EFFECT OF KNOCKDOWN BARD1 EXPRESSION ON THE PROLIFERATION, INVASION AND MIGRATION OF HEPATOCELLULAR CARCINOMA CELLS AND ITS POSSIBLE MECHANISM

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ABSTRACT

Objective: *To investigate the effect of reduced BRCA1-related RING domain 1 (BARD1) expression on the proliferation, invasion, and migration of hepatocellular carcinoma cells and its possible mechanism.*

Methods: *The expression levels of BARD1 in liver cancer and normal adjacent tissues were determined by immunohistochemistry. Liver cancer cell line SMMC7721 was selected for culture, siRNA was selected to silence the BARD1 gene in SMMC7721 cells (BARD1 silencing group), and non-related sequence sirna-nc was selected to transfection SMMC7721 cells as the negative control group (control group). The effect of the BARD1 knockdown on the proliferation of hepatocellular carcinoma cells was observed through cell cloning. Teanswell cell assay was used to determine the changes of cell invasion and migration. Finally, a western blot was used to determine the expression of Akt, mTOR and MMP-9 in each group.*

Results: *The expression of BARD1 in hepatocellular carcinoma was significantly higher than that that of paracancer. Compared with the control group, the proliferation, migration and invasion abilities of the BARD1 silencing group were significantly lower ($P<0.01$). Compared with the control group, the expression levels of BARD1, Akt, mTOR and MMP-9 in the BARD1 silencing group were significantly lower ($P<0.05$).*

Conclusion: *BARD1 knockdown may inhibit the proliferation, invasion, and migration of HCC cells by inhibiting the activation of the Akt/ mTOR signaling pathway.*

Keywords: *BARD1, hepatocellular carcinoma, proliferation, invasion, migration, mechanism.*

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Introduction

Primary hepatocellular carcinoma (HCC) is one of the most common malignancies with a high incidence. More than 600,000 people in the world die of liver cancer every year. With the progress of medical science and technology, treatments from liver cancer are increasing, but the recurrence and metastasis rates of liver cancer patients are still high. In addition, the 5-year survival rate of patients is only about 35%, which seriously affects the life and health of patients[1-2]. The continuous proliferation, invasion, and metastasis of cells are the most significant characteristics of malignant tumors, and the most prevalent cause of death in patients. The BRCA1 RING structure of the related Domain 1 (BARD1) is a kind of S phase of cell division and DNA damage. After interacting with the BRCA1 nucleoprotein, the BARD1 can join with breast cancer susceptibility gene 1 (BRCA1) to form stable heterologous dimers and maintain the organism genome integrity[3-4]. Previous studies have shown that BARD1 is prominently expressed in a variety of cancers, especially in certain aggressive, highly specific malignancies, and there are relatively few studies on its expression in liver cancer cells[5]. In

this study, the changes of proliferation, invasion, and metastasis of liver cancer cells and their possible influencing mechanisms after BARD1 expression were discussed and analyzed.

Materials and methods

Main materials, instruments and reagents

This experiment utilized the following:

• 40 cases of liver cancer tissues and normal paracancer tissues (liver cancer tissues and normal paracancer tissues removed during surgery for liver cancer patients in the hospital); the SMMC7721 human liver cancer cell line (Shanghai Hongshun Biotechnology Co., LTD.);

• A thermostatic water bath box (Shanghai Zhichu Instrument Co., LTD. model: ZHSY-50V);

• A high-speed centrifuge (Yancheng Kitt Experimental Instrument Co., LTD., model number: KS50R);

• A paraffin slicing machine (Hubei Xiaogan Kuohai Medical Technology Co., LTD., model: KH-Q320);

• A fluorescence microscope (Thermo Fisher Technology [China] Co., LTD., model: AMAFD2000);

• An electronic balance (Shanghai Yixin Scientific Instrument Co., LTD., model: fa-a);

• An ultra-low temperature refrigerator (Haier Bio-medical Co., LTD., model: DW-86L728);

• An immunohistochemical kit (Bhaode Biotechnology Co., LTD.);

• aPCR instrument (Shanghai Bayu Industrial Co., LTD., model: Veriti96);

• Flow cytometry (Suzhou Taomaison Scientific Instrument Co., LTD., model: qlc-1);

• A DMEM medium (Shanghai Syme Fisher Biotechnology Co., LTD.);

• A fetal bovine serum (Shanghai Leichuang Biotechnology Co., LTD., specification: Z7185FBS-100);

• Xylene (Nanchang Lanxiang Chemical Co., LTD.);

• Rabbit anti-human BARD1 polyclonal antibody (Emmett Technologies, LTD.).

