SLC7A5 Promotes Colorectal Cancer Progression by Regulating Cell Cycle and Migration

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Research

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Abstract

Background: Solute carrier family 7 member 5 (SLC7A5) was identified highly expressed and as a key participant in various tumor development; however, the role it played in colorectal cancer remains unclear.

Methods and Results: In the current study, the expression of SLC7A5 were systematically mined in public databases and validated by real-time PCR in colon cancer and normal tissues. And then, the co-expression and pathway analysis got from public database, which indicated the potential influence of SLC7A5 for the etiology of colorectal cancer, were evaluated in the colon cancer cell lines by loss of SLC7A5 function experiment, flow cytometry, western blot, and wound healing assay. The results showed that the mRNA expression of SLC7A5 was significantly higher in colorectal cancer tissues than that in the non-tumor controls for GEO and TCGA datasets as well as 40 pairs of Xiangya clinical samples. The functional enrichment analysis based on public database showed that the pathways enriched most were cell cycle and epithelial-to-mesenchymal transition (EMT), and Cyclin D1 (CCND1) were the only gene that had a significant positive correlation with SLC7A5. Loss of SLC7A5 function in colon cancer cell lines could arrest cell cycle at G1 phase by down-regulating CCND1 and CDK2 protein expression, and may reduce cell migration by reversing EMT though upregulation of E-Cadherin and downregulation of zonula occludens-1.

Conclusion: SLC7A5 is likely associated with the progression of colon cancer.

Introduction

Colorectal cancer poses a significant threat in human health worldwide [1]. Clinically, colorectal cancer ranks the top three among all diagnosed malignant tumors [2], and its mortality rate also ranks the same place in all cases of malignant tumor diagnosis [3]. There're over 49,700 people dead for colorectal cancer in U.S. every year [4, 5]. However, the mechanism of how the colorectal cancer developing was still not fully understood.

The disposition of nutrients in tumor cells is critical as lots of them are needed to support tumor cells for rapid proliferation [6, 7], and proteins transporting nutrients into cells, just as amino acid transporters, are essential for the maintenance of amino acid nutrition and survival of tumor cells [8]. Nowadays, many amino acid transporters have been found to be highly expressed in cancers: such that, one of them, solute carrier family 7 member 5 (SLC7A5) has been reported over-expressed in most cancers [9-11].

SLC7A5, also known as LAT1 (L-type amino acid transporter 1), is a member of the solute carrier (SLC) 7 family genes with a 12-transmembrane spanning protein responsible for Na⁺ independent transport of large neutral essential amino acids [12, 13]. By combining with 4F2hc (also named SLC3A2 or CD98, a small type II membrane glycoprotein) [14], SLC7A5 functions to transport large neutral amino acids into cells, such as leucine, isoleucine, valine, tyrosine, phenylalanine, and histidine [15, 16]. In other words, one of the functions of SLC7A5 is to ensure the supply of nutrition in tumor cells. SLC7A5 has been found to be overexpressed during the development of many different cancers such as prostate cancer [17], breast

cancer [18], gastric cancer [19, 20], lung cancer [21, 22] and pancreatic cancer [23]. Several studies suggested that it may be important for cell cycle progression of cancer cells [13, 24]. It was also reported that after knockdown of SLC7A5, cell cycle progression can be arrested at G1 phase in oral cancer [25], gastric cancer [26], and esophageal cancer [24, 27]. Studies also indicated that SLC7A5 played a significant role in growth and proliferation of cancer cells [24, 28-30] since cell proliferation was limited after SLC7A5 knockdown in prostate cancer [28], melanoma [24], gastric cancer [30] and small cell lung cancer [29]. Therefore, SLC7A5 may contribute to the oncogenesis and development in various tumors.

