High Percentage of Cancer Stem Cells in Metastatic Locations: Upregulation of circBIRC6 in Highly Metastatic Breast Cancer Subline

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Abstract

Metastasis is a devastating complication in breast cancer. Cancer relapse and metastasis are associated with cancer stem cells. CircBIRC6 is a circular RNA that proposed to be involved in stemness of stem cells. In breast cancer, metastatic tumor cells have higher stem cell properties. In the present study we evaluate the expression of circBIRC6 in these cells. After development of syngenic animal model of TNBC, primary breast cancer cells named 4T1T were isolated from tumor mass. Highly metastatic tumor cells named 4T1B and 4T1L were isolated and expanded from brain metastasis lesions and lung of cancerous mice respectively. Sphere formation ability in metastatic and primary tumor cells was evaluated separately. Quantitative real-time polymerase chain reaction was performed to analyze the expression of circBIRC6 in primary and metastatic tumor cells. Our data revealed that, sphere formation ability among metastatic tumor cells was significantly higher. Surprisingly expression of circBIRC6 was significantly upregulated in these metastatic tumor cells. In comparison with 4T1T, circBIRC6 was upregulated 5.7 and 3.5 time in 4T1B and 4T1L respectively. These findings provided important insights regarding the molecular properties of metastatic tumor cells and can be used to design targeted therapeutic strategies to combat these cells.

Introduction

Breast cancer is the most common cancer in women worldwide [1]. Among all type of breast cancer, triple-negative breast cancer (TNBC) is the most aggressive and invasive type with poor prognosis [2]. Metastases account for 90% of human cancer deaths. Bone, lung, liver and brain are the main sites of metastases in breast cancer [3]. Metastasis and resistance to chemotherapy are linked phenomenon. The molecular mechanisms that lead to therapeutic resistance are diverse and are still incompletely understood [4].

In breast cancer numerous studies demonstrate the existence of breast cancer stem cells (BCSCs). They emphasized that BCSCs have fundamental role in tumour progression, metastasis and resistance to current cancer treatment [5, 6]. It has been demonstrated that CSCs have the ability to form spheres in vitro when grown in special culture conditions [7]. Such spheres that allow the growth and propagation of CSCs, applied as a standard experimental test for evaluating the potential of stemness in cancer cells, and is the best tool for characterizing cancer stem cells [8].

In recent years a new member of noncoding RNAs, named Circular RNAs (circRNAs) was discovered. Length of circRNAs range from a few hundred to thousands of nucleotides [9]. In circRNAs covalent bonding of 3' and 5' (head-to-tail) ends create a circular structure [10]. In contrast to other type of RNA, circRNAs are stable in cells. This is due to the absence of open sites at the 5' and 3' ends exempts circRNAs from endonuclease degradation [11]. CircRNAs have been implicated in a variety of biological and pathological processes including cancers ; they have been shown to play an important role in cancer initiation and progression and may function as molecular markers for cancer diagnosis and treatment

[12]. Discovery and in-depth study of circRNAs may provide ideas for the search for new methods for the treatment of malignant tumors.

CircBIRC6 (hsa_circ_0000989) is a new member of circRNAs that participate in stem cell properties[13]. Expression and potential role of circBIRC6 in cancer have been proposed in some research. In a recent work, result showed that circBIRC6 promotes cancer cell progression [13]. Our previous work indicated that, in compare with primary tumor cells, metastatic breast cancer cells have higher stem cell properties. In this regard, the present study was designed to investigate the expression of circBIRC6 in these cells.

Methods

Cell culture

4T1 cell line was obtained from the cell bank of Pasteur Institute of Iran (C604). The cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS (fetal bovine serum) and 2% Penicillin-Streptomycin (all from Gibco, USA) in humidified atmosphere of 5% CO2 at 37°C.

