

# Involvement of SLC39A6 in gastric adenocarcinoma and correlation of the SLC39A6 polymorphism rs1050631 with clinical outcomes after resection

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## Research article

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## Abstract

The single-nucleotide polymorphism *SLC39A6* rs1050631 is strongly implicated in esophageal squamous cell carcinoma, leading us to question whether it may also play a role in gastric adenocarcinoma (GA). We genotyped the *SLC39A6* rs1050631 in 512 patients who underwent GA resection. All study subjects lived in an area of China with high GA incidence. Genotypes were examined for possible correlation with survival and recurrence. The potential involvement of *SLC39A6* in gastric cancer was explored in clinical samples and cell culture studies. Multivariable analysis showed that patients with the CT+TT genotype at *SLC39A6* rs1050631 were at greater risk of recurrence (hazard ratio, HR 1.387,  $p = 0.004$ ) and death (HR 1.429,  $p = 0.002$ ) than patients with CC genotype. Median recurrence-free and overall survival were significantly shorter in patients with the CT+TT genotype (20, 27 months) than in patients with the CC genotype (36, 43 months,  $p = 0.001$ ,  $p < 0.001$ ). Patients with the CT+TT genotype who were male or  $\geq 60$  years, or who had a tumor  $\geq 5$  cm or a moderately differentiated tumor were at significantly higher risk of recurrence and death. *SLC39A6* was overexpressed in tissues from GA patients and in GA cell lines, and *SLC39A6* knockdown in GA cell lines inhibited their proliferation, migration and invasion. In conclusion, *SLC39A6* rs1050631 correlates with post-resection prognosis of GA patients and *SLC39A6* may participate in GA onset or progression.

## Introduction

Gastric cancer is one of the most common causes of cancer-related deaths worldwide [1]. Most gastric cancer cases occur in Asia, particularly in China [2,3]. The incidence of gastric cancer, its progression and patient prognosis differ across geographic regions and ethnic groups, and the reasons for these variations are poorly understood. A high-salt diet may stimulate gastric mucosa excessively, leading to chronic gastric inflammation and gastric carcinogenesis [4]. Consumption of *N*-nitroso-containing foods may contribute to gastric cancer, reflecting the fact that the *N*-nitroso group is a well-known carcinogen [5]. Genetic factors also appear to contribute to gastric cancer development and progression [6-8]. To clarify the factors contributing to gastric cancer, it may be beneficial to study populations living in areas with a high incidence of this disease, since such groups may have unique genetic backgrounds linked to the molecular mechanisms behind the illness. Therefore we examined patients who underwent surgical resection to treat gastric adenocarcinoma (GA) in the Chinese province of Fujian, one of the areas with the highest incidence of gastric cancer in China [9,10].

Briefly, we examined whether polymorphism in the gene encoding solute carrier family 39 (zinc transporter) member 6, often referred to as *SLC39A6* or LIV-1, is associated with GA. This gene is known to promote the development and metastasis of several human cancers [11,12]. Studies involving patients from different parts of China have generated strong evidence linking *SLC39A6* overexpression with risk of esophageal squamous cell carcinoma (ESCC) and poor survival [13,14], and linking the single-nucleotide polymorphism *SLC39A6* rs1050631 with survival [14]. The esophagus is connected physically and functionally to the stomach, yet we are unaware of studies exploring a potential link between *SLC39A6* rs1050631 and gastric cancer. Therefore we decided to focus on this polymorphism, although other polymorphisms may also be important in gastric cancer.

We obtained clinical samples from patients in Fujian province following resection to treat GA, and examined potential association between *SLC39A6* rs1050631 with clinical outcomes. We also examined the relationship between *SLC39A6* expression and *SLC39A6* rs1050631. Potential effects of knocking down *SLC39A6* expression were examined in representative GA cell lines.

## Materials And Methods

**Patients.** This retrospective study included 512 Han Chinese patients living in Fujian, China. All patients were diagnosed with primary GA. Surgical resection of the primary gastric tumors was performed between July 2003 and December 2009 at 900 Hospital of the Joint Logistics Team (Fujian, China). Pathologists confirmed the diagnosis of GA following histopathological examination of the tumor tissues. All patients had complete medical records, including detailed clinical pathological features. Recurrence was defined based on our previously described method [15]. Survival was defined as the interval from the date of surgery to the date of death or the last follow-up (November 2014). Survival information was obtained primarily through telephone interview and the Social Security Death Index system. Of the 512 patients, 329 received postoperative chemotherapy with epirubicin, cisplatin, fluorouracil, or one or two of these three drugs plus the remaining one or two drugs. All 512 patients had T3 GA at the time of surgery. This disease stage was selected because it is associated with worse prognosis than T1 or T2 disease, and with better prognosis than T4 disease; therefore it may provide more information about the prognostic influence of genetic factors. The following data were extracted from medical records in the hospital database: age, sex, tumor differentiation grade, tumor size, tumor-node-metastasis (TNM) stage, lymph node metastasis, distant metastasis, chemotherapy status, and other clinicopathological information. TNM staging and histologic classification were performed by experienced pathologists as described [16].

**Immunohistochemical detection.** *SLC39A6* expression was examined in a subset of 198 randomly selected GA tissue blocks and 83 non-cancerous gastric tissues using standard immunohistochemical method. The anti-*SLC39A6* antibody was from Abcam (Cambridge, MA).

Immunostaining was assessed as described [16,17]. Tissues showing scores of  $\geq+$  for SLC39A6 staining were defined as positive; scores of  $\geq 2+$  were defined as high expression and  $< 2+$  as low expression.

**SNP Selection and Genotyping.** *SLC39A6* rs1050631 was selected as the focus of the present study because of the strong evidence linking this gene to proliferation and invasion of ESCC cells, and this polymorphism to survival outcomes in ESCC patients, based on analysis of different groups of individuals from different parts of China [13,14]. The esophagus and stomach are physically and functionally connected in the digestive tract, and *SLC39A6* rs1050631 has never been investigated in GA. Genomic DNA was extracted from 512 GA tissue samples using a QIAamp DNA Kit (Qiagen GmbH). The tissue samples had been formalin-fixed and paraffin-embedded immediately after surgical resection. Genotyping and analysis of the single-nucleotide polymorphism were performed as described [15-17]. The assay involved PCR to amplify the DNA, PCR product extension using a single primer, and product identification using MassARRAY SpectroCHIP and matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (Sequenom). Data were analyzed using TYPER software (Sequenom) [15-17].