Many studies revealed that SLC7A5 was related to some important signaling pathways [31-36] because it may either regulate many signaling pathway or be regulated by them. However, the mechanisms of SLC7A5 to regulate or to be regulated are not well understood so far. Keitaro Hayashi's study demonstrated c-MYC was able to enhance the activity of SLC7A5 promoter activity in vitro [32]. Pascal Hafliger revealed the role of the RAS-MEK-ERK pathway in SLC7A5 regulation in mouse thyroid tumors model [35]. Many studies found that the knockdown of SLC7A5 led to increasing in autophagy and decreasing in cell growth through downregulation of mTORC1 [36]. But there is little literature which detail the mechanism of SLC7A5 in the development of colorectal cancer.

There have been few researches on SLC7A5 in colorectal cancer over the years. Some studies found that high expression of SLC7A5 occurred in the colorectal cells [37, 38]. Hiroomi Ogawa's research revealed that positive expression of SLC7A5 was associated with lymphatic permeation and the expression of 4F2hc. [37] Arafath reported that SLC7A5 was related to efficient growth of KRAS-mutant colorectal cancer. [39] However, a systematic and comprehensive study on the function of SLC7A5 in colorectal cancer has not been done.

In the current study, we systematically mined public databases to discover the potential association between SLC7A5 and colorectal cancer and verified it in clinical samples and cancer cell lines. Then the mechanism of SLC7A5 on colorectal cancer at the cellular and molecular levels was investigated. Our study reveals the promising roles SLC7A5 in the occurrence and development of colorectal cancer.

Materials And Methods

Clinical samples

Tumor tissue samples and individual-matched adjacent mucosa samples were obtained from 40 patients with colorectal cancer who underwent reset surgery at Xiangya Hospital between 2014 and 2016. The adjacent mucosa samples were acquired 2-5cm away from the tumor. Tissue samples were stored immediately in liquid nitrogen after surgery [40].

TCGA and GEO data mining

The Cancer Genome Atlas (TCGA) gene expression data were obtained from the TCGA data portal (<http://www.cancergenome.nih.gov/>). The datasets included 380 primary tumor samples and 50 normal

solid tissue samples. The gene expression profiles of **GSE5206**, **GSE8671**, **GSE9348** and **GSE20916** were downloaded from the Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) database. The UCSC Cancer Browser (<http://gepia.cancer-pku.cn/>) was used to mine the co-expression genes of SLC7A5 [40]. The Science 2012 dataset was obtained from cBio Cancer Genomics Portal (cBioPortal, <http://www.cbioportal.org/>). The Science 2012 dataset was processed using the software Funrich and got the pathways and genes co-expressed with SLC7A5.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Tissue specimen and cells total RNAs were extracted with RNAiso Plus reagent (Takara). Then, reverse transcription was done by the PrimeScript 1st strand complementary DNA Synthesis kit (Takara). The real-time PCR assay was performed on the LightCycler 480 (Roche) platform with SYBR Primix Dimer Eraser kit (Takara) [41]. PPIA and B2M were set as the internal controls in tissue specimens. Tubulin served as the internal controls in cell samples. Relative quantification of gene expression was calculated by the comparative cycle-threshold (CT) ($2^{-\Delta\Delta CT}$) method. The primer sequences were as following: SLC7A5 forward primer, 5'-CCGTGAACTGCTACAGCGT-3' and reverse primer, 5'-CTTCCCGATCTGGACGAAGC-3'; PPIA forward primer, 5'-GTGGTATAAAAGGGGCGGGAG-3' and reverse primer, 5'-GTGGGGTTGACCATGGCTAATAGTA-3'; B2M forward primer, 5'-ATGCCTGCCGTGTGAACCATGTGA-3' and reverse primer, 5'-TCCAAATGCGGCATCTTCAAACCTC-3'. Primers for SLC7A5, PPIA and B2M were designed and synthesized by Biosune Biotechnology (Shanghai) Co, Ltd.

