Elevated expression of TrpC5 and GLUT1 is associated with chemoresistance in colorectal cancer

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Abstract. Reprogramming of energy metabolism (aerobic glycolysis) is thought to play an essential role in cancer. Compared to oxidative phosphorylation, aerobic glycolysis consumes more glucose through the upregulation of glucose transporters, notably glucose transporter 1 (GLUT1). Elevated glycolysis occurs in chemoresistant cancer cells, but the detailed mechanism is not well understood. The upregulation of the Ca^{2+}-permeable transient receptor potential channel 5 (TrpC5) activates the Wnt/β-catenin signaling pathway in 5-fluorouracil (5-Fu)-resistant human colorectal cancer (CRC) HCT-8 (HCT-8/5-Fu) cells. In the present study, TrpC5 was overexpressed at the mRNA and protein levels along with GLUT1 in HCT-8/5-Fu cells. Suppression of TrpC5 expression with a TrpC5-specific shRNA reduced the induction of GLUT1 in the HCT-8 cells. The inhibition of the Wnt/β-catenin signaling pathway with XAV939 resulted in a decreased GLUT1 and nuclear c-Myc expression. Further study using clinical specimens validated the positive correlation between TrpC5 and GLUT1 protein levels and showed that a high TrpC5/GLUT1 expression was significantly correlated with chemoresistance. Taken together, we demonstrated the essential role of TrpC5 in GLUT1 induction and revealed that a high TrpC5/GLUT1 expression is associated with chemoresistance in human CRC.

Introduction

Colorectal cancer (CRC) is the fifth most common malignant tumor and the fifth cause of cancer-related deaths in China (1). As the major treatment for CRC, chemotherapy fails in most cases due to chemoresistance, and this is the major cause of cancer-related death. Understanding of the detailed mechanisms behind CRC chemoresistance is important for improving the prognosis of CRC.

As one of the 10 hallmarks of cancer (2), aerobic glycolysis plays an important role in cancer proliferation, metastasis and angiogenesis (3-9). Moreover, aerobic glycolysis is elevated in chemoresistant cancer cells compared with wild-type cancer cells and glycolytically derived ATP is crucial for the maintenance of chemoresistance (10,11). Compared with oxidative phosphorylation in normal cells, aerobic glycolysis in cancer cells consumes more glucose. Cancer cells do this by upregulating glucose transporters, notably glucose transporter 1 (GLUT1), which substantially increases glucose import into the cytoplasm (2,12,13).

Intracellular Ca^{2+} [$(Ca^{2+}i)$] is involved in almost all cellular functions. The Ca^{2+}-permeable transient receptor potential canonical (TrpC) family of channel proteins has received increasing attention in cancer research (14,15). For example, TrpC1 and TrpC3 are involved in the proliferation of breast and ovarian cancer cells, respectively (16,17), while TrpC6 is involved in the proliferation of liver and prostate tumor cells (18,19). Previously, we found that TrpC5-mediated Ca^{2+}-entry induced chemoresistance in CRC via activating the Wnt/β-catenin signaling pathway (20), and the latter was demonstrated to upregulate glycolysis in CRC (21). To date, there is still no study concerning the role of Ca^{2+} signaling in the regulation of glycolysis, and the detailed mechanism by which TrpC5 induces chemoresistance also deserves exploration. In the present study, we designed experiments to explore the possible involvement of TrpC5 in regulating GLUT1 expression.

Materials and methods

Cells and cell culture. The human CRC cell line HCT-8 and 5-fluorouracil (5-Fu)-resistant HCT-8 cells (HCT-8/5-Fu) were purchased from KeyGen Biotech Co. Ltd. (Nanjing, Jiangsu, China). HCT-8 cells were cultured in RPMI-1640 medium supplemented with 10% inactivated fetal calf serum. HCT-8/5-Fu cells were cultured in RMPI-1640 medium supplemented with 15 mg/l 5-Fu.

