Int J Clin Exp Pathol 2017;10(7):7940-7946
www.ijcep.com /ISSN:1936-2625/IJCEP0053361

Original Article

IL-9 promotes proliferation and metastasis of hepatocellular cancer cells by activating JAK2/STAT3 pathway

Rong-E Lei, Cheng Shi, Pei-Ling Zhang, Bang-Li Hu, Hai-Xing Jiang, Shang-Yu Qin

Department of Gastroenterology, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi, China

Received March 21, 2017; Accepted May 24, 2017; Epub July 1, 2017; Published July 15, 2017

Abstract: The role of IL-9 in hepatocellular carcinoma (HCC) remains unknown. This study was designed to investigate the effect of IL-9 on HCC cells and the underlying signaling pathway. HCC cell lines SMMC-7721 was treated by IL-9, and the activities of cells were tested. The expression of JAK2, STAT3, p-JAK2 and p-STAT3 was detected by Western Blot assay. RT-PCR was used to detect the expression of MMP-2, MMP-9 and VEGF. SMMC-7721 cells were pre-treated with AG490, which is the inhibitor of JAK2/STAT3 pathway, and then incubated with IL-9. The expression of STAT3, p-STAT3, VEGF, MMP-2 and MMP-9 was detected, and the activities of SMMC-7721 cells was tested. The data showed that IL-9 significantly promoted the proliferation, invasion and migration of SMMC-7721 cells in a concentration dependent manner. Exposure to IL-9 increased the activation of p-STAT3 and p-JAK2, and increased the expression of MMP-2, MMP-9 and VEGF at the same time. Suppression of JAK2/STAT3 pathway by AG490 attenuated the promotive effects of IL-9 on SMMC-7721 cells, and reduced the expression of VEGF, MMP-2 and MMP-9. The present study demonstrated that IL-9 promotes the proliferation and metastasis in HCC cells and the effect may partly through the regulation of JAK2/STAT3 pathway.

Keywords: Hepatocellular carcinoma, IL-9, JAK2, STAT3, AG490

Introduction

Hepatocellular carcinoma (HCC) is one of the most common primary malignancies and the third most frequent cause of cancer deaths [1]. At present hepatectomy is the most effective treatment for early HCC, however, most HCC is not diagnosed until the disease is at an advanced stage [2, 3]. Therefore, prevention and treatment of HCC is of great importance. Emerging evidence has shown that signaling pathways changes immensely during the pathogenesis of HCC. The Janus-activated kinases/signal transducer and activator of transcription 3 (JAK/STAT3) pathway is involved in the pathogenesis of different types of solid tumor [4, 5]. After activated by the JAKs, STAT3 translocate to nucleus and induce expression of downstream molecules, such as vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP)-2, and MMP-9 [6], which are reported to mediate hepatic tumor cell progression and metastasis [7, 8]. STAT3 participates in immune evasion, oncogenesis, cell proliferation, apoptotic resistance, and angiogenesis [4, 9]. Growing evidence has shown that the STAT3 signaling pathway is involved in the carcinogenesis, including HCC [10, 11].

STAT3 could be triggered by several extracellular cytokines and growth factors, such as interleukins (ILs) [6]. IL-9 is an important immunoregulatory cytokine, and plays various roles through the combination with its receptors (IL-9R). Evidences have demonstrated that IL-9 has been involved in allergic inflammation, parasitic infection and tumor immunity [12]. Up to date, fewer studies focused on the role of IL-9 in tumor, and the relationship between IL-9 and tumor remains controversial. Study of melanoma suggested that IL-9 could promote the activation and proliferation of mast cells, which have cytotoxic function against tumor cells [13].

Table 1. Sequence-specific primers for PCR amplification

Gene	Forward primer	Reverse primer
MMP-2	5'-AAAAGCTGACCCACCCAATAAC-3'	5'-AAAGAACATAGCACGACCCAGAA-3'
MMP-9	5'-CAAGCTGGACTCGGTCTTTGA-3'	5'-GCCTGTGTACACCCACACCT-3'
VEGF	5'-AGCACGAGCTACCTCAGCAAGAC-3'	5'-TTTAGACATGCATCGGCAGGAA-3'
GADPH	5'-GCACCGTCAAGGCTGAGAAC-3'	5'-TGGTGAAGACGCCAGTGGA-3'

But Ye et al [14] demonstrated that IL-9 could promote the proliferation of lung cancer cells.

