Effects of Glut1 gene silencing on proliferation, differentiation, and apoptosis of colorectal cancer cells by targeting the TGF-β/PI3K-AKT-mTOR signaling pathway

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Abstract
This study aims to investigate the effects of glucose transport 1 (Glut1) gene on proliferation, differentiation, and apoptosis of colorectal cancer (CRC) cells by regulating the TGF-β/PI3K-AKT-mTOR signaling pathway. Immunohistochemistry was conducted to detect the positive Glut1 expression. Normal human CRC epithelial cells (CCD-18Co) and CRC cell line HCT116 were grouped into the control, blank, negative control (NC), and shGlut1-1 groups. RT-qPCR and Western blotting were performed to detect the expressions of Glut1, TGF-β1, PI3K, AKT, PTEN, mTOR, Bcl-2, and Bax. Protein expression of phosphorylated-PI3K (p-PI3K), p-S473-AKT, p-S389-S6K1, p-T70-4EBP1, Cleaved caspase-3 and Cleaved-PARP were detected. MTT assay, flow cytometry, and colony formation assay were performed in order to detect cell viability, cell cycle, and apoptosis, respectively. The positive expression rate of Glut1 in CRC tissues was 75% ± 8%, while in the adjacent normal tissues it was 0%. In comparison to adjacent normal tissues, CRC tissues had increased Glut1, TGF-β1, PI3K, AKT, mTOR, and Bcl-2 expressions, and p-PI3K, p-S473-AKT, p-S389-S6K1, and p-T70-4EBP1 expressions; and decreased PTEN, Bax, Cleaved caspase-3, and Cleaved-PARP expressions. In comparison with the blank and NC groups, cells in the shGlut1-1 group showed decreased Glut1, TGF-β1, PI3K, AKT, mTOR, and Bcl-2 expressions, and p-PI3K, p-S473-AKT, p-S389-S6K1, and p-T70-4EBP1 expressions; and increased PTEN, Bax, Cleaved caspase-3, and Cleaved-PARP expressions; along with more arrested cells in C0/C1 phase than in S phase and slower cell growth. These results suggested that silencing the Glut1 gene inhibited proliferation and promoted apoptosis of CRC cells by inactivating TGF-β/PI3K-AKT-mTOR signaling pathway.

Keywords
apoptosis, colorectal cancer, Glut1 gene silencing, proliferation, TGF-β/PI3K-AKT-mTOR signaling pathway
1 | INTRODUCTION

Colorectal cancer (CRC) is the third greatest contributor of cancer-related deaths, which accounts for 10% morbidity and mortality of cancers related deaths all over the world.\(^1\) As one of the most common cancers in developed countries, CRC has a 5-year survival rate of about 60%.\(^2\) Various prognostic factors (clinical and pathological) have been proposed for CRC, such as tumor size, differentiation of tumor, location of tumor, distant metastasis, depth of invasion, and lymph node metastasis.\(^3\) Presently, the major treatment options for CRC are radiotherapy, chemotherapy, and surgery.\(^4\) Although there has been significant improvement in CRC treatment, yet CRC still remains as a crucial public health concern, resulting in approximately 608,000 deaths per year.\(^5\) Therefore, in depth researches on CRC are of high clinical value.

Glucose transporters play a critical role in supplying sugars to metabolically active cells, and glucose transporter protein-1 (Glut1), the most prominent glucose transporter, has a significant role in cancer development stages including early tumor growth, cancer metastasis, and invasiveness.\(^6,7\) Glut1 is a member of homologous sugar transporters or cotransporters family, which has been discovered in both eukaryotes and prokaryotes.\(^8\) The elevated expression or activation of Glut1 has been demonstrated to be the main contributor in many malignancies.\(^9\) Furthermore, Glut1 was reported to play a vital part in the treatment of CRC involving tumor-node-metastasis (TNM) stage, subtype, therapeutic schedule, and early-stage diagnosis.\(^10\) Transforming growth factor beta (TGF-\(\beta\)) is a secreted ligand that has been intimately linked to the regulation of tumor initiation, progression, and metastasis.\(^11\) The TGF-\(\beta\) signaling pathway exhibits a crucial effect on controlling cell proliferation and differentiation of CRC.\(^12\) Congruently, it has been reported that the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K-AKT-mTOR) signaling pathway is closely associated with resistance to apoptosis, cells proliferation, angiogenesis, and metastasis, which contributes to the development and maintenance of CRC.\(^13\) Currently, the activation of AKT signaling and impaired expression of AKT regulator has been reported in about 60-70% of CRC cases, indicating that the inhibitors of PI3K-AKT signaling can be considered as potential therapeutic agents in CRC.\(^14\) mTOR is a direct substrate for AKT kinase which promoted protein translation, growth, angiogenesis, and metabolism, and inhibited mTOR represses cell growth and induced apoptosis of CRC.\(^13\) Moreover, the inhibition of mTOR signaling pathway has also reported to induce autophagy in colorectal cancer cells.\(^14\) The role of Glut1 gene involving TGF-\(\beta\)/PI3K-AKT-mTOR signaling pathway has not investigated. Therefore, this study aims to explore the effects of Glut1 gene silencing-mediated TGF-\(\beta\)/PI3K-AKT-mTOR signaling pathway on CRC cells with hope to find a novel therapeutic strategy for CRC patients.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