Antibodies, siRNA and reagents. Anti-TrpC5 (ACC-020) was purchased from Alomone Labs (Jerusalem, Israel) and

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Table I. Real-time PCR primers.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
β-actin	GCCCTTGCTCCTTCCACTATC	CCGGACTCTTCGTACTCATCCT
TrpC5	CCACCAGCTATCAGATAAGG	CGAAACAAGCCACTTATACC
GLUT1	CTTTGTGGCCTTCTTTGAAGT	CCACACAGTTGCTCCACAT

TrpC5, transient receptor potential canonical 5; GLUT1, glucose transporter 1.

anti-glucose transporter 1 (GLUT1) (ab115730) and anti-c-Myc (ab32) were purchased from Abcam Biotechnology (Cambridge, MA, USA), and anti-histone (AH433) and anti-β-actin (AA128) were from Beyotime Biotechnology (Nantong, Jiangsu, China). The secondary antibodies, a goat anti-rabbit IgG (A0208) and a goat anti-mouse IgG (A0216), were purchased from Beyotime Biotechnology. The Nuclear and Cytoplasmic Protein Extraction kit (P0027) was purchased from Beyotime Biotechnology. TRIzol (10296-010) and Lipofectamine 2000 (11668-019) were purchased from Invitrogen (Camarillo, CA, USA). TrpC5-shRNA (sc-42670) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Dimethyl sulfoxide (DMSO), 3-BrPA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and XAV939 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 5-Fu was purchased from Jinyao Amino Acid Co., Ltd. (Tianjin, China).

Cell transfection. When the cells reached 50-70% confluency, the HCT-8/5-Fu cells were treated with a TrpC5-shRNA (indicated as HCT-8/5-Fu/RNAi) (scrambled siRNA was used as a control and was indicated as HCT-8/5-Fu/scrambled). XAV939 (an inhibitor of the canonical Wnt/β-catenin signaling pathway) was used in the present study. HCT-8/5-Fu cells were treated with XAV939 (10 μM, overnight) (indicated as HCT-8/5-Fu/XAV939) (DMSO was used as a control and was indicated as HCT-8/5-Fu/DMSO). Real-time PCR and western blotting were used to determine the expression of TrpC5, GLUT1 and c-Myc.

Western blotting. The whole-cell protein lysate was obtained using RIPA buffer containing 1 mM phenylmethylsulphonyl fluoride (PMSF). The nuclear proteins were isolated using a Nuclear and Cytoplasmic Protein Extraction kit. The same quantity of total proteins was electrophoresed on a 10% polyacrylamide gel containing 0.1% SDS. The resolved proteins were semi-dry-transferred to a polyvinylidene difluoride (PVDF) membrane. The primary antibodies, anti-TrpC5 (1:500), anti-GLUT1 (1:1,000) and anti-c-Myc (1:500), were used to detect the expression of the proteins of interest. β-actin and histone were the internal references. The antigen-antibody complexes were visualized by an enhanced chemiluminescent reaction. The protein bands were analyzed using ImageJ software (NIH, Bethesda, MD, USA).

Real-time PCR. Total RNA was extracted from the cells using TRIzol. The procedure for the real-time PCR was previously reported (20). The primer pairs are listed in Table I.

MTT assay. Briefly, 10^4 cells (200 μl) were seeded into 96-well plates. Twelve hours later, the cells were treated with medium containing different concentrations of 5-Fu for 48 h. Then, the medium in each well was replaced with 200 μl of fresh RPMI-1640 containing 5 mg/ml MTT for 4 h. DMSO (150 μl) was added to each well and then, the absorbance was determined at 490 nm.

