1.021–2.875	0.041*	1.888	1.059–3.363	0.031*
TNM stage	2.035	1.349–3.071	0.001*	1.426	0.860–2.365	0.169
Tumor capsule	0.719	0.477–1.085	0.116			
Vascular invasion	2.214	1.250–3.923	0.006*	1.397	0.712–2.742	0.331

HR hazard ratio, *CI* confidence interval

* *p* value < 0.05

similar to those of IL-36α-overexpressing human HCC cell line (Fig. 4f, g). These data demonstrated that the antitumor activity of IL-36α was mediated by recruiting $CD3^+$ and $CD8^+$ T lymphocytes to tumor sites in vivo.

Discussion

Accumulating evidence suggests that IL-1 family members play a significant role in tumor immunity [23, 24]. It has been shown that members of the IL-1 family not only affect the proliferation and metastasis of malignant cells, but also induce antitumoral responses in tumor sites [23, 25–27]. IL-36α is a newly identified IL-1 family member and has been shown to play a potential role in immune-mediated diseases [1, 3–5]; however, little is known about the relationship between IL-36α and cancer.

In the present study, we detected intratumoral IL-36α expression in primary HCC samples and analyzed its prognostic value in HCC patients. We found that IL-36α was primarily expressed in normal hepatocytes and well-differentiated HCC cells, but weakly in moderately and poorly differentiated HCC cells. Clinical pathological analysis further showed that low IL-36α expression was significantly correlated with tumor size, histological differentiation, tumor stage, and vascular invasion. These data suggested that decreased IL-36α expression may contribute to tumor progression. Kaplan–Meier analysis revealed that positive intratumoral IL-36α expression was significantly linked to better OS and DFS in HCC patients, which further indicated that IL-36α may be a cytokine that was in favor of antitumor response.

To explore the role of IL-36α in HCC progression, we measured $CD3^+$, $CD8^+$, and $CD4^+$ TILs in the same HCC tissues using serial tissue sections. We found that high levels of IL-36α expression significantly correlated with high numbers of $CD3^+$ and $CD8^+$ TILs, but not with $CD4^+$ TILs. In vitro transwell chemotaxis assays also confirmed the same tendency. To further explore the role of IL-36α in tumor pathogenesis in vivo, we introduced IL-36α cDNA into mouse HCC cell line and investigated the impact of IL-36α expression on tumor growth in vivo. We found that overexpression of IL-36α in mouse HCC cell lines significantly delayed tumor growth in vivo and enhanced $CD3^+$ and $CD8^+$ T cell infiltration into the tumors, consistent with our analysis of human HCC samples. Our results are in agreement with Blumberg et al., who also found that IL-36α could enhance the infiltration of $CD3^+$ T lymphocytes into sites of inflammation [4]. Taken together, these results indicate that IL-36α might help to recruit effector T cells to tumor sites and activate antitumor immunity.

Previous studies have reported that several IL-1 family members were capable of activating antitumoral immunoresponse. For example, overexpression of IL-1α in tumor cells would induce the massive infiltration of mononuclear cells in the tumor mass [28]. IL-1β was able to enhance the cytolytic activity of T lymphocytes, monocyte, and NK cells [29]. Intratumoral injection of IL-18 gene could markedly enhance the activity of NK cells and CTL cells [30]. Our study showed that IL-36α played a role in the antitumor immunity via the similar mechanism to these IL-1 family members. But, more detailed investigations will be needed to further clarify the potential function of IL-36α.

