



# B7-H4 facilitates proliferation and metastasis of colorectal carcinoma cell through PI3K/Akt/mTOR signaling pathway

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Received: 3 July 2019 / Accepted: 22 October 2019 / Published online: 29 October 2019  
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## Abstract

B7-H4 is over-expressed in various tumors and may affect many aspects of cancer biology. Our previous studies have reported that the over-expressed B7-H4 in serum or tumor tissue of colorectal carcinoma (CRC) patients was closely related to CRC progression. However, B7-H4 in cell biological characteristics of CRC is not well studied. Here, we investigate the effect of the B7-H4 on cell proliferation, migration and its expression regulated by PI3K/Akt/mTOR signaling pathway in CRC. Firstly, pSilencer 4.1-B7-H4-shRNA vector was constructed and stable transfection was performed on HT-29 cells. Secondly, cell proliferation, cell cycle, cell apoptosis and cell migration were evaluated after B7-H4 silencing, and the expression of Bcl-2, caspase-3, MMP-2 and MMP-9 was also measured. Finally, the regulation of B7-H4 by PI3K/Akt/mTOR signaling pathway was measured followed by treatment with or without PI3K/Akt and mTOR inhibitor. The results showed that the viability of HT-29 cells was significantly decreased after B7-H4 silencing ( $P < 0.05$ ). B7-H4 silencing significantly increased the apoptosis rate and caspase-3 protein expression while decreased Bcl-2 protein expression ( $P$  all  $< 0.05$ ). B7-H4 silencing also significantly reduced the migration of HT-29 cells ( $P < 0.01$ ) and the secretion of MMP-2 or MMP-9 ( $P$  all  $< 0.05$ ). Following treatment with PI3K/Akt and mTOR inhibitor in HT-29 cells, the expression of B7-H4 was significantly down-regulated compared with untreated group ( $P$  all  $< 0.05$ ). Our results strongly suggest that B7-H4 may be involved in cell proliferation and migration by PI3K/Akt/mTOR signaling pathway. Therefore, blocking B7-H4 signaling might be a novel treatment strategy for CRC.

**Keywords** B7-H4 · Colorectal carcinoma · Proliferation · Metastasis · PI3K/Akt/mTOR signaling pathway

## Introduction

Colorectal carcinoma (CRC) is one of the most frequent malignancies and the leading cause of cancer-related mortality worldwide [1, 2]. Due to the changes in lifestyle and dietary behaviors, the incidence of CRC has recently been increasing in China [3]. With the development of diagnostic technology, increasing number of patients is diagnosed in CRC early stage, and the proper treatment is taken in

time; the CRC prognosis has obviously improved. However, CRC metastasis, especially colorectal liver metastasis, is still the main cause of cancer death [4, 5]. Therefore, there is an urgent need for development of better predictive target-based therapies for improving the treatment of this aggressive disease.

B7-H4, also known as B7x or B7S1, is a new member of the B7 family. It has been identified as a cytoplasmic-nuclear shuttling protein [6]. A study indicated that B7-H4 mRNA expression was widely distributed in the peripheral tissues including kidney, liver, lung, spleen, thymus and placenta. In contrast to constitutive expression of the B7-H4 mRNA, B7-H4 protein was not found in the above-mentioned normal tissues [7]. These results suggest that B7-H4 expression is tightly controlled in the translational level in normal peripheral tissues and may have important functions. In contrast, B7-H4 is highly expressed in tissue samples from various cancer patients, including ovarian serous [8], gallbladder [9], renal cell [10], hepatocellular [11], gastric [12],

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bladder urothelial [13] and thyroid carcinomas [14]. The expression level of B7-H4 is in tumor cells of these malignancies mostly associated with tumor incidence, progression and prognosis. Our previous study has found that B7-H4 expression is positively related to the infiltration depth and lymph node metastasis in CRC [15]. The B7-H4 level in serum is positively correlated with infiltration depth, tumor masses and the lymph node metastasis [16]. Based on these findings, we conclude that B7-H4 is closely related to cancer progression and metastasis. As a consequence, we attempt to explore the effect of the B7-H4 short hairpin RNA (shRNA) silencing on cell proliferation, cell cycle, cell apoptosis and cell migration in human CRC HT-29 cells. We also study the correlation between B7-H4 and PI3K/Akt/mTOR signaling pathway.