Patients and tumor specimens. We retrospectively screened advanced CRC patients with unresectable metastasis who underwent a biopsy and/or surgery for a primary lesion at the Affiliated Hospital of Jiangnan University (The Fourth People's Hospital of Wuxi) from January 2009 to December 2014. Patients who postoperatively received 5-Fu-based first-line systematic chemotherapy were enrolled in the present study. The following were the exclusion criteria: i) patients receiving a localized treatment to the target lesions, such as radiotherapy or radiofrequency ablation; ii) patients receiving preoperative cytotoxic treatment, such as radiation or chemotherapy; iii) patients with a diameter of the maximum target lesion was <1 cm by CT-scan; and iv) patients receiving postoperative systematic chemotherapy for <2 cycles. Tumor assessment was performed after every 2 cycles of chemotherapy according to the Response Evaluation Criteria in Solid Tumors 1.1 (RECIST 1.1) criteria (22), and the assessment was classified as a complete response (CR), a partial response (PR), stable disease (SD) and progressive disease (PD). Ethical permission was obtained from the Ethics Committee at the Affiliated Hospital of Jiangnan University (The Fourth People's Hospital of Wuxi).

Immunohistochemical staining. CRC tissue slides were de-paraffinized with xylene and rehydrated through a graded alcohol series. After incubation with 10% bovine serum albumin, the slides were then incubated with the primary antibodies [TrpC5 (1:2,000) and GLUT1 (1:100)] overnight at 4˚C in a humidified chamber and were subsequently incubated with the secondary antibodies. The results of immunostaining were assessed by two pathologists, respectively. Five visual fields were selected from each slide. The results were judged according to the German semi-quantitative scoring system (23) (no staining, 0; weak staining, 1; moderate staining, 2; and strong staining, 3), and the extent of stained cells (0%=0; 1-24%=1; 25-49%=2; 50-74%=3; and 75-100%=4). The final immunoreactive score was determined by multiplying the intensity score with the extent of score of the stained cells, ranging from 0 (the minimum score) to 12 (the maximum score) and was defined as follows: ± (0-3), + (3.1-6), ++ (6.1-9)

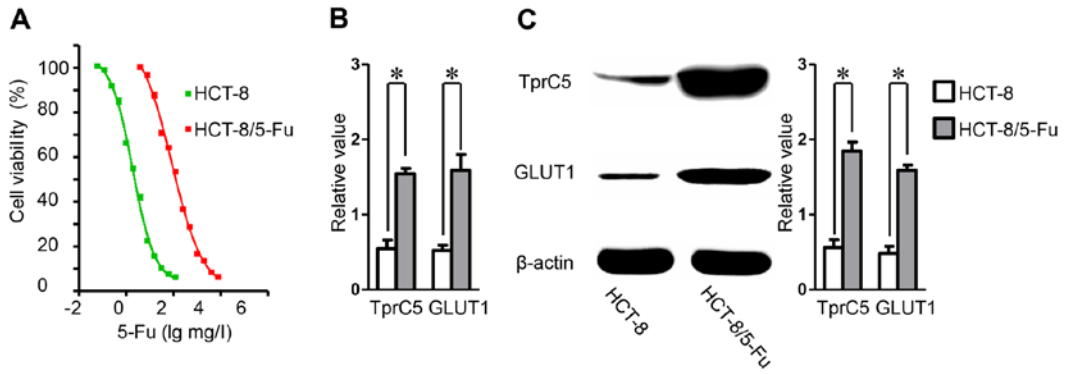

Figure 1. Upregulated expression of TrpC5 and GLUT1 in 5-Fu chemoresistant human CRC cells. (A) MTT assay showed that HCT-8/5-Fu were much more resistant to 5-Fu-induced cell death than HCT-8 cells. (B) Real-time PCR and (C) western blotting showed much higher expression of both TrpC5 and GLUT1 at the mRNA and protein levels in the HCT-8/5-Fu cells than levels in HCT-8 cells (*p<0.05, Student's t-test).