**Cell culture and siRNA transfection.** One normal gastric cell line (GES-1) and four GA cell lines (BGC-823, SGC-7901, AGS and MGC-803) were used in this investigation. The lines GES-1, BGC-823, and AGS were obtained and cultured as described. The lines SGC-7901 and MGC-803 were purchased from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China) and cultured in the same way as BGC-823. Two siRNAs targeting different sites in the *SLC39A6* gene were designed and synthesized by GenePharma (Shanghai, China). siRNA1 targeted nt 1213-1231 in the *SLC39A6* cDNA (sense: 5' –GCGAGGAAGUUAUCUGUAATT–3'; anti-sense: 5'–UUCACAGUAACUCCUCGCTT–3'). siRNA2 targeted nt 658-676 in the *SLC39A6* cDNA (sense: 5' –GCAAUUUCCACACGGCAAUTT–3'; anti-sense: 5'–AUUGCCGUGUGGAAAUUGCTT–3'). A scrambled siRNA (sense: 5'–UUCUCCGAACGUGUCACGUTT–3'; anti-sense: 5'–ACGUGACACGUUCGGAGAATT–3') was used as the negative control (NC). siRNAs were transfected into BGC-823 and SGC-7901 cells at a final concentration of 100 nM using Lipofectamine 2000.

**Quantitative RT-PCR assay.** Relative levels of *SLC39A6* mRNA in GES-1 cells and four GA cell lines (AGS, BGC-823, SGC-7901 and MGC-803) were determined by quantitative RT-PCR as described for lncRNA HNF-AS1 [18], with the following primers: *SLC39A6*, 5'-GCCTGCAGTCTTGAAGAAG-3' and 5'-GCCGAGTGTATCGTGGAAT-3'; GAPDH, 5'-GGGAGCCAAAAGGGTCA-3' and 5'-GAGTCCTTCCACGATACCAA-3'.

**Cell proliferation.** BGC-823 and SGC-7901 cells were transfected for 48 h with anti-*SLC39A6* siRNA, then 2,000 cells in 100  $\mu$ l medium were seeded into each well of a 96-well plate. At 2 h (0 d), 1 d, 3 d and 5 d after seeding, CCK-8 solution (10  $\mu$ l per well; Boster, Wuhan, China) was placed into each well and cells were incubated at 37 °C for 1 h. Absorbance at 450 nm was measured using a SpectraMax microplate reader (Molecular Devices, Thermo Fisher, Waltham, MA, USA). Culture dish containing no cells and only medium was used as the blank control. BGC-823 and SGC-7901 proliferation were plotted with time based on absorbance at 450 nm.

**Cell migration and invasion.** BGC-823 and SGC-7901 cell migration and invasion were examined almost as described [19], except that we performed measurements at 36 h and 48 h, respectively.

**Statistical analysis.** Statistical analysis was performed using SPSS 17.0 (IBM, Chicago, IL, USA). All statistical tests were two-sided, and  $p < 0.05$  was considered significant. The chi-square test was used to identify associations of *SLC39A6* rs1050631 genotypes with clinico-demographic characteristics, tumor pathology and outcomes (recurrence and survival). The variables used in uni- and multivariable Cox regression were age, sex, tumor size, differentiation grade, gross findings, lymph node metastasis, distant metastasis, and chemotherapy regimen. Only sex, age and common variables or those with  $p < 0.2$  in chi-square testing were adjusted in subsequent multivariable Cox models. Risks are presented as hazard ratios (HRs) and 95% confidence intervals (CIs). Chi-square partitioning was used to assess the significance of relationships between *SLC39A6* rs1050631 genotypes and survival or recurrence. Log-rank testing of Kaplan–Meier curves was used to assess associations of genotypes with recurrence-free and overall survival. Relationships among *SLC39A6* rs1050631 genotypes and recurrence/survival were assessed using multivariable Cox models that adjusted for age, sex, and lymph node metastasis status. Stratification was also performed based on certain clinico-demographic characteristics in an effort to identify factors that strongly influenced recurrence or survival; Cox models in stratified analyses did not adjust for the variable that was being tested. Two-way ANOVA was used to evaluate differences in cell proliferation among different treatment groups. One-way ANOVA and Dunnett's multiple comparison test was used to assess the significance of differences in cell expression level, cell migration and invasion among different groups.

## Results

**Associations between *SLC39A6* rs1050631 genotype and clinico-demographic characteristics.** Among the 512 tissue samples from patients resected for GA, 3.9% (20 cases), 27.1% (139 cases), and 69.0% (353 cases) had the TT, CT, or CC genotype, respectively (Table 1). Genotype frequencies did not differ significantly (based on the chi-squared test) with age ( $< 60$  vs.  $\geq 60$  years), degree of differentiation, gross findings (apophysis vs. invasion), Lauren classification (intestinal type, diffuse type, uncertain type), tumor location (upper third, middle third, lower

third), tumor size ( $\geq 5$  vs.  $< 5$  cm), distant metastasis status or chemotherapy status (Table 1). Potential association was observed between genotype and lymph node metastasis status ( $p < 0.2$ ). Genotype showed a significant association with post-resection survival ( $p = 0.002$ ) and recurrence ( $p = 0.004$ ) (Table 1).

*Associations between SLC39A6 rs1050631 genotype and GA recurrence.* Of all 512 patients, 334 (65.2%) experienced recurrence. Of the 353 patients with the CC genotype, 214 (60.6%) experienced recurrence (Tables 1 and 2). A significantly higher proportion of patients with the CT (104 of 139, 74.8%) or TT genotype experienced recurrence (16 of 20, 80.0%) (Table 2A).

Univariable analysis showed risk of recurrence to be markedly higher in patients with the CT genotype (HR 1.813,  $p = 0.022$ ) or the CT+TT genotype (HR 1.440,  $p = 0.001$ ) (Table 2B). Similar results were obtained with multivariable analysis: CT genotype, HR 1.379,  $p = 0.007$ ; CT+TT genotype, HR 1.387,  $p = 0.004$ . In order to isolate factors that may influence post-resection outcomes in patients with GA, we stratified our patients by sex, age, tumor size, histologic grade and postoperative chemotherapy status. Multivariable analysis showed that patients with the CT genotype were at increased recurrence risk if they were male (HR 1.564,  $p = 0.001$ ), aged  $\geq 60$  years (HR 1.512,  $p = 0.009$ ), with a tumor  $\geq 5$  cm (HR 1.807,  $p = 0.001$ ) or with a moderately differentiated tumor (HR 1.830,  $p < 0.001$ ) than those patients with CC genotype had (Table 3). Similar results were obtained for patients with the or CT+TT genotype: male, HR 1.545,  $p = 0.001$ ; age, HR 1.529,  $p = 0.005$ ; tumor  $\geq 5$  cm, HR 1.789,  $p = 0.001$ ; and moderately differentiated tumor, HR 1.780,  $p < 0.001$  (Table 3). In terms of the associations among the genotypes and recurrence risk involved chemotherapy status, Multivariable analysis exhibited that patients with CT, TT or CT+TT had higher recurrence risk only appeared in the group of patients without performing postoperative chemotherapy (Table 3).