Previously, IL-9 and IL-9R was found to be increased in HCC tissue compared with peritumor tissue, suggesting that IL-9 may involve in the pathogenesis of HCC [15]. However, few studies have investigated the signaling pathway underlying the effect of IL-9 on HCC. Thus, the present study was aim to examine the role of IL-9 in regulating the proliferation, migration, and invasion of HCC and the relevant signaling pathway.

Materials and methods

Cell lines and reagents

The human HCC cell line (SMMC-7721) was purchased from Shanghai Institute of Cell and Biology, Chinese Academy of Science. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) were supplied by Gibco BRL (Rockville, IN, USA). Rabbit anti-p-JAK2 (Y-1007/Y-1008), JAK2, p-STAT3 (Y-705) and STAT3 antibodies were purchased from Abcam (Cambridgeshire, England). Total RNA Kit, PrimeScript RT Reagent kit and SYBR Green Real-time PCR Master Mix were obtained from TaKaRa (Dalian, China). Cell lysis buffer, proteases inhibitor, CCK-8 kit were obtained from Beyotime Institute of Biotechnology (Jiangsu, China).

Cell culture

The cell lines SMMC-7721 were routinely cultured in high glucose DMEM medium supplemented with 10% heat-inactivated FBS and 100 units of penicillin-streptomycin at 37°C in a humidified incubator with 5% CO_2.

Cell viability assay

The viability of SMMC-7721 cells which were treated with different concentration IL-9 for 24 h were assessed by cell counting kit-8 (CCK8) assay. 1×10^4 cells were seeded in each well of 96-well plates; 24 h later, SMMC-7721 cells in each well were incubated with 10 μL of CCK8 at 37°C for 2 h. Then, the absorbance for each well was measured using a microplate reader (Tecan, Austria) at 450 nm. Experiments were performed at three times.

Transwell invasion and migration assays

A Transwell system with 8-μm pore (Corning, Grand Island, NY, USA) was used in the cell invasion and migration assays, according to the manufacturer's protocol. In the migration assay, approximately 5×10^4 cells were seeded to the upper chamber and cultured in serum free media. And DMEM medium containing 10% FBS was added into the lower chamber. After incubation at 37°C for 24 h, the cells on the upper surface were carefully removed, then the upper chambers were fixed in 4% methanol for 20 min, followed by 0.1% crystal violet staining for 20 min. Cells that migrated to the bottom surface were observed by an microscope. Five fields were randomly selected and the mean number of cells per field was calculated. Each individual experiment had triplicate inserts. In the invasion assay, the same experimental design was used, but Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were precoated onto the membranes.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from SMMC-7721 cells by using Total RNA Kit according to the manufacturer's instruction. Next, a PrimeScript RT Reagent kit was used to synthesize cDNA. a SYBR green PCR master mix was used to performed Real-time quantitative PCR. The sequence-specific primers used for PCR amplification were showed in **Table 1**. Thermal cycling conditions included pre-incubation at 95°C for 30 s followed by 40 PCR cycles at 95°C for 3 s and 60°C for 30 s. The gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. All reactions were run in triplicate.

Western blot analysis

SMMC-7721 cells were harvested after inducting by IL-9 for 24 h. Protein was extracted from

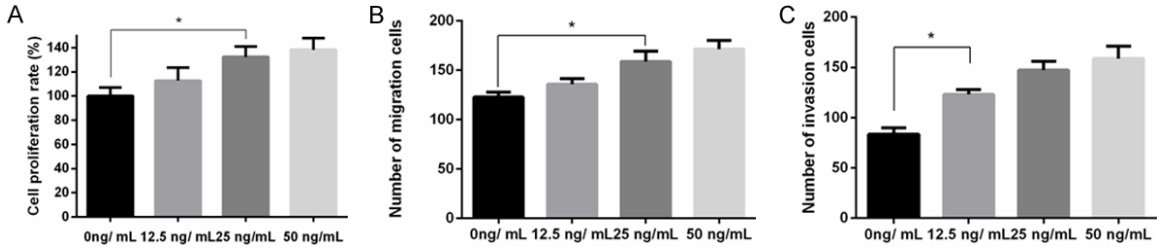

Figure 1. IL-9 promoted proliferation, migratory and invasion of SMMC-7721 cells. A: IL-9 promoted proliferation of SMMC-7721 cells; B: IL-9 promoted migratory of SMMC-7721 cells. C: IL-9 promoted invasion of SMMC-7721 cells. Data expressed as mean ± SD. The differences among multiple groups were analyzed using one-way ANOVA/LSD post-hoc test. $^{*}P<0.05$.

