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ORIGINAL ARTICLE

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Exosomal GPT2 derived from triple-negative breast cancer cells promotes metastasis by activating BTRC

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Abstract

Background: There have been reports of increased glutamate pyruvate transaminase 2 (GPT2) expression in certain cancers including breast cancer. Although the role of GPT2 as a metabolic enzyme is well understood in breast cancer progression, little is known about the other roles of GPT2, especially exosomal GPT2.

Methods: BT549 and BT474 Cells were cultured and their exosomes were isolated by using ultracentrifugation. Cells migrated through the membrane were stained with crystal violet, and then were observed by microscope. Total RNA was extracted from culture cells and transcribed into cDNA, quantitative real-time RT-PCR was used to detect mRNA expression of ICAM1, VCAM1, and MMP9 using SYBR Green qPCR Mix with a 7500 Fast Real-time PCR system. Western blot was used to detect the gene expression of p-lkBa and TSG101 and GPT2 in breast cancer cells. Immunohistochemistry was used to detect the protein expression of GPT2 and BTRC in cancer cells, animal models loaded with metastasis breast cancer cells were established via tail vein injections. Interaction between GPT2 and BTRC in breast cancer cells was investigated via Co-immunoprecipitation.

Results: GPT2 was up-regulated in TNBC. Exosomes were isolated effectively from TNBC cells, and confirmed that GPT2 was overexpressed inexosomes. QRT-PCR showed that mRNA expression levels of ICAM1, VCAM1, and MMP9 in TNBC were high. Exosomal GPT2 derived from TNBC enhanced migration and invasion of breast cancer via in vitro cell experiment and in vivo animal model experiment. Exosomal GPT2 binds with BTRC to degrade p-lkBa, and improved metastasis of breast cancer cells.

Conclusion: We demonstrated that GPT2 was upregulated in TNBC as well as in exosomes derived from triple-negative breast cancer (TNBC) cells. GPT2 expression was associated with the malignancy of breast cancer and promoted metastasis of breast cancer cells. Moreover, exosomal GPT2 derived from TNBC cells was verified to increase the capacity of breast cancer cells to metastasize through activating beta-transducin repeat containing E3 ubiquitin protein ligase (BTRC). This suggested that exosomal GPT2 may be useful for breast cancer patients as a potential biomarker and treatment target.

KEYWORDS
breast cancer, BTRC, exosomal GPT2

INTRODUCTION

Breast cancer (BC) is one of the most commonly diagnosed cancers among women worldwide accounting for 30% of female cancers.[1] A major factor in death from breast cancer is high metastasis and recurrence. About one-third of breast cancer patients develop distant metastasis to vital organs via the circulation. Bone, lung, liver, and brain are common

target sites of breast cancer metastasis.[2,3] Inflammatory factors, chemokines, and cell adhesion molecules have been reported to enhance cancer metastasis. For example, circEZH2 upregulated KLF5 through adsorbing miR-217-5p, thus leading to activate CXCR4 transcriptionally to induce epithelial and mesenchymal transition in breast cancer.[4,5]

Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype defined by the lack of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expression.[6] Chemotherapy is the only option for the treatment of TNBC. Intratumor heterogeneity leads to drug resistance and tumor recurrence, resulting in poor prognosis in patients with TNBC.[7] Therefore, understanding the mechanism of metastatic heterogeneity of breast cancer is essential.

Intercellular communication is of great importance in tumor progression and metastasis. Exosomes (30–150 nm in diameter) are nano-sized vesicles secreted by many kinds of cells carrying various bioactive molecules, such as lipids, proteins, and nucleic acids.[8,9] Exosomes are considered as mediators to regulate metastasis because they transport biomolecules to recipient cells in the tumor microenvironment or at specific distant sites.[10–12] Sun et al. reported high S100A4 expression in exosomes isolated from highly metastatic hepatocellular carcinoma cells (HCC). Exosomal S100A4 increased the capacity of metastasis in HCC through activating STAT3, indicating exosomal S100A4 may be a treatment target.[13] Exosomal ENO1 has been reported to regulate integrin α6β4 expression and promote HCC growth and metastasis.[14] However, there have been few reports about the intracellular crosstalk via exosomes between breast cancer cells with different subtypes.

