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Apatinib regulates the cell proliferation and apoptosis of liver cancer by regulation of VEGFR2/STAT3 signaling

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ABSTRACT

Liver cancer is the third most common cause of cancer related death worldwide. Apatinib showed satisfactory efficacy in various types of cancers, including breast cancer, malignant fibrous histiocytoma and liver cancer. However, how did Apatinib function in liver cancer is largely unknown. mRNA levels of vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor 2 (VEGFR2) as well as protein levels of VEGF and p-VEGFR2 were obviously higher in liver cancer cell line SMCC7721 than in normal liver cell LO2. Apatinib significantly inhibited the mRNA levels of VEGF and VEGFR2 as well as protein levels of VEGF and p-VEGFR2 compared with those in control group. At 12, 24 and 48 h after corresponding treatments, compared with the control group, Apatinib significantly lowered the cell viability of SMCC7721 cells, while transfection of si-signal transducer and activator of transcription 3 (siSTAT3) further augmented the effects of Apatinib. At 48 h after treatment, compared with the control group, Apatinib significantly upregulated the cell apoptosis rate of SMCC7721 cells, which was further induced by the transfection of siSTAT3. Compared with control group, Apatinib significantly induced BAX/Bcl-2 ratio elevation, reduced p-STAT3 and p-VEGFR2 expression, which were significantly augmented by the treatment of siSTAT3. In conclusion, Apatinib inhibited the cell proliferation and promoted the cell apoptosis of liver cancer by inhibiting the activation of VEGFR2/STAT3.

1. Introduction

Liver cancer is the 5th most common cancer burden in men, the 7th most common cancer burden in women, and the 3rd most common cause of cancer related death worldwide [1]. Regarding the huge burden of liver cancer and its low overall survival rate [2], early diagnosis is of great importance in decreasing the treatment cost and mortality. Cirrhosis is the main cause of liver cancer, due to hepatitis B, hepatitis C, or alcohol [3]. In 2015, a large quantity of mortality was caused by liver cancer, for instance, hepatitis B led to 263,000 deaths, hepatitis C resulted in 167,000 deaths and alcohol generated 245,000 deaths [4].

Recently, a few of angiogenesis inhibitors are available for the treatment of lung, breast, renal, hepatic, and colon cancers in clinical, agents such as Sunitinib and Sorafenib target many receptor tyrosine kinases [5,6]. Also, Apatinib shows satisfactory efficacies on extrahepatic bile duct carcinoma [7], breast cancer [8], and malignant fibrous histiocytoma [9]. Recently, there has been a study shows the efficacy and safety of Apatinib in the treatment of advanced liver cancer [10].

As a novel small-molecule tyrosine kinase inhibitor, Apatinib selectively inhibits the activity of VEGFR2, which inhibits the cell migration, proliferation, and tumor microvascular density that are mediated by VEGF [11]. It is known that Apatinib targets VEGFR2 in chemoresistant gastric cancer, thus improving the overall survival of gastric cancer patients [12]. Clinical trials have proved the effects of Apatinib on hepatocellular carcinoma [13] and gastric cancer [14]. Despite Apatinib has an antitumor effect in human liver cancer [15], our knowledge about the molecular mechanism of Apatinib is still limited. Consequently, we aimed to investigate how does Apatinib function in liver cancer.

2. Materials and methods

2.1. Cell culture

The human normal liver cell LO2 and liver cancer cell line SMCC7721 was purchased from the American Type Culture Collection (Manassas, VA, USA). LO2 and SMCC7721 cells were cultured in RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences, USA),

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supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and 1% penicillin-streptomycin (EMD Millipore, Billerica, MA, USA) at 37 °C in a humidified chamber with 5% CO_2.

The cells were incubated with Apatinib (Hengrui, Jiangsu, China) at the condition of 10 μM as a previous study reported [16].

2.2. Gene knockdown using siRNA

siSTAT3 and negative control siRNAs (siNC) were purchased from GenePharma (Suzhou, Jiangsu, China). SMCC7721 cells were transfected with siRNA by Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's protocol. Briefly, SMCC7721 cells were seeded into 6-well plates in RPMI1640 medium at 37 °C in a humidified chamber with 5% CO_2. When the cells achieved 80% confluent, the culture medium was changed by OPTI-MEM (Thermo Fisher Scientific). The cells were cultivated for 48 h for the following experiments.