Cell culture and transfections

SW480 was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and HCT116 was cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum. Both cells were incubated at 37°C in a humidified incubator with 5% CO₂. The transfection of short interfering RNA (siRNA) was carried out using RNAiMAX (Invitrogen). The final concentrations of the siRNA were 10nM. After transfection for 48 hours, the cells were utilized for experiments [41]. The sequences of the siRNA were as follows: for the negative control (NC): 5'-UUCUCCGAACGUGUCACGUTT-3', for siRNA-1 (si-1): 5'-GGAAGGGUGAUGUGUCCAATT-3', and for siRNA-2 (si-2): 5'-CCUCCAUCCUCUCCAUGAUTT-3'.

Cell cycles analysis by flow cytometry

SW480 and HCT116 cells were serum starved for 12 hours before transfection. After transfection for 24 hours, the transfected cells were trypsinized, washed, resuspended in precooled 70% ethanol solution. The cells were resuspended using PBS and incubated with RNase and propidium iodide (PI) for 30 minutes at room temperature before testing [42]. The cells were detected with a (fluorescence activated cell sorter) Calibur flow cytometry system (BD, San Jose, CA, USA) equipped with a Macintosh PowerMac G4 personal computer (Apple Computer Inc. Cupertino, CA, USA) using BD CellQuest software version 3.3 (BD). This experiment was repeated in triplicate and the data were analyzed with ModFitLT software.

Western blot

After transfection for 48 hours, total proteins were extracted using RIPA lysate buffer with the protease inhibitor. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride membranes. The primary antibodies were incubated at 4°C overnight. After incubation with secondary antibodies at room temperature for 1 hour, the proteins were visualized and quantified by Quantity One software (Bio-Rad). Antibody against Cyclin D1, Cyclin Dependent Kinase 2 (CDK2) and SLC7A5 was obtained from Cell Signaling Technology. Tubulin antibody was obtained from Selleck. This experiment was repeated in triplicate.

Wound healing assay

Transfected SW480 cells were cultured in 6- well plates. When cells reached greater than 90% confluence, a pipette tip was used to create the scrape. The generated floating cells were washed away using PBS. Wound healing was imaged at 0 and 48 hours [42].

Statistical analysis

Statistical analysis was performed by GraphPad Prism 6.01 software (GraphPad Software Inc). The real-time PCR data of tissue samples and cells were analyzed with $2^{-\Delta Ct}$, respectively. Student's t-test was used to evaluate the significance of the groups, and a value $p < 0.05$ was considered statistically significant.

Results

The mRNA expression of SLC7A5 is upregulated in colorectal cancer

To investigate the potential association between SLC7A5 and colorectal cancer development, firstly, the gene expression of it in several GEO datasets of colorectal cancer and TCGA database was investigated. It was found that the mRNA expression of SLC7A5 was significantly increased in the cancer samples compared to that in the non-tumor controls for the datasets **GSE8671**, **GSE9348**, **GSE20916**, and **GSE5206** (Fig. 1a-d) with all the P-value ≤ 0.0001 , and the result was validated by TCGA database (Fig. 1e) ($P \leq 0.0001$), with a 7.5 folds difference between colorectal cancers and non-tumor controls.

To further verify the results discovered in the samples of public datasets, the mRNA expression of SLC7A5 were investigated in 40 pairs of colorectal cancer and adjacent mucosa samples from Xiangya hospital (Table. 1) by real-time PCR. It was showed that the relative expression of SLC7A5 was significantly upregulated in the colorectal cancer tissues compared with the non-tumor tissues (Fig. 1f) ($P = 0.0001$), which is consistent with the previous results of TCGA and GEO databases.

SLC7A5 may affect cell cycle and migration in colorectal cancer

In order to investigate the potential mechanism that SLC7A5 affect the etiology of colorectal cancer, co-expression network of the gene was mined in the public database. PanCancer datasets, Provision datasets and Nature2012 datasets from TCGA were used to propose genes co-expressing with SLC7A5. A total of 238 genes were found in these three datasets (Figure. 2a), and FunRich was used to functional enrichment analysis of those genes. FunRich analysis showed that the pathways enriched most were cell cycle and EMT (Figure. 2c), and Cyclin D1 (CCND1) were the only gene that had a significant positive correlation with SLC7A5 (Figure 2b) in TCGA COADREAD gene expression dataset.