Induction of syngeneic animal model of breast cancer

Female BALB/c mice weighing 20 to 25 gram obtained from Royan institute (Iran). The animals were housed in cages at 12-h photoperiod while they had free access to food and water. All animal experiments were in compliance with the relevant laws, and this study was approved by the Ethics Committee of Shahroud University of Medical Sciences (registration number: IR.SHMU.REC.1400.265). 4T1 cells were subcutaneously injected to the flank (or the right hind limb) of the mice (10^5 cells suspended in 100 µL PBS) using an insulin syringe with 32G needle. The mice were monitored daily for the appearance and behavior characteristics.

Primary and metastatic breast tumor cell extraction

Primary and metastatic tumor cell extraction, was performed according to our and other group previous works [14, 15]. Briefly primary tumor, lung and brain of cancerous mice were excised after 35 days of tumor induction in mice, and surface blood was removed by rinsing it in PBS. After mincing with scissors, fragments were placed to 50 ml conical tube. For enzymatic digestion, primary tumor, lung and the brain were digested in 10 mg ⁄ ml collagenase type IV at 37°C for 75 min on a platform rocker. All enzymes were purchased from Sigma (St Louis, MO, USA). The digested organ filtered through 70-um cell strainers, and washed with PBS. In the next step, washed cells were resuspended in medium containing 10% FBS, 100 U/ml Penicillin, and 100 ug/ml Streptomycin (all from Gibco, USA). Ultimately, the cells were cultured at 37°C in 5% CO2.

Sphere formation ability

Heterogeneous population of primary and metastatic tumor cells were separately cultured in DMEM containing 10% FBS, 100 U/ml Penicillin, and 100 ug/ml Streptomycin (all from Gibco, USA) at 37°C in

5% CO2. For Sphere formation, cells were seeded at 2.5 × 10^4 cells in petri dish in DMEM supplemented with 10 % FBS. After 24 h cell culture media replaced with fresh medium comprised of DMEM supplemented with 2% FBS. Again, after 72 h cell culture media replaced with fresh medium comprised of DMEM supplemented with 1% FBS. Primary/1° and secondary/2° mammosphere formation was achieved after 8 days. The sphere formation index was determined by two independent investigators by counting sphere formed in 10 high power fields per petri dish.

Quantification of circBIRC6 by RT-qPCR

Primary and metastatic tumor cells (1×10^4) were seeded in each well of 24-well plates in complete medium. After 48 hours Total RNA was extracted from these cells using QIAzol Lysis Reagent (QIAGEN). The quality, yield, and size of extracted RNA were analyzed using spectrophotometry (NanoDrop-ThermoFisher) and electrophoresis. The first strand cDNA synthesis was performed using reverse transcription system (Easy cDNA Synthesis Kit for RNA or mRNA to cDNA - pars tous). Real-time PCR procedure was executed based on the 1 ul cDNA in all samples. Quantization of all gene transcripts was done by SYBR Green Real time PCR Master Mix (Amplicon A/S, Denmark) using StepOnePlus™ Real-Time PCR System, according to the manufacturer's instruction. The amplification procedure was as follows: 1 cycle of 95°C for 15 min, 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. The exact mRNA expression was normalized to the expression level of GAPDH. Relative changes of gene expression were calculated by the following formula, and the data was represented as fold up-regulation/down-regulation.

Fold change = $2^{-\Delta\Delta Ct}$, where ΔΔCt = [Ct of circBIRC6 (in 4T1B/4T1L cells) - Ct of GAPDH (in 4T1B/4T1L cells)] - [Ct of circBIRC6 (in 4T1T cells) - Ct of GAPDH (in 4T1T cells)].

Primers were designed using AlleleID version 6 software (Premier Biosoft Inc.).

The used primers are as follows:

For circBIRC6, Forward 5'-ACCATAGATGAACCATTGACAC-3', Reverse 5'- CTAACTGCGGCTCATTCAC -3';

For GADPH, Forward 5'-CCTGGAGAAACCTGCCAAGTA-3', Reverse 5'-GGCATCGAAGGTGGAAGAGT -3'.