## Materials and methods

### Cell culture

The human CRC HT-29 cell line was purchased from American Type Culture Collection (ATCC, Shanghai, China). Cells were cultured in complete DME/F-12 medium (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Sijiqing Co., Zhejiang, China) at 37 °C in a humidified incubator with 5% CO<sub>2</sub>.

### Construction of pSilencer 4.1-B7-H4-shRNA plasmid

Plasmid pSilencer 4.1-CMV neo was purchased from Shanghai Genetimes Technology, Inc. (Shanghai China). Short-chain oligonucleotide was designed according to the B7-H4 mRNA sequence provided by GenBank and chemosynthesized by Shenggong (Shanghai, China). The following two oligonucleotides were selected: forward, 5'-GATCCGACA CTCCATCACAGTCACTTCAAGAGAGTGACTGTGATG GAGTGTCTCA-3'; reverse, 5'-AGCTTGAGACACTCC ATCACAGTCACTCTTGAAGTGACTGTGATGGAGT GTCG-3'. The recombinant pSilencer 4.1-B7-H4-shRNA vector was confirmed by DNA sequencing. A scramble shRNA was used as negative control: forward, 5'-AGGCGA TTAAGTTGGGTA-3'; reverse, 5'-CGGTAGGCGTGTACG GTG-3'.

### Stable transfection

Transfection reagent x-tremeGENE HP DNA was purchased from Roche (Mannheim, Germany). Transfection process was performed according to the manufacturer's protocol. G418 (600 µg/ml) was used as antibiotic selection after transfection for 24 h, and stable clones were obtained in 3 weeks.

### RT-qPCR

Total RNA isolation and reverse transcription were performed using RNAiso Plus kit and PrimeScript™ RT reagent Kit (both from TaKaRa Biotechnology co., Dalian, China) according to the manufacturer's protocol. The expression level of B7-H4 mRNA was examined by RT-qPCR with SYBR Premix Ex Taq™ II (Takara) and a Piko-Real 24 Real-Time PCR system (Thermo Fisher Scientific, America). In brief, a 5 µl aliquot of 1:10 diluted cDNA was mixed with 10 µl SYBR Premix Ex Taq II and 0.4 µM of the designated primers. The primer sequence for qPCR were as follows: B7-H4 primers: forward, 5'-TAT TAG CAG CCG CTC TGT GC-3'; reverse, 5'-TCA GGA TTC CAT CCT CCC CA-3'; GAPDH primers: forward, 5'-ATG GGG AAG GTG AAG GTC G-3'; reverse, 5'-GGG TCA TTG ATG GCA ACA ATA TC-3'. The reaction procedure was as follows: 95 °C for 15 min and 40 cycles of 95 °C for 10 s and 60 °C for 10 s. The relative expression of the target gene was determined using the 2<sup>-ΔΔC<sub>t</sub></sup> method, with GAPDH as an internal control.

### Western blotting

Cells were washed with PBS and then incubated in RIPA buffer (Beyotime, Jiangsu, China) containing protease inhibitor on ice for 30 min, and centrifuged at 15,000 × g for 15 min at 4 °C. Equal amounts of proteins (30 µg) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% BSA for 1 h at room temperature and then incubated with primary monoclonal antibodies for B7-H4 (1:1000, Cell Signaling Technology, USA), caspase-3, Bcl-2, Akt, p-Akt, mTOR, p-mTOR and β-actin (1:1000; all from Beyotime) on a 4 °C shaker overnight. The next day, the membrane was washed five times with TBST buffer and incubated with horseradish peroxidase (HRP)-conjugated IgG (1:2000, Beyotime) for 1 h at room temperature. The immunoreactive proteins were detected using a chemiluminescence reagent (BeyoECL Star Kit, Beyotime). β-Actin was considered as an internal reference.

### Cell viability assay

Cell viability was measured using Cell Counting Kit-8 (CCK-8; Beyotime, Jiangsu, China). Stable transfected cells in the logarithmic growth phase were seeded into 96-well plates at a concentration of 5000 cells per well and incubated for 24 h, 48 h, 72 h and 96 h, respectively.

Then, 100  $\mu$ l DMEM/F-12 and 10  $\mu$ l of CCK-8 solution was added to each well at the measuring time point, and the plates were continuously incubated for 2 h. Experimental cells were then placed on a Tecan Infinite 200 Pro microplate reader (Tecan, Switzerland) to measure the optical density at 450 nm.