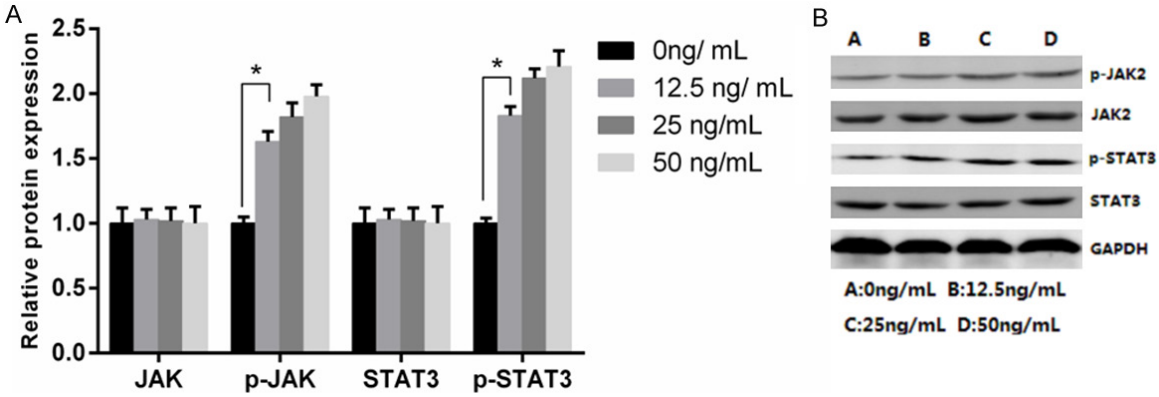

Figure 2. Expression of JAK2/STAT3 in SMMC-7721 cells after IL-9 treatment. A: Relative proteins expression of JAK2, p-JAK2, STAT3 and p-STAT3 in SMMC-7721 cells. One-way ANOVA/LSD post-hoc test showed that the expression of p-JAK2 and p-STAT3 was significantly elevated in IL-9 treatment groups compared with control group, $^{*}P<0.05$ compared with control; B: Western-blot results of JAK2, p-JAK2, STAT3 and p-STAT3.

cells. Equal amounts of extracted proteins were separated by 10% SDS-PAGE and then transferred to PVDF membranes. After being blocked with 5% nonfat milk, the membranes were incubated with primary antibodies against p-JAK2 (Y-1007/Y-1008), JAK2, p-STAT3 (Y-705), STAT3 and GAPDH overnight at 4°C for 12 h and then incubated with Li-cor AlexaFluor® 680 secondary antibodies for 1 h at room temperature. The signals were detected using Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE) and quantitated by Fluorchem 8900 system (Alpha Innotech, San Leandro, CA). The expression level of p-JAK2, JAK2, p-STAT3, STAT3 was normalized to that of GAPDH, the reference protein.

Statistical analysis

Statistical analyses were performed by the Statistical Package for the Social Sciences version 17.0. Data were expressed as mean values ± standard deviation (SD), and analysis using the one-way analysis of variance (ANOVA) followed by LSD post-hoc test. The level of significance was set at $P<0.05$.

Results

IL-9 promoted proliferation, migratory and invasion of SMMC-7721 cells

SMMC-7721 cells were treated with IL-9 (12.5 ng/mL, 25 ng/mL, 50 ng/mL) for 24 h. CCK8 assay indicated that the proliferation of SMMC-7721 cells was promoted by IL-9, and one-way ANOVA test showed that this effect was elevated accompany with increased concentrations of IL-9 ($P<0.05$). Similar to the results of cell proliferation, the Transwell assay showed that migration and invasion activity of SMMC-7721 cells were promoted by IL-9, and the metastasis of cells was increased with the elevated of IL-9 ($P<0.05$). See **Figure 1**.