Statistical analysis

Results are expressed as the mean ± standard deviation. Data were analyzed with GraphPad Prism statistical software 6.0 (GraphPad Software, La Jolla, CA, USA) using one-way ANOVA. $P < 0.05$ was considered statistically significant.

Result

Primary and metastatic tumor cells extraction

Metastatic animal model of breast cancer was generated after 35 days following tumor induction in BALB/c mice (Fig. 1A). When injected into BALB/c mice, 4T1 spontaneously produces highly metastatic tumors that can metastasize to the brain and lung while the primary tumor is growing in situ. The primary tumor does not have to be removed to induce metastatic growth. H and E staining and pathological confirmation were performed on tumor tissues and metastatic lesions (Fig. 1B, C, D). We properly extracted primary and metastatic tumor cells from subcutaneous primary tumor, brain and lung of cancerous mice, respectively (Fig. 1C, B, D). The metastatic tumor cells, after isolation, form colonies in the culture medium. Due to the high rate of growth and proliferation, the tumor cells in these colonies are purified after 3 passages. These tumor cells are called 4T1B for brain metastatic tumor cells and 4T1L for lung brain metastatic tumor cells while tumor cells that are obtained in the same way, from the original tissue of the tumor, are primary tumor cells called 4T1T (Fig. 1C, B).

Higher capacity of metastatic tumor cells in sphere formation

After 6 day of culturing primary and metastatic tumor cells in sphere forming media, results indicated that metastatic tumor cell have greater ability in sphere formation. As shown in fig.2 metastatic tumor cell sphere formation ability was higher and greater than primary tumor cell in term of number and size respectively.

Significant Upregulation of circBIRC6 in metastatic tumor cells in mRNA level

The expression of circBIRC6 was analyzed in 4T1T, 4T1B and 4T1L. The quality, yield, and size of extracted RNA, synthesized cDNA, and PCR products were confirmed using nanodrope and gel electrophoresis. As shown in fig 2 the expression of circBIRC6 was up-regulated 3.5 times in 4T1L compared with 4T1T. About 4T1B, the expression of this circular RNA was involved in higher alteration in metastatic cascade of breast cancer and up-regulated 5.7 times in 4T1B compared with 4T1T (Fig. 3).

Discussion

In previous work we determined that sphere formation ability in lung metastatic tumor cells is significantly higher than primary tumor cells [16]. In the present study, in addition to lung, brain metastatic tumor cells were also isolated from mouse model of TNBC. Result indicated that compared with primary and lung metastatic tumor cell, these cells have higher capacity to sphere formation. In molecular analysis we detected that expression of circBIRC6 was significantly upregulated in metastatic tumor cells. We proposed that these high level of circBIRC6 in metastatic tumor cells may be correlate with high stem properties of these cells.

BCSCs are increasingly thought to play a major role in breast cancer growth and the formation of metastases. Sphere forming ability of BCSCs have been demonstrated in tumor cells isolated from pleural effusions of breast cancer patients. These cells were tumorigenic when transplanted into SCID mice [17]. Numerous studies have investigated the role of cancer stem cells in cancer initiation, spread,

and metastasis [18]. In accordance with our results, Britton etal clarified that the percentage of CSCs increases during breast cancer development and spread [19].

Analysis of CSCs roles in metastasis has been mainly conceptual and speculative, and the reasons for a higher number of CSCs in the metastatic loci are questionable. Analysis of molecular properties of metastatic tumor cells can be used for answering these questions.

Recently, Yu et al. identified a novel circRNA, BIRC6 (circBIRC6) that was found to be enriched in undifferentiated human embryonic stem cells (hESCs). They also found that circBIRC6 participates in the regulating the expression of target genes maintaining pluripotency [13]. Expression and potential role of circBIRC6 in cancer has not been yet well studied and needs further investigation.