Kaplan–Meier and log-rank analyses showed that median recurrence-free survival time was only 20 months in patients with the CT+TT genotype, significantly shorter than the 36 months in patients with the CC genotype ( $p = 0.001$ , Fig. 1A). Stratification analyses based on sex, age, differentiation grade, tumor size, histologic grade and postoperative chemotherapy status showed that patients with the CT+TT genotype who were male, aged  $\geq 60$  years or who had a tumor  $\geq 5$  cm or had a moderately differentiated tumor or no matter patients who whether performed postoperative chemotherapy had significantly shorter median recurrence-free survival time than patients with the CC genotype had (sex: 18 vs. 38 months,  $p < 0.001$ ; age, 13 vs. 35 months,  $p = 0.001$ ; tumor size, 9 vs. 35 months,  $p < 0.001$ ; moderately differentiated tumor, 22 vs. 56 months,  $p < 0.001$ ; had performed postoperative chemotherapy: 48.2 vs. 64.3 months,  $p = 0.031$ ; or had not perform postoperative chemotherapy: 27.4 vs. 53.2 months,  $p = 0.004$ . Fig. 1B, 1C and Fig. 2).

*Associations between SLC39A6 rs1050631 genotype and overall survival.* In the entire cohort of 512 patients, 330 (64.5%) died, similar to the proportion of patients with the CC genotype who died (210 of 353, 59.5%;  $p = 0.125$ ). A significantly higher rate of death occurred among patients with the CT (104 of 139, 74.8%) or TT genotype (16 of 20, 80%), based on the chi-squared test and chi-square partitioning (Tables 1 and 4A).

Univariable Cox analysis revealed markedly increased risk of death in patients with the CT genotype (HR 1.440,  $p = 0.002$ ) and also in patients with the CT+TT genotype (HR 1.492,  $p < 0.001$ ). Similarly, multivariable Cox analysis revealed markedly increased risk of death in patients with the CT genotype (HR 1.416,  $p = 0.004$ ) or the CT+TT genotype (HR 1.429,  $p = 0.002$ ), after adjusting for age, sex, and lymph node metastasis status (Table 4B). Stratification by sex showed that risk of death was significantly higher among male patients with the CT genotype (HR 1.601,  $p = 0.001$ ) or CT+TT genotype (HR 1.586,  $p < 0.001$ ) than among the patients with CC genotype (Table 5). Similarly, stratification by age, tumor size, differentiation grade, or postoperative chemotherapy status revealed significantly increased risk of death among patients with the CT or CT+TT genotype when they were aged  $\geq 60$  years (HR 1.531,  $p = 0.007$ ; HR 1.542,  $p = 0.004$ ) or had a tumor  $\geq 5$  cm (HR 1.928,  $p < 0.001$ ; HR 1.904,  $p < 0.001$ ) or a moderately differentiated GA (HR 1.735,  $p = 0.001$ ; HR 1.706,  $p = 0.001$ ) (Table 5). In terms of the associations among the genotypes and death risk involved chemotherapy status, no matter patients had or had not performed postoperative chemotherapy, who carried CT or CT+TT genotypes showed higher death risk than those carried CC genotype (Table 5).

Patients with the CC genotype showed median overall survival time of 43 months, compared to 27 months for patients with the CT+TT genotype ( $p < 0.001$ ; Fig. 3A). Stratification analyses showed that survival time was significantly shorter in patients with the CT+TT genotype if they had any of the following characteristics: male (26 vs. 45 months,  $p < 0.001$ ), aged  $\geq 60$  years (20 vs. 42.0 months,  $p = 0.001$ ), had a tumor  $\geq 5$  cm (16 vs. 42 months,  $p < 0.001$ ), had moderately differentiated GA (31 vs. 59 months,  $p < 0.001$ ) or no matter patients who whether performed postoperative chemotherapy: had performed postoperative chemotherapy: 51.7 vs. 70.6 months,  $p = 0.05$ , which almost equal to have statistically significant difference; or had not perform postoperative chemotherapy: 32.5 vs. 54.4 months,  $p = 0.012$ . (Fig. 3B, 3C and Fig. 4).

*SLC39A6 overexpression in GA.* The results of the above mentioned experiments suggest an association between SLC39A6 rs1050631 and post-resection outcomes in patients with GA. This raised the question of whether SLC39A6 expression might be associated with GA. We used quantitative PCR and immunohistochemistry to assess expression levels in four GA cell lines (AGS, BGC-823, SGC-7901 and MGC-803) and our cohort of GA tissues. We found that the protein was overexpressed in all four cell lines relative to the normal gastric cell line GES-1, and

that the protein was present (immunopositivity scores of  $\geq 1+$ ) in 150 of 198 (75.76%) GA tissues, compared to only 48 of 83 (57.83%) non-cancerous gastric tissues ( $p = 0.003$ ; Fig.5A and 5B).

*SLC39A6 knockdown inhibits proliferation, migration and invasion of GA cells.* We designed two short interfering siRNAs to inhibit SLC39A6 expression in the GA cell lines BGC-823 and SGC-7901 (Fig. 6A and 6B), and this knockdown led to significantly less proliferation in both lines than in untransfected cells (Mock) or cells transfected with scrambled control siRNA (Fig. 6C and 6D). Knockdown also significantly reduced migration (Fig. 7A and 7B) and invasion by both cell lines (Fig. 7C and 7D). These results suggest that *SLC39A6* may contribute to GA by functioning as a typical oncogene.

*Association of different genotype at SLC39A6 rs1050631 with SLC39A6 expression in GA.* Immunohistochemistry of 198 GA tissues from our patient cohort detected high SLC39A6 protein expression ( $\geq 2+$ ) in 86 tissues (43.43%; Fig. 8); high expression was detected in 6 of 14 (42.86%) patients with TT, 50 of 92 (54.35%) patients with CT, 56 of 106 (52.83%) patients with CT+TT, and 31 of 92 (33.70%) patients with CC genotype. Though the high positive expression rate among the three different genotypes groups showed no statistically significantly different from that in the whole group ( $p > 0.05$ ), the rate of high SLC39A6 expression was significantly greater among patients with CT genotype than among those with CC genotype ( $p = 0.005$ ) based on chi-square partitioning (Table 6). We speculate that the *SLC39A6* rs1053631 genotype is associated with post-resection prognosis of patients with GA because the CT genotype leads to higher SLC39A6 expression.