### Cell cycle and cell apoptosis assay

Stable transfected cells in the logarithmic growth phase were seeded in 6-well plates at a concentration of  $1 \times 10^6$  cells per well and cultured for 48 h. The cell cycle was detected using Muse™ cell cycle kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. The cell apoptosis was measured using Annexin V/PI double staining provided by ApopNexin™ FITC apoptosis detection kit (Millipore, Billerica, MA, USA). Finally, the cells were filtered using a nylon mesh filter and then detected by Muse Cell Analyzer (Millipore, Billerica, MA, USA).

### Cell migration assay

Stable transfected cells with serum-free medium were placed into the upper chamber of an insert (8  $\mu$ m pore size; Corning, USA), and media containing 10% FBS were added to the lower chamber. After incubation for 24 h, the cells remaining on the upper membrane were removed with cotton wool, and the migrated cells through the membrane were stained with crystal violet for 5 min. Finally, migrated cells were counted using an inverted microscope (Olympus, Japan).

### ELISA assay

The supernatant of stable transfected cells was harvested by centrifugation ( $2000 \times g$  for 10 min) following incubation for 72 h. The expression levels of MMP-2 and MMP-9 in the supernatant were detected using ELISA kits (EK0459 and EK0465; Boster Biological Technology, Wuhan, China) according to the manufacturer's instructions. The plates were read on a microplate reader (Tecan, Switzerland) at 450 nm. The content of MMP-2 and MMP-9 was calculated according to the standard curve.

### Inhibition test for PI3K/Akt/mTOR signaling pathway

HT-29 cells in the logarithmic growth phase were seeded into 6-well plates at a concentration of  $5 \times 10^5$  cells per well and incubated overnight. Cells were treated with/without the PI3K/Akt inhibitor LY294002 at 10  $\mu$ M or mTOR inhibitor rapamycin at 50 nM for 24 h, and then the expression of B7-H4 mRNA and protein was detected by RT-qPCR and western blotting.

### Statistical analysis

Statistical analysis was conducted in SPSS 17.0 (SPSS, IBM, Chicago, IL, USA). All analysis data were presented as the mean  $\pm$  standard deviation (SD) from at least three independent experiments. The differences among groups were analyzed by 2-tailed Student's *t* test.  $P < 0.05$  indicated that the difference was statistically significant.

## Results

### Stable B7-H4 silencing in HT-29 cells by shRNA

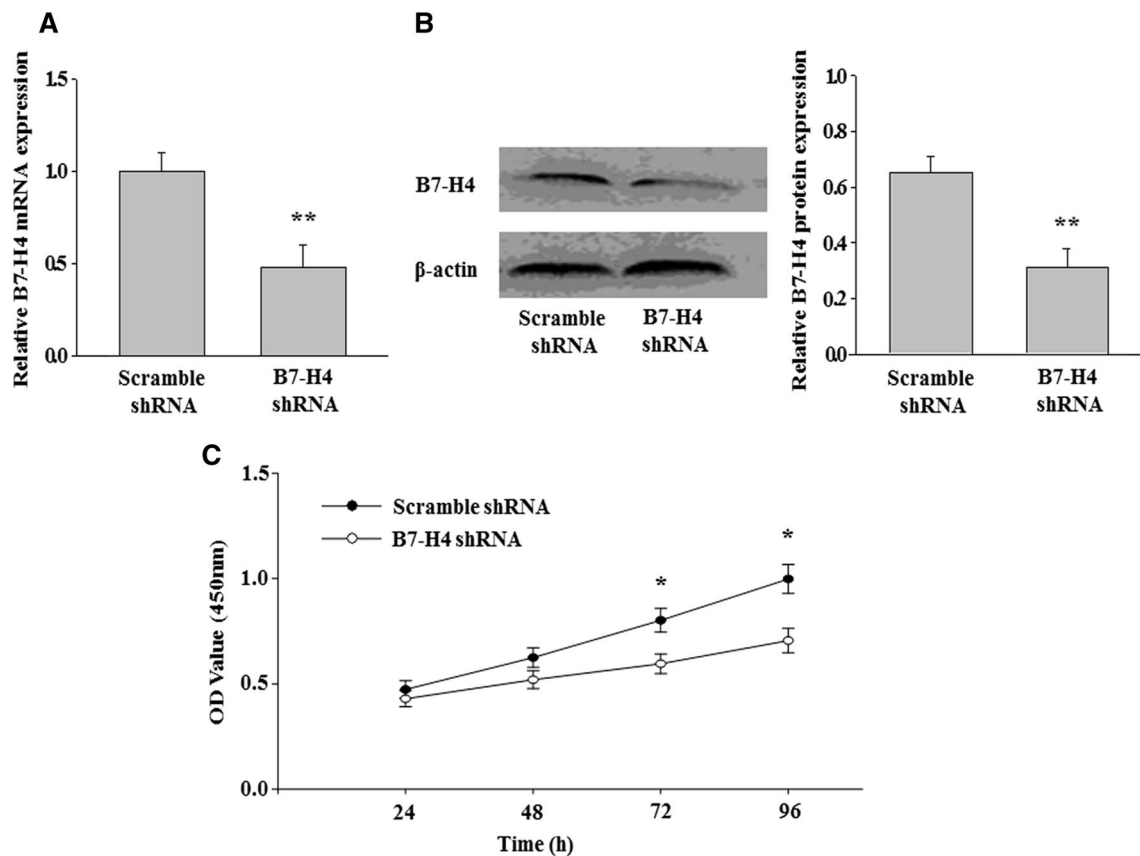
To assess the efficiency of stable B7-H4 silencing in HT-29 cells, RT-qPCR and western blotting assays were used to detect the expression of B7-H4 mRNA and protein. As shown in Fig. 1a, the expression level of B7-H4 mRNA in B7-H4 shRNA group was significantly downregulated compared with scramble shRNA group ( $P < 0.01$ ). Western blotting results also showed a significant reduction of B7-H4 protein in B7-H4 shRNA group compared with scramble shRNA group ( $P < 0.01$ ; Fig. 1b).