In summary, our study found that decreased intratumoral IL-36α expression correlates with HCC progression and a poor prognosis in HCC patients. Intratumoral IL-36α expression may constitute a novel prognostic marker for HCC. Furthermore, in vitro and in vivo studies

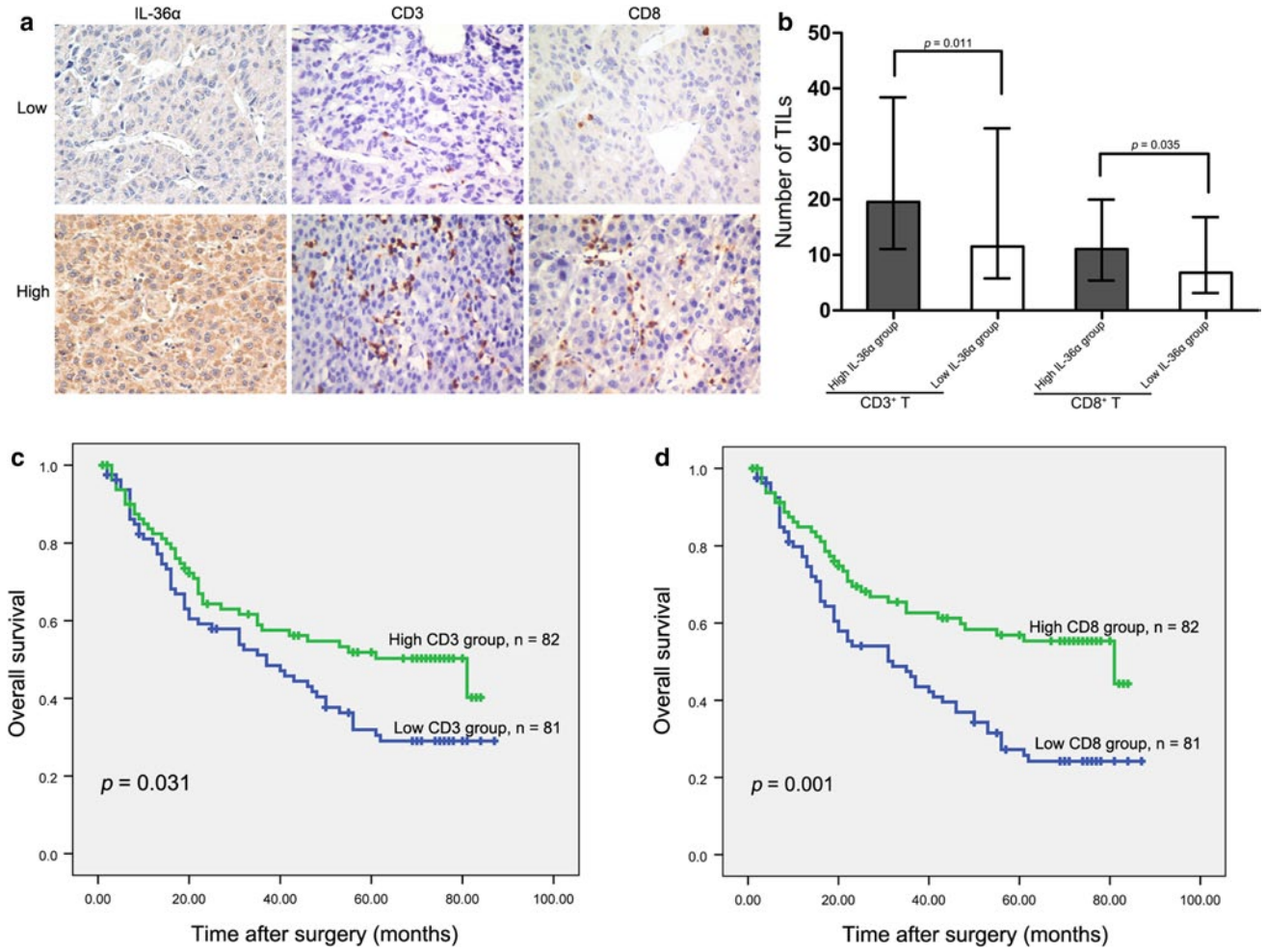

Fig. 2 Relationship between intratumoral IL-36α expression and intratumoral T cell number. Representative photomicrographs showing immunohistochemical staining of IL-36α, CD3, and CD8 in the same primary HCC tumors are shown (**a**). The low-intratumoral-IL-36α-expressing group had a low density of $CD3^+$ and $CD8^+$ TILs. The high-intratumoral-IL-36α-expressing group had a relatively high density of $CD3^+$ and $CD8^+$ TILs. Original image magnification ×400. Intratumoral IL-36α expression positively correlated with the number of