Experimental methods and observation indicators

Liver cancer tissues and normal paracancer tissues were collected, and the expression levels of BARD1 in liver cancer tissues and normal paracancer tissues were determined by immunohistochemistry.

Cell culture

The liver cancer cell line SMMC7721 was selected, removed from the liquid nitrogen, and thawed in a constant temperature water bath at 37 oC. After thawing, it was cultured in the DMEM medium at 37 oC with 15% fetal bovine serum and 5% CO_2. A cell culture was conducted when the cells grew to a fusion state of approximately 75%, and the cells were cultured after two passages.

When the cell density reached about 85% and the growth conditions were favorable, the cells were inoculated into a 6-well plate or 30 cm2 culture medium, and the cells could be frozen and stored for subsequent experiments.

Cell transfection

The cells were removed and thawed, the SMMC7721 cells with appropriate density were digested with trypsin and centrifuged, the cell suspension was made, the cells were seeded with 60,000 cells/well in 96-well plates for culture, and the transfection experiment was carried out when the cell density was approximately 70%. SiRNA was selected to silence the BARD1 gene in SMMC7721 cells (BARD1 silencing group). In addition, the non-correlated sequence SiRNA-NC was selected to transfect SMMC7721 cells as the negative control group (control group).

Cell cloning experiment

The SMMC7721 human hepatocellular carcinoma cells were digested, centrifuged, resuscitated, and counted with trypsin after treatment. The cells were seeded at 6×10^3 cells/well into a six-well plate and cultured in a medium of 5% CO_2 at 37 oC for 2 weeks.

After washing with phosphate buffer solution, fixing with formalin solution, and washing with distilled water, staining with 0.1% crystal violet, the effect of the knockdown BARD1 expression on the proliferation of HCC cells was observed.

Teanswell cell migration experiment

A 200-h, serum-free medium was added to the upper chamber of Teanswell cell, and a 600-h, serum-free medium was added to the lower chamber and cultured in a DMEM medium of 10% FBS, and 5% CO_2 at 37 oC for 48 h.

The cells that had not migrated in the past were stained and washed, and the effect of a BARD1 knockdown on the migration ability of liver cancer cells was observed by microscope.

Teanswell cell invasion experiment

200 μL of a serum-free medium was added to the upper chamber of the Teanswell cell, and 600 μL of a serum-free medium was added to the lower chamber. It was then cultured in a DMEM medium of 10% fetal bovine serum, and 5% CO_2 at 37°C for 48 h. The non-penetrating cells were stained and washed, and the effect of a BARD1 knockdown on the invasion ability of liver cancer cells was observed by microscope. The expression of a mammalian target of rapamycin (mTOR) and matrix metalloproteinase-9 (MMP-9) in serine-threonine kinase (Akt) was determined by western blotting.

Statistical methods

In this study, the measurement data were compared by one-way anova and an LSD t-test. The effect of the BARD1 knockdown on the proliferation of hepatocellular carcinoma cells was observed by cell cloning. A Teanswell cell assay was used to determine the changes caused by cell invasion and migration. In addition, a western blot was used to determine the expression of Akt, mTOR, and MMP-9 in each group, and P<0.05 was considered statistically significant. In this study, a SPSS20.0 software package was used for statistical data analysis.

Results

Observing the expression of BARD1 in liver cancer tissues and normal paracancer tissues

In this study, the expression of BARD1 in liver cancer tissues was significantly higher than that in normal para-carcinoma tissues, as shown in Figure 1.

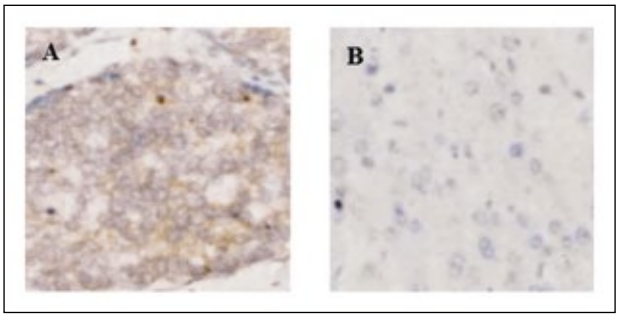

Figure 1: BARD1 expression in liver cancer cells and normal paracancer tissues.
A: liver cancer tissue; B: normal paracancer tissue.