Glutamate pyruvate transaminase 2 (GPT2) encodes a mitochondrial alanine transaminase, a pyridoxal enzyme that catalyzes the reversible transamination between alanine and 2-oxoglutarate to generate pyruvate and glutamate. It is known that GPT2 is widely expressed in certain cancers including breast cancer and promotes tumorigenesis and stemness of breast cancer cells.[15,16] However, whether exosomal GPT2 participates in breast cancer progression remains unknown.

Our findings in the study suggest that exosomal GPT2 derived from TNBC cells enhanced the metastatic potential of breast cancer cells through activating beta-transducin repeat containing E3 ubiquitin protein ligase (BTRC), indicating that exosomal GPT2 may be a novel biomarker and therapeutic target to treat breast cancer.

METHODS

Cells and reagents

Human breast cancer cells BT549 and BT474 were obtained from ATCC. Cells were grown in Dulbecco's modified Eagle medium (DMEM) (Gibco, C11885500BT) with 10% fetal bovine serum (FBS) (Gibco, 16170078) and 1% penicillin–streptomycin (Adamas life, C8021). Cells were incubated at 37°C with 5% CO_2. The following antibodies were employed in the study: GPT2 (16757-1-AP, Proteintech), BTRC (37-3400, Invitrogen), IgG (30000-0-AP, Proteintech) and β-actin (81115-1-RR, Proteintech).

Isolation and characterization of exosomes

Sequential ultracentrifugation was employed to collect the exosomes. BT549 and BT474 cells were grown in DMEM with 10% exosome-free FBS for 72 h. When the cell confluence reached about 80%, the supernatants were centrifuged in the sequence of 300 × *g* for 10 min, 2000 × *g* for 15 min, and 10 000 × *g* for 30 min. The supernatants were then filtered and the exosomes collected by 100 000 × *g* ultracentrifugation for 60 min. We resuspended the pellets in 5 mL phosphate buffered saline (PBS) and repeated 100 000 × *g* ultracentrifugation for 60 min to collect the exosomes. We adopted transmission electron microscopy, nanoparticle tracking analysis and western blots (WB) to verify the morphology, size and markers of the exosomes.

Transwell assay

A cell density of 10^5 cells per well was used for plating cells into the top chambers. The media in the top chambers was serum-free. Then, 10% FBS was added as supplements to the media in the lower chambers. The following day, cells in the lower chambers were gently cleaned with PBS before fixing with 4% paraformaldehyde. Then, cells on the upper layer of membrane were carefully wiped off with a cotton swab. Crystal violet was used to stain the cells. A microscope was used to visualize images.

Wound healing assay

A cell density of 10^6 cells per well was used for plating cells into six-well plates. When the cell confluence reached about 90%, we scratched the cell monolayer using a pipette tip. The cells were gently cleaned with PBS. Images were visualized at 0, 24, and 48 h.

Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent and transcribed into cDNA following All-in-one reverse transcription kit instructions (G8041-0100, Adamas life). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR Green qPCR Mix (D7262, Beyotime) with a 7500 fast real-time PCR system. The primers used in the study were as follows: ICAM1 forward primer: ACGGAGCTCCCAGTCCTAAT, ICAM1 reverse primer:

CTCCTTCTGGGGAAAGGCAG, VCAM1 forward primer: GGACCACATCTACGCTGACA, VCAM1 reverse primer: TTGACTGTGATCGGCTTCCC, MMP9 forward primer: TCTATGGTCCTCGCCCTGAA, MMP9 reverse primer: CATCGTCCACCGGACTCAAA.