In a recent work on non-small cell lung cancer (NSCLC), Yan et al showed that circBIRC6 promotes NSCLC cell progression, possibly by sponging miR-145. This group found that after the introduction of circBIRC6-specific siRNA into human lung cancer cells (A549 cells) the proliferation and invasion ability of A549 cells decreased, and the apoptosis rate increased significantly. This experiment confirmed that circBIRC6 played an essential role in the proliferation and invasion of lung cancer cells in vitro [20]. In another work on NSCLC result indicated that circ-BIRC6 functions as a critical regulator of growth and apoptosis in NSCLC cells via sponging miR-4491. Depletion of this CircRNA suppressed NSCLC growth by targeting miR-4491 [21].

In bladder cancer findings demonstrated that circ-BIRC6 knockdown suppressed tumorigenesis and progression via regulation of the miR-495-3p/XBP1 signaling axis, offering a promising therapeutic target for the treatment of this cancer [22].

Paclitaxel is an effective chemotherapeutic agent for the treatment of cancer patients. In hepatocellular carcinoma (HCC), Liu etal showed that Paclitaxel inhibited the expression of circ-BIRC6 and YWHAZ while promoted expression of miR-877-5p. they concluded that paclitaxel suppressed HCC tumorigenesis by modulating circ-BIRC6/miR-877- 5p/YWHAZ axis [22]. In another study on HCC, Yang etal in 2019 Showed that circBIRC6 promote HCC progression by targeting the miR-3918/Bcl2 axis [23]

Interestingly, circBIRC6 has been found to be significantly increased in gastric cancer patients (Patent PCT/CN2018/084506) , where it could maintain the pluripotency of the CSCs [24].

In our work, higher potential of breast metastatic tumor cells in spheroid formation that represent higher stem cell properties of this cells, accompanied with significant upregulation of circBIRC6 in these cells. These results indicated that circBIRC6 may be involved in cancer stem cell properties of metastatic breast cancer cells.

Information about circRNA roles in cancer is still in its first stages and more research is needed. Our work focuses on the participation of circBIRC6 in the BCSCs, which are considered one of the main causes of failure of cancer treatments, the appearance of metastases, and the occurrence of relapses. Accordingly, this circRNA have been suggested as markers for the identification of BCSCs, as well as therapeutic

targets for their elimination, in order to find a cure or to improve the life expectancy of people suffering from breast cancer.

Declarations

Conflict of Interest Statement

The author declares that they have no competing interests.

Ethical statement

This study was approved by the Ethics Committee of Shahroud University of Medical Sciences (registration number: IR.SHMU.REC.1400.265).

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Informed Consent

Written informed consents have been signed by the participants.

Data availability

All data generated or analysed during this study are included in this published article.