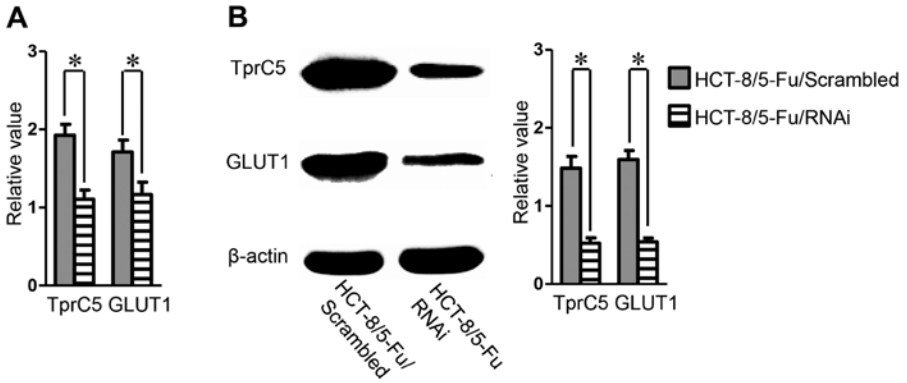

Figure 2. Effect of TrpC5 suppression on GLUT1 expression. (A) Real-time PCR and (B) western blotting showed that both TrpC5 and GLUT1 expression at the mRNA and protein levels were significantly downregulated in the HCT-8/5-Fu/RNAi cells when compared with these levels in the in HCT-8/5-Fu/Scrambled cells (*p<0.05, Student's t-test).

and +++ (9.1-12). Each grade of TrpC5 and GLUT1 was from the same sample. The expression levels were categorized as low (± and +) or high (++ and +++).

Statistical analyses. The results are presented as the mean ± standard error. Statistical significance was determined by a Student's t-test and a Pearson's Chi-squared test as applicable. A value of $p<0.05$ was considered to indicate a statistically significant result.

Results

Upregulated expression of TrpC5 and GLUT1 in 5-Fu chemoresistant human CRC cells. In the present study, GLUT1 expression was examined to represent the glycolytic level. 5-Fu-resistant human CRC cells (HCT-8/5-Fu) and HCT-8 parental cell lines were used in the present study. An MTT assay revealed that the half maximal inhibitory concentration of 5-Fu (5-Fu IC_{50}) for the HCT-8/5-Fu and HCT-8 cells was 86.0 mg/l [95% confidence interval (CI), 72.2-102.5 mg/l] and 1.9 mg/l (95% CI, 1.8-2.1 mg/l) ($p<0.05$), respectively (Fig. 1A). Real-time PCR analysis showed that the expression of both TrpC5 and GLUT1 at the mRNA level in the HCT-8/5-Fu cells was higher than the levels in the HCT-8 cells (Fig. 1B). This finding was validated by western blotting. The protein expression of TrpC5 and GLUT1 was higher in the HCT-8/5-Fu cells, whereas only a low level was detected in the HCT-8 parental cell line (Fig. 1C).

Effect of TrpC5 suppression on GLUT1 expression. The possible role of TrpC5 in controlling GLUT1 expression was explored. The real-time PCR analysis demonstrated that, when the HCT-8/5-Fu cells were treated with TrpC5-siRNA, the TrpC5 and GLUT1 mRNA levels were both reduced compared with the cells treated with the scrambled siRNA (Fig. 2A). Furthermore, a western blot assay gave similar results. The TrpC5 and GLUT1 protein expression levels in the HCT-8/5-Fu/RNAi group were significantly lower than these levels in the HCT-8/5-Fu/scrambled cells (Fig. 2B).

The Wnt/β-catenin signaling pathway is involved in the regulation of GLUT1 by TrpC5. In our previously study, upregulated expression of TrpC5 in human CRC cells activated the Wnt/β-catenin signaling pathway (20), and the latter was demonstrated to regulate GLUT1 expression through its target gene c-Myc (24,25). We next explored the mechanism of the Wnt/β-catenin signaling pathway and how it is involved in the regulation of GLUT1 by TrpC5. The Wnt/β-catenin signaling pathway was found to be activated in HCT-8/5-Fu cells (20), and in the present study, XAV939 was used to inhibit