The effect of knockdown of BARD1 expression on the proliferation of hepatocellular carcinoma cells

Compared with the control group, the proliferation ability of the BARD1 silencing group was significantly lower (P<0.01) (see Figure 2 and Table 1).

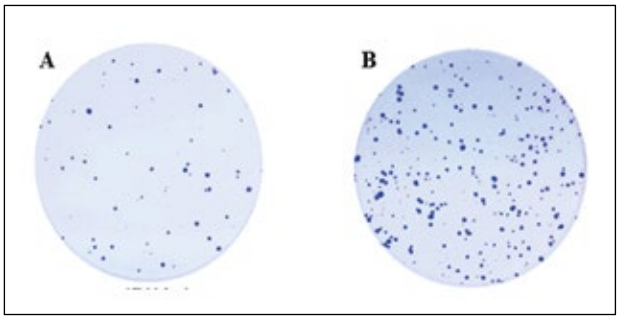

Figure 2: The effect of BARD1 expression on the proliferation of hepatocellular carcinoma cells.
A: BARD1 silence group; B: control group.

Group	Changes in cell proliferation
Control group	276.69±22.37
BARD1 silent group	83.15±18.55
t	16.313
P	<0.001

Table 1: Effect of BARD1 expression on the proliferation of hepatocellular carcinoma cells ($\bar{x}\pm s$).

The effect of knockdown of BARD1 expression on the migration ability of hepatocellular carcinoma cells

Compared with the control group, the cell migration ability of the BARD1 silencing group was significantly lower (P<0.01) (see Figure 3 and Table 2).

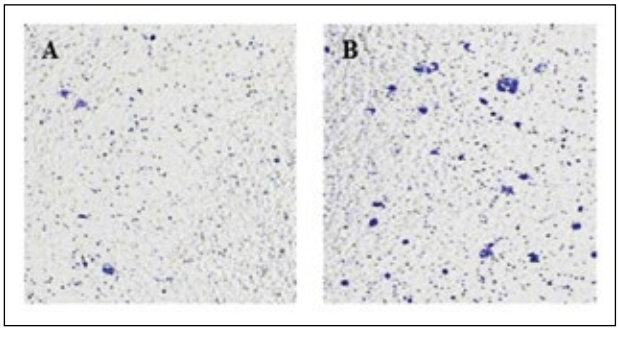

Figure 3: The effect of BARD1 expression on the migration ability of hepatocellular carcinoma cells.
A: BARD1 silence group; B: control group.

Group	Changes in cell migration
Control group	36.22±11.54
BARD1 silent group	5.19±1.22
T	6.551
P	<0.001

Table 2: The effect of BARD1 expression on the migration ability of hepatocellular carcinoma cells.

Effect of knockdown BARD1 expression on the invasion ability of liver cancer cells

Compared with the control group, the invasion ability of the BARD1 silencing group was significantly reduced (P<0.01) (see Figure 4 and Table 3).

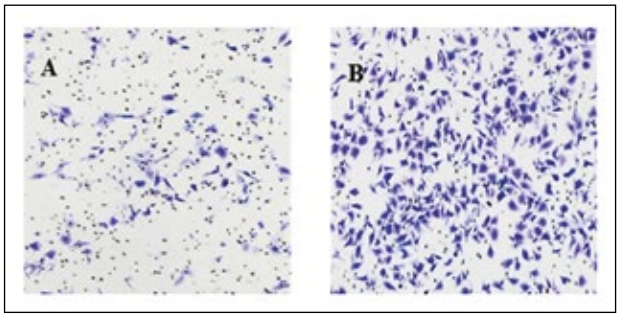

Figure 4: The effect of BARD1 expression on the invasion ability of hepatocellular carcinoma cells.
A: BARD1 silence group; B: control group.

Group	Changes in cellular invasiveness
Control group	437.62±36.39
BARD1 silent group	109.67±24.36
t	18.344
P	<0.001

Table 3: Effect of BARD1 expression on the invasion ability of hepatocellular carcinoma cells.

Expression of BARD1, Akt, mTOR, and MMP-9 in each group

Compared with the control group, the expression levels of BARD1, Akt, mTOR, and MMP-9 in the BARD1 silencing group were significantly reduced (P<0.05), as shown in Figure 5.