Western blot

Radioimmunoprecipitation assay (RIPA) buffer was used to lyse the cells. Protein concentration was determined by a bicinchoninic acid assay (BCA) protein quantity kit

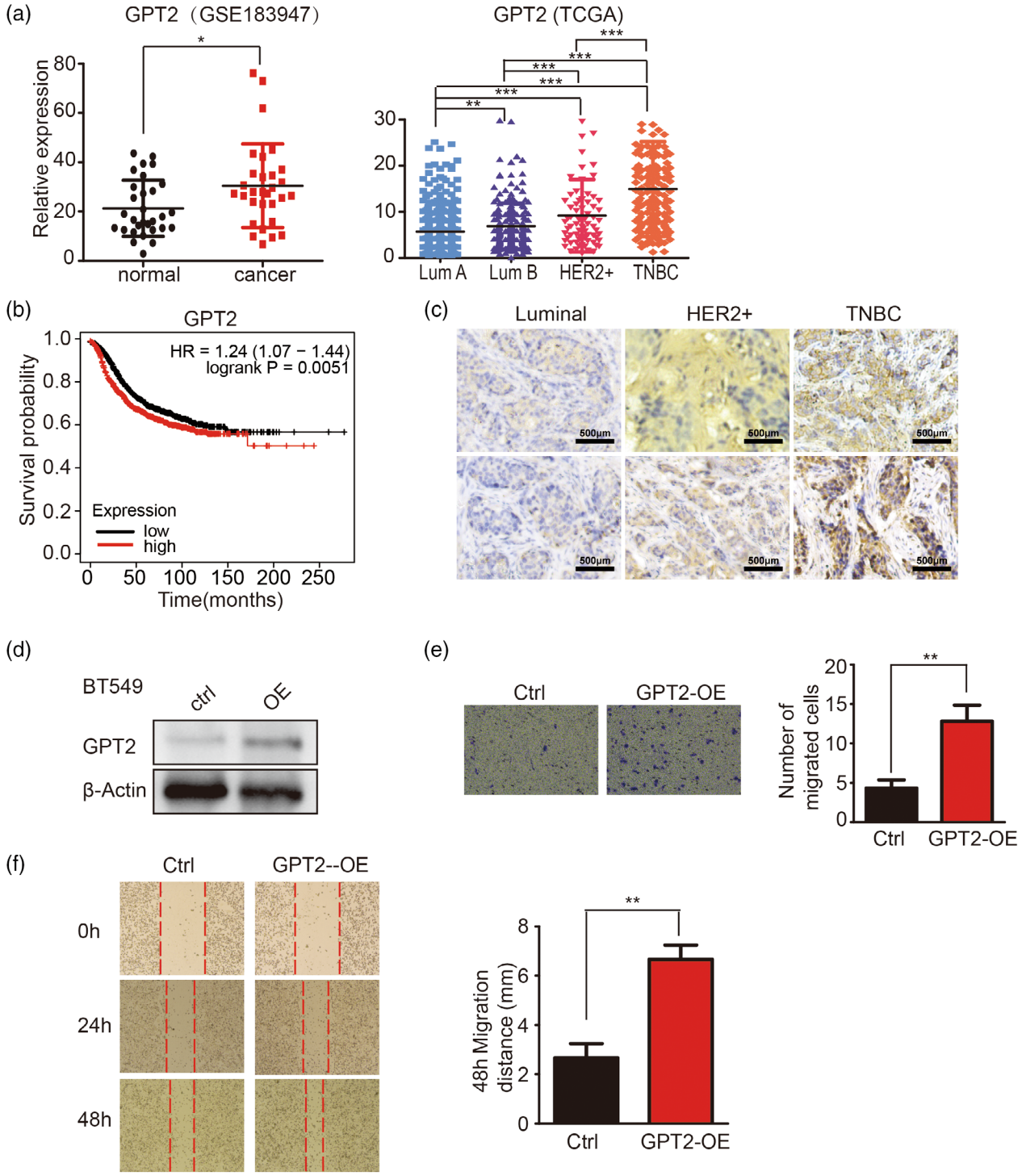

FIGURE 1 Glutamate pyruvate transaminase 2 (GPT2) is upregulated in triple-negative breast cancer (TNBC). (a) Diagrams showing GPT2 expression according to GSE183947 database and TCGA databases. (b) Kaplan–Meier analysis of overall survival on GPT2 expression in breast cancer patients. (c) Immunohistochemistry staining of GPT2 in breast cancer samples. Scale bar = 500 μm. (d) A stably overexpressed GPT2 breast cancer cell line detected by western blot. (e) The effect of GPT2 overexpression on migratory capacity by wound healing assay in breast cancer cells (**$p < 0.01$). (f) The effect of GPT2 overexpression on invasive capacity by transwell assay in breast cancer cells (**$p < 0.01$).