### Effect of B7-H4 on the proliferation of HT-29 cells

CCK-8 assay was used to analyze the effect of B7-H4 silencing on the proliferative ability of HT-29 cells. Results showed that silencing of B7-H4 inhibited cell viability in a time-dependent manner and exhibited a significant difference at 72 h and 96 h compared with control group ( $P$  all  $< 0.05$ ; Fig. 1c). These data indicate that silencing of B7-H4 could inhibit the proliferation of HT-29 cells.

### Effect of B7-H4 on the cell cycles and cell apoptosis of HT-29 Cells

Cell cycle and cell apoptosis analysis were evaluated by flow cytometry. Cell cycle analysis showed that the cell proportion in the  $G_0/G_1$  phase and S phase between B7-H4 shRNA and scramble shRNA group had no significant difference ( $P$  all  $> 0.05$ ; Fig. 2a). Cell apoptosis analysis showed a significant increase in the apoptosis rates of cells in B7-H4 shRNA group ( $24.55 \pm 2.81\%$ ) compared with scramble shRNA group ( $8.74 \pm 1.62\%$ ) ( $P < 0.01$ ; Fig. 2b). Furthermore, apoptosis-related proteins Bcl-2 and caspase-3 were detected by western blotting. Results showed that Bcl-2 protein was significantly decreased, while caspase-3 protein was significantly increased in B7-H4 shRNA group than that in scramble shRNA group ( $P$  all  $< 0.05$ ; Fig. 2c). The above results suggest that silencing of B7-H4 could increase cell apoptosis rates by regulating Bcl-2 and caspase-3 expression in HT-29 cells.



**Fig. 1** Silencing of B7-H4 inhibits the proliferation of HT-29 cells. **a** qPCR was carried out to confirm stable silencing of B7-H4 mRNA in HT-29 cells. **b** Western blotting was performed to confirm stable

silencing of B7-H4 protein in HT-29 cells. **c** The effect of B7-H4 silencing on the proliferation of HT-29 cells was evaluated by CCK-8 assays. Data were presented as mean  $\pm$  SD (\* $P$  < 0.05, \*\* $P$  < 0.01)