## Discussion

Occurrence and prognosis of gastric cancer are influenced by genetics, lifestyle and the environment [20,21]. Single-nucleotide polymorphisms in certain genes may alter the levels or activity of proteins in a way that leads to cancer [22]. Here we analyzed the potential association of a particular polymorphism with GA and post-resection recurrence and death in a population living in a part of China with one of the highest incidences of gastric cancer in this country. This population may contain genetic elements that predispose them to GA, offering unique opportunities for studying genetic risk factors and prognostic markers, especially since most genetic risk studies are not conducted in high-incidence areas. Our data may also strengthen our understanding of risk factors in a high-risk environment.

Although several polymorphisms have been linked to gastric cancer, we focused on polymorphism in the *SLC39A6* (*LIV-1*) gene because it is a downstream target of the STAT3 oncoprotein and promotes cancer progression by increasing cell growth and differentiation [23]. SLC39A6 can influence the epithelial–mesenchymal transition in pancreatic cancer cells, making their phenotype more aggressive [24]; and treatments that target SLC39A6 can reduce metastasis and predict prognosis of patients with hepatocellular carcinoma [25], and it can inhibit progression of metastatic breast cancer [26]. In particular, we focused on the *SLC39A6* rs1050631 polymorphism. This polymorphism shows a strong association with death risk of ESCC in extensive studies involving more than a thousand of Chinese from at least two different parts of the country [14]. We are unaware of reports exploring a possible association between this polymorphism and GA.

Our study suggests that GA patients with the CT+TT genotype at rs1050631 are at significantly higher risk of recurrence and death than patients with CC genotype. Male gender, age older than 60 years, having a tumor  $\geq 5$  cm or having a moderately differentiated tumor are associated with worse prognosis in patients with the CT+TT genotype than in those with the CC genotype. It is still unknown the reason why *SLC39A6* rs1050631 polymorphism had different effect on affecting the recurrence risk and death risk in GA patients with and without performing postoperative chemotherapy. Patients with CT or CT+TT genotypes were only associated with increased recurrence risk in the group of patients no carrying out postoperative chemotherapy. Whereas, patients with CT or CT+TT genotypes were associated with increased death risk both in the groups of patients carry and no carrying out postoperative chemotherapy. However, patients with CT+TT genotypes had both significantly decreased recurrence-free survival time and overall survival time than those with CC genotype. About these, it needs further investigation to reveal the reasons.

This observation of an association between *SLC39A6* rs1050631 and post-resection outcomes in patients with GA prompted to ask whether SLC39A6 expression is associated with GA. Therefore we compared expression levels in a panel of GA cell lines as well as in our cohort of GA patients. The results show an association between SLC39A6 up-regulation and GA. We further found that the CT+TT genotype at *SLC39A6* rs1050631, which was associated with worse clinical outcomes in our cohort. Consistent with a role of *SLC39A6* in GA, we found that knocking down the protein in GA cell lines inhibited their proliferation, migration and invasion. These results illustrating SLC39A6 overexpression in GA tissues and GA cell lines as well as anti-cancer effects of SLC39A6 down-regulation mirror results published for SLC39A6 in ESCC [14]. These findings indicate that, as in ESCC and several other cancers, SLC39A6 appears to participate in GA, such that polymorphisms affecting its expression may be useful prognostic markers. Our results further suggest a model in which *SLC39A6* rs1050631 alters the expression of SLC39A6 and thereby influences post-resection outcomes.

We identified several clinico-demographic characteristics associated with worse post-resection outcomes, including male gender, older age and having larger, moderately differentiated tumors or chemotherapy status.

In conclusion, our study provides preliminary evidence that *SLC39A6* is involved in GA, and that genotype at *SLC39A6* rs1050631 can predict post-resection prognosis of GA patients, at least in a population in an area with high GA incidence. Although we focused here on *SLC39A6* rs1050631 because of the strong evidence in favor of a linkage with ESCC [14], future research is needed to explore other single-nucleotide polymorphisms that may be involved in gastric cancer. Due to this study has its limitation: all the clinical samples were obtained from single center in Fujian Province, further studies are also needed to examine whether our findings in this Chinese population can be generalized to other ethnic groups.

## Declarations

## Acknowledgments

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

WJR and JG performed the experiments including immunohistochemical staining, cell culture, cell transfection, and cell function assays. WJR, JG and CHX conducted the statistical analysis. WYW and ZZZ collected the data. YD and LYL analyzed and interpreted the data. QJH and RHW wrote the manuscript. LW and SQC revised the manuscript. LYL and SQC designed the project. All authors have read and approved the final manuscript.

## Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Fuzhou General Hospital(Dongfang Hospital), and informed written consent was obtained from all patients. Tumor tissues of GA patients were obtained based on the Declaration of Helsinki.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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## Tables

Table 1. Patient characteristics and distribution of *SLC39A6* rs1050631 genotypes

Variables	Patients N (%)	SLC39A6 rs1050631 genotype (N)			<i>P</i> <sup>*</sup>	<i>P</i> <sub>dominant</sub> <sup>a</sup>	<i>P</i> <sub>additive</sub> <sup>b</sup>
		TT	CT	CC			
Total	512	20(3.9%)	139(27.1%)	353(69.0%)			
Age					0.26	0.25	0.14
<60	235(45.9)	6	61	168			
≥60	277(54.1)	14	78	185			
Gender					0.38	0.18	0.25
Male	383(74.8)	15	110	258			
Female	129(25.2)	5	29	95			
Grade of differentiation					0.85	0.93	0.86
Well differentiated	21(4.1)	1	6	14			
Moderately differentiated	263(51.4)	9	75	179			
Poorly differentiated	228(44.5)	10	58	160			
Gross findings					0.79	0.96	0.81
Apophysis	35(6.8)	2	9	24			
Invasion	477(93.2)	18	130	329			
Lauren classification					0.65	0.45	0.22
Intestinal type	420(82.1)	18	119	283			
Diffuse type	81(15.8)	2	18	61			
Uncertain type	11(2.1)	0	2	9			
Tumor location					0.32	0.38	0.42
Upper third	179(35.0)	4	52	123			
Middle third	78(15.2)	4	16	58			
Lower third	252(49.2)	12	69	171			
Total	3(0.6)	0	2	1			
Tumor size					0.99	0.88	0.92
≥5cm	210(41.0)	8	58	144			
<5cm	302(59.0)	12	81	209			
Survival					0.002	<0.001	<0.001
Alive	182(35.5)	4	35	143			
Dead	330(64.5)	16	104	210			
Recurrence					0.004	<0.001	<0.001
Yes	334(65.2)	16	104	214			
No	178(34.8)	4	35	139			
Distant metastasis					0.43	0.87	0.81
Yes	63(12.3)	4	15	44			
No	449(87.7)	16	124	309			
Lymph node metastasis					0.12	0.21	0.08
Yes	398(77.7)	19	110	269			
No	114(22.3)	1	29	84			
Chemotherapy					0.61	0.78	0.69
Yes	329(64.3)	13	93	223			
No	183(35.7)	7	46	130			