(E8053-500 T-PKG, Adamas life). To separate the proteins, sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Then, protein gels were transferred to polyvinylidene difluoride (PVDF) membranes. We used 5% nonfat milk to block the PVDF membranes to avoid nonspecific protein interference. Gels were incubated with the specific primary antibody overnight. After incubation with secondary antibody, gels were visualized using a Tanon 4600 chemiluminescence imaging system.

Immunohistochemistry

Formalin was used to fix the tumor tissues, which were then selected and embedded for immunohistochemistry. The slices were subjected to hydration in the order of xylene, 100% alcohol, 95% alcohol, and 75% alcohol. Slices were treated with 10% goat serum to avoid nonspecific protein interference following heat-mediated antigen repair. Specific antibody was added to the slices and they were incubated at 4°C overnight. The next day, after washing gently with PBS, horseradish peroxidase (HRP)-conjugated antibody was added to the slices, which were subsequently visualized with a 3,3'-diaminobenzidine (DAB) substrate.

Coimmunoprecipitation

Cells were lysed using single detergent cleavage solution. The samples were incubated with nonspecific IgG or anti-BTRC antibody overnight. The samples were examined using WB.

Animal experiments

Four-week-old female BALB/c nude mice were purchased from Shanghai Animal Laboratory. All mice were raised in a specific pathogen-free (SPF) environment with sterilized feed and water. All animal experiments were approved by Shanghai Jiao Tong University's institutional Animal Use and Care Committee. Approximately 1×10^6 cells were suspended in 50 μL PBS or 50 μL exosomes (50 μg/mL) and were injected into the tail vein. Livers of the mice were removed from mice sacrificed 4 weeks after the injection of tumor cells.

Statistical analysis

All results are expressed as means ± SD. A two tailed student's *t*-test was used to evaluate the variation across groups. *p*-values < 0.05 were considered statistically significant.

RESULTS

GPT2 is upregulated in TNBC

The GSE183947 GEO database showed higher expression of GPT2 in breast cancer than normal tissues. The TCGA databases showed higher expression of GPT2 in TNBC than other subtypes of breast cancer (Figure 1a). Kaplan–Meier survival analysis also showed an inverse relationship between GPT2 expression and breast cancer prognosis, with patients with lower GPT2 expression having a better prognosis ($p = 0.05$, Figure 1b). In addition, our immunohistochemistry data found