The study was performed with the approval of the ethics committee of the First Affiliated Hospital of Hebei North University, and all experiments were conducted in accordance with the Helsinki Declaration. Informal signed consents were acquired from the participating patients or their families before specimen collection.

2.2 | Study subjects

A total of 50 patients with primary CRC who underwent surgery in the First Affiliated Hospital of Hebei North University from January 2015 to January 2017 were enrolled in our study. CRC tissues and adjacent normal tissues were collected from these patients. All participating CRC patients were diagnosed by the fourth edition of definition of CRC in Pathology and Genetics of Tumors of the Digestive System in 2010 released by the World Health Organization (WHO), and the First Affiliated Hospital of Hebei North University provided their complete clinical data. There were 26 males and 24 females aging from 35 to 78 years (mean age: 61.96 ± 16.26). There were 15 cases in TNM stage I, 18 cases stage II, 8 cases stage III, and 9 cases stage IV.\(^15\) There were 24 cases in which tumor diameter was >5 cm, 26 cases ≤ 5 cm; 30 cases with lymph node metastasis (LNM) and 20 cases without; 36 cases with well/moderate differentiation and 14 cases with poor differentiation; 37 cases with venous invasion and 13 cases without. Inclusion criteria were as follows: CRC patients confirmed by Pathology and Genetics of Tumors; the tumor could be surgically resected after clinical evaluation; patients were able to undergo abdominal surgery or thoracic surgical procedures; patients without any other digestive tumors or tumor history: patients did not undergo chemotherapy or radiotherapy before surgery.\(^16\) Exclusion criteria were as follows: patients with severe injury of vital organs like heart, liver, and kidney; patients with a history of autoimmune diseases; patients with chronic infectious diseases, or acute infectious diseases.

2.3 | Immunohistochemistry

Frozen experimental specimens (CRC tissues and adjacent normal tissues) were acquired, after which they were fixed with 10% formalin, embedded in paraffin and sliced into 3-4-µm-thick serial sections. Paraffin sections were placed in 3% H\(_2\)O\(_2\), followed by dewaxing with xylene I and II (each for
10 min), dehydration with 100, 95, 80, and 70% gradient ethanol (each for 2 min), followed by washing with distilled water twice (each for 5 min) on a swing table. The sections were submerged in 3% H2O2 for 10 min after which they were washed with distilled water. After high-pressure antigen repair for 90 s and cooling at room temperature, the sections were washed with phosphate-buffered saline (PBS). Drops of 5% bovine serum albumin (BSA) blocking solution were added and the sections were incubated at 37°C for 30 min. This was followed by addition of mouse anti-human Glut1 (NBPI-35926, Shanghai Xuan Ling Biotec Ltd., Shanghai, China) monoclonal antibody (1:100) at 4°C overnight, the sections were incubated with secondary antibody of biotinylated goat anti-mouse IgG labeled by horse radish peroxidase (HRP) (No.: SE134; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 30 min, and nucleus was re-stained with hematoxylin (No. C0105, Beyotime Biotechnology Co., Ltd., Shanghai, China) monoclonal antibody (1:100) at 4°C overnight, the sections were incubated with secondary antibody of biotinylated goat anti-mouse IgG labeled by horse radish peroxidase (HRP) (No.: SE134; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) for 30 s, then the sections were treated with DAB (No. P0202; Beyotime Biotechnology Co. Ltd.). After dehydrated by hydrochloric acid ethanol to clear, sections were mounted with gum and then observed and photographed under the microscope. The criteria for assessing the immunohistochemical staining results were as follows:17 the cells with obvious brown or brownish yellow granules in cytoplasm and the staining degree more than 25% were considered as Glut1 positive cells. Positive expression rate = the number of positive cells/total cells. The experiment was repeated three times.