To validate the results got from public dataset analysis, the loss of SLC7A5 function experiments were performed in colorectal cancer cell lines, and factors important for cell cycle and migration were examined.

Knockdown of SLC7A5 mRNA may arrest cell cycle progression by downregulation of cyclin D1 and CDK2 in SW480 and HCT116 cell lines

Western Blot Assays were used in different colorectal cancer cells to evaluate the expression levels of SLC7A5 in SW480, Caco 2, HCT116, and HT-29 cell lines. The expression levels of SLC7A5 in SW480 and HCT116 cells were much higher than that in Caco 2 and HT-29 cells (Fig. S1). Therefore, HCT116 and SW480 cell lines were used for the following loss-of-function assays.

In SW480 cells (Fig. 3b), the cell percentages of si SLC7A5- 1 and si SLC7A5- 2 groups were raised significantly compared with normal control (NC) group at G0/G1 phase, while the cell percentage of NC group was considerably higher than the si SLC7A5- 1 and si SLC7A5- 2 groups at G2/M phase. Moreover, the S phases cell percentages of si SLC7A5- 1 and si SLC7A5- 2 groups were reduced significantly in comparison to the NC group. Coincidentally, the similar result showed in HCT116 cells (Fig. 3c). These results suggest that knockdown of SLC7A5 might arrest cell cycle at G1 phase in colorectal cancer cells.

In SW480 cells (Fig. 3e), cyclin D1 and CDK2 protein expression were down-regulated when SLC7A5 was dramatically knockdown in si SLC7A5- 1 and si SLC7A5- 2 group. Also, the same situation occurred in HCT116 cells (Fig. 3d) as well. These similar tendencies might suggest cyclin D1 and CDK2 were regulated by SLC7A5 in colorectal cancer cells with a positive correlation, which is consistent with the results got from public dataset mining for co-expression genes for SLC7A5 in TCGA.

Knockdown of SLC7A5 mRNA limits migration in colorectal cancer cells

To examine whether SLC7A5 affect the cell migration, the wound healing assay was performed in SW480 cells after knocking down of the gene. The gap area of NC group was much smaller than that of si SLC7A5- 1 and si SLC7A5- 2 groups (Fig. 4a), which revealed that loss of SLC7A5 function on mRNA level could significantly weaken the ability of migration of SW480 cells.

To further confirm the change of migration in SW480 cells, the protein expression levels of zonula occludens (ZO)-1 and E-Cadherin in SW480 cells were detected. When SLC7A5 was knock down, the epithelial marker E-Cadherin was upregulated, while the mesenchymal marker ZO-1 was downregulated

(Fig. 4c). Similar changes were also observed in HCT116 cells (Fig. 4b). Therefore, SLC7A5 may regulated migration of colorectal cancer cells by reversing EMT pathway.

Discussion

Lots of researchers had found that SLC7A5 was overexpressed in various solid cancers [43-46], however, there were few data on the function of SLC7A5 in colorectal cancer cells. Currently, a systematic and comprehensive study on the function of SLC7A5 in the occurrence and development of colorectal cancer has been performed.

The present study demonstrated that the content of SLC7A5 in colorectal cancer was upregulated in the colorectal cancer dataset and confirmed that the mRNA expression levels in colorectal cancer was significantly upregulated than that in non-tumor tissues. Therefore, it can be speculated that SLC7A5 may play an important role in the occurrence and development of colorectal cancer. However, there are so many phenotypes occurring in the development of colorectal cancer, such as proliferation, cell cycle, apoptosis, motility, chemosensitivity and so on. Further research was conducted on this next.