### Effect of B7-H4 on the migration of HT-29 cells

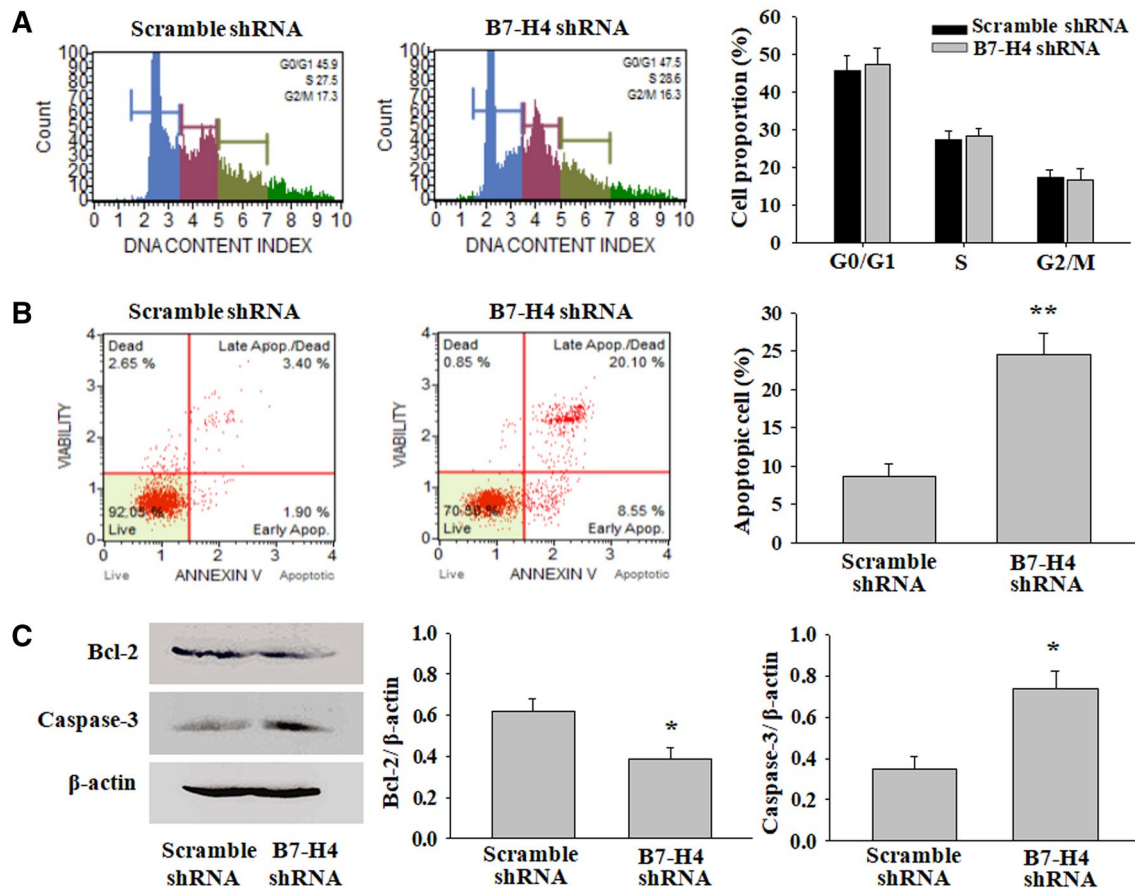
Transwell assay was used to investigate the effect of B7-H4 silencing on the migratory capacity of HT-29 cells. Results showed that the numbers of migrated cells in B7-H4 shRNA group were significantly reduced compared with scramble shRNA group ( $P$  < 0.01; Fig. 3a). These findings indicate that silencing of B7-H4 could inhibit the migration of HT-29 cells.

### Effect of B7-H4 on MMP-2 and MMP-9 expression

To investigate the possible mechanisms of B7-H4 inhibiting the migration of HT-29 cells, the levels of MMP-2 or MMP-9 were measured by ELISA assay. Results showed that the levels of MMP-2 or MMP-9 in culture supernatants of B7-H4 shRNA group were both significantly decreased compared with scramble shRNA group ( $P$  all < 0.05; Fig. 3b, c). These data suggest that silencing of B7-H4 could inhibit the migratory capacity of HT-29 cells through reducing the secretion of MMP-2 and MMP-9.

### The regulation of B7-H4 expression by PI3K/Akt/mTOR signaling pathway

To explore the effect of PI3K/Akt/mTOR signaling pathway on B7-H4 expression, HT-29 cells were pre-treated with/without specific PI3K/Akt inhibitor LY294002 at 10  $\mu$ M or mTOR inhibitor rapamycin at 50 nM for 24 h. The expression of B7-H4 mRNA and protein was detected by RT-qPCR and western blotting. As shown in Fig. 4, HT-29 cells treatment with LY294002 and rapamycin obviously inhibited the activation of p-Akt and p-mTOR protein, respectively ( $P$  all < 0.05). Moreover, the expression of B7-H4 mRNA and protein was significantly downregulated following treatment with LY294002 and rapamycin compared with untreated group ( $P$  all < 0.05). These findings suggest that B7-H4 expression could be regulated by the PI3K/Akt/mTOR signaling pathway in HT-29 cells.