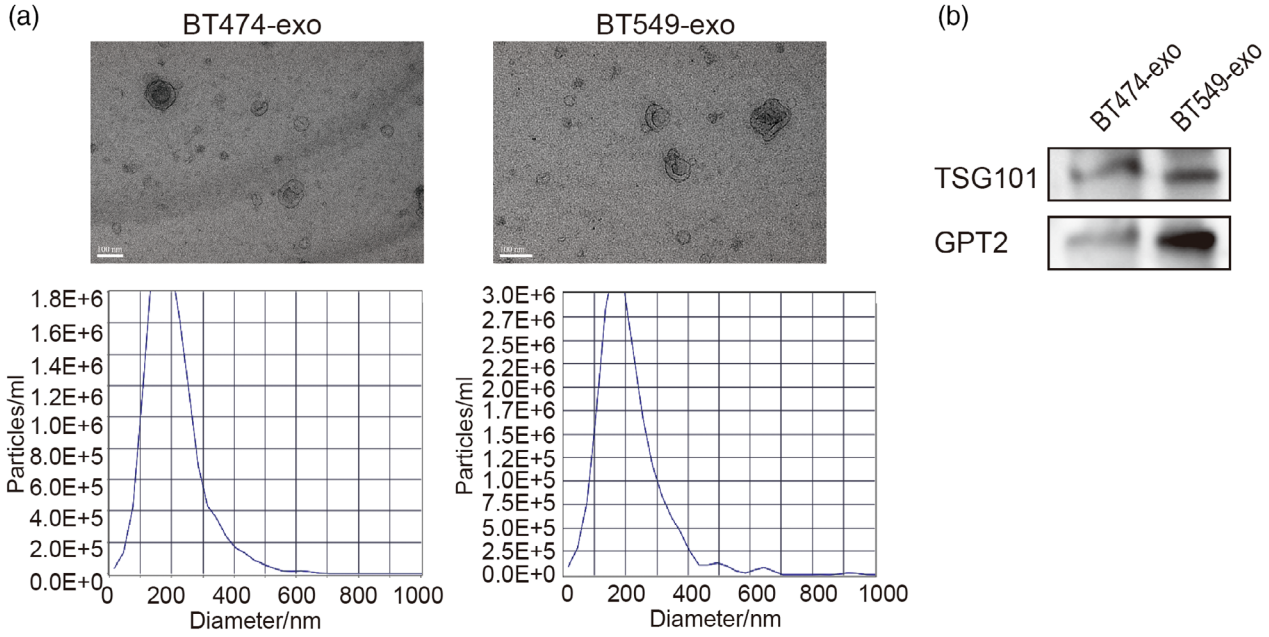

FIGURE 2 Isolation and identification of exosomes. (a) Transmission electron microscopy and NTA analysis of exosomes derived from BT474 and BT549. Scale bar = 100 nm. (b) Western blot analysis of exosomal positive protein marker TSG101 and glutamate pyruvate transaminase 2 (GPT2).

that GPT2 was significantly upregulated in TNBC samples compared with other subtypes of breast cancer tissues (Figure 1c). These findings suggest that GPT2 is upregulated in TNBC.

It has been reported that GPT2 promoted tumorigenesis and stemness in breast cancer in previous research.[15,16] We speculated that GPT2 may play other roles in cancer progression. To investigate whether GPT2 promotes cancer metastasis, we constructed a stably overexpressed GPT2 cell line and conducted functional experiments (Figure 1d). Wound healing assay indicated that GPT2-OE cells had greater migratory capacities than control cells (Figure 1e). Transwell assay also indicated that GPT2-OE cells were more aggressive (Figure 1f). The results suggest that GPT2 promotes metastasis of breast cancer cells.

Isolation and identification of exosomes

To study GPT2 expression in breast cancer cells-derived exosomes, we isolated exosomes by differential ultracentrifugation