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Figures

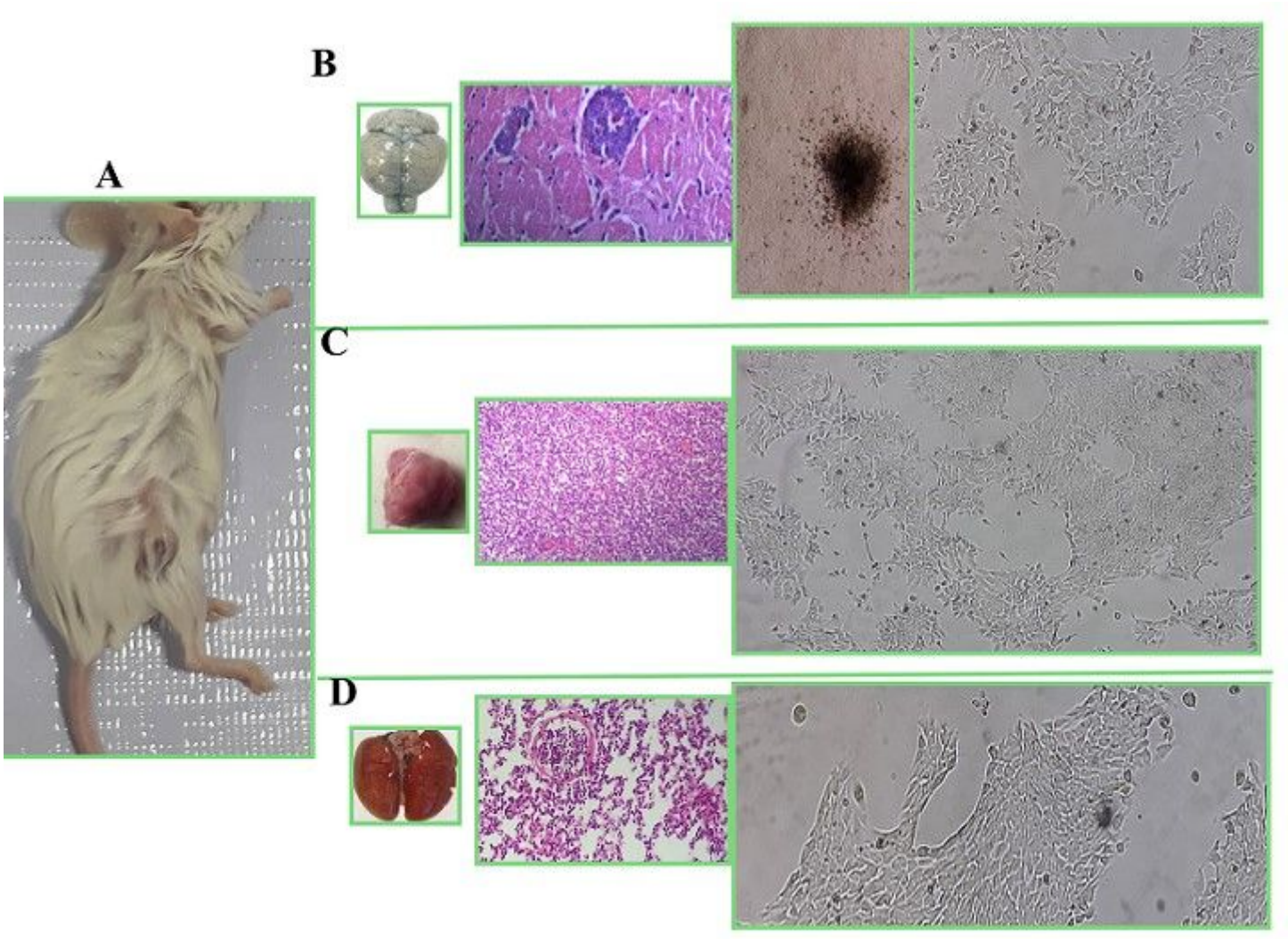

Figure 1

Primary and Metastatic Tumor Cells Isolation. A. Metastatic animal model of triple negative breast cancer was generated after 35 days of tumor induction in BALB/c mice. B. Brain metastatic tumor isolation, H&E staining and metastatic tumor cell extraction was performed on brain of cancerous mice. C. Primary tumor isolation, H&E staining and primary tumor cell extraction was performed on primary tumor tissues. D. Lung metastatic tumor isolation, H&E staining and metastatic tumor cell extraction was performed on lung of cancerous mice