2.3. Reverse transcription-quantitative PCR (RT-qPCR)

SMCC7721 cells were washed twice with ice-cold TBS, and RNA was extracted with TRIzol reagent (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. RNA was reverse transcribed into cDNA by PrimeScript RT Reagent kit (Takara Bio, Inc., Otsu, Japan). RT-qPCR was carried out by the SYBR Green kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and ABI Prism 7500 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Primers used in the present study were as listed: VEGF forward: 5′-TCACCAA GGCCAGCACAT

AG-3′; reverse: 5′-GAGGCTCCAGGGCATTAGAC-3′. VEGFR2 forward: 5′-CGTCAACAAAGTCGGGAGA-3′; reverse: 5′-CAGTGCACCACA AAGACAC

G-3′. GAPDH forward: 5′-ACACCCACTCCTCCACCTTT-3′; reverse: 5′-TTACTCCTTGGAGGCCATGT-3′. STAT3 forward: 5′-ACCTGCAGCAA TACC

ATTGAC-3′; reverse: 5′-AAGGTGAGGGACTAAACTGC-3′. The amplification conditions were as listed: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s. GAPDH was used as an internal control for STAT3, VEGF and VEGFR2. The relative expressions of STAT3, VEGF and VEGFR2 were analyzed using the $2^{-\Delta\Delta Cq}$ method.

2.4. Western blot

For protein detection, the SMCC7721 cells were lysed by ice-cold RIPA buffer (Thermo Fisher Scientific) and centrifuged at 4 °C, 12,000 × *g*, for 5 min. Protein concentrations were determined by BCA (Beyotime Biotechnology, Shanghai, China). Total proteins (20 μg/lane) were subjected to electrophoresis in 12% polyacrylamide gel, followed by transferring to a polyvinylidene difluoride membrane. Following transfer, the membrane was blocked at 37 °C for 1 h with 5% non-fat milk in tris-buffered saline, with 0.05% Tween-20. The blots were then separately incubated with primary antibodies for VEGF (1:1000; cat. no. ab53465; Abcam, Inc. Cambridge, MA, USA), P-VEGFR (1:1000; cat. no. ab194806; Abcam, Inc. Cambridge, MA, USA), STAT3 (1:1000; cat. no. 12,640; Cell Signaling Technology, Inc. Danvers, MA, USA), p-STAT3 (1:1000; cat. no. 9145; Cell Signaling Technology, Inc. Danvers, MA, USA), p-VEGFR2 (1:1000; cat. no. 4991; Cell Signaling Technology, Inc. Danvers, MA, USA), Bcl-2 (1:1000; cat. no. 4223; Cell Signaling Technology, Inc. Danvers, MA, USA), BAX (1:1000; cat. no. 5023; Cell Signaling Technology, Inc. Danvers, MA, USA), and GAPDH (1:1000; cat. no. 5174; Cell Signaling Technology, Inc. Danvers, MA, USA), and then with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:5000; cat. no. ab6721; Abcam, Inc. Cambridge, MA, USA). Signals were detected by enhanced chemiluminescence (Thermo Fisher Scientific). Western blot images were analyzed semi-quantitatively using QuantityOne software (Bio-Rad Laboratories). The relative intensity values of bands were normalized to GAPDH.

2.5. Cell viability assay

Briefly, cell viability was detected by the Cell Counting Kit (CCK-8). SMCC7721 cells (1×10^5) were cultured in Biocoat™ 24-well plates (BD Biosciences, San Jose, CA, USA) in RPMI1640 medium and treated with Apatinib for 12, 24 and 48 h, respectively.

2.6. Flow cytometric assays

To detect the apoptotic rate, SMCC7721 cells were seeded in 12-well plates at the seeding density of 3×10^5/well and cultured for 48 h. Thereafter, the SMCC7721 cells were harvested by 0.025% trypsin (Thermo Fisher Scientific). After washing with PBS, 5 μl fluorescein isothiocyanate-labeled Annexin V (FITC) and 5 μl PI was added into the cells and incubated in the dark for 15 min at 37 °C. The cell apoptosis rate was analyzed by flow cytometry within 1 h.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc.). Data are expressed as the mean ± standard deviation. Values are expressed as the mean ± standard error of the mean. Data between two groups were compared with the Student's t-test, while data among 3 groups were compared by one-way analysis of variance followed by Newman-Keuls analysis. $P < 0.05$ was considered to indicate a statistically significant result.