2.4 | Cell culture

Normal human CRC epithelial cells (CCD-18Co, ATCC, Shanghai Yong Chuang Biotechnology Co., Ltd., Shanghai, China) and human CRC cell line HCT116 (Shanghai Institute of Biotechnology, Shanghai, China) were cultured in RPMI1640 medium containing 10% fetal bovine serum (FBS, 22400089, Gibco, Grand Island, NY). Cells were seeded in a six-well plate at a density of $1 \times 10^5$/well and incubated at 37°C under 5% CO2 incubator conditions along with saturated humidity. Culture medium was replaced every 2-3 days according to the condition of cell growth. The cells were subcultured when they reach confluence close to 80-90%. The medium was discarded, and the cells were washed with PBS twice, digested with 0.25% trypsin for 2-5 min, resuspended with 5 mL Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% FBS, after which subculture was performed again.

2.5 | Plasmid construction

According to mRNA sequence of Glut1 (No. 005983) reported in GenBank, the shRNA sequence was designed according to the target sequence from the Glut1 encoding region, and the BLAST software detected no homology with other unrelated genes. In accordance with the requirements of the Pg-Pu6/Neo vector, the oligonucleotide arrays (Table 1) that can encode siRNA were designed and all oligonucleotide fragments were synthesized by Shanghai Sangon Biotech Company (Shanghai, China). The vector of pSIREN (NO.: HAB 2-9, Beijing Hua Ao Bio Technology Co., Ltd., Beijing, China) linearized by BamHI and EcoRI were inserted into it, and after annealing, it was transformed into Escherichia coli through recombinant plasmid pEGFP-C2. The colonies were selected and cultured for amplification. The plasmid was prepared promptly in small amounts to detect nucleic acid sequencing detection. The cells with correct sequence were selected for clonal expansion.

2.6 | Cells transfection and grouping

Cells in the logarithmic growth phase were selected. The collected cell suspension was inoculated in a six-well plate ($5 \times 10^4$ cells/well) with fresh complete medium, and were then transfected until cells reached confluence between 50-80%. Liposome transfection was conducted according to the manufacturer’s instructions of lipofectamin 2000 (No.: 11668-027, Invitrogen, Inc., Carlsbad, CA). The cells were divided into control group (normal human colon epithelial cells CCD-18Co), blank group (human CRC cell line HT29), negative control (NC) group (CRC cells transfected with unrelated siRNA sequences), shGlut1-1 group (CRC cells transfected with shGlut1-1 sequence), shGlut1-2 group (CRC cells transfected with shGlut1-2 sequence), and shGlut1-3 group (CRC cells transfected with shGlut1-3 sequence). Before transfection, cells in the logarithmic growth phase were seeded in a six-well plate. When cell density reached 80-90%,

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>shGlut1-1</td>
<td>Forward: 5′-CACCCGGAGTGACAAAGACTTTTGTCAAGGCA-3′</td>
</tr>
<tr>
<td>shGlut1-2</td>
<td>Forward: 5′-ACAAAGTCCAGATTTGTCAAGGAC-3′</td>
</tr>
<tr>
<td>shGlut1-3</td>
<td>Forward: 5′-TTCTAAGCGCATGTCATCAAGTCTGCA-3′</td>
</tr>
<tr>
<td>Negative  control</td>
<td>Forward: 5′-ATCCGACTTCAATAAGCGCAGC-3′</td>
</tr>
</tbody>
</table>
the cells were incubated in a serum-free medium Opti-MEM (Gibco, Gaithersburg, MD), and then transfected according to lipofectamin 2000 instructions (Invitrogen, Inc.). Lipo solution, total volume of 250 µL with 240 µL of serum-free medium and 10 µL of lipo, was incubated at room temperature for 5 min. Plasmid solution was of 24 µL, out of which 20 µL was serum-free medium, and 4 µL was plasmid. The abovementioned two solutions were mixed and placed at room temperature for 20 min, and then the mixture was dripped by droplet method into the well, and the culture plate was shaken for even mixing. Cells were incubated at 37°C with 5% CO2 for 5-6 h, and cultured in complete medium for 24-48 h for the following experiment.