\* The  $\chi^2$  test was used to calculate p values. Fisher's exact test was used in the analysis of contingency tables when sample size was smaller than 5.

<sup>a</sup> Logistic regression analysis for the dominant model (TT+CT vs CC) was used to calculate the p value.

<sup>b</sup> Logistic regression analysis for the log-additive model was used to calculate the p value by comparing the effect of each additional variant allele.

**Table 2 A.** Chi-square partitioning of the association between *SLC39A6* rs1050631 and recurrence

SNP	Genotype	Recurrence		Case-total <sup>a</sup>	Case-case <sup>b</sup>
		No(n)/ Yes(n)	Recurrence(%)	p<0.00833	p<0.0125
Total(n=512)		178/334	65.2		
rs1050631	CC	139/214	60.6	0.167	1vs2:0.003
	CT	35/104	74.8	0.033	2vs3:0.784
	TT	4/16	80.0	0.0578	1vs3:0.100

<sup>a</sup>Case-total: p value for the comparison between the group with the indicated genotype and the total group of 512 samples;

<sup>b</sup>Case-case: p value for the pairwise comparisons between groups with different genotypes.

**Table 2 B.** Associations between *SLC39A6* rs1050631 genotypes and recurrence

SNP	Genotype	Recurrence No(n)/Yes(n)	Unadjusted			Adjusted <sup>a</sup>		
			HR	95%CI	p	HR	95%CI	p
Total (n=512)		178/334						
rs1050631	CC	139/214	1 (reference)			1 (reference)		
	CT	35/104	1.813	1.09-3.01	0.022	1.379	1.090-1.744	0.007
	TT	4/16	1.400	1.11-1.77	0.005	1.441	0.864-2.403	0.161
	CT+TT	39/120	1.440	1.15-1.80	0.001	1.387	1.108-1.735	0.004

The bold values indicate p value < 0.05.

<sup>a</sup>Data were calculated using multivariable Cox models that adjusted for gender, age and lymph node metastasis.

Abbreviations: HR, hazard ratio; CI, Confidence Interval.

Table 3. Associations between *SLC39A6* rs1050631 genotypes and recurrence, after stratification by sex, age, tumor size, differentiation grade or chemotherapy status

SNP	Genotype	Gender							
		Male				Female			
		Recurrence				Recurrence			
	No(n)/Yes(n)	HR	95%CI	$p^a$	No(n)/Yes(n)	HR	95%CI	$p^a$	
Total		134/249				44/85			
(n=512)	CC	105/153				34/61			
	CT	1.564	1.197-2.044	0.001	9/20	0.951	0.572-1.581	0.846	
	TT	1.424	0.788-2.573	0.241	1/4	1.456	0.524-4.048	0.471	
	CT+TT	1.545	1.196-1.996	0.001	10/24	1.008	0.626-1.624	0.973	

SNP	Genotype	Age							
		<60				≥60			
		Recurrence				Recurrence			
	No(n)/Yes(n)	HR	95%CI	$p^b$	No(n)/Yes(n)	HR	95%CI	$p^b$	
Total		90/145				88/189			
(n=512)	CC	70/98				69/116			
	CT	1.242	0.866-1.782	0.238	17/61	1.512	1.108-2.065	0.009	
	TT	1.127	0.414-3.071	0.814	2/12	1.620	0.890-2.948	0.115	
	CT+TT	1.232	0.868-1.747	0.243	19/73	1.529	1.139-2.052	0.005	

SNP	Genotype	Tumor Size							
		<5cm				≥5cm			
		Recurrence				Recurrence			
	No(n)/Yes(n)	HR	95%CI	$p^c$	No(n)/Yes(n)	HR	95%CI	$p^c$	
Total		113/189				65/145			
(n=512)	CC	84/125				55/89			
	CT	1.131	0.820-1.558	0.453	8/50	1.807	1.269-2.575	0.001	
	TT	1.333	0.696-2.554	0.386	2/6	1.657	0.720-3.811	0.235	
	CT+TT	1.158	0.856-1.567	0.341	10/56	1.789	1.273-2.516	0.001	

SNP	Genotype	Histologic grade											
		Poorly differentiated				Moderately differentiated				Well differentiated			
		Recurrence				Recurrence				Recurrence			
	No(n)/Yes(n)	HR	95%CI	$p^c$	No(n)/Yes(n)	HR	95%CI	$p^c$	No(n)/Yes(n)	HR	95%CI	$p^c$	
Total		70/158				103/160				5/16			
(n=512)	CC	48/112				87/92				4/10			
	CT	1.052	0.73-1.52	0.789	14/61	1.830	1.32-2.54	<0.001	1/5	1.827	0.54-6.22	0.335	
	TT	1.468	0.71-3.03	0.298	2/7	1.421	0.65-3.10	0.376	0/1	NA <sup>d</sup>	NA <sup>d</sup>	NA <sup>d</sup>	
	CT+TT	1.107	0.78-1.56	0.565	16/68	1.780	1.29-2.45	<0.001	1/6	2.131	0.67-6.78	0.200	

SNP	Genotype	Chemotherapy							
		Yes				No			
		Recurrence							
		No(n)/Yes(n)	HR	95%CI	<i>p</i> <sup>c</sup>	No(n)/Yes(n)	HR	95%CI	<i>p</i> <sup>c</sup>
Total		128/201				50/131			
(n=512)	CC	95/128	1(reference)			44/84	1(reference)		
	CT	29/64	1.27	0.94-1.72	0.981	6/40	1.79	1.21-2.63	0.003
	TT	4/9	1.01	0.51-2.01	0.571	0/7	2.78	1.26-6.13	0.012
	CT+TT	33/73	1.24	0.92-1.65	0.153	6/47	1.89	1.30-2.73	<0.001

The bold values indicate p value < 0.05.