3. Results

3.1. mRNA levels of VEGF/VEGFR2 and protein levels of VEGF/p-VEGFR2 are elevated in liver cancer cell line

The mRNA levels of VEGF and VEGFR2 were examined in normal liver cell LO2 and liver cancer cell line SMCC7721 using RT-qPCR. mRNA levels of VEGF and VEGFR2 were obviously higher in liver cancer cell line SMCC7721 than in normal liver cell LO2 (Fig. 1A and B).

The protein levels of VEGF and p-VEGFR2 were also tested in normal liver cell LO2 and liver cancer cell line SMCC7721 using western blot. Protein levels of both VEGF and p-VEGFR2 were obviously higher in liver cancer cell line SMCC7721 than in normal liver cell LO2 (Fig. 1C and D).

3.2. Apatinib inhibits expression of VEGF and p-VEGFR2 in liver cancer cell line

The effects of Apatinib in mRNA levels of VEGF and VEGFR2 were examined in liver cancer cell line SMCC7721 using RT-qPCR. Apatinib significantly inhibited the mRNA levels of VEGF and VEGFR2 (Fig. 2A and B).

The effects of Apatinib in protein levels of VEGF and p-VEGFR2 were also tested in liver cancer cell line SMCC7721 using western blot. Apatinib significantly inhibited the protein levels of both VEGF and p-VEGFR2 (Fig. 2C and D).

3.3. siSTAT3 inhibits protein levels of STAT3 in liver cancer cell line SMCC7721

Apatinib was reported to suppress p-STAT3/BCL-2 signal path in osteosarcoma [16]. More importantly, STAT3 has become a promising target for the treatment of cancer [17]. We are eager to know the effects of STAT3 in liver cancer. Thereafter, we first conducted siSTAT3 knockdown in liver cancer cell line SMCC7721. To verify the successful knockdown of STAT3, western blot was carried out to examine the

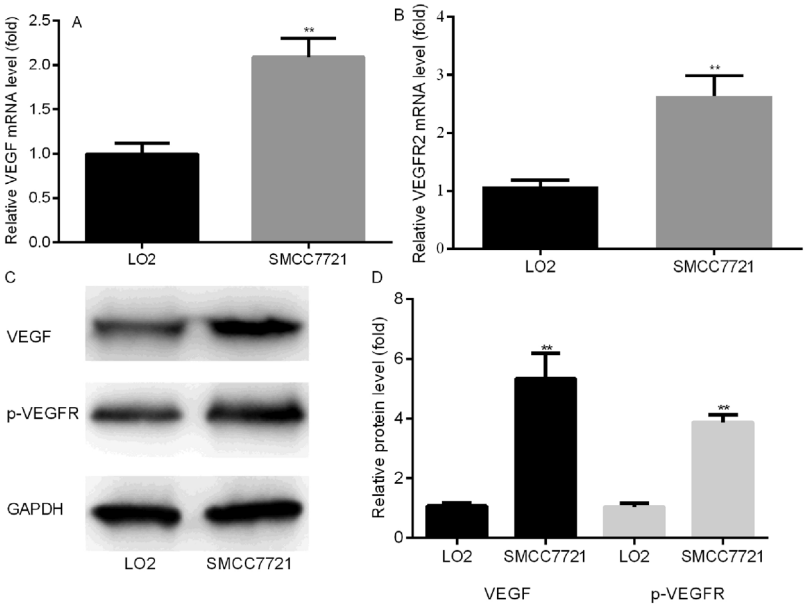

Fig. 1. VEGF and p-VEGFR2 expression levels are elevated in vitro.
mRNA levels of VEGF (A) and VEGFR2 (B) were was obviously higher in SMCC7721 than in LO2. The protein levels of VEGF and p-VEGFR2 showed the similar pattern (C and D). **p < 0.01, SMCC7721 vs. LO2.

mRNA and protein expression levels of STAT3 in different groups. And results showed that, compared with siNC group, both siSTAT3-1 and siSTAT3-2 successfully inhibits STAT3 mRNA and protein levels, and siSTAT3-1 showed a relatively more obvious knockdown effect (Fig. 3A–C). Therefore, it was used for the following experiments.