Cell cycle is important for cancer cell progression. If cell cycle is arrested, cell proliferation, apoptosis, and even the development of tumor will be influenced, eventually [47]. SLC7A5 might cause different effects in different cell lines since it was reported that after knockdown of SLC7A5, cell cycle progression had been detected to be arrested at G1 phase in oral cancer [25], gastric cancer [26], and esophageal cancer [24, 27]. Moreover, the cell number of G2 phase showed a significant increase while SLC7A5 was knockdown in T-cell acute lymphoblastic leukemia [48]. In the results of FACS, cell cycle was arrested in G1 phase after SLC7A5 knockdown obviously. Further researches were done to verify this phenotype. Control of the G1/S phase transition is largely a matter of regulating a set of specific cyclin dependent kinase (CDK) activities [49]. The G1 phase specific CDK activities are composed of complexes among cyclin D1, either CDK4 or CDK6, and between cyclin E and CDK2. Cyclin D1 is the most important cyclin that binds to CDK4 or CDK6 in G1 phase. Downregulation of cyclin D1 can lead to cell cycle arrest in G1 phase [25, 50]. In our study, it was found that cyclin D1 and CDK2 were down-regulated after SLC7A5 silence in SW480 and HCT116 cells. It represented decrease of the G1 phase specific CDK activities in SW480 and HCT116 cell lines. These data implied that SLC7A5 played an important role in cell cycle, as inducing cell cycle arrested at G1 phase in colorectal cancer.

Metastatic ability is an important characteristic of tumor cells undergo EMT. The metastatic cascade is a complex, highly inefficient, but deadly process [51]. During tumor metastasis, epithelium-derived tumor cells must migrate in order to segregate from the primary tumor and spread into the circulation [51, 52]. The hallmark of the EMT is the loss of cell-to-cell contacts and actin cytoskeletal rearrangements, leading to filopodia formation and the progressive up-regulation of a mesenchymal gene expression pattern enabling cell migration [52, 53]. E-Cadherin is an adhesion molecule through which cells interact with each other [51, 53]. While EMT occurring, EMT transcription factors (EMT-TFs) are activated to orchestrate the EMT program and capable for binding to the E-Box sequences in the promoters of E-Cadherin to

repress transcription [51]. At the same time, expression of mesenchymal genes such as vimentin and Fibronectin and N-Cadherin is up-regulated [54-56]. From the results of wound healing assay, it revealed that the gap area of NC group was much smaller than that of si SLC7A5- 1 and si SLC7A5- 2 groups. It showed that the ability of migration in colorectal cells was weakened when SLC7A5 was knockdown. In other word, SLC7A5 knockdown limited migration in colorectal cancer cells. How would it happen? As we knew, migration ability is an important characteristics of tumor cells undergo EMT process. As shown on WB, E-Cadherin was significantly up-regulated and vimentin was down-regulated when SLC7A5 knockdown. It implied that EMT was reversed after SLC7A5 silenced. In this case, cells lose some mesenchymal phenotypes and acquired some epithelial features, such as cell-to-cell interactions increasing and cells migration limited. In one word, SLC7A5 may alter the ability of cells migration through regulating EMT pathway.

As limitations, knockdown of SLC7A5 was tried in order to study the function of SLC7A5 in colorectal cancer cells. However, the mechanism for the regulation of CDK2 and E-Cadherin by SLC7A5 is not still clear, whether it is a direct interaction of SLC7A5 with CDK2 and E-Cadherin or indirect regulation through bypassing signals. Another study on it has been planning to perform after study of the function of SLC7A5 in colorectal cancer cells.

Conclusion

In summary, SLC7A5 not only participates in cell cycle regulation, but also controls cell motility in colorectal cancer. Therefore, it might play an important role in the occurrence and development of colorectal cancer and has great potential as a therapeutic target for colorectal cancer.

