Interleukin-35 Play Tumor Promoting Roles in Hepatocellular Carcinoma

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Abstract

Backgroud: A long-term hepatic inflammatory response is a risk factor for liver cancer initiation and progression. Interleukin (IL)-35 is the newest member of the IL-12 cytokine family, and has been reported to play an essential role in the immunosuppressive liver microenvironment. Herein we focus on the expression profiles of IL-35 in hepatocellular carcinoma (HCC) and the effect on local immune status. Methods: HCC transcriptome array data were downloaded from Gene Expression Omnibus. The bioinformatics analysis was performed by the BRB array tools and online Ingenuity Pathway Analysis software. The serum IL-35 level was detected by AimPlet bead-based immunoassay. In situ IL-35 expression detection was performed by immunohistochemical staining and western blot. Results: Our results showed that there were large amounts of IL-35 expressed in HCC serum and tumor tissues. IL-35 expression affects the transcript of thousands of genes, most of which correlated with T-cell immunity. This study proved that enhancement of regulatory T cells (Tregs) and impairment of cytolytic T cells are prominent effects of IL-35. Conclusions: Elevated IL-35 played critical roles in HCC patients through affecting the balance between Tregs and cytotoxic T cells. Dissection of the precise targets and the underlying molecular mechanisms will lead to alternative treatments for HCC patients.

Key words: Hepatocellular carcinoma; Interleukin-35; T cell exhaustion; Liver immune

Introduction

Liver cancer mainly includes hepatocellular carcinoma (HCC), intrahepatic cholangiocarcinoma, hepatoblastoma, hepatocellular adenoma, and pediatric neoplasms according to the origin of liver tumorigenesis and molecular features [1]. HCC is the most common primary liver cancer and accounts for $> 80\%$ of liver tumors. HCC exacts a heavy disease burden and is the sixth most common cancer and the fourth leading cause of cancer-related deaths worldwide [2, 3].

The incidence of HCC and mortality rate have been increasing, with almost 800,000 newly diagnosed cases each year in recent decades.[1] The prognosis of HCC is poor with a 3-year survival rate of 12.7% and a median survival of 9 months. HCC can be treated with surgical resection, liver transplantation, liver-directed therapy, and systemic therapy [4]. Unfortunately, $> 50\%$ of systemic therapies available for HCC patients are minimally effective and might exert considerable toxic damage to the remaining normal liver, further limiting clinical outcomes [5]. Given the poor prognosis, attempts to explore new alternatives to HCC therapy are necessary.

The liver microenvironment contains a large population of lymphocytes with strong anti-tumor function, including T cells, natural killer cells, natural killer T cells, mucosal-associated invariant T cells, and gamma delta T cells [6]. Most HCCs are a consequence of chronic infection with hepatitis B virus and hepatitis C virus or different metabolic and inflammatory disorders related to non-alcoholic steatohepatitis and alcoholic steatohepatitis (5). Long-term hepatic inflammatory responses, characterized by continued cytokine expression and immune cell infiltration, might lead to the changes in the liver immunologic microenvironment,

which are essential risk factors for hepatocarcinogenesis [7]. The decrease in cytotoxic function and increase in the frequency of Tregs and release of suppressive cytokines lead to HCC tolerance and growth.

Interleukin (IL)-35 is the newest member of the IL-12 family and is predominantly produced by Tregs (8). As an anti-inflammatory and immune inhibitory cytokine, IL-35 has been shown to have potent immunosuppressive effects in immune evasion [8]. Research focusing on the presence or geographic location of IL-35 has uncovered a much larger tissue distribution. Subunits of IL-35, EBI3, and IL-12 p35 have been detected in placental trophoblasts, Hodgkin lymphoma cells, acute myeloid leukemia cells, lung cancer cells, esophageal carcinoma, HCC, pancreatic ductal adenocarcinoma, cervical carcinoma, and colorectal cancer [9]. Detection in HCC patients indicated that high liver IL-35 expression correlates with tumor aggressiveness and post-operative recurrence [10]. Similar research in intrahepatic cholangiocarcinoma patients showed that a high IL-35 level is positively associated with aggressiveness and can serve as a prognostic factor [11]. In contrast, Long et al. (13) revealed decreased expression of IL-35 in HCC patients with increasing AJCC TNM stage, worse histologic grade, larger tumor size, and histologic identification with micro-vascular invasion and lymph node/distant metastasis. Therefore, more studies are needed to concentrate on the relationship between immune status and the level of IL-35 expression. In this study, we determined the expression of IL-35 in serum and tumor samples from HCC patients. Furthermore, our results proposed that IL-35 might play essential roles in tumor growth by inhibiting T cell cytotoxicity and T cell exhaustion.

Materials and methods

Data source for bio-informatics analysis

A total of 488 mRNA expression profiles for patients with HCC were downloaded from Gene Expression Omnibus (GEO; accession number GSE14520), which were detected using a single-channel platform (Affymatrix Genechip HG-U133A 2.0 arrays) (14). All of the patients had transcriptomic data form the tumor and peri-tumor samples.

Human sample collection

The peripheral blood and tissue samples of 30 cases pathologically-diagnosed with HCC in the General Surgery Department at Tianjin Medical University General Hospital were collected prior to surgery from October 2015 to July 2017. Tumor tissues and paired non-tumor control tissues were at distances > 5 cm. All specimens were divided into two aliquots: one aliquot was frozen at -80 °C for protein extraction; and the other specimen was fixed with 4% formalin for immunohistochemical (IHC) detection. Peripheral venous blood (2–3ml) were obtained from 20 volunteers who served as healthy controls. The study was planned according to the ethical guidelines following the Declaration of Helsinki. This project was approved by the Ethics Committee of Tianjin Medical University General Hospital. All subjects gave informed consent prior to enrollment in the investigation.

Cytokine detection

The serum was obtained by centrifugation at 1000 $\times$ g for 10 min and the content of IL-35 was detected by an AimPlex bead-based immunoassay (Quanto Bio, Beijing, China) according to the manufacturer's instructions. All samples were analyzed by a FACS Calibur instrument and data were collected by Cell Quest Pro software (BD Biosciences, San Diego, CA, USA). Data were analyzed by FCAP Array V3.0 software (BD Biosciences).

IHC detection

IHC was used to detect the *in situ* expression of EBI3 and IL12A in HCC tumor and peri-tumor samples. After paraffin-embedded tissue sections were deparaffinized with xylene and graded alcohols, heat-activated antigen retrieval was performed in EDTA antigen retrieval solution (Beyotime, Shanghai China). A standard two-step protocol was applied with the following primary antibodies incubated overnight at 4 °C: rabbit anti-human EBI3 polyclonal antibody (1:100, ab8396; Abcam, Cambridge, MA, USA) and rabbit anti-human IL12A monoclonal antibody (1:1000, ab131039; Abcam) Then, the tissue sections were serially rinsed and

antibody binding was detected using a peroxidase-conjugated secondary antibody (Absin, Shanghai, China) for 30 min at room temperature. Finally, slides were counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene and covered with slips. IHC slides were scanned using an Automatic Digital Slice Scanning System(PRECICE 500, Beijing, China).

Western blotting

Human HCC tumor and peri-tumor tissues were lysed in RIPA lysis buffer (Solarbio, Beijing, China) for 30 min and total protein was harvested by centrifugation (12,000 rpm for 10 min) at 4 degC. A total of 25 ug of collected protein were separated by SDS-PAGE and transferred to PVDF membranes. After the membranes with blotted proteins were incubated with rabbit anti-human EBI3 antibody (1:250) and rabbit anti-human IL12A antibody (1:1000) overnight at 4 degC , a horse radish peroxidase)-conjugated goat anti-rabbit IgG antibody (1:6000; Cell Signaling Technology, Danvers, MA, USA) for 30 min at 37 degC. The targeted proteins were detected and quantified using a Chemi Scope 6300 Touch chemiluminescence imaging system (Clinx, Shanghai, China). The membranes were stripped and reblotted with HSP90 antibody to verify the equal loading of protein in each lane. Signals of target or reference protein bands were quantified using Image J software (developed by NIH, MD, USA).

Expression profile analysis of mRNAs in HCC tumor and peripheral non-tumor samples

Class comparison of the gene expression data was done with BRB-Array Tools software (version 4.6.0, Bethesda, MD, USA). Unsupervised hierarchical clustering analysis was performed using GENESIS software (version 1.8.1, IBMT-TUG, Graz, Austria).

Ingenuity Pathway Analysis (IPA)

The biological functions and pathway analysis for the list of genes were performed by the online software, IPA. IPA is a web-based functional analysis tool for comprehensive omic data that can be approached on the internet (https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/).

Data analysis

Data are expressed as the mean +- standard deviation. For continuous variables, normality was assessed by the Kolmogorov-Smirnov test. Data were analyzed using both parametric and non-parametric tests. Analyses were performed using GraphPad Prism (version 5.0; San Diego, CA, USA). A p-value < 0.05 was considered to be statistically significant.

Results

IL-35 is highly expressed in peripheral blood and tumor tissues of HCC patients

The transcriptome dataset of GSE14520 has 249 HCC tumor and 239 non-tumor samples. We detected IL-35, represented by the mean expression of subunits IL-12p35 and Ebi3, in the HCC tumor and paired non-tumor samples. The results showed that level of IL-35 expression in tumor tissues were significantly higher than paired non-tumor tissues ($P < 0.0001$, Fig.1A). A similar trend was detected in the IL-35R gene expression examination, which was calculated by the mean expression of IL-12Rβ2 and gp130 ($P < 0.0001$, Fig.1B). To further validate the bioinformatics analysis results, we detected the level of serum IL-35 expression by AimPlex bead-based immunoassays and performed IHC analyses of IL-35 by the level of p35 and Ebi3 co-expression. Our results showed that the level of serum IL-35 expression was significantly higher in HCC patients compared with health control volunteers (HC) (P=0.0006, Fig.1C). IHC staining results showed that p35 and Ebi3 co-expressed in the hepatic cytoplasm and both subunits were highly expressed in the tumor tissues compared with the paired non-tumor samples (Fig.2A). The same results were observed through western blot analysis (Fig.2B and 2C).

IL-35 expression affected the gene expression profiles

We stratified both the tumor (249 cases) and non-tumor (239 cases) samples into IL-35 low and high groups according to its gene expression level. Then, class comparison analyses of transcriptome data between IL-35-

low and -high groups were performed to investigate the changes in mRNA expression profiles. A total of 1625 differentially expressed genes (DEGs) were found (univariate $P < 0.001$, false discovery rate < 0.05; Fig.3B) in tumor samples, whereas 4238 DEGs were detected in the non-tumor group (Fig.3A). Venny plot analysis showed that there were a total of 1270 genes changed in both tumor and non-tumor tissues (Fig.4A). To determine the biological functions for these genes, we performed IPA analysis for the overlapped 1270 gene list and found that the top 20 [log(p-value) > 5] signal pathway were immune function related, especially the T cell pathway (Fig.4B).

IL-35 expression correlated with the decrease in T cell cytotoxicity and T cell exhaustion

Published reports have indicated that IL-35 induces tumor tolerance and evasion mainly through an increase in Tregs frequency or function, but decreased cytotoxic T cell function [9, 12, 13]. We then compared the level of Foxp3 expression, the main transcription factor of Tregs; the level of expression was significantly associated with IL-35 content (Fig.5A). Similarly, the level of IL-10 expression in the IL-35 high sample group was significantly higher than the IL-35 low expression group (Fig.5B). Hacohen et al. (17) developed a quantitative measure of the activity of tumor-infiltrating lymphocytes, especially $CD8^+$T cells, based on two key effector genes (GZMA and PRF1), referred to as cytolytic activity (CYT). We demonstrated that the "CYT" significantly decreased in the IL-35 high group sample and there was less IFN-γ expressed (Fig.5C and D), which indicated impaired cytotoxic immunity. Furthermore, we detected the expression of some T cell exhaustion markers and found that PD1 and LAG3 were positively correlated with the IL-35 level, which showed a significant increase in the IL-35 high expression group (Fig.5E and F).

Discussion

IL-35 has been reported to have a role in liver disease, including viral hepatitis, hepatic fibrosis, liver cirrhosis, chemical-induced liver injury, as well as HCC [10, 14-18]. Additionally, a large amount of HCC is associated with impairment of the proliferation, cytokine production, and cytotoxic effector functions accompanied by a chronic hepatitis virus-specific T cell immune reaction [19]. In this study we found large amounts of IL-35 in HCC patient serum and tumor tissues, which indicated that IL-35 might be involved in HCC initiation and progression. Thus, a better understanding of the relationship between the level of IL-35 expression and the anti-tumor immune changes seemed necessary.

The immune cells and secreted cytokines present in the tumor microenvironment contribute to tumor progression or active repression of tumor development. A total of 1270 DEGs were detected in the tumor and non-tumor tissues. Through online IPA pathway analysis we found that most of the overlapped DEGs were involved in T cell immune activities. Based on our previous work in which over-expressed IL-35 was shown to exert strong inhibition on $CD4^+$T cells through Tregs-derived IL-10, we determined the levels of Foxp3 and IL-10 expression to explore the changes in Tregs [20]. We found that there were higher Foxp3 and IL-10 expression in IL-35 high group cases, which suggested that IL-35 was positively correlated with Tregs frequency and function.

Peripheral and tissue resident $CD8^+$T cells have roles in the anti-tumor effect and IL-35 suppressed cytotoxicity and pro-inflammatory cytokine secretion [21]. Hacohen et al. (17) developed cytolytic activity as a quantitative measure of the activity of tumor-infiltrating lymphocytes, especially $CD8^+$T cells, based on two key effector genes (GZMA and PRF1[22]. In this study we calculated the index "CYT" and detected the level of IFN-γ expression to reflect the cytotoxic status and anti-tumor cytokine secretion capability between the two compared groups. Our results showed that both the cytolytic activity and IFN-γ secretion were significantly decreased when there was a large amount of IL-35 present in the liver microenvironment.

In the past few years, immune inhibitory checkpoints have been increasingly recognized as important elements in the immunosuppression of chronic inflammation and tumor immunity. There are studies that have shown Tregs-derived IL-35 induce CD8+T cell exhaustion that limits effective anti-tumor immunity [13]. We checked the expression of T cell inhibitory receptor to reflect the intratumoral T cell exhaustion status. Our results revealed that both PD1 and LAG3 are highly expressed in the IL-35 high group samples, which indicated that IL-35 might promote inhibitory receptor induction and exhaustion.

Conclusion

In summary, we found increased IL-35 expression in HCC serum and tumor tissues. Elevated IL-35 has a critical role in the immunosuppressive tumor environment by affecting the balance between Tregs and cytotoxic T cells. Further studies are required to determine the regulatory network of IL-35 to effector T cells and how to modulate it. Dissection of the precise mechanism and corresponding management will hopefully lead to the development of more effective treatment methods for HCC patients.

List of Abbreviations

IL, interleukin; HCC, hepatocellular carcinoma; GEO, Gene Expression Omnibus; IHC, immunohistochemical; Tregs, regulatory T cells; AJCC, American Joint Committee on Cancer; TNM, Tumor Node Metastasis; IPA, Ingenuity Pathway Analysis; DEGs, differentially expressed genes.

Conflict of interests

The authors declare that they have no conflicts of interest regarding the publication of this article.

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References:

1. Sia D, Villanueva A, Friedman SL, Llovet JM. Liver Cancer Cell of Origin, Molecular Class, and Effects on Patient Prognosis. GASTROENTEROLOGY 2017;152(4):745-61.

2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018;68(6):394-424.

3. Jiang Y, Han QJ, Zhang J. Hepatocellular carcinoma: Mechanisms of progression and immunotherapy. World J Gastroenterol 2019;25(25):3151-67.

4. Kim DW, Talati C, Kim R. Hepatocellular carcinoma (HCC): beyond sorafenib-chemotherapy. J Gastrointest Oncol 2017;8(2):256-65.

5. Hou J, Zhang H, Sun B, Karin M. The Immunobiology of Hepatocellular Carcinoma in Humans and Mice: Basic Concepts and Therapeutic Implications. J HEPATOL 2019.

6. Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. NAT IMMUNOL 2013;14(10):1014-22.

7. Makarova-Rusher OV, Medina-Echeverz J, Duffy AG, Greten TF. The yin and yang of evasion and immune activation in HCC. J HEPATOL 2015;62(6):1420-9.

8. Chaturvedi V, Collison LW, Guy CS, Workman CJ, Vignali DA. Cutting edge: Human regulatory T cells require IL-35 to mediate suppression and infectious tolerance. J IMMUNOL 2011;186(12):6661-6.

9. Xue W, Yan D, Kan Q. Interleukin-35 as an Emerging Player in Tumor Microenvironment. J CANCER 2019;10(9):2074-82.

10. Fu YP, Yi Y, Cai XY*, et al.* . Overexpression of interleukin-35 associates with hepatocellular carcinoma aggressiveness and recurrence after curative resection. Br J Cancer 2016;114(7):767-76.

11. Zhang MX, Gan W, Jing CY*, et al.* . Overexpression of interleukin-35 in intrahepatic cholangiocarcinoma is a prognostic indicator after curative resection. CANCER SCI 2018;109(4):1195-206.

12. Wei X, Zhang J, Gu Q, Huang M, Zhang W, Guo J, Zhou X. Reciprocal Expression of IL-35 and IL-10 Defines Two Distinct Effector Treg Subsets that Are Required for Maintenance of Immune Tolerance. CELL REP 2017;21(7):1853-69.

13. Sawant DV, Yano H, Chikina M, *et al.* . Adaptive plasticity of IL-10(+) and IL-35(+) Treg cells cooperatively promotes tumor T cell exhaustion. NAT IMMUNOL 2019;20(6):724-35.

14. Shi YY, Dai MJ, Wu GP, Zhou PP, Fang Y, Yan XB. Levels of interleukin-35 and its relationship with regulatory T-cells in chronic hepatitis B patients. VIRAL IMMUNOL 2015;28(2):93-100.

15. Luo M, Peng H, Chen P, Zhou Y. The immunomodulatory role of interleukin-35 in fibrotic diseases. Expert Rev Clin Immunol 2019;15(4):431-9.

16. Shi M, Wei J, Dong J, Meng W, Ma J, Wang T, Wang N, Wang Y. Function of interleukin-17 and -35 in the blood of patients with hepatitis B-related liver cirrhosis. MOL MED REP 2015;11(1):121-6.

17. Zheng XF, Hu XY, Ma B, *et al.* . Interleukin-35 Attenuates D-Galactosamine/Lipopolysaccharide-Induced Liver Injury via Enhancing Interleukin-10 Production in Kupffer Cells. FRONT PHARMACOL 2018;9:959.

18. Wang W, Guo H, Li H, Yan Y, Wu C, Wang X, He X, Zhao N. Interleukin-35 Gene-Modified Mesenchymal Stem Cells Protect Concanavalin A-Induced Fulminant Hepatitis by Decreasing the Interferon Gamma Level. HUM GENE THER 2018;29(2):234-41.

19. Trehanpati N, Vyas AK. Immune Regulation by T Regulatory Cells in Hepatitis B Virus-Related Inflammation and Cancer. SCAND J IMMUNOL 2017;85(3):175-81.

20. Zhao N, Li H, Yan Y, Jiang R, He X. Mesenchymal stem cells overexpressing IL-35 effectively inhibit CD4(+) T cell function. CELL IMMUNOL 2017;312:61-6.

21. Yang L, Shao X, Jia S, Zhang Q, Jin Z. Interleukin-35 Dampens CD8(+) T Cells Activity in Patients With Non-viral Hepatitis-Related Hepatocellular Carcinoma. FRONT IMMUNOL 2019;10:1032.

22. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. CELL 2015;160(1-2):48-61.

Figure legends

Figure 1. IL-35 is abundantly expressed in HCC patients. (A-B) The level of mRNA expression of IL-35 and IL-35R was calculated by the log2 array data downloaded from GSE14520. We used the mean expression of IL-12p35 and Ebi3 to reflect IL-35 and the mean expression of IL-12Rβ2 and gp130 to reflect IL-35R. (C) Serums from 30 HCC cases and 30 heath volunteers were isolated by centrifuging and IL-35 level was detected using an AimPlex bead-based immunoassay.

Figure 2. Detection of IL-35 in HCC tissues. (A) Representative images of immunohistochemical (IHC) staining for of IL-12p35 and Ebi3 in HCC tumor and non-tumor tissues (50× and 200× respectively). The left panel was tumor samples and the right panels were non-tumor samples. (B) Western blot analysis of IL-12p35 and Ebi3 in 30 paired HCC tumor and non-tumor tissues. Three representative results were shown. (C) Quantitative data represent the mean ± standard deviation. * represent $p < 0.05$, ** represent $p < 0.01$.

Figure 3. Differentially expressed genes in IL-35-high and -low expressed groups. (A) The hierarchical clustering of the 4238 genes differentially expressed in non-tumor samples. (B) The hierarchical clustering of the 1625 genes differentially expressed in tumor samples. Each column represents individual tissue samples. Genes and samples were ordered by centered correlation and complete linkage. The scale represents the level of gene expression from -3.0 to 3.0 on a log2 scale.

Figure 4. Majority of the differentially expressed genes correlated with T cell immune function. (A) Venny plot of the differentially expressed genes showed that there were 1270 genes overlapped between

the tumor and non-tumor samples. (B) Input of all 1270 genes into the online IPA software and the analysis results showed that most of the canonical pathway was related to T cell immunity. The top 20 signal pathway names and the log(p-value) are listed.

Figure 5. Characteristics of IL-35 high and low subgroups determined by the comparison of the gene array transcriptome data.(A–B) The comparison of Foxp3 and IL-10 expression levels between the IL-35 high and low subgroups. (C) Immune cytolytic activity which reflected T cell cytotoxic capability was calculated based on transcript levels of two key cytolytic effectors [granzyme A (GZMA) and perforin (PRF1)]. (D) Comparison of IFN-γ, the main T cell-derived effective cytokine, expression between the IL-35 high and low subgroups. (E–F) The expression comparisons of T cell inhibitory receptors, which reflect the T cell exhaustion status.

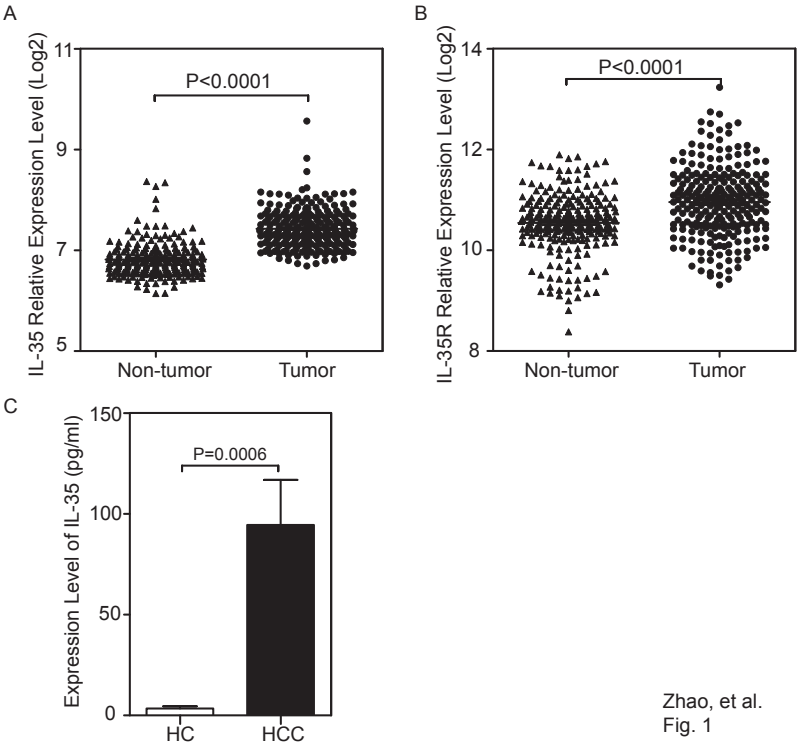

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Fig. 1

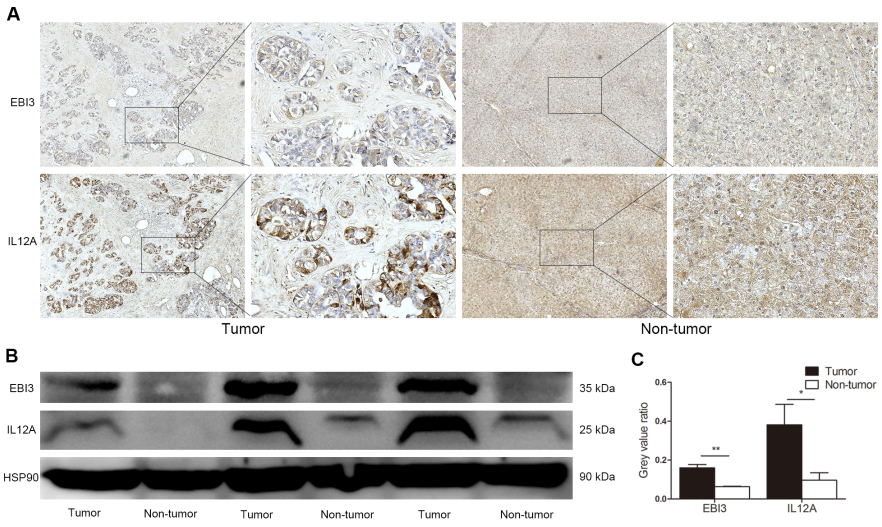

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Fig.2

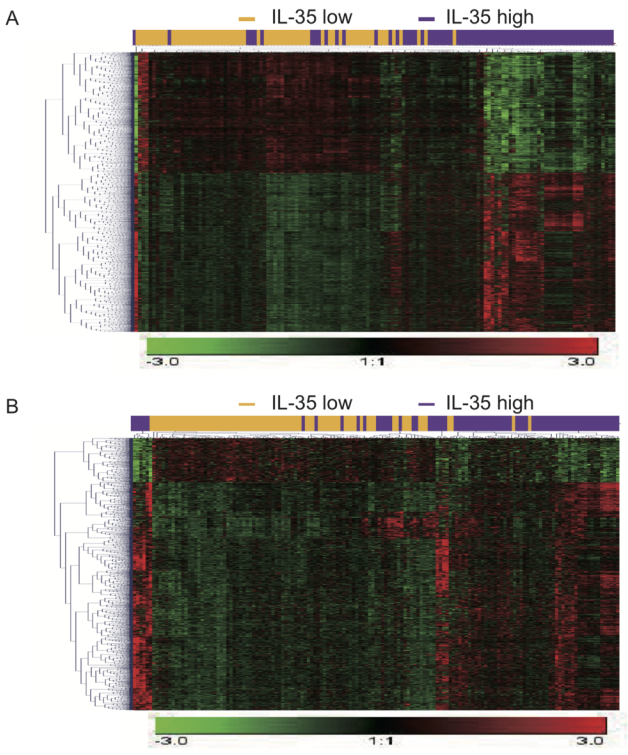

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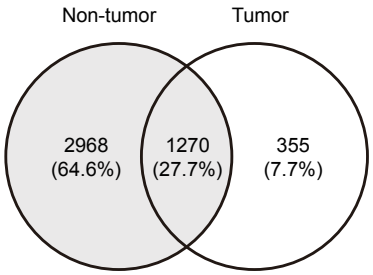
Fig. 3

A



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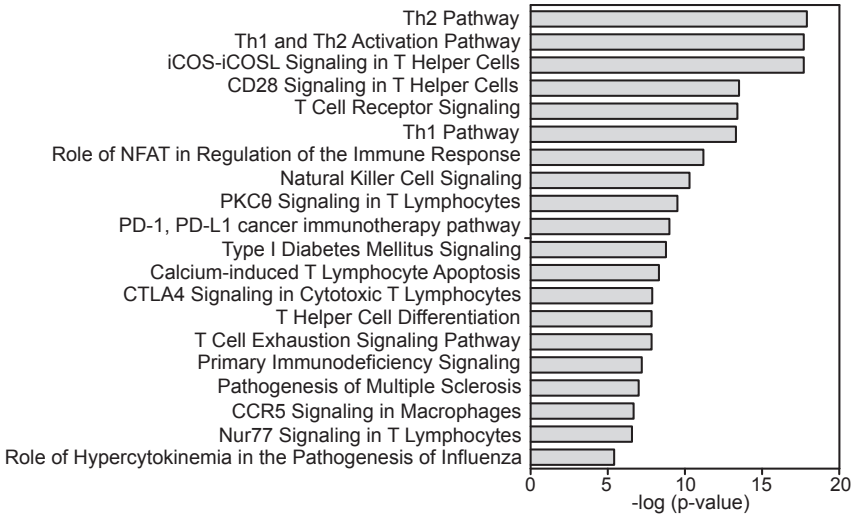

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Fig. 4

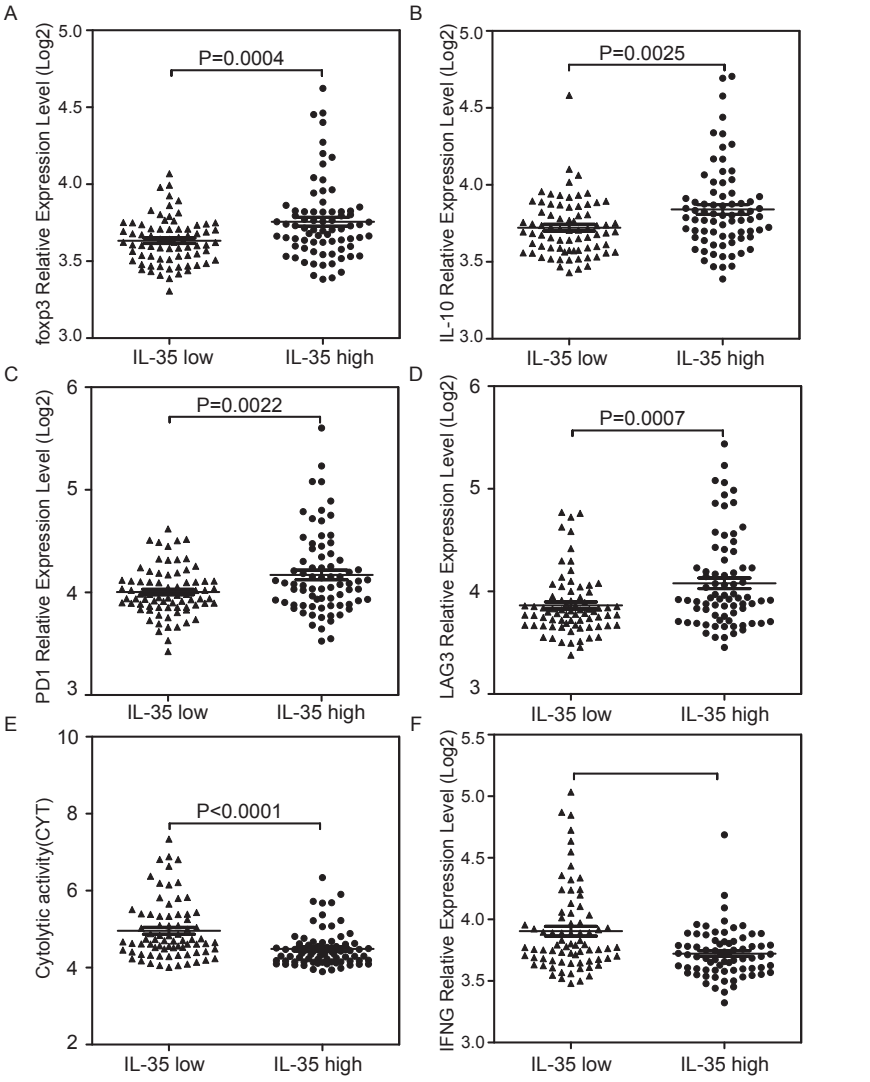

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Fig. 5