2.7 Immunofluorescence assay

Cells were seeded into a six-well plate, 2 mL of complete medium was added, and incubated at 37°C with CO2 overnight. When cell density reached 50-80%, the solution was prepared in a sterilized centrifugation tube as follows: solution A: 4 µg pure DNA which had to be transfected was diluted in 250 mL serum-free medium standing for 5 min; solution B: 2-25 mL Lipofectamine was diluted in 250 mL serum-free medium, standing for 5 min. Solutions A and B were gently mixed for an even mixture and placed at room temperature for a time period of 15-45 min. The cells were washed with 2 mL serum-free medium, and 0.8 mL serum-free medium was added to each well. Liposome complex was added into wells, gently mixed, and incubated at 37°C with CO2 for 20-24 h. After DAPI (No.: C1005, Beyotime Biotechnology Co., Ltd.) incubation for 30 min devoid of light, the transfection solution was replaced with complete medium Opti-MEM (Gibco, Gaithersburg, MD), and total RNA was extracted according to the Trizol method and three replicates were made for each gene of each sample. The experiment was repeated three times.

2.8 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RT-qPCR was performed in order to detect Glut1 expression in the shGlut1-1, shGlut1-2, and shGlut1-3 groups. The CRC tissues and adjacent normal tissues (each for 30 mg) were added with 1 mL Trizol reagent (Invitrogen), crushed in an ice bath, and total RNA was extracted according to the Trizol method (this method has been also performed in subsequent cell experiments to extract the total RNA of cells in each group). Ultraviolet spectrophotometry (UV1901, Shanghai Science Instrument Co., Ltd., Shanghai, China) was employed to detect RNA purity and concentration in tissues and cells. The purity of all samples was A260/A280 = 1.8-2.0.

Next, the concentration of all samples was adjusted to 0.1 g/µL. RNA was reversely transcribed into cDNA (50 ng/µL) using the PrimeScript™ RT reagent Kit (Takara, RR047A, Beijing Think-Far Technology Co., Ltd, Beijing, China) in 10 µL system according to the instructions. Reaction conditions were as follows: 37°C, 15 min for three times (RT reaction), 80°C for 5 s (reverse transcriptase inactivation reaction) followed by freezing at −80°C. Primers were designed using the Premier 5.0 software and synthesized by TSINGKE Biological Technology Company (Beijing, China) (Table 2). The reaction was conducted on ABI 7900HT RT-qPCR instrument (ABI 7900, Shanghai PuDi Biotech Co., Ltd. Shanghai, China) by two-step approach. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was referred to as internal control. The reaction conditions were as follows: pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, annealing at 58°C for 30 s, and extension at 72°C for 15 s, 40 cycles. Relative mRNA expression was calculated by 2−ΔΔCt method and three replicates were made for each gene of each sample.

2.9 Western blotting

The 30 g of tissue samples obtained were grounded to uniform powder form at low temperature. The samples were washed

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences for RT-qPCR</th>
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<tbody>
<tr>
<td>Glut1</td>
<td>Forward: 5'-TCTCTGGTAAACGGAGATCAAAC-3′</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Forward: 5'-CCAAGGAACGGAATA-3′</td>
</tr>
<tr>
<td>PI3K</td>
<td>Forward: 5'-TGAGAGCGAGCAAGGTG-3′</td>
</tr>
<tr>
<td>Akt</td>
<td>Forward: 5'-GGAGCGGGAGAATTGAGGAA-3′</td>
</tr>
<tr>
<td>PTEN</td>
<td>Forward: 5'-CACCGCAAAATTTACAGCAG-3′</td>
</tr>
<tr>
<td>mTOR</td>
<td>Forward: 5'-TACGGCCTCTGACTCGAG-3′</td>
</tr>
<tr>
<td>Bax</td>
<td>Forward: 5'-GGGCAATTTGGAGATGACTG-3′</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Forward: 5'-GGGGAGAGTGGATACAGCAG-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5'-GGTGAAGGTCGGAGTCAACGG-3′</td>
</tr>
</tbody>
</table>