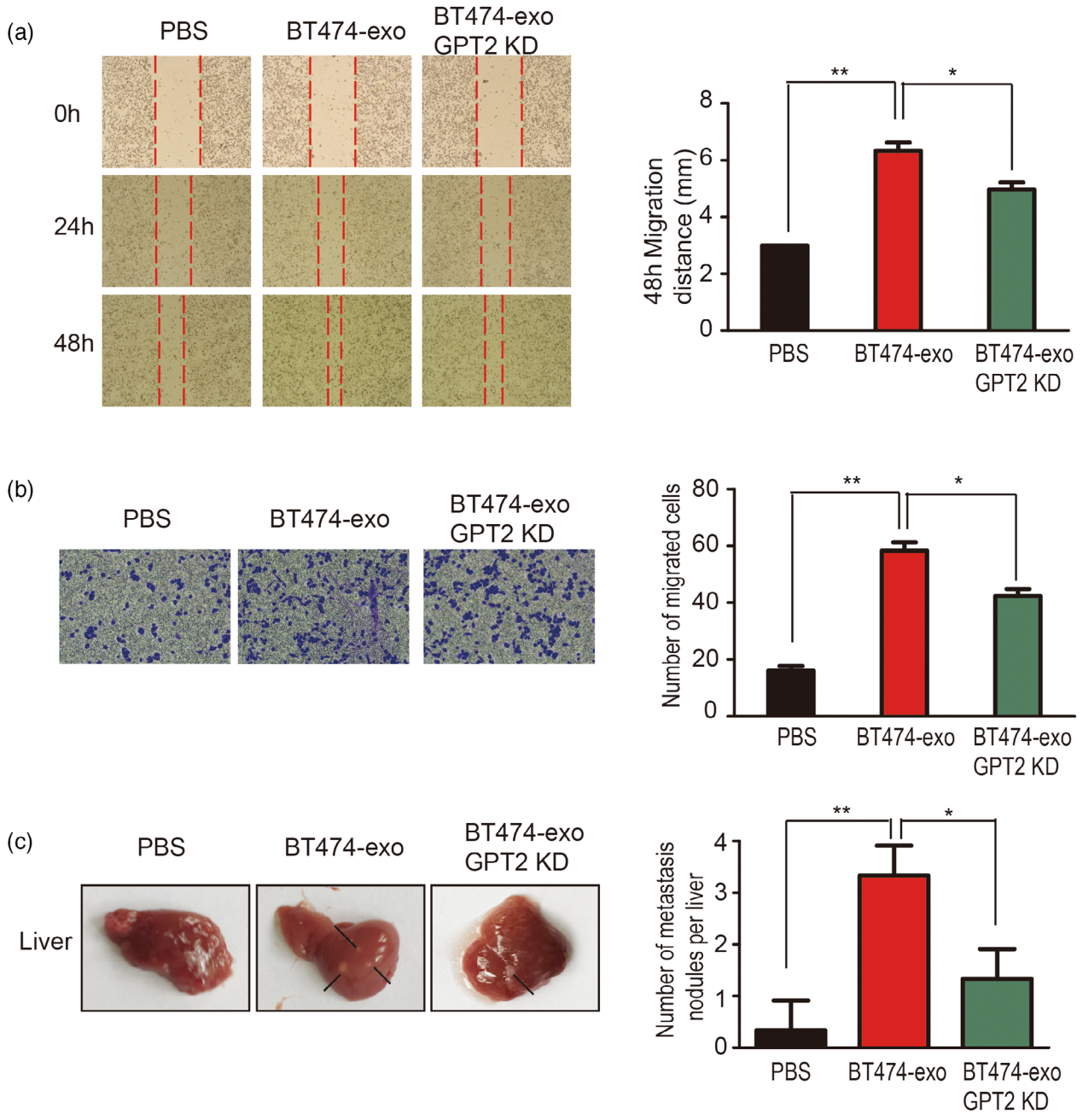

FIGURE 3 Exosomal glutamate pyruvate transaminase 2 (GPT2) enhances the metastatic capacity of breast cancer cells. (a) Wound healing assay of BT474 and BT474-shGPT2 treated with BT549-exosomes (50 μg/mL) (**$p < 0.01$; *$p < 0.05$). (b) Transwell assay of BT474 and BT474-shGPT2 treated with BT549-exosomes (50 μg/mL) (**$p < 0.01$; *$p < 0.05$). (c) Exosomal GPT2 promoted liver metastasis of BT474 cells. Metastatic sites in the liver decreased in BT474-shGPT2 cells treated with BT549-exosomes (50 μg/mL) (**$p < 0.01$; *$p < 0.05$).