**Fig. 2** The effect of B7-H4 silencing on the cell cycle and cell apoptosis in HT-29 cells. **a** The effect of B7-H4 silencing on the cell cycle in HT-29 cells. **b** The effect of B7-H4 silencing on the cell apopto-

sis in HT-29 cells. **c** The effect of B7-H4 silencing on the apoptosis-related proteins Bcl-2 and caspase-3. Data were presented as mean  $\pm$  SD (\* $P$  < 0.05, \*\* $P$  < 0.01)

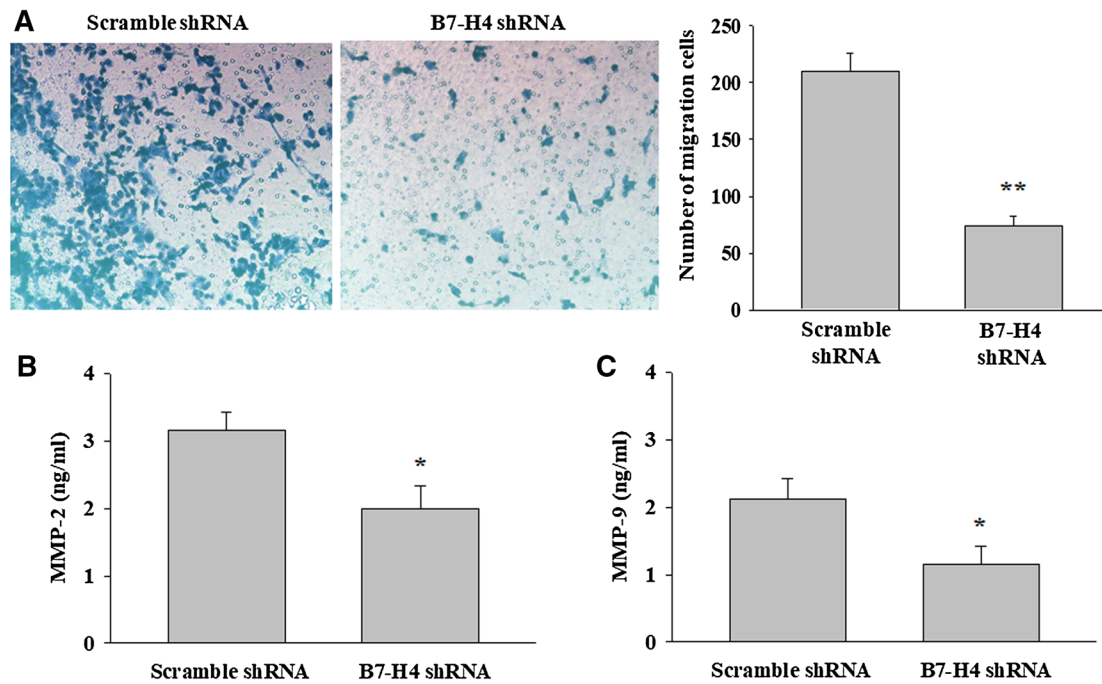
## Discussion

B7-H4, a new member of the inhibitory B7 family, is regarded as a negative regulatory molecule of the T cell-mediated immune response [17]. A number of studies have demonstrated that B7-H4 is over-expressed in various tumors and mostly associated with tumor incidence, progression and prognosis [8–14, 18]. Recently, soluble B7-H4 (sB7-H4) has been detected in blood samples from various tumor patients and high level of sB7-H4 was a significant diagnostic and prognostic marker [19–21]. Our previous studies have proved that the elevated B7-H4 levels in both CRC tissues and serum samples are positively correlated with tumor progression [15, 16]. These findings suggest that B7-H4 may promote the development and progression of human tumors by some underlying mechanisms. Therefore, B7-H4 might be an important tumor promoter and a novel therapeutic target for human malignancies.