<sup>a</sup>Data were calculated using a multivariable Cox model that adjusted for age and lymph node metastasis.

<sup>b</sup>Data were calculated using a multivariable Cox model that adjusted for gender and lymph node metastasis.

<sup>c</sup>Data were calculated using a multivariable Cox model that adjusted for gender, age and lymph node metastasis.

<sup>d</sup>Data were not available.

Abbreviations: HR, hazard ratio; CI, Confidence Interval.

Table 4 A Chi-square partitioning of the association between *SLC39A6* rs1050631 and survival

SNP	Genotype	Survival		Case-total <sup>a</sup> p<0.00833	Case-case <sup>b</sup> P<0.0125
		Ali(n)/Dea(n)	Death(%)		
Total(n=512)		182/330	64.5		
rs1050631	CC	143/210	59.5	0.125	1vs2:0.001
	CT	35/104	74.8	0.021	2vs3:0.784
	TT	4/16	80.0	0.231	1vs3:0.098

<sup>a</sup>Case-total: p value for the comparison between the group with the indicated genotype and the total group of 512 samples;

<sup>b</sup>Case-case: p value for the pairwise comparison between groups with different genotypes.

Table 4 B Associations between *SLC39A6* rs1050631 genotypes and survival

SNP	Genotype	Survival Ali(n)/Dea(n)	Unadjusted			Adjusted <sup>a</sup>		
			HR	95%CI	<i>p</i>	HR	95%CI	<i>p</i>
Total(n=512)		182/330						
rs1050631	CC	143/210	1(reference)			1(reference)		
	CT	35/104	1.440	1.138-1.823	0.002	1.416	1.119-1.792	0.004
	TT	4/16	1.947	1.170-3.239	0.010	1.523	0.912-2.542	0.107
	CT+TT	39/120	1.492	1.192-1.868	<0.001	1.429	1.141-1.790	0.002

The bold values indicate p value < 0.05.

<sup>a</sup>Data were calculated using a multivariable Cox model that adjusted for gender, age and lymph node metastasis.

Abbreviations: HR, hazard ratio; CI, Confidence Interval.

Table 5 Associations between *SLC39A6* rs1050631 genotypes with survival, after stratification by sex, age, tumor size, differentiation grade or chemotherapy status

SNP	Genotype	Gender							
		Male				Female			
		Ali(n)/Dea(n)	HR	95%CI	$p^a$	Ali(n)/Dea(n)	HR	95%CI	$p^a$
Total		138/245				44/85			
(n=512)	CC	109/149	1(reference)			34/61	1(reference)		
	CT	26/84	1.601	1.224-2.093	0.001	9/20	0.985	0.592-1.640	0.954
	TT	3/12	1.486	0.822-2.688	0.190	1/4	1.654	0.595-4.600	0.335
	CT+TT	29/96	1.586	1.226-2.051	<0.001	10/24	1.055	0.654-1.701	0.826

SNP	Genotype	Age							
		<60				≥60			
		Ali(n)/Dea(n)	HR	95%CI	$p^b$	Ali(n)/Dea(n)	HR	95%CI	$p^b$
Total		95/140				87/190			
(n=512)	CC	74/94	1(reference)			69/116	1(reference)		
	CT	19/42	1.282	0.889-1.847	0.183	16/62	1.531	1.123-2.087	0.007
	TT	2/4	1.290	0.473-3.517	0.618	2/12	1.605	0.880-2.926	0.123
	CT+TT	21/46	1.282	0.900-1.827	0.169	18/74	1.542	1.150-2.068	0.004

SNP	Genotype	Tumor Size							
		<5cm				≥5cm			
		Ali(n)/Dea(n)	HR	95%CI	$p^c$	Ali(n)/Dea(n)	HR	95%CI	$p^c$
Total		116/186				66/144			
(n=512)	CC	86/123	1(reference)			57/87	1(reference)		
	CT	28/53	1.141	0.826-1.577	0.423	7/51	1.928	1.355-2.742	<0.001
	TT	2/10	1.433	0.747-2.749	0.279	2/6	1.726	0.750-3.974	0.200
	CT+TT	30/63	1.179	0.869-1.600	0.289	9/57	1.904	1.355-2.675	<0.001

SNP	Genotype	Histologic grade											
		Poorly differentiated				Moderately differentiated				Well differentiated			
		Ali(n)/Dea(n)	HR	95%CI	$p^c$	Ali(n)/Dea(n)	HR	95%CI	$p^c$	Ali(n)/Dea(n)	HR	95%CI	$p^c$
Total		75/153				102/161				5/16			
(n=512)	CC	54/106	1(reference)			85/94	1(reference)			4/10	1(reference)		
	CT	19/39	1.209	0.84-1.75	0.315	15/60	1.735	1.25-2.41	0.001	1/5	1.731	0.48-6.26	0.403
	TT	2/8	1.479	0.72-3.06	0.291	2/7	1.482	0.68-3.23	0.322	0/1	NA <sup>d</sup>	NA <sup>d</sup>	NA <sup>d</sup>
	CT+TT	21/47	1.248	0.88-1.76	0.209	17/67	1.706	1.24-2.35	0.001	1/6	2.192	0.66-7.34	0.203

SNP	Genotype	Chemotherapy							
		Yes				No			
		Ali(n)/Dea(n)	HR	95%CI	<i>p</i> <sup>c</sup>	Ali(n)/Dea(n)	HR	95%CI	<i>p</i> <sup>c</sup>
Total		135/194				47/136			
(n=512)	CC	102/121	1(reference)			41/89	1(reference)		
	CT	29/64	1.40	1.03-1.89	0.032	6/40	1.66	1.13-2.43	0.010
	TT	4/9	1.40	0.58-2.30	0.693	0/7	2.47	1.12-5.46	0.025
	CT+TT	33/73	1.36	1.01-1.83	0.040	6/47	1.74	1.20-2.53	0.004

The bold values indicate p value < 0.05.

<sup>a</sup>Data were calculated using a multivariable Cox model that adjusted for age and lymph node metastasis.

<sup>b</sup>Data were calculated using a multivariable Cox model that adjusted for gender and lymph node metastasis.

<sup>c</sup>Data were calculated using a multivariable Cox model that adjusted for gender, age and lymph node metastasis.

<sup>d</sup>Data were not available.

Abbreviations: HR, hazard ratio; CI, Confidence Interval.