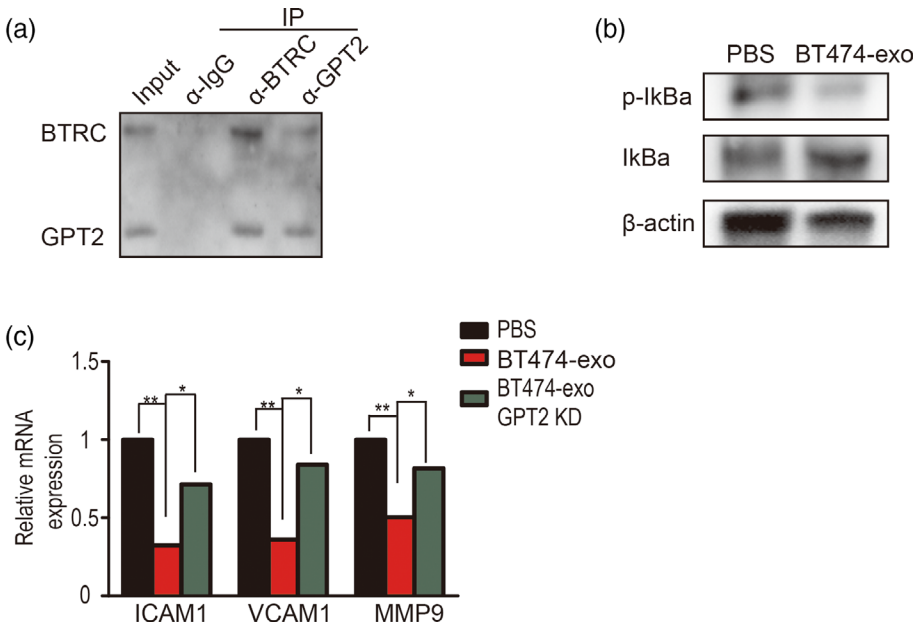

FIGURE 4 Glutamate pyruvate transaminase 2 (GPT2) binds with beta-transducin repeat containing E3 ubiquitin protein ligase (BTRC) to degrade p-IkBa. (a) BTRC detection following coimmunoprecipitation of GPT2. (b) Western blot analysis of p-IkBa. (c) Quantitative real-time polymerase chain reaction (qRT-PCR) showed mRNA expression of ICAM1, VCAM1, and MMP9 (**$p < 0.01$; *$p < 0.05$).

from luminal breast cancer cell BT474 and TNBC cell BT549. From the perspective of morphology, exosomes showed a typical cup-shaped structure through transmission electron microscope (Figure 2a). From the perspective of size, exosomes were around 150 nm in diameter through nanoparticle tracking analysis (Figure 2a). Furthermore, we detected specific exosomal marker TSG101 to verify exosomes. Western blot results showed that GPT2 expression was higher in BT549-exosomes than in BT474-exosomes (Figure 2b). Therefore, we successfully isolated exosomal GPT2 from BT549 cells and continue to explore the role of exosomal GPT2 in cancer metastasis.

Exosomal GPT2 enhances the metastatic capacity of breast cancer cells

As GPT2 promotes metastasis of breast cancer cells, we speculated that exosomal GPT2 may mediate cell-to-cell communication in cancer metastasis. A luminal breast cancer cell BT474 cell line with relatively low metastatic potential was used to explore the effect of exosome education. We used BT549-exosomes to pretreat BT474 for 24 h. After pretreatment, wound healing and transwell assays were carried out. It was noted that BT474 cells treated with BT549-exosomes had greater migratory and invasive capacities than those treated with PBS. Meanwhile, we knocked down GPT2 in BT474 cells treated with BT549-exosomes. Consequently, the abilities of migration and invasion were both attenuated (Figure 3a,b).

To determine whether exosomal GPT2 plays a vital role in breast cancer progression in vivo, we used animal models that received tumor cells via tail vein injections. As expected, liver metastases were detected in the BT549-exosomes-educated group compared with the PBS-educated group. GPT2 knockdown in the BT549-exosomes-educated group decreased liver metastases (Figure 3c). These findings suggest that exosomal GPT2 has a crucial role in breast cancer cell metastasis. Exosomal GPT2 derived from TNBC could enhance migration and invasion in vitro and in vivo.

GPT2 binds with BTRC to degrade p-IkBa

Next, we sought to identify proteins that associate with GPT2 to promote cell migration from the online database (thebiogrid.org). Among the candidate proteins binding GPT2, BTRC targeting IκBα for ubiquitination and degradation was reported to drive migration and invasion in TNBC.[17] To validate the association of GPT2 with BTRC, we collected BT474 cell lysates treated with GPT2-exosomes and performed a coimmunoprecipitation assay. As shown in Figure 4a, BTRC coimmunoprecipitated with GPT2, indicating BTRC forms a complex with GPT2. Then, BTRC was knocked down in BT474 cells treated with GPT2-exosomes to verify whether BTRC participates in metastasis. GPT2 interacted with BTRC to decrease the protein level of p-IκBα evidenced by Figure 4b. It is reported that degradation of IκBα leads to nuclear translocation of NF-kB complexes. Therefore, we detected NF-kB regulated genes by qRT- PCR. Metastasis-related genes including ICAM1, VCAM1, and MMP9 were significantly upregulated in BT474 cells treated with GPT2-exosomes. BTRC knockdown showed an opposite trend (Figure 4c), suggesting that GPT2 interacts with BTRC to promote cell migration and invasion.