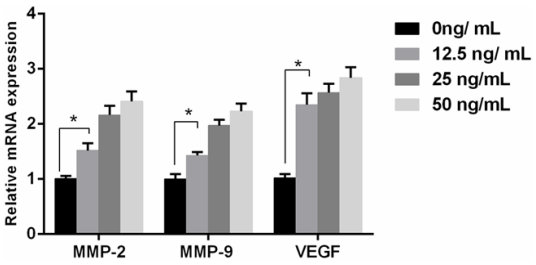

Figure 3. Relative mRNA expression of MMP-2, MMP-9 and VEGF in SMMC-7721 cells. Data expressed as mean ± SD. One-way ANOVA/LSD post-hoc test showed significantly differences in the expression of MMP-2, MMP-9 and VEGF among IL-9 treatment groups and control group. *P<0.05 compared with control group.

Expression of JAK2/STAT3 in SMMC-7721 cells after IL-9 treatment

In order to explore the mechanism of IL-9 promoted SMMC-7721 cells proliferation and metastasis, western blot assay was used to detect the expression of JAK2, p-JAK2, STAT3 and p-STAT3. One-way ANOVA test showed significant differences in the expression of p-JAK2 and p-STAT3 (P<0.05), and these changes were more prominent with the elevated of IL-9 concentration, but no significantly difference on expression of total JAK2 and STAT3 (P>0.05), indicating that phosphorylated of JAK2/STAT3 pathway involved in this process. See **Figure 2**.

Expression of MMP and VEGF in SMMC-7721 cells after IL-9 treatment

MMP-2, MMP-9 and VEGF are the downstream molecules of STAT3 pathway, to study the underlying mechanism of IL-9 promoted SMMC-7721 cells invasion, the mRNA of MMP-2, MMP-9 and VEGF was tested by qRT-PCR. One-way ANOVA test showed that, after treated by IL-9 for 24 h, the expression of MMP-2, MMP-9 and VEGF mRNA in the cells was significantly increased (P<0.05). See **Figure 3**.

Inhibition of STAT3 abrogated the effect of IL-9 on SMMC-7721 cells

STAT3 inhibitor AG490 (40 μmol/L) was added into the medium of SMMC-7721 cells in the presence of IL-9 (50 ng/mL), the results showed that, the expression of p-STAT3 protein in the cells was decreased compared with those without AG490 treatment. Similarly, the proliferation, invasion and migration of SMMC-7721 cells in AG490+IL-9 treatment group were greatly decreased compared with IL-9 treatment group (P<0.05). Further, the expression of MMP-2, MMP-9 and VEGF mRNA was decreased significantly (P<0.05). See **Figure 4**.

Discussion

Owing to the identification of its expression by multiple T helper (Th) cells, such as Th2 cells, Th9 cells and Th17 cells, IL-9 has attracted renewed interest. In this study, we showed that IL-9 could promote the proliferation, migration and invasion activity of HCC cells via the JAK2/STAT3 pathway, and inhibition of the phosphorylation of STAT3 abolished the promoting effect, and the downstream molecules of JAK2/STAT3 pathway were also down-regulated. Taken together, these results demonstrated that IL-9 promotes HCC cells proliferation and metastasis, and this effect is via JAK2/STAT3 pathway, which provided a therapy target for HCC.

IL-9 functions both as positive and negative regulator of immune responses depending on the type of diseases. For example, IL-9 has detrimental roles during allergy and autoimmunity [16]. However, during parasitic infections, IL-9 can help to clear the pathogen [17]. At present there are limited researches focused on the IL-9 in tumor, especially in solid tumors. Our previous study showed that frequency of Th9 cells and the IL-9 levels were significantly higher in malignant ascites (MA) from HCC patients than those in cirrhotic patients and healthy control. Moreover, increasing peritoneal Th9 cells predicted shorter survival time of patients with MA [18], indicating that IL-9 might play a role during occurrence and progression of HCC. In the present study, we found that IL-9 could significantly promote the proliferation and metastasis of SMMC-7721 cells, which was in agree with the results in the study of lung cancer cells [14]. However, some studies have shown the IL-9 was able to suppress tumor growth. Purwar et al [19] treated melanoma-bearing RORγt-deficient mice with IL-9-neutralizing antibody, and observed that the growth of melanoma and the activation and proliferation of mast cells were promoted. Lu et al [20] demonstrated that IL-9 could attracts $CCR6^+$ dendritic cell (DC) and $CD8^+$ CTL into the tumor bed, which favored tumor antigen uptake, presentation and eradication of cancer cells. The different effect of IL-9 on tumor cells may