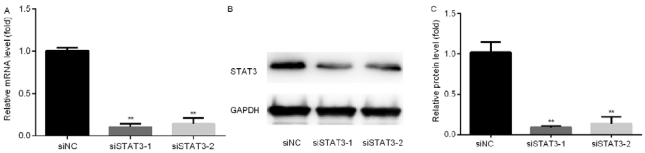

Fig. 3. siSTAT3 inhibits protein levels of STAT3.
Compared with siNC group, both siSTAT3-1 and siSTAT3-2 successfully inhibit STAT3 mRNA (A) and protein levels (B and C), and siSTAT3-1 showed a relatively more obvious effect. **p < 0.01, siSTAT3 vs. siNC.

3.4. siSTAT3 promotes Apatinib-induced cell viability inhibition

Thereafter, we investigated the effects of siSTAT3 in Apatinib-induced cell viability. CCK-8 assay showed that, compared with control group, Apatinib significantly reduced the cell viability of liver cancer cell line SMCC7721, which was further augmented by the treatment of siSTAT3 (Fig. 4).

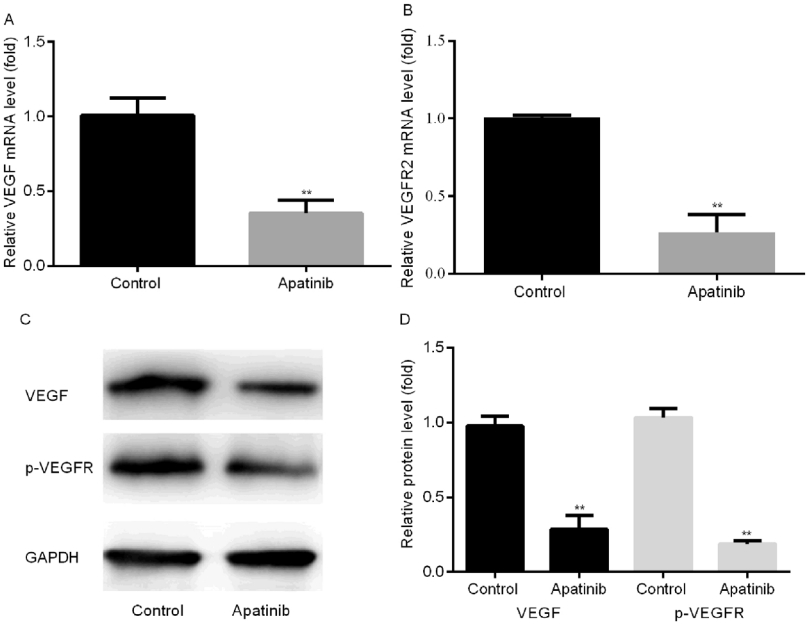

Fig. 2. Apatinib inhibits expression of VEGF and p-VEGFR2 in vitro.
Apatinib significantly inhibited the mRNA levels of VEGF (A) and VEGFR2 (B) and protein levels of VEGF and p-VEGFR2 (C and D). **p < 0.01, Apatinib vs. Control.

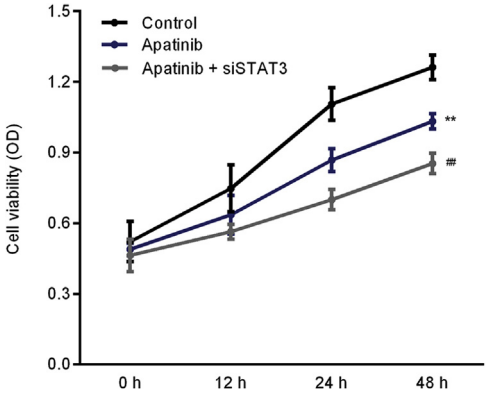

Fig. 4. siSTAT3 promotes Apatinib-induced cell viability inhibition.
Compared with control group, Apatinib significantly reduced the cell viability of SMCC7721, which was further augmented by siSTAT3. **p < 0.01, Apatinib vs. Control; ##p < 0.01 Apatinib + siSTAT3 vs. Apatinib.