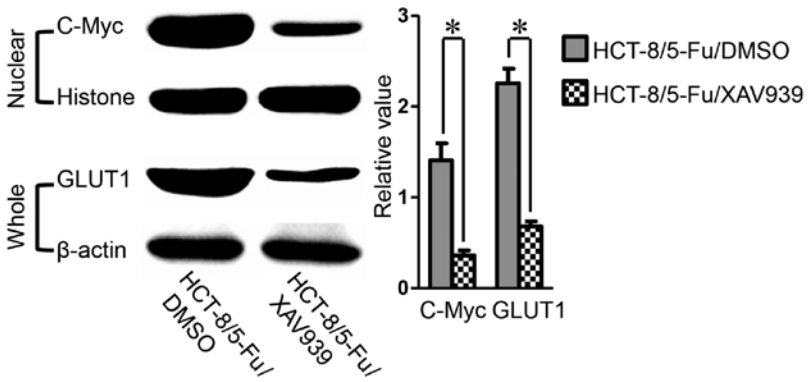

Figure 3. Inhibition of the activated Wnt/β-catenin signaling pathway in HCT-8/5-Fu cells resulted in decreased GLUT1 repression. Western blotting showed that decreased nuclear c-Myc expression, and a markedly decreased GLUT1 repression in the HCT-8/5-Fu cells treated with XAV939 (*p<0.05, Student's t-test).

Table II. Clinicopathological characteristics of the 72 CRC patients.

Characteristics		n	(%)
Age (years)			
Mean ± SD	60.2±8.9		
≤60		37	(51.4)
>60		35	(48.6)
Gender			
Male		43	(59.7)
Female		29	(40.3)
Primary tumor location			
Colon cancer		39	(54.2)
Rectal cancer		33	(45.8)
Tumor differentiation			
Well or moderate		48	(66.7)
Poor		24	(33.3)
Outcome of chemotherapy[a]			
CR		0	(0.0)
PR		28	(38.9)
SD		19	(26.4)
PD		25	(34.7)

[a]Outcome of first-line 5-Fu chemotherapy in 72 CRC patients was classified according to the Response Evaluation Criteria in Solid Tumors 1.1 (RECIST 1.1) categories [complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD)]. CRC, colorectal cancer.

Table III. Relationship between TrpC5 and GLUT1 expression level in CRC tissues.

	GLUT1[a]			
TrpC5[a]	High	Low	Wald	P-value[b]
High	25	15	13.9	<0.01
Low	6	26		

[a]The staining intensity of TrpC5 and GLUT1 in CRC tissues was classified into 4 grades (±, +, ++ and +++). Expression levels were categorized as low (± and +) or high (++ and +++); [b]p<0.05 by the Chi-squared test. TrpCs, transient receptor potential channel 5; GLUT1, glucose transporter 1; CRC, colorectal cancer.

the Wnt/β-catenin pathway in the HCT-8/5-Fu cells. Western blot analysis showed decreased nuclear c-Myc expression and a markedly decreased GLUT1 expression in the HCT-8/5-Fu cells treated with XAV939 (Fig. 3).

Elevated TrpC5 and GLUT1 expression is associated with chemotherapy failure in advanced CRC patients. Table II shows the clinical and pathological characteristics of the 72 CRC patients enrolled in the present study. After the first 2 cycles of 5-Fu chemotherapy, 28 patients achieved CR or PR (these were considered responders) and the remaining 44 patients achieved SD or PD (these were considered non-responders). The immunostaining showed that TrpC5 and GLUT1 were mostly localized near the cell surface (Fig. 4). TrpC5 and GLUT1 were found to be highly co-expressed in 21 of the 44 non-responders and in only 4 of the 28 responders. A Pearson's Chi-squared test showed a significant correlation between TrpC5 expression and GLUT1 expression (Table III), and a higher TrpC5/GLUT1 expression was closely related with chemotherapy failure in the advanced CRC patients (Table IV).

Discussion

Warburg first observed the reprogramming of energy metabolism in cancer cells, such that cancer cells consume glucose avidly and prefer to metabolize glucose by glycolysis even in the presence of sufficient oxygen (aerobic glycolysis), and thus phenomenon is referred to as the Warburg effect (26). It is believed that aerobic glycolysis provides metabolic intermediates that serve as precursors for anabolic biosynthesis (27-29) and adenosine triphosphate (ATP) (28,29) required by the rapidly proliferating cancer cells. The essential role of glycolysis in the regulation and progression of cancer makes it a promising target.