Abbreviations

SLC7A5: The solute carrier family 7 member 5; GEO: the Gene Expression Omnibus; TCGA: The Cancer Genome Atlas; FACS: Flow Cytometric Analysis Cell Scan; WB: Western Blot; EMT: Epithelial-to-mesenchymal Transition; LAT1: L-type amino acid transporter 1; SLC: The solute carrier; GEPIA: The Gene Expression Profiling Interactive Analysis; cBioPortal: cBio Cancer Genomics Portal; qRT-PCR: quantitative real-time polymerase chain reaction; FBS: Fetal Bovine Serum; SiRNA: Short Interfering RNA; NC: Negative control; PI: Propidium Iodide; ZO-1: Zonula Occludens -1; EMT-TFs: EMT transcription factors; CDK2: Cyclin Dependent Kinase 2; CCND1: Cyclin D1

Declarations

Ethical approval and consent to participate

Our study was approved by the Institutional Review Board of Department of Clinical Pharmacology, Xiangya Hospital, Central South University (registration no. CTXY-150001-2).

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Availability of data and materials

The datasets analyzed during the current study are available in the GEO and TCGA database.

Authors' contributions

Conceptualization of this study: Baiyou Tang and Hong-hao Zhou; Experimental design: Baiyou Tang, Lihua Zhang and Hong-hao Zhou; Sample collection: Baiyou Tang, Lihua Zhang and Hong-hao Zhou; Data collection: Baiyou Tang, Jing Yu and Yongbin Liu; Data analysis: Baiyou Tang, Jing Yu, Lihua Zhang and Dongli Hu; Manuscript writing: Baiyou Tang and Hong-hao Zhou; Article revision: Mingjing Peng, Ying Guo and Hong-hao Zhou; Achieve funding: Hong-hao Zhou and Yu Cheng. All authors read, commented and approved the final manuscript.

Consent for publication

Not applicable.

Conflict of Interest Policy

The authors have no potential conflicts of interest.

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Table

Table 1.

Summary of 20 patients' cohort information

Characteristic	NO.	%
Number of patients	35	100
Sex		
Male	16	46
Female	19	54
Age (years)		
<60	22	63
≥60	13	37
Clinical stage		
Stage II	26	74
Stage III	7	20
Stage IV	2	6
Tumor size (cm)		
T2	1	3
T3	6	17
T4	28	80
Regional lymph node involvement		
N0	27	77
N1	6	17
N2	1	3
N3	1	3
Distant metastasis		
M0	32	91
M1	1	3
MX	1	3
NO	1	3

Figures

Figure 1

SLC7A5 is upregulated in colorectal cancer. (a-d) The mRNA expression of SLC7A5 in the GSE5206, GSE8671, GSE9348 and GSE20916; (e) The mRNA expression of SLC7A5 in colorectal cancers in TCGA; (f) The expression levels of SLC7A5 in 40 pairs of colorectal cancer and adjacent mucosa samples by real-time PCR

Figure 2

SLC7A5 is related to cell cycle and EMT in colorectal cancer dataset. (a) A total of 238 genes were found in PanCancer datasets, Provision datasets and Nature2012 datasets from TCGA to speculate genes co-expressing with SLC7A5; (b) Expression level correlation analysis between SLC7A5 and CCND1 in TCGA COADREAD gene expression dataset; (c) The result of FunRich used for functional enrichment analysis of 238 genes.

Figure 3

SLC7A5 knockdown arrests cell cycle progression in HCT116 and SW480 cell lines. (a-b) Phases of the cell cycle were assessed by flow cytometry after 24h of SLC7A5 knockdown in SW480 and HCT116 cells; (c-d) Protein expression changes of CDK2 and Cyclin D1 in HCT116 and SW480 cells after transfection with SLC7A5 siRNA.

Figure 4

SLC7A5 knockdown limits migration in colorectal cancer: (a) SW480 cells were seeded at 800000 cells/well in 6-well plates and transfected with SLC7A5 siRNA. Then a cell-free space was created by scraping; (b-c) HCT116 and SW480 cells were transfected with SLC7A5 siRNA for 24h. Total protein was isolated and expression of E-Cadherin and Vimentin was detected by western blot assay

Supplementary Files

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