3.5. siSTAT3 promotes Apatinib-induced cell apoptosis elevation

Thereafter, we investigated the effects of siSTAT3 in Apatinib-induced cell apoptosis. Flow cytometric assays showed that, compared with control group, Apatinib significantly induced the cell apoptosis of liver cancer cell line SMCC7721, which was further augmented by the treatment of siSTAT3 (Fig. 5A and B).

3.6. siSTAT3 promotes Apatinib-induced BAX/Bcl-2 ratio elevation, p-STAT3 and p-VEGFR2 reduction

Eventually, we tested the molecules that might be responsible for the above changes. Results in western blot showed that, compared with control group, Apatinib significantly induced BAX/Bcl-2 ratio elevation, reduced p-STAT3 and p-VEGFR2 expression, which were significantly augmented by the treatment of siSTAT3 (Fig. 6A–D).

4. Discussion

Liver cancer is the 3rd most common cause of cancer related death worldwide [1], consequently, it is of great importance to find out novel therapeutic target and the potential molecules that take part in the process of function.

Clinical trials have proved the effects of Apatinib on advanced liver cancer [10], hepatocellular carcinoma [13] and gastric cancer [14]. Despite Apatinib has an antitumor effect in human liver cancer [15], our knowledge about the molecular mechanism of Apatinib is still limited. Consequently, we aimed to investigate it. Apatinib selectively inhibits the activity of VEGFR2, which inhibits cell migration, proliferation, and tumor microvascular density that are mediated by VEGF [11]. Therefore, we tested the expression changes of VEGF and VEGFR2. We found that, both mRNA levels of VEGF/VEGFR2 and protein levels of VEGF and p-VEGFR2 were obviously higher in liver cancer cell line SMCC7721 than in normal liver cell LO2. These findings were consistent with a previous study which showed the upregualted expression levels of VEGF and VEGFR2 in liver cancer tumor tissues than in normal liver tissues [18]. Thereafter, we tested the effects of Apatinib in VEGF and VEGFR2 expression levels. The results showed that, both mRNA levels of VEGF/VEGFR2 and protein levels of VEGF and p-VEGFR2 were obviously lower in Apatinib treatment group than in control group. These results were consistent with the previous report [11].

Since Apatinib is reported to suppress p-STAT3/BCL-2 signal path in osteosarcoma [16]. More importantly, STAT3 has become a promising target for the treatment of cancer [17]. We would like to explore whether STAT3 influences liver cancer cell line behaviors. Thereafter, we investigated the effects of siSTAT3 in Apatinib-induced cell viability and cell apoptosis. Compared with control group, Apatinib significantly reduced the cell viability and obviously induced the cell apoptosis of liver cancer cell line SMCC7721, which was further augmented by the treatment of siSTAT3. The above results were in line with a previous study that exhibits Apatinib suppresses cell growth and induces cell apoptosis of osteosarcoma [16], B and T lineage ALL cells [19], as well as a study which demonstrates that Apatinib induces cell apoptosis of intrahepatic cholangiocarcinoma [18]. We demonstrated that, STAT3 was involved in the process of Apatinib induced cell viability and cell apoptosis changes. Afterwards, the potential mechanisms about how STAT3 did lead to the above changes were explored.

Recent researches have indicated that STAT3 is regularly activated in multiple types of cancers, including breast cancer [20], esophageal squamous cell carcinoma [21], hepatocellular carcinoma [22], and lung cancer [23]. Bcl-2 protein families induce or repress cell apoptosis in humans, including Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) [24].