In addition to the postulation that increased aerobic glycolysis is a compensatory mechanism for the mitochon-

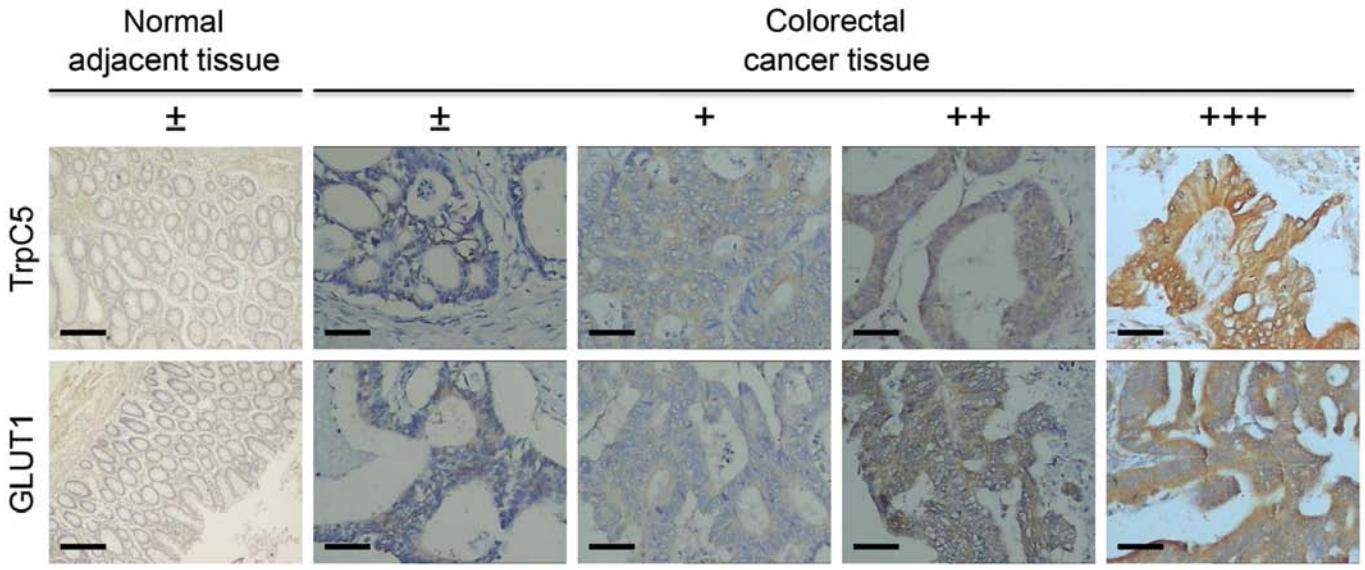

Figure 4. Representative images from the immunohistochemical staining of TrpC5 and GLUT1 expression in human CRC tissues and normal adjacent tissues. Scale bars, 100 μM.

Table IV. Characteristics of the CRC patients according to TrpC5/GLUT1 expression status.

	TrpC5[a]			GLUT1[a]		
Characteristics	High (n=40)	Low (n=32)	P-value[b]	High (n=31)	Low (n=41)	P-value[b]
Age (years)			0.09			0.66
≤60	17	20		15	22	
>60	23	12		16	19	
Gender			0.96			<0.01
Male	24	19		13	30	
Female	16	13		18	11	
Primary tumor location			0.87			0.29
Colon cancer	22	17		19	20	
Rectal cancer	18	15		12	21	
Tumor differentiation			0.50			0.40
Well or moderate	28	20		19	29	
Poor	12	12		12	12	
Chemotherapy outcome			<0.01			<0.01
Responders	9	19		6	22	
Non-responders	31	13		25	19	