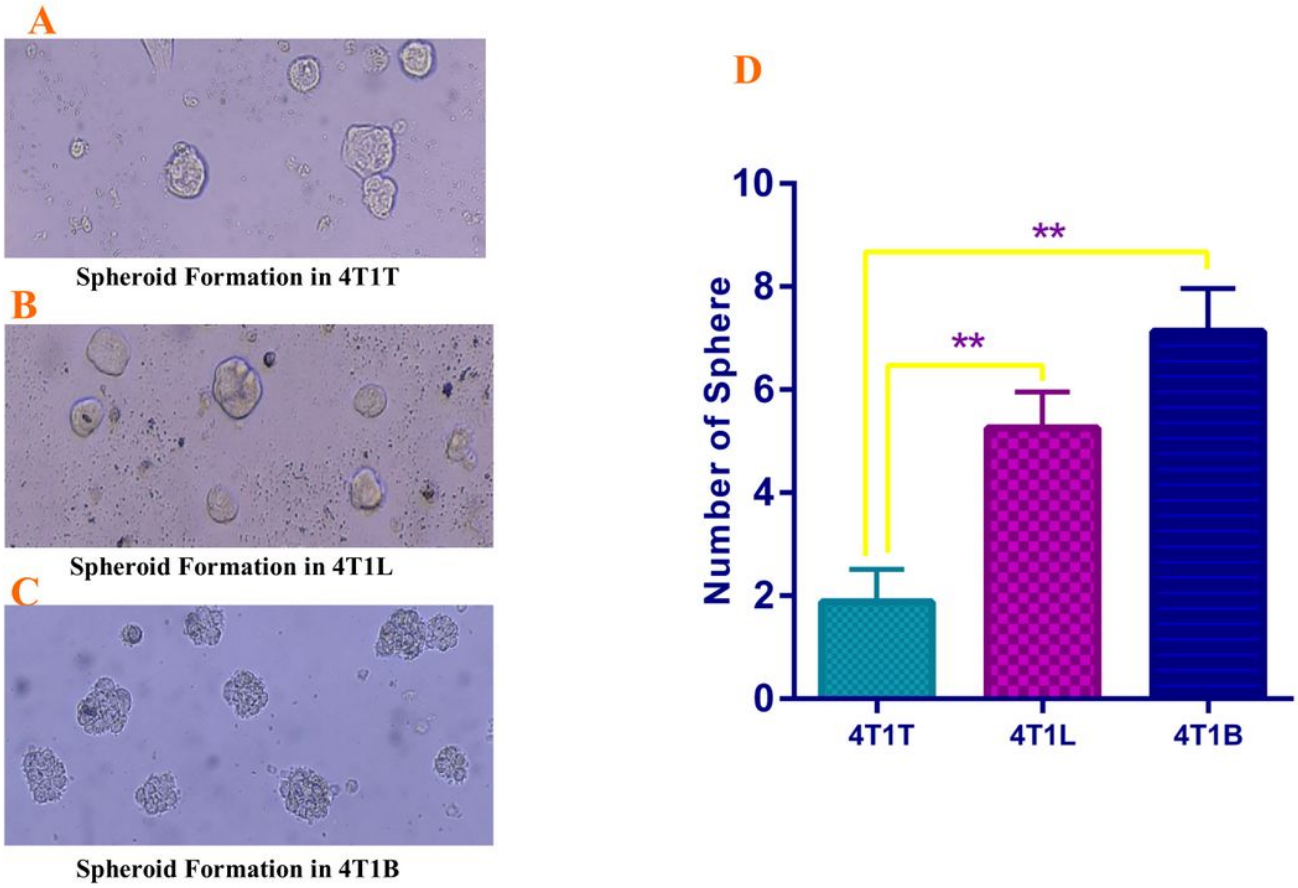

Figure 2

Higher capacity of metastatic breast tumor cells in sphere formation. A, B and C. Sphere formation in primary and metastatic tumor cells after serum starvation in culturing media. D. Sphere formation capability was significantly higher in metastatic tumor cells. All results are expressed as mean ± SD from at least three independent experiments analyzed by one-*way ANOVA*. **P < 0.0001