The development of RNA interference (RNAi) technology has made it possible to suppress the function of specific molecular targets. It is also an attractive approach for

the analysis of gene function and gene therapy [22]. In this study, three small interfering RNA (siRNA) molecules targeting B7-H4 were first designed, which were connected to pSilencer 4.1-CMV neo vector and transiently transfected into HT-29 cells, respectively. The recombinant plasmid with the best silencing effect was chosen to construct stably silencing expression of B7-H4 cells by RNA interference (data not shown). Further results demonstrated that the established HT-29 cells stably expressing shRNA against B7-H4 displayed relatively low levels of B7-H4 on both B7-H4 mRNA and protein expression. This should be particularly useful for the analysis of B7-H4 gene of phenotypic effects on the growth or differentiation characteristics of CRC cells.

Next, we investigated the role of B7-H4 expression in cell proliferation, cell cycle and cell apoptosis. B7-H4 gene silencing assays revealed that the downregulation of B7-H4 inhibited the proliferation of HT29 cells and increased the apoptosis of HT29 cells but did not induce cell cycle arrest of HT29 cells. Apoptosis is a critical step for tumor development. The induction of apoptosis represents a powerful



**Fig. 3** Silencing of B7-H4 inhibits cell migration and MMP-2 and MMP-9 expression in HT-29 cells. **a** The effect of B7-H4 silencing on the migration of HT-29 cells. **b** The effect of B7-H4 silencing on

the expression of MMP-2 and MMP-9 in HT-29 cells. Data were presented as mean  $\pm$  SD (\* $P$  < 0.05, \*\* $P$  < 0.01)

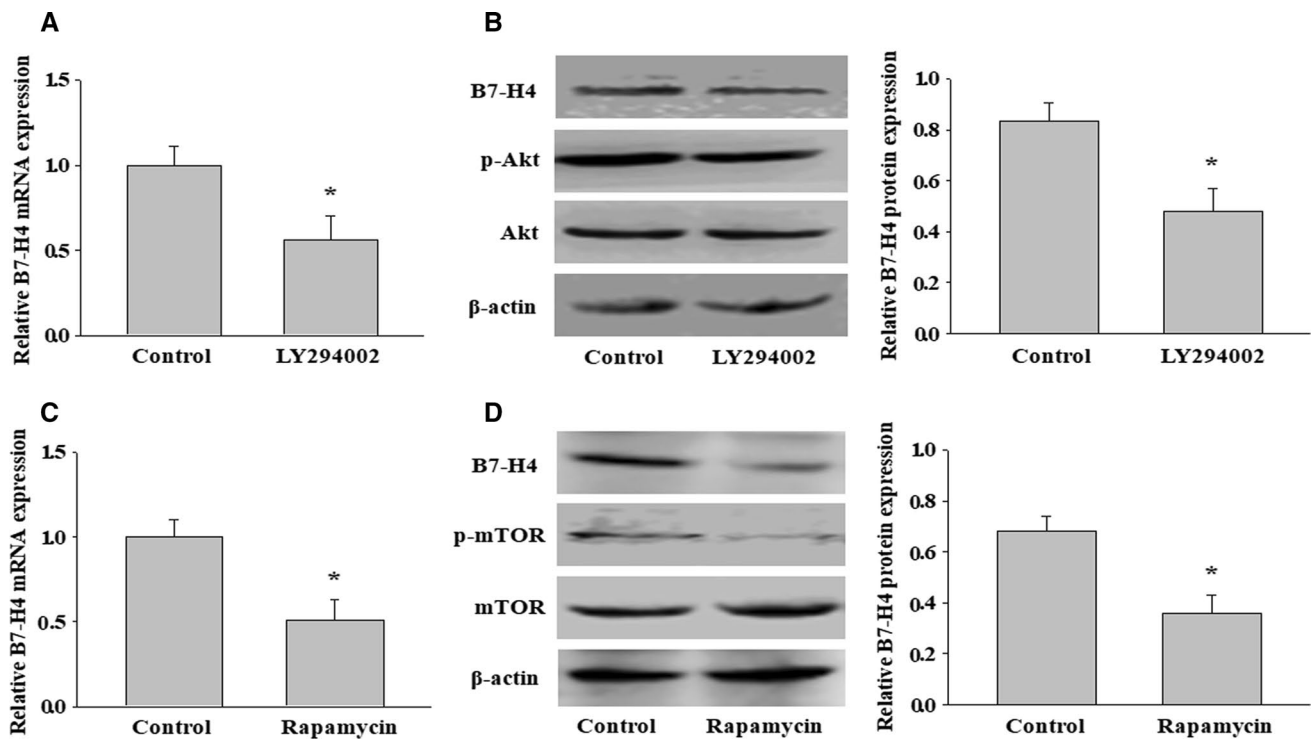
alternative therapeutic strategy for the treatment of malignant tumors [23]. Apoptosis can be induced either through an extrinsic pathway, which is mediated by the binding of apoptosis-inducing ligands to cell surface receptors, or through an intrinsic pathway, which is adjusted by the balance between pro-apoptotic and anti-apoptotic Bcl-2 family proteins in the mitochondria [24]. The balance change is responsible for the caspase-8 and caspase-3 activation and the induction of apoptosis [25]. In this study, HT-29 cells transfection with B7-H4 shRNA resulted in decreasing of anti-apoptotic Bcl-2 protein and increasing of caspase-3 protein. These data suggest that the caspase-dependent mitochondrial pathway is involved in the mechanism underlying the B7-H4 silencing-induced apoptosis of HT-29 cells.