Glut1, glucose transporter 1; TGF-β1, transforming growth factor-beta; PI3K, phosphatidylinositol 3 kinase; PTEN, phosphatase and tensin homolog deleted on chromosome 10; mTOR, mechanistic target of rapamycin; Bax, Bcl-2 associated X protein; Bcl-2, B-cell lymphoma-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
with PBS twice, added with protein lysate, centrifuged at 4°C for 20 min at 12,000 r/min. The supernatant was collected. The total protein concentration was tested using bicinchoninic acid (BCA) protein assay kit (P0012-1, Beyotime Biotechnology Co., Ltd.). Cells in the logarithmic growth phase underwent centrifugation at 4°C for 20 min at 3000 r/min and the supernatant was removed. Packed cell volume (PCV) was estimated (volume of compacted cells after centrifugation). In total, 100 µL of lysate and 1 µL enzyme inhibitors (Beijing Jianmay Biotechnology Co., Ltd., Beijing, China) were added to each 20 µL of PCV sample, followed by ice splitting for 30 min. The samples were centrifuged for 10 min at 12,000 r/min at a low temperature. The supernatant was obtained in order to conduct quantitative detection of protein. A total of 50 µg protein was dissolved in 2× SDS sample buffer. After boiling at 100°C for 5 min, these samples were transferred to PVDF membrane by SDS-PAGE gel of 10% mass concentration and blocked with skimmed milk powder of 5% mass concentration at room temperature for 1 h. The primary antibodies of rabbit anti-human (Glut1: ab115730, 1:1000; TGF-β1: ab92486, 1:1000; PI3 K: ab182651, 1:2000; AKT: ab8805, 1:500; p-PI3 K: ab182651, 1:800; p-T308-AKT: ab38449, 1:1000; p-S473-AKT: ab8932, 1:1000; p-T386-S6K1: ab2571, 1:500; p-T389-S6K1: ab75831, 1:500; phosphatase and tensin homolog deleted on chromosome ten (PTEN): ab32199, 1:600; mTOR: ab87540, 1:500; Bax: ab32503, 1:800; Bcl-2: ab32503, 1:1000; Cleaved caspase-3: ab136812, 1:1000; and Cleaved-PARP: ab136812, 1:1000; Shanghai, China) were added for incubation. Samples were washed with TBST three times. The membrane and HRP labeled goat anti secondary antibody (1:5000; No.: SE134, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) were incubated for 1 h. After washing with TBST and ECL developing, X film exposure and photography were performed. Image Acquisition and Analysis System was employed for absorbance analysis of color banding (JS series, Shanghai Dobio Biotech Co., Ltd., Shanghai, China). The relative content of the sample proteins = average absorbance of samples/absorbance of internal reference. The experiment was repeated three times.

2.10 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

After 24 h of transfection, cells in logarithmic growth phase were used to prepare 2.5 × 10^3/mL cell suspension with RPMI 1640 medium containing 10% FBS, and then were seeded into a 96-well plate. Eight wells were set in each group (100 µL per well) and cells were incubated at 37°C with 5% CO₂. Culture plate was removed for observation at 24 h, 48 h, and 72 h, after which 10 µL of 5 mg/mL MTT solution (Sigma, St Louis, MO) was added to culture for 4 h. After culture, the supernatant was discarded, 150 µL of DMSO was added into each well and then shaken for 10 min for complete dissolution. The OD value was measured at 490 nM by an automatic microplate reading instrument (Bio-Rad, Hercules, CA). The experiment was repeated three times.

2.11 Flow cytometry

After cells transfection for 48 h, the cells were collected and digested with 0.25% trypsin. Cell number was adjusted to 1 × 10^6/mL. Then 1 mL of cells was centrifuged for 10 min at 1500 r/min and the supernatant was discarded. Per milliliter of cell suspension 2 mL PBS was added. After centrifugation, the supernatant was discarded. The cells were fixed after addition of precooking 70% ethanol overnight at 4°C. The next day, the cells were washed twice with PBS. Cell suspension of 100 µL was added with 50 µg PI dye containing RNAase (40710ES03, Shanghai Qian Chen Bioscience & Technologies Co., Ltd., Shanghai, China). After allowing the samples to rest in dark for 30 min, the samples were filtered under a nylon mesh (100 meshes). Red fluorescence at 488 nM excitation wavelength was recorded by the flow cytometer (BD Biosciences, Franklin Lakes, NJ) in order to detect cell cycle. Annexin V-FITC/PI double staining was conducted to detect cell apoptosis. The treated cells were cultured in an incubator (37°C, 5% CO₂) for 48 h, and then the cells were collected, washed twice with PBS, centrifuged and resuspended in 200 µL of binding buffer. To the cells 10 µL of Annexin V-FITC (ab14085, Abcam Inc., Cambridge, MA) and 5 µL of PI were added. 300 µL of binding buffer was added after mixing gently in the dark at room temperature for 15 min. Cell apoptosis was detected at the excitation wavelength of 488 nM by flow cytometry. The experiment was repeated three times.