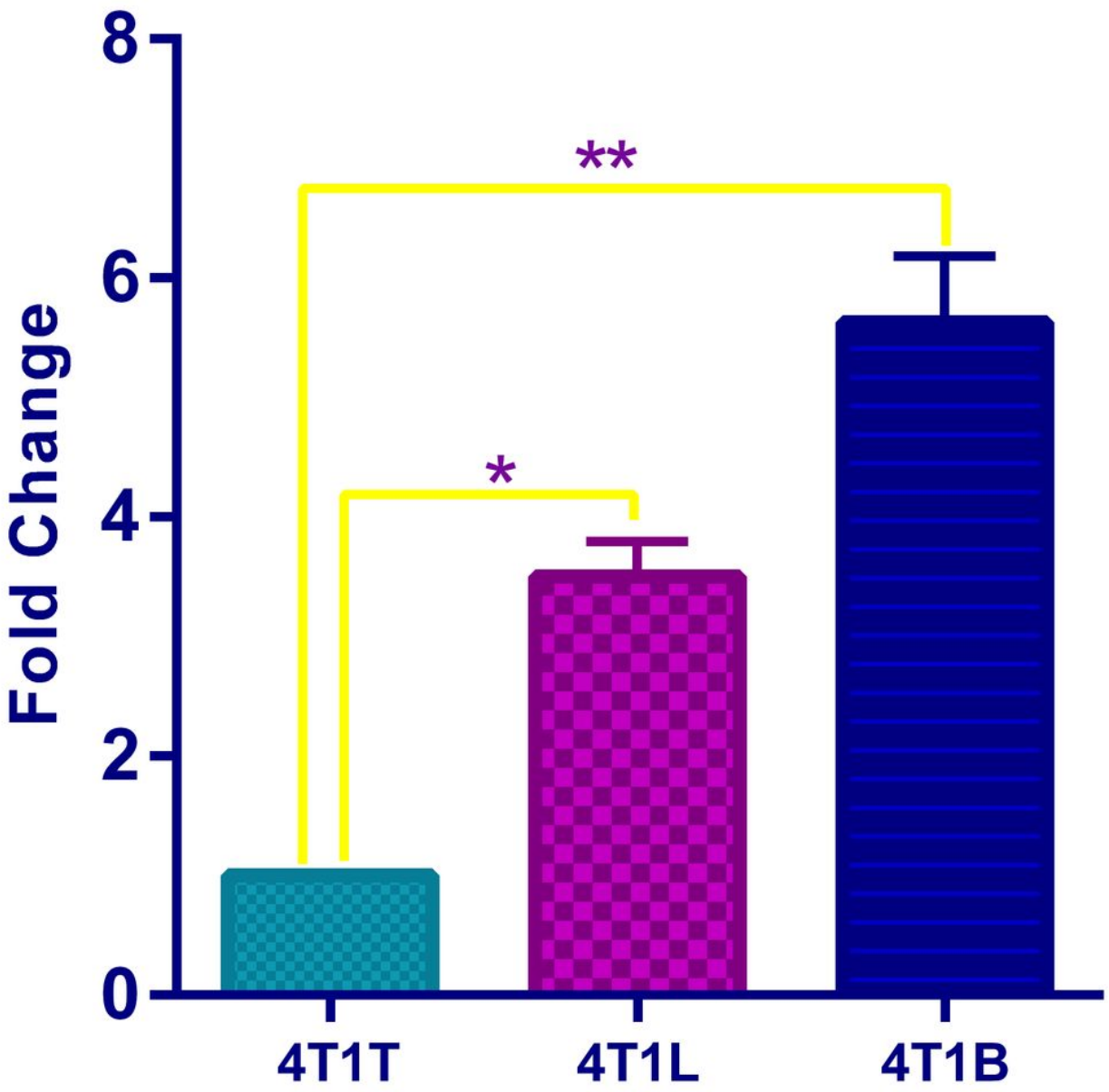

Figure 3

Enhanced Expression of circBIRC6 in Metastatic Tumor Cells Using Real-Time PCR. CircBIRC6 was significantly upregulated in Metastatic Tumor Cells. All results are expressed as mean ± SD from at least three independent experiments analyzed by one-*way ANOVA*. **P < 0.001