TILs (**b**). High-IL-36α-expressing tumors exhibited a significantly higher proportion of intratumoral $CD3^+$ and $CD8^+$ TILs compared to low-IL-36α-expressing tissue specimens. Kaplan–Meier survival curves for primary HCC patients ($n = 163$) after surgical resection. The survival rate for patients in the high $CD3^+$ (**c**) or $CD8^+$ (**d**) TILs group was significantly better than that for patients in the low-expressing group

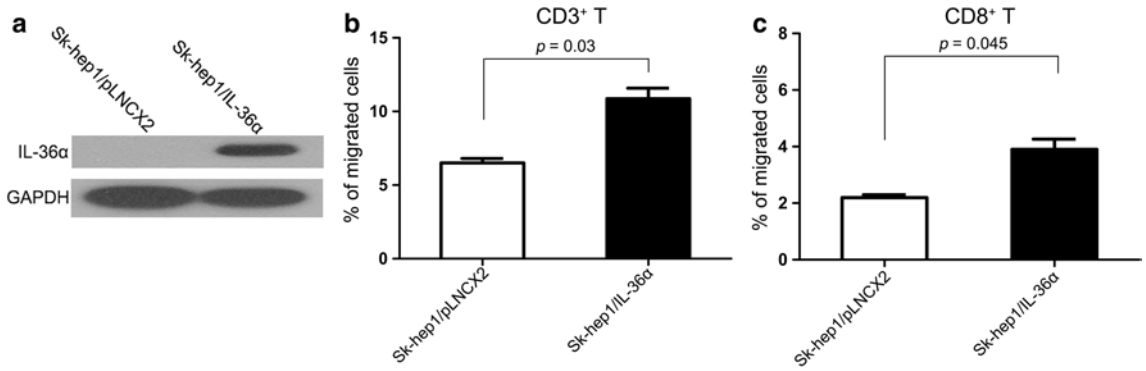

Fig. 3 IL-36α promotes $CD3^+$ and $CD8^+$ T lymphocyte chemotaxis and migration. The IL-36α stably expressing clone, Sk-hep1/IL-36α, was confirmed by Western blotting (**a**). Supernatant from Sk-hep1/IL-36α cells significantly enhanced the migration of $CD3^+$ cells (**b**) and $CD8^+$ T cells (**c**) when compared to the supernatant from Sk-hep1/pLNCX2 cells