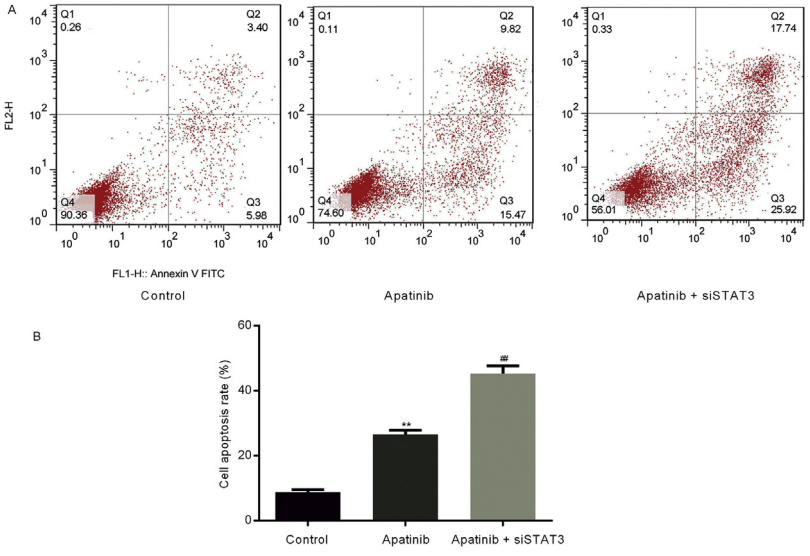

Fig. 5. siSTAT3 promotes Apatinib-induced cell apoptosis.
Compared with control group, Apatinib significantly induced the cell apoptosis of SMCC7721, which was further augmented by siSTAT3 (A and B). **p < 0.01, Apatinib vs. Control; ##p < 0.01 Apatinib + siSTAT3 vs. Apatinib.

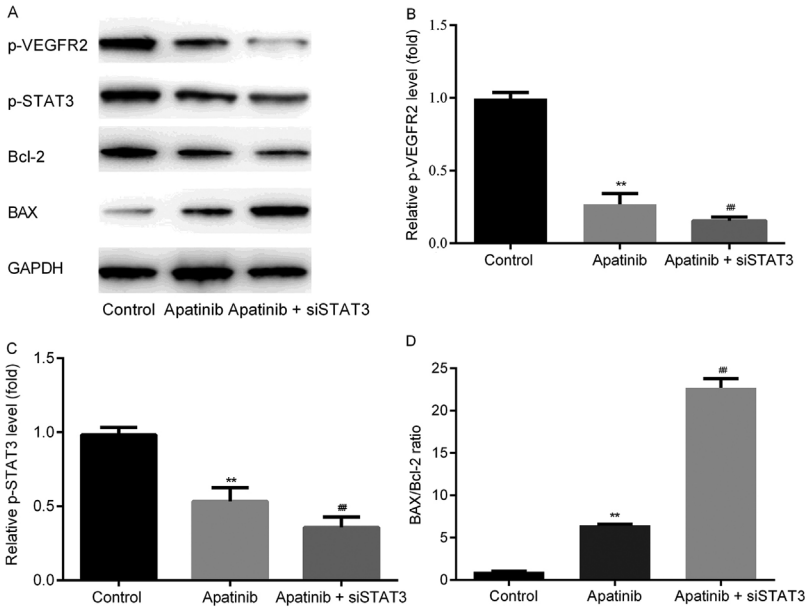

Fig. 6. siSTAT3 promotes Apatinib-induced BAX/Bcl-2 ratio elevation, p-STAT3 and p-VEGFR2 reduction.
Compared with control group, Apatinib significantly induced BAX/Bcl-2 ratio elevation, reduced p-STAT3 and p-VEGFR2 expression, which were significantly augmented by siSTAT3 (A–D). **p < 0.01, Apatinib vs. Control; ##p < 0.01 Apatinib + siSTAT3 vs. Apatinib.

In current study, Apatinib was found to inhibit the expression of p-STAT3/p-VEGFR2 and induce BAX/Bcl-2 ratio elevation, which were in line with a previously reported study [16], siSTAT3 further inhibited the Apatinib-induced inhibition of p-STAT3/p-VEGFR2 and augmented BAX/Bcl-2 ratio elevation.

In conclusion, Apatinib regulates the cell proliferation and apoptosis of liver cancer by regulation of VEGFR2/STAT3 signaling. However, there was a limitation in our present study, ie, we did not clarify the relationship of STAT3 and VEGF/VEGFR2 pathway in Apatinib therapy on liver cancer, which will be further investigated in our future work.

Disclosure

None.

Availability of materials and data

They are available on special request.

Conflict of interest

None.

Acknowledgements

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