DISCUSSION

TNBC is a highly heterogeneous disease with high recurrence rates, high incidence of distant metastases, and poor

overall survival.[6,18] Breast cancer metastasizes to bone, lung, liver, and brain.[3] Although scientists have improved therapeutic strategies by studying the molecular mechanisms of metastasis, there is still a need to study tumor heterogeneity and explore in-depth mechanisms to provide novel insight for the effective treatment of TNBC.

It has been reported that GPT2 contributes to cancer development as a metabolic enzyme.[19,20] The abnormal expression of GPT2 decreased cellular effective α-ketoglutarate to sustain the tricarboxylic acid cycle anaplerosis, thus promoting cell survival and proliferation.[21,22] GPT2 has also been reported to promote tumorigenesis in breast cancer.[15,16] As reported here, we found that GPT2 expression was positively correlated with the malignancy of breast cancer. High GPT2 expression predicted a worse survival, suggesting that GPT2 may serve as a biological marker of breast cancer prognosis. Moreover, we showed that overexpression of GPT2 promoted metastasis of breast cancer cells. In the present study, our results indicate a positive role of GPT2 in breast cancer metastasis.

Growing evidence indicates that exosomes play indispensable roles in intercellular communication through transporting their cargo, including nucleic acids, proteins and metabolites to corresponding cells.[23,24] For example, it has been previously reported that exosomes isolated from breast cancer cells could damage vascular endothelial barriers and promote metastasis.[25] Given the crucial role of exosomes in cancer progression, we consider that exosomal GPT2 may participate in cancer metastasis. Our present study was intended to determine the function of exosomal GPT2 in breast cancer metastasis. Consistently with our speculation, GPT2 expression was higher in exosomes from TNBC cells than exosomes from luminal breast cancer cells. In our study, GPT2 expression was upregulated in luminal breast cancer cells when educated with exosomes derived from TNBC cells. Exosomal GPT2 increased the metastatic capabilities of breast cancer cells validated by wound healing and transwell assays. Moreover, animal experiments were performed to confirm that exosomal GPT2 promoted liver metastasis in vivo. Consequently, exosomal GPT2 derived from TNBC can act as tumor promoters and instigate cancer metastasis.

GPT2 is reported to regulate cancer progression as a metabolic enzyme in previous studies. Our findings verify the interaction between GPT2 and BTRC by co-IP. Beta-transducin repeat containing E3 ubiquitin protein ligase (BTRC) belongs to the F-box and WD40 repeat family of proteins and negatively regulates EMT-related protein.[26] Recognition by BTRC stimulates IκBα ubiquitination and induces subsequent proteasomal degradation of the substrate, hence facilitating the release of NF-kB dimers into the nucleus.[27,28] Our findings revealed that GPT2 could bind to BTRC and degraded IκBα, thus activating the transcription of metastasis-related genes, which explains how exosomal GPT2 engages in cancer metastasis. The limitation of our study was the way that GPT2 binds with BTRC needs further investigation.

In conclusion, our study demonstrated that exosomal GPT2 derived from TNBC promotes the metastatic potential of breast cancer cells by activating BTRC, indicating exosomal GPT2 may be a novel biomarker and therapeutic target to treat breast cancer patients.

AUTHOR CONTRIBUTIONS

Mingqing Cui finished main experiments, and wrote the primary manuscript; Jiawei Peng finished PCR primer designing and QRT-PCR experiment; Yuanyuan Zhou finished the cell culture and exosome isolation; Xixi Wang was responsible for reagent purchase and animal model experiment; Daxiang Cui Put forward the research content and designed this experiment.

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CONFLICT OF INTEREST STATEMENT

The author reports no conflicts of interest in this work.

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