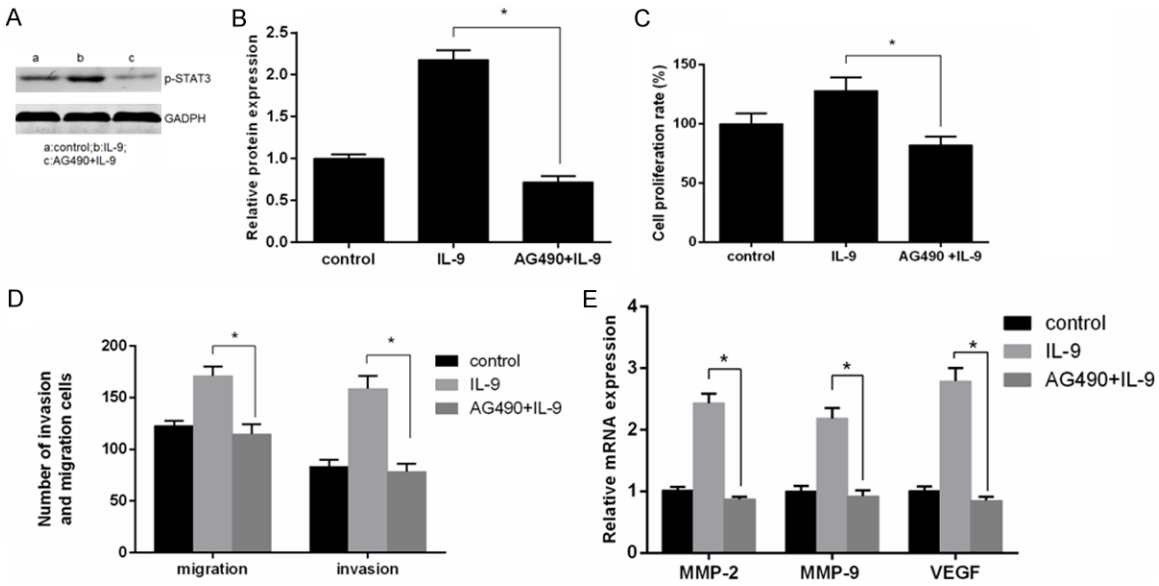

Figure 4. Inhibition of STAT3 abrogated the effect of IL-9 on SMMC-7721 cells. A: Western-blot results of STAT3 and p-STAT3; B: Relative proteins expression of STAT3 and p-STAT3 in SMMC-7721 cells. C: Cells proliferation of the control, IL-9 and IL-9+AG490 group. D: Migratory and invasion of SMMC-7721 cells of the control, IL-9 and IL-9+AG490 group. E: Relative mRNA expression of MMP-2, MMP-9 and VEGF in SMMC-7721 cells of the control, IL-9 and IL-9+AG490 group. Data expressed as mean ± SD. One-way ANOVA/LSD post-hoc test showed that the expression of p-STAT3 in IL-9+AG490 treatment group were greatly decreased compared with IL-9 treatment group. Similarly, the proliferation, invasion and migration of SMMC-7721 cells in AG490+IL-9 group were greatly decreased compared with IL-9 group. $^{*}P<0.05$.

due to the different of cell type and genetic background.