2.12 Colony formation assay

Cells were digested using the enzyme trypsin. The cells were suspended and counted after termination of serum. Samples were seeded into a six-well plate at a density of 1000 cells/well. The cells were incubated in semisolid culture medium at 37°C with 5% CO₂. After 2 weeks, cells were stained with crystal violet. Changes in cell number and cell size were observed. The experiment was repeated three times.

2.13 Statistical analysis

All data were analyzed using SPSS 21.0 statistical software (IBM Corp., Armonk, NY). The measurement data were presented as mean ± standard deviations. Comparisons between CRC tissues and adjacent normal tissues were
highlighted using t-tests, and for comparing among multiple groups one-way analysis of variance was highlighted. $P < 0.05$ value signified statistically significant difference.

3 | RESULTS

3.1 | Immunohistochemical results of adjacent normal tissues and CRC tissues

Immunohistochemistry results (Figure 1) showed that CRC tissues were darkly stained with more brownish yellow granules, while the normal tissues were lightly stained with few brownish yellow granules. Glut1 was positively expressed at about 75% $\pm$ 8% in CRC tissues, while it was not expressed in adjacent normal tissues.

3.2 | Expressions of TGF-β, PI3K-AKT- mTOR signaling pathway—and apoptosis—related proteins in adjacent normal tissues and CRC tissues

Results of RT-qPCR and Western blot (Figure 2) demonstrate that compared to adjacent normal tissues, mRNA and protein expressions of Glut1, TGF-β1, AKT, mTOR, and Bcl-2 in

![FIGURE 1](https://example.com/figure1.png)

**FIGURE 1** Immunohistochemistry results of adjacent normal tissues and CRC tissues ($\times 200$). CRC, colorectal cancer

![FIGURE 2](https://example.com/figure2.png)

**FIGURE 2** Comparison of mRNA and protein expressions of TGF-β, PI3K-AKT-mTOR signaling pathway- and apoptosis-related proteins in adjacent normal tissues and CRC tissues. A, a histogram for mRNA expression; B, band patterns for Western blotting; C, a histogram for protein expression; $t$-test was applied; $\ast$, compared to adjacent normal tissues, $P < 0.05$; TGF-β, transforming growth factor β; PI3K-AKT- mTOR, phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin; Glut1, glucose transporter protein-1; PTEN, phosphatase and tensin homolog deleted on chromosome ten; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CRC, colorectal cancer
CRC tissues up-regulated (all $P < 0.05$); whereas the mRNA and protein expressions of PTEN and Bax reduced; the expressions of p-PI3 K, p-S473-AKT, p-T389-S6K1, and p-T70-4EBP1 proteins increased; and the expressions of Cleaved caspase-3 and Cleaved-PARP protein decreased (both $P < 0.05$); no significant difference was observed regarding the expression of p-T308-AKT protein ($P > 0.05$).

3.3 | Results of transfection efficiency detection

After 72 h of transfection, the fluorescence was observed under a fluorescence microscope. The average transfection efficiency of the NC sequence was $75.2 \pm 3.1\%$, shGlut1-1 sequence $92.5 \pm 4.2\%$, shGlut1-2 sequence $88.4 \pm 4.1\%$, and shGlut1-3 sequence $86.2 \pm 3.2\%$ (Figures 3A and 3B). RT-qPCR results (Figure 3D) showed that the Glut1 expression in the shGlut1-1 group was the lowest among three groups (all $P < 0.05$). Therefore, shGlut1-1 sequence was selected for the following experiments.