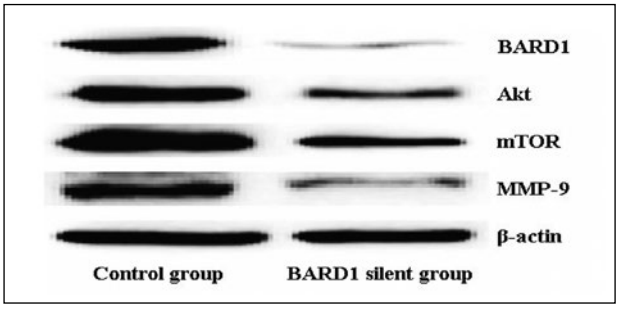

Figure 5: Expression of BARD1, Akt, mTOR, and MMP-9 in each group.

Discussion

In the world, primary liver cancer is still one of the important diseases threatening human health. With the development of modern medical technology and the deepening of the research on malignant tumors, treatments for liver cancer have made important progress. Since early symptoms are often less obvious, most of the patients at the time of diagnosis are in the middle–late stage, when incidence of malignancy is higher. Thus, they soon lose the chance of having an optimal operation, and there is currently no cure for advanced liver cancer[6]. High rates of metastasis and recurrence are still major obstacles to long-term survival of HCC patients. With the continuous development of molecular biology technology and tumor medicine, studies have found that the occurrence and development of liver cancer are closely related to the regulation of various intercellular and extracellular factors and multiple signaling pathways[7]. Therefore, it is important to search for new molecular markers to improve the therapeutic effect and improve the prognoses of patients with HCC.

The BARD1 gene is located in 2q34-35, and the BARD1 protein encoded by this gene has a RING domain at the -NH2 end and a BRCT domain at the -COOH end. BARD1 binds to BRCA1 to form a stable heterodimer, which can inhibit the cell cycle and participate in cell cycle regulation, gene transcriptional regulation, and DNA damage repair[8]. Choudhary et al.[9] found that there was a significant positive correlation between the overexpression of BARD1 and the clinical characteristics of patients with liver cancer, and this increased expression level may lead to liver injury and further promote the occurrence and development of liver cancer. In this study, the expression of BARD1 in liver cancer tissues was significantly higher than that in normal paracancer tissues. These results echoed those of Choudhary et al. Some researchers have believed that a tumor is a disease with abnormal cell proliferation and differentiation and that the proliferation, invasion, and migration of cancer cells are the most important processes in liver cancer metastasis[10]. In this study, compared with the control group, the proliferation ability, migration ability, and invasion ability of the BARD1 silencing group were significantly reduced. These results suggested that BARD1 knockdown could significantly inhibit the proliferation, invasion, and metastasis of HCC cells. Invasion and metastasis are important biological characteristics of malignant tumors, and they are multi-step and multi-factor complex processes. They involve a variety of factors, such as the change of tumor cell adhesion, the shedding of tumor cells at the primary site, the degradation of interstitial cells and basement membrane, the invasion of vasculature, and the formation of new blood vessels[11]. It has

been reported that the occurrence and development of malignant tumors are related to the abnormal expression of a variety of genes—that is, the process of over-activation of oncogenes and inhibition of tumor suppressor genes—which is regulated by a variety of complex cell signaling pathways[12]. In order to explore the applicable mechanism of action, the expression levels of BARD1, Akt, mTOR, and MMP-9 in liver cancer cells were determined by western blot by knocking down the expression levels of BARD1 in SMMC7221 cells. This study revealed that the expression levels of BARD1, Akt, mTOR, and MMP-9 were significantly reduced in the BARD1 silencing group compared with those of the control group ($P<0.05$). It showed that the knockdown of BARD1 expression could significantly down-regulate the expression of Akt, mTOR, and MMP-9 and inhibit the conduction pathway of Akt (a serine/threonine kinase) and mTOR. A number of studies have found that the level of Akt is significantly increased in most patients with liver cancer, and the activated Akt can induce a large amount of downstream expression of related molecules, such as blood vessel formation, cell survival, and cell proliferation[13-14]. In addition, mTOR and MMP-9 are downstream molecules of Akt, and the catalytic domain encoding phosphoinositol-3 kinase gene is mutated, promoting the increase of Akt expression. In turn, this activates mTOR to further regulate the expression of the vascular endothelial factor and the formation of MMP-9 and other related proteins[15].

In summary, knockdown of BARD1 may inhibit the proliferation, invasion, and migration of hepatocellular carcinoma cells by inhibiting the death activation of the Akt/mTOR signaling pathway.

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