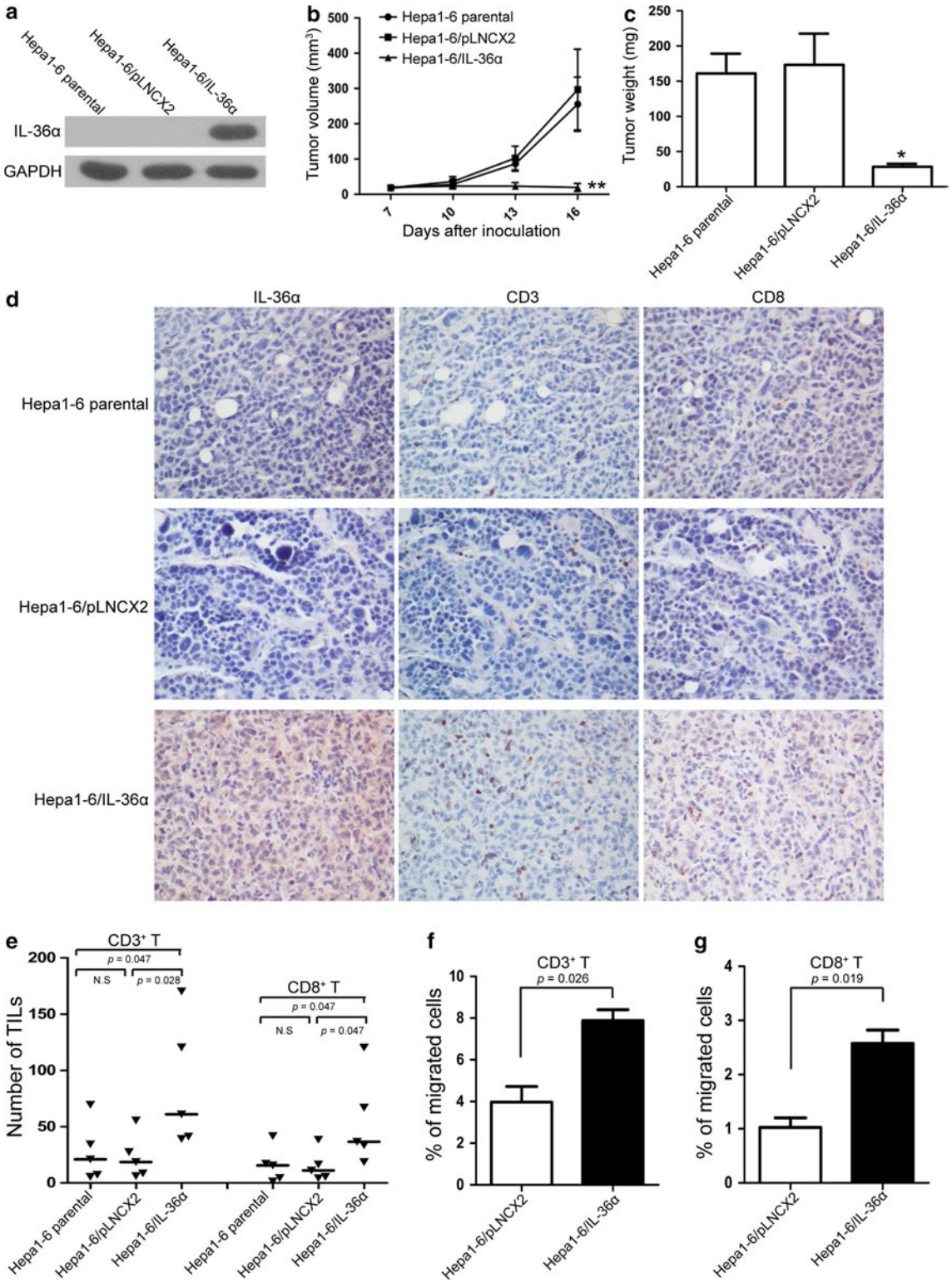

Fig. 4 Involvement of T lymphocytes in the induction of antitumor activity by IL-36α in mice. Expression of IL-36α in parental and transfected Hepa1-6 cells was confirmed by Western blotting (**a**). The tumor growth rate was slower in the tumors that overexpressed IL-36α (**b**). The final tumor weights were lower in tumors that overexpressed IL-36α (**c**). Photomicrographs of representative sections from the respective groups are shown at ×400 magnification (**d**). The density of $CD3^+$ and $CD8^+$ T cell infiltration was estimated by calculating the mean number of positively stained cells per randomly chosen high-power field (×400 magnification) from ten fields per section (**e**). Hepa1-6/IL-36α cells recruited a significantly higher portion of mouse $CD3^+$ T cells (**f**) and $CD8^+$ T cells (**g**) compared with Hepa1-6/pLNCX2 cells. *NS* not significant; $*p < 0.05$; $**p < 0.01$

suggested that IL-36α might mediate antitumor immune responses through recruiting $CD3^+$ and $CD8^+$ T lymphocytes to the tumor site and activating adaptive immunity. Thus, our findings may serve as the basis for novel immunotherapy strategies aimed at the induction of IL-36α expression by cells comprising the neoplasm or alternatively by local application of the cytokine in the vicinity of the tumor.

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Conflict of interest The authors declare that they have no conflict of interest.

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