Metastasis is a complex process involving multiple genes, stages and factors. One of the most important steps in the metastatic cascade is the intravasation of tumor cells into the circulation, which is related to cell migration and invasion. The processes are dependent on the tumor microenvironment [26]. Cytoskeletal rearrangements within the cancer cells, combined with the action of adhesive interactions, secreted extracellular matrix metalloproteinases (MMPs), are the main events prior to metastasis [27]. MMPs, especially MMP-2 and MMP-9, degrade a variety of extracellular matrix (ECM) macromolecules and facilitate tumor cell invasion [28]. Furthermore, the over-expression of MMP-2 and MMP-9 is also correlated with a worse prognosis in

patients with tumors [29, 30]. In our study, the cell migration ability in B7-H4 shRNA group was significantly decreased when compared with the control cells. Meanwhile, B7-H4 shRNA significantly attenuated expression levels of MMP-2 and MMP-9 proteins. Therefore, we speculate that B7-H4 gene silencing may inhibit the migration activity of HT-29 cells due to inhibition of MMPs.

The abnormal activation of signal transduction pathway plays key roles in the occurrence and development of tumor [31]. PI3K/Akt/mTOR signal pathway is one of the most widely researched intracellular signaling pathways in the last two decades. The activated signal pathway is associated with multiple tumors, and it plays important roles in the proliferation, apoptosis, invasion, migration and autophagy of tumor cells [32–35]. The activation of PI3K/Akt/mTOR pathway has also been found in CRC, which may contribute to the growth and progression of CRC [36–38]. Results in this study showed that the expression of B7-H4 in HT-29 cells could be regulated by the PI3K/Akt/mTOR signaling pathway, suggesting that B7-H4 may affect the cell proliferation and migration through PI3K/Akt/mTOR signaling pathway.

In conclusion, we revealed that shRNA targeting of B7-H4 effectively suppressed the proliferation and migration of HT-29 cells by inhibiting the effect of MMPs. B7-H4-targeted tumor therapy will be useful for mitochondria-mediated apoptotic HT-29 cell death. This work can supply important evidence



**Fig. 4** The regulation of B7-H4 expression by PI3K/Akt/mTOR signaling pathway. **a** The expression of B7-H4 mRNA was detected by qPCR following treatment with/without LY294002 at 10  $\mu$ M for 24 h in HT-29 cells. **b** The expression of B7-H4 protein was detected by western blotting following treatment with/without LY294002 at 10  $\mu$ M for 24 h in HT-29 cells. **c** The expression of B7-H4 mRNA

was detected by qPCR following treatment with/without rapamycin at 50 nM for 24 h in HT-29 cells. **d** The expression of B7-H4 protein was detected by western blotting following treatment with/without rapamycin at 50 nM for 24 h in HT-29 cells. Data were presented as mean  $\pm$  SD (\* $P$  < 0.05)

for PI3K/Akt/mTOR being a novel target signaling pathway of B7-H4.

**Acknowledgments** This research was supported by the Science and Technology Department of Jilin province (20170414030GH; 20190201220JC) and the Education Department of Jilin Province (JJKH20180353 KJ).

### Compliance with ethical standard

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

**Ethical approval** This study was conducted in accordance with the approved institutional guidelines of Beihua University in China. The Ethics Committee of Beihua University (2018015) approved this study and all experimental protocols.

**Informed consent** All enrolled participants of this study provided written informed consent.

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