**Table 6** Chi-square partitioning to test the association between *SLC39A6* rs1050631 and *SLC39A6* expression detected by immunohistochemistry

SNP	Genotype	expression of <i>SLC39A6</i>		Case-total <sup>a</sup> p<0.00833	Case-case <sup>b</sup> p<0.0125
		Low(n)/ High(n)	High expression rate (%)		
Total(n=198)		112/86	43.4		
rs1050631	CC	61/31	33.7	0.116	1vs2:0.005
	CT	42/50	54.3	0.083	2vs3:0.422
	TT	8/6	42.9	0.966	1vs3:0.712

<sup>a</sup>Case-total: p value for the comparison between the group with the indicated genotype and the total group of 198 samples;

<sup>b</sup>Case-case: p value for the pairwise comparisons between groups with different genotypes.

## Figures

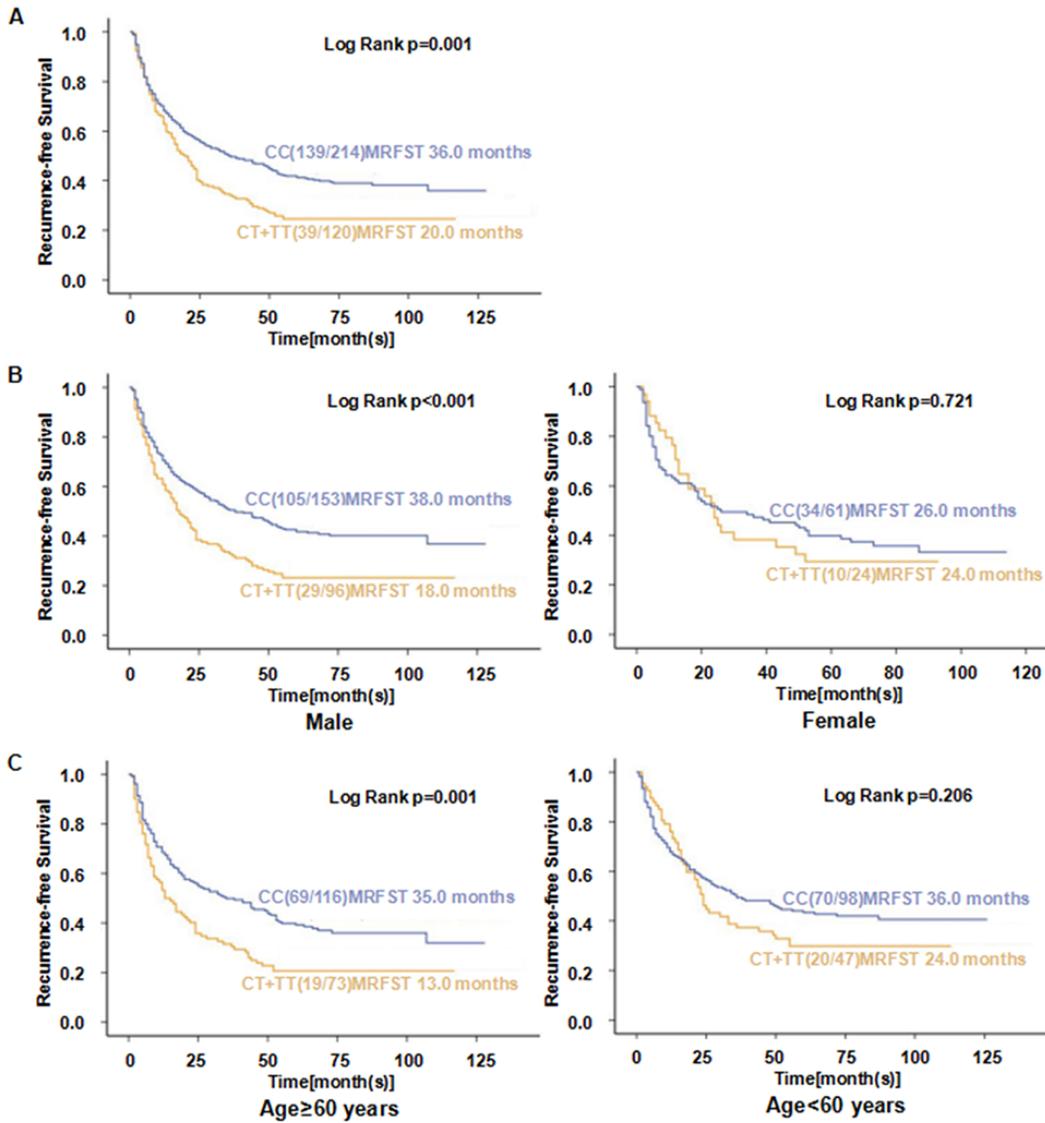


Figure 1

Kaplan–Meier curves of median recurrence-free survival time (MRFST) based on SLC39A6 rs1050631 genotype. (A) Curves calculated for the whole cohort. (B-C) Curves calculated for subgroups stratified by (B) sex and (C) age. The log-rank test was used to calculate p values.

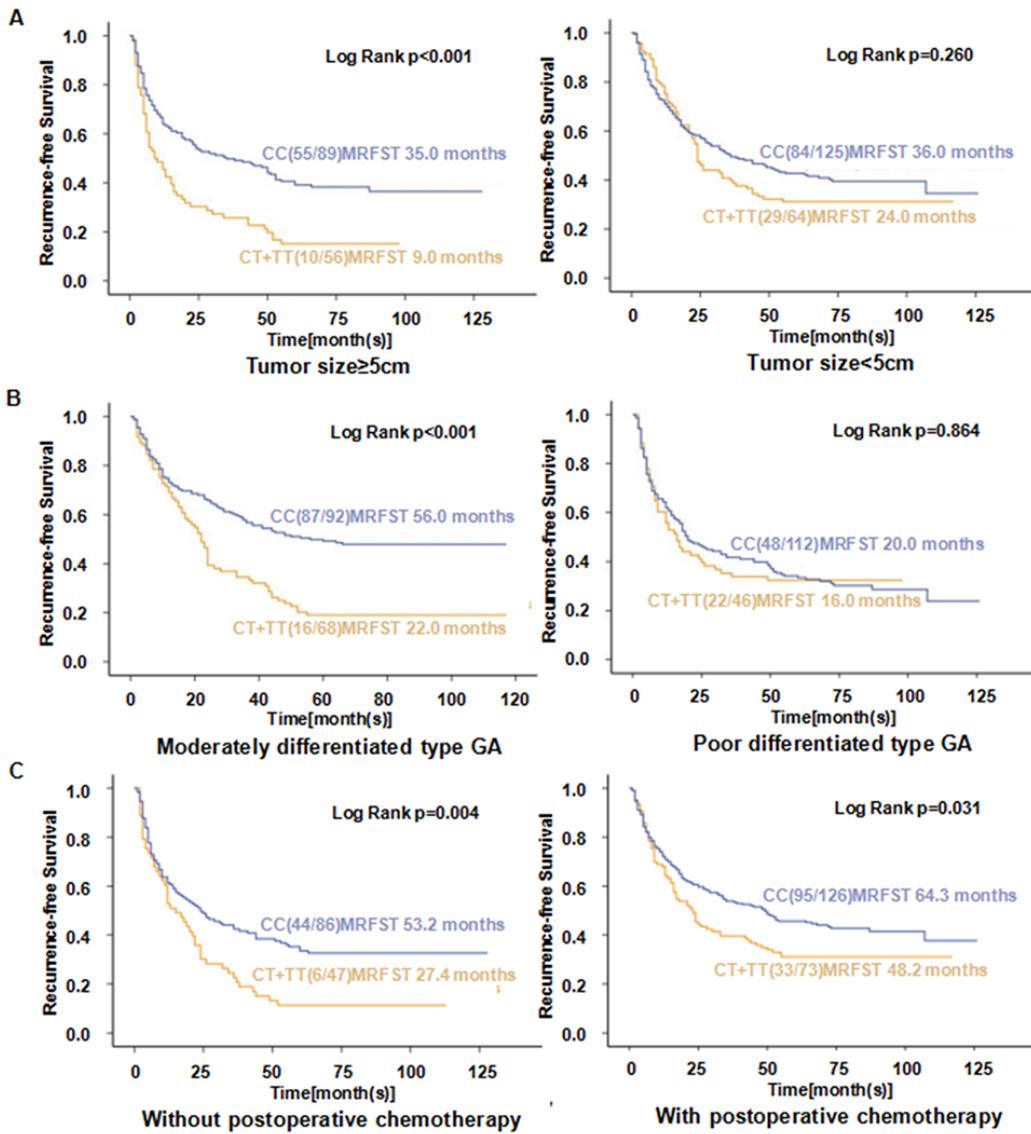


Figure 2

Subgroup Kaplan–Meier curves of median recurrence-free survival time (MRFST) based on SLC39A6 rs1050631 genotype. Curves calculated for subgroups stratified by (A) tumor size (B) differentiation grade and (C) chemotherapy status. The log-rank test was used to calculate p values.

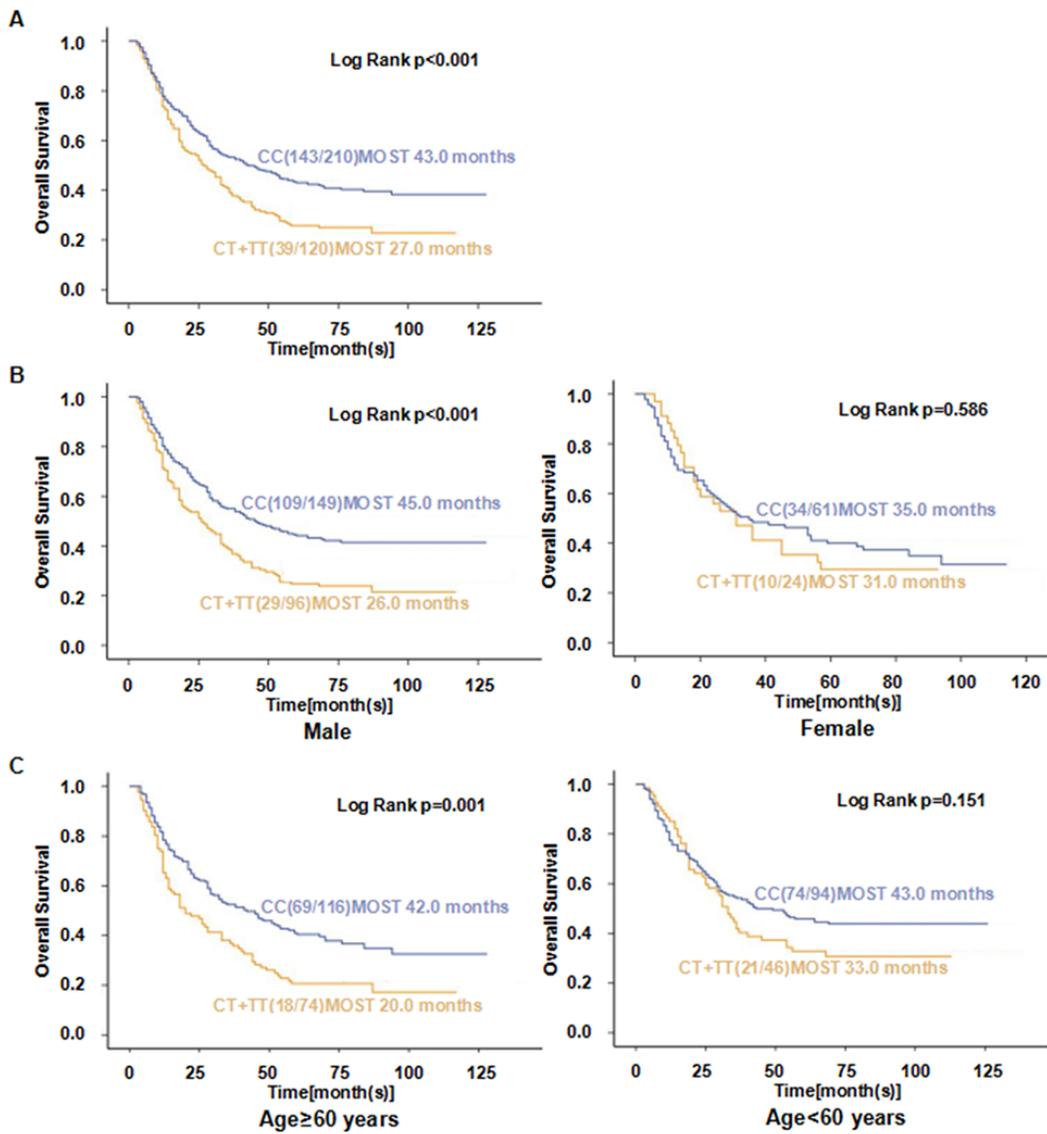


Figure 3

Kaplan–Meier curves of median overall survival time (MOST) based on SLC39A6 rs1050631 genotype. (A) Curves calculated for the whole cohort. (B-C) Curves were calculated for subgroups stratified by (B) sex and (C) age. The log-rank test was used to calculate p values.