Previous studies have demonstrated that STAT3 signaling pathway has been involved in the pathogenesis of liver fibrosis, cirrhosis and HCC. Activation of STAT3 has been implicated in the proliferation and metastasis of liver cancer cells [21, 22]. The cross-phosphorylation of JAKs could be promoted by IL-9-induced receptor activation and leads to the activation of STAT complexes, specifically STAT1-STAT3 heterodimers [23]. In order to explore possible pathway involving in the process of IL-9-induced HCC cells proliferation and metastasis, we examined the expression of JAK, p-JAK, STAT3 and p-STAT3 in SMMC-7721 cells. Our data showed that, IL-9 significantly increased the phosphorylation levels of JAK2 and STAT3 in SMMC-7721 cells, and the downstream molecules of JAK2/STAT3 pathway (MMP-2, MMP-9 and VEGF) were increased simultaneously. Furthermore, this promotive effect on SMMC-7721 cells could be abrogated by STAT3 inhibitors, indicating JAK2/STAT3 pathway participated in the regulation of IL-9 promote SMMC-7721 cells.

To our knowledge, this study was the first research that identified that IL-9 promoted HCC cells proliferation and metastasis via the regulation of JAK2/STAT3 pathway. However, in this study, we've just only used SMMC-77211 cell lines, which are more suitable for the investigation of cells metastasis; however, these results remained need to confirm by studies using other cell lines and animal models. In addition, although we concluded that IL-9 regulated JAK2/STAT3 pathway, but how IL-9 and its receptor, IL-9R, perform this effect remains unknown. Finally, the pathogenesis of HCC involves in many pathways, but we only selected JAK2/STAT3 pathway, other pathway need to be examined in the future studies.

Conclusions

Our data showed that by activating JAK2/STAT3 pathway, IL-9 promoted HCC cell proliferation, migratory and invasion. These data provide the basis for developing novel immune- regulating strategies for HCC therapy.

Acknowledgements

This study was partially supported by research funding from the National Natural Science Foundation of China (No. 31360221; 31560257).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hai-Xing Jiang and Shan-Yu Qin, Department of Gastroenterology, The First Affiliated Hospital of Guangxi Medical University, 6 Shuangyong Road, Nanning 530021, Guangxi, Guangxi, China. Tel: +86-771-5356725; Fax: +86-771-5356725; E-mail: gxjianghx@163.com (HXJ); qsy0511@163.com (SYQ)

References

[1] Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ and He J. Cancer statistics in China, 2015. CA Cancer J Clin 2016; 66: 115-132.

[2] Han ZB, Chen HY, Fan JW, Wu JY, Tang HM and Peng ZH. Up-regulation of microRNA-155 promotes cancer cell invasion and predicts poor survival of hepatocellular carcinoma following liver transplantation. J Cancer Res Clin Oncol 2012; 138: 153-161.

[3] Llovet JM, Burroughs A and Bruix J. Hepatocellular carcinoma. Lancet 2003; 362: 1907-1917.

[4] Marotta LL, Almendro V, Marusyk A, Shipitsin M, Schemme J, Walker SR, Bloushtain-Qimron N, Kim JJ, Choudhury SA, Maruyama R, Wu Z, Gonen M, Mulvey LA, Bessarabova MO, Huh SJ, Silver SJ, Kim SY, Park SY, Lee HE, Anderson KS, Richardson AL, Nikolskaya T, Nikolsky Y, Liu XS, Root DE, Hahn WC, Frank DA and Polyak K. The JAK2/STAT3 signaling pathway is required for growth of CD44(+)CD24(-) stem cell-like breast cancer cells in human tumors. J Clin Invest 2011; 121: 2723-2735.

[5] Sansone P and Bromberg J. Targeting the interleukin-6/Jak/stat pathway in human malignancies. J Clin Oncol 2012; 30: 1005-1014.

[6] Yu H, Pardoll D and Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer 2009; 9: 798-809.

[7] Huang YK, Fan XG and Qiu F. TM4SF1 promotes proliferation, invasion, and metastasis in human liver cancer cells. Int J Mol Sci 2016; 17: 661.

[8] Yan JJ, Zhang YN, Liao JZ, Ke KP, Chang Y, Li PY, Wang M, Lin JS and He XX. MiR-497 suppresses angiogenesis and metastasis of hepatocellular carcinoma by inhibiting VEGFA and AEG-1. Oncotarget 2015; 6: 29527-29542.

[9] Heo JY, Kim HJ, Kim SM, Park KR, Park SY, Kim SW, Nam D, Jang HJ, Lee SG, Ahn KS, Kim SH, Shim BS, Choi SH and Ahn KS. Embelin suppresses STAT3 signaling, proliferation, and survival of multiple myeloma via the protein tyrosine phosphatase PTEN. Cancer Lett 2011; 308: 71-80.