3.4 | Expressions of TGF-β, PI3K-AKT-mTOR signaling pathway—and apoptosis—related proteins of cells in each group

Results of RT-qPCR and Western blot (Figure 4) showed that compared with the control group, the mRNA and protein expressions of Glut1, AKT, mTOR, TGF-β1, and Bcl-2 in other groups increased while the mRNA and protein expressions of PTEN and Bax decreased; the expressions of p-PI3 K,
p-S473-AKT, p-S389-S6K1, and p-T70-4EBP1 protein increased; and the expressions of Cleaved caspase-3 and Cleaved-PARP protein decreased (all $P < 0.05$). In comparison with the blank group and NC group, $P < 0.05$; NC, negative control; TGF-β1, transforming growth factor β; PI3K-AKT-mTOR, phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin; Glut1, glucose transporter protein-1; PTEN, phosphatase and tensin homolog deleted on chromosome ten; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

3.5 | Cell proliferation in each group

Results of MTT assay (Figure 5) highlighted significant differences in OD values of each group at 48 h and 72 h compared with the value at 24 h; compared with the control group, the other three groups had significantly higher OD values while compared with the blank and NC groups, OD values were lower in the shGlut1-1 group (all $P < 0.05$).

3.6 | Cell cycle and apoptosis in each group

The result of PI staining (Figure 6) showed that compared with the control group, the other groups exhibited less cells in G0/G1 phases and more cells in S phases in (all $P < 0.05$); compared with the blank and NC groups, cells in G0/G1 phase in the shGlut1-1 group increased, yet cells in S phase decreased (all $P < 0.05$). There was no significant difference regarding the number of cells in G2 phase in each group (all $P > 0.05$).

3.7 | Results of colony formation assay

The results (Figure 8) showed that compared with the control group, other groups exhibited more clone cells (all $P < 0.05$); there was no significant difference in the number of clone

The result of Annexin and V-FITC/PI double staining (Figure 7) showed that the apoptosis rate of control group was $21.3 \pm 3.2\%$, blank group $12.5 \pm 1.2\%$, NC group $12.9 \pm 0.03\%$, and shGlut1-1 group $18.5 \pm 1.1\%$; in comparison to the control group, the apoptosis rate was significantly lower in the other groups (all $P < 0.05$); compared with the blank and NC groups, the apoptosis rate of the shGlut1-1 group was significantly higher (both $P < 0.05$).

FIGURE 4 Comparison of mRNA and protein expressions of TGF-β1, PI3K-AKT-mTOR signaling pathway- and apoptosis-related proteins of cells in the control, blank, negative control (NC), and shGlut1-1 groups. A, a histogram for mRNA expression; B, band patterns for Western blotting; C, a histogram for protein expression; one-way analysis of variance was applied; *, compared to the control group, $P < 0.05$; #, compared to the blank group and NC group, $P < 0.05$; NC, negative control; TGF-β1, transforming growth factor β; PI3K-AKT-mTOR, phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin; Glut1, glucose transporter protein-1; PTEN, phosphatase and tensin homolog deleted on chromosome ten; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

FIGURE 5 Comparison of cell proliferation at 24, 48, and 72 h in the control, blank, NC, and shGlut1-1 groups. One-way analysis of variance was applied; *, compared to the control group, $P < 0.05$; #, compared to the blank group and NC group, $P < 0.05$; NC, negative control; OD, optical density; Glut1, glucose transporter protein-1.
FIGURE 6  Comparison of cell cycle in the control, blank, NC, and shGlut1-1 groups. A, cells cycle detected by flow cytometry; B, a histogram for cell cycle distribution; one-way analysis of variance was applied; *, compared to the control group, $P < 0.05$; #, compared to the blank group and NC group, $P < 0.05$; NC, negative control; Glut1, glucose transporter protein-1

FIGURE 7  Comparison of cell apoptosis in the control, blank, NC, and shGlut1-1 groups. A, cell apoptosis detected by flow cytometry; B, a histogram for cell apoptosis rate; one-way analysis of variance was applied; *, compared to the control group, $P < 0.05$; #, compared to the blank group and NC group, $P < 0.05$; NC, negative control; Glut1, glucose transporter protein-1
cells between the blank group and NC group \( (P > 0.05) \); compared with the blank group and NC group, the shGlut1-1 group had less clone cells, and the slowest tumor growth rate (both \( P < 0.05 \)).

4 | DISCUSSION

With rising morbidity and mortality rates, CRC is one of the most prevalent cancers worldwide, and its development is considered to be a multi-step process. Presently, Glut1 glucose transporter expression is believed to associated strongly with neoplastic progression in the colon, and assessment in CRC identifies patients with a poorer prognosis. A better understanding of the signaling pathways involved in the multi-step development of CRC will contribute to the development of novel treatment options for CRC and further enhance patients’ survival. Therefore, the study investigated the effects of Glut1 gene on CRC cells through the TGF-\( \beta \)/PI3K-AKT-mTOR signaling pathway.

First, it was detected that Glut1 positive expression rate in CRC tissues was about 75%, while Glut1 was not expressed in adjacent normal tissues. Cancerous cells exhibit an acute demand for energy, resulting in increased levels of Glut1, which suggested that Glut1 was a target for therapeutic inhibitors that manage a variety of cancer types. As a molecular feature of various malignancies, increased mRNA expression of Glut1 was detected in CRC as compared to the expression in normal colon. Correspondingly, it has been reported that Glut1 was expressed in all colonic cancer cells, while Glut1 was not expression in the normal colon, which clearly indicated that Glut1 was closely associated with CRC. Ciampi et al reported that Glut1 was an important glucose transporter in the thyroid tumoral cells. Shao et al suggested that an abnormal expression of Glut1 may involved in serous ovarian tumor occurrence and progression and may function as a biomarker reflecting tumor malignant behavior. Endo et al highlighted that the overall survival of bone and soft

**FIGURE 8**  The results of colony formation assay the control, blank, NC, and shGlut1-1 groups. A, colony formation of cells in each group; B, a histogram for number of clone cells; one-way analysis of variance was applied; *, compared to the control group, \( P < 0.05 \); #, compared to the blank group and NC group, \( P < 0.05 \); NC, negative control; Glut1, glucose transporter protein-l
tissue sarcomas with Glut1 overexpression was significantly poor compared to those without.

Moreover, it was observed that the mRNA and protein expressions of Glut1, AKT, mTOR, and TGF-β1 in the shGlut1-1 group decreased. PI3K/AKT signaling has been associated to the progression and development of CRC. Elevated mTORC1 activity is observed in the majority of human tumors, due to activation of upstream oncogenes (PI3K, AKT) and/or loss of tumor suppressors (PTEN, LKB1). Highly activation of PI3K1/AKT/mTOR signaling in glandular elements of CRC and colorectal adenomas with advanced intraepithelial neoplasia suggested that the inhibition of PI3K/AKT/mTOR signaling components might serve as promising anti-CRC targets. As a recognized downstream effector of PI3K/AKT signaling, mTOR regulates the tumorigenesis of CRC, as the increased mRNA expression of mTOR, mTORC1, and mTORC2 were regarded with CRC at advanced stages, while the inhibited mTOR signaling can attenuate the migration and invasion of CRC by rapamycin or stable inhibition of mTORC1 and mTORC2. TGF-β emerged with the evolution of multi-cellular organisms, and it plays a crucial role in embryogenesis and is essential for tissue homeostasis. It has been reported that TGF-β can mediate the response to glucose through the activation of the PI3K/Akt/mTOR signaling pathway. A previous study also evidenced the decisive effect of TGF-β signaling pathway in controlling the proliferation and differentiation of CRC cells.

In addition, we observed that the proliferative ability of CRC cells decreased in the shGlut1-1 group, with an increase in cell apoptosis. The overexpressed Glut1 has been associated with a poor prognosis and aggressive course of CRC, while down-regulated Glut1 has been associated with sensitized tumor cells to apoptosis, which supported the result that the inhibited Glut1 expression decreases cell growth thereby increasing cell apoptosis. Gene silencing by interfere RNA has been reported that couls inhibit certain genes for treatment of various disease. For example, the inhibited Glut1 expression was found to have an anti-colon tumor effect via PI3K/Akt/mTOR signaling pathway, which suggested the inhibition of Glut1 by gene silencing inhibits CRC by inactivating TGF-β/PI3K-AKT-mTOR signaling pathway.

Over the past years, although the conventional therapies for CRC have been developed to an extent, the curative effects were still on a satisfactory level, and the underlying mechanisms regarding the regulation of growth and progression of CRCs are not entirely known. Therefore, the current study explored the effects of Glut1 on CRC cells by regulating TGF-β/PI3K-AKT-mTOR signaling pathway, and we demonstrated that Glut1 gene silencing-mediated TGF-β/PI3K-AKT-mTOR signaling pathway inhibited proliferation and differentiation whereas it promoted apoptosis of CRC cells, thus providing a novel basis for targeted therapy and development of decisive drugs for CRC.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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