[a]The staining intensity of TrpC5 and GLUT1 in CRC tissues was classified into four grades (±, +, ++ and +++). Expression levels were categorized as low (± and +) or high (++ and +++). [b]p<0.05 by the Chi-squared test. CRC, colorectal cancer; TrpCs, transient receptor potential channel 5; GLUT1, glucose transporter 1.

drial defects observed in cancer to drive tumorigenesis (30), accumulating evidence indicates that activated oncogenes or inactivated tumor suppressors upregulate aerobic glycolysis in cancer (2,9,31-35). For example, the aberrant activation of Wnt/β-catenin signaling, which is observed in many human cancers, was identified to promote glycolysis through its target gene c-Myc, which upregulates GLUT1 expression (21).

Ca^{2+} signaling is important for maintaining the physiological functions of all living cells. Studies linking Ca^{2+} signaling to non-malignant tumors have emerged over the years (36,37). Recently, increasing studies concerning Ca^{2+} signaling in malignant tumors have demonstrated that it plays an important role in cancer. Among these, Trp channels have received the most attention. The abnormal expression of

Trp channels contributes to the abnormal biological behavior of cancer cells, including invasion (38) and proliferation. In contrast, little is known concerning the involvement of Ca^{2+} signaling in the regulation of aerobic glycolysis.

In the present study, we found overexpression of TrpC5 in chemoresistant human CRC cells, which was in accordance with our previous study (20). In addition, GLUT1, the key glucose transporter for elevated aerobic glycolysis in cancer cells, was also overexpressed in chemoresistant CRC cells. Further studies using clinical specimens validated these results. Both TrpC5 and GLUT1 protein levels in CRC tissues were positively correlated with chemoresistance in advanced CRC patients, and the expression of TrpC5 and GLUT1 was significantly higher in non-responders compared to responders. We next knocked down TrpC5 expression in the HCT-8/5-Fu cells using a TrpC5-shRNA. As a result, the mRNA and protein levels of GLUT1 were significantly decreased. Immunochemical analysis of the clinical specimens showed similar results. TrpC5 and GLUT1 were found to be highly co-expressed in 25 patients and lowly co-expressed in 26 patients, while inconsistently co-expressed in only 21 patients. A Pearson's Chi-squared test revealed a positive correlation between TrpC5 and GLUT1 protein levels. These results suggest that GLUT1 is regulated by TrpC5 in human CRC.

The detailed mechanism by which TrpC5 regulates GLUT1 was further investigated. In the present study, XAV939 was used to inhibit the Wnt/β-catenin signaling pathway, which is activated in chemoresistant cancer cells (20). Western blotting showed that nuclear c-Myc expression was decreased, and importantly, we found the GLUT1 expression was also significantly decreased.

To cope with cytotoxic pressure, various molecular events are essential for chemoresistant cancer cells, which include mutation of survival-related genes, increased expression of anti-apoptotic genes and/or activation of intracellular survival signaling (41). In spite of the numerous findings regarding the mechanism of chemoresistance, little is known concerning the initial step. As sensors for a wide variety of factors, the functional Trp channels enable the body to react and adapt to different forms of environmental changes (42). Recently, TrpC5, one member of the Trp superfamily, was found to act as a novel sensor in cancer. The increased expression of TrpC5 in cancer cells was determined to 'sense' the cytotoxic pressure from chemotherapeutics and subsequently, activate different intracellular survival signaling pathways to enable the cancer cells to adapt to the extracellular cytotoxic pressure (20,43-45). In the present study, we identified a novel mechanism related to TrpC5. Glycolysis, which is essential for chemoresistance maintenance, was found to be involved in TrpC5-induced chemoresistance.

In conclusion, the abnormal expression of Trp channels results in the remodeling of the calcium homeostasis of the cell, and this is linked to many abnormal processes in cancer cells. In the present study, we demonstrated that TrpC5 regulates GLUT1 expression in chemoresistant CRC cells. The underlying mechanism involved activation of the Wnt/β-catenin signaling pathway. These findings contribute to the understanding of the complicated underlying mechanism of chemoresistance induced by TrpC5.

Acknowledgements

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