[10] Lee TK, Castilho A, Cheung VC, Tang KH, Ma S and Ng IO. CD24(+) liver tumor-initiating cells drive self-renewal and tumor initiation through STAT3-mediated NANOG regulation. Cell Stem Cell 2011; 9: 50-63.

[11] Lin L, Amin R, Gallicano GI, Glasgow E, Jogunoori W, Jessup JM, Zasloff M, Marshall JL, Shetty K, Johnson L, Mishra L and He AR. The STAT3 inhibitor NSC 74859 is effective in hepatocellular cancers with disrupted TGF-beta signaling. Oncogene 2009; 28: 961-972.

[12] Stassen M, Schmitt E and Bopp T. From interleukin-9 to T helper 9 cells. Ann N Y Acad Sci 2012; 1247: 56-68.

[13] Fang Y, Chen X, Bai Q, Qin C, Mohamud AO, Zhu Z, Ball TW, Ruth CM, Newcomer DR, Herrick EJ and Nicholl MB. IL-9 inhibits HTB-72 melanoma cell growth through upregulation of p21 and TRAIL. J Surg Oncol 2015; 111: 969-974.

[14] Ye ZJ, Zhou Q, Yin W, Yuan ML, Yang WB, Xiong XZ, Zhang JC and Shi HZ. Differentiation and immune regulation of IL-9-producing CD4+ T cells in malignant pleural effusion. Am J Respir Crit Care Med 2012; 186: 1168-1179.

[15] Li HJ, Sun QM, Liu LZ, Zhang J, Huang J, Wang CH, Ding R, Song K and Tong Z. High expression of IL-9R promotes the progression of human hepatocellular carcinoma and indicates a poor clinical outcome. Oncol Rep 2015; 34: 795-802.

[16] Noelle RJ and Nowak EC. Cellular sources and immune functions of interleukin-9. Nat Rev Immunol 2010; 10: 683-687.

[17] Licona-Limon P, Henao-Mejia J, Temann AU, Gagliani N, Licona-Limon I, Ishigame H, Hao L, Herbert DR and Flavell RA. Th9 cells drive host immunity against gastrointestinal worm infection. Immunity 2013; 39: 744-757.

[18] Yang XY, Jiang H, Huang XL, Ma SJ and Qin SY. Role of Th9 cells and Th17 cells in the pathogenesis of malignant ascites. Asian Pac J Trop Biomed 2015; 5: 6.

[19] Purwar R, Schlapbach C, Xiao S, Kang HS, Elyaman W, Jiang X, Jetten AM, Khoury SJ, Fuhlbrigge RC, Kuchroo VK, Clark RA and Kupper TS. Robust tumor immunity to melanoma mediated by interleukin-9-producing T cells. Nat Med 2012; 18: 1248-1253.

[20] Lu Y, Hong S, Li H, Park J, Hong B, Wang L, Zheng Y, Liu Z, Xu J, He J, Yang J, Qian J and Yi Q. Th9 cells promote antitumor immune responses in vivo. J Clin Invest 2012; 122: 4160-4171.

[21] Hedvat M, Huszar D, Herrmann A, Gozgit JM, Schroeder A, Sheehy A, Buettner R, Proia D, Kowolik CM, Xin H, Armstrong B, Bebernitz G, Weng S, Wang L, Ye M, McEachern K, Chen H, Morosini D, Bell K, Alimzhanov M, Ioannidis S,

McCoon P, Cao ZA, Yu H, Jove R and Zinda M. The JAK2 inhibitor AZD1480 potently blocks Stat3 signaling and oncogenesis in solid tumors. Cancer Cell 2009; 16: 487-497.

[22] Xia N, Tang Z, Wang C, Xu G, Nie X, Zhang W, Zhao Y, Wang S, Zhu X and Cui S. PCBP2 regulates hepatic insulin sensitivity via HIF-1alpha and STAT3 pathway in HepG2 cells. Biochem Biophys Res Commun 2015; 463: 116-122.

[23] Buchert M, Burns CJ and Ernst M. Targeting JAK kinase in solid tumors: emerging opportunities and challenges. Oncogene 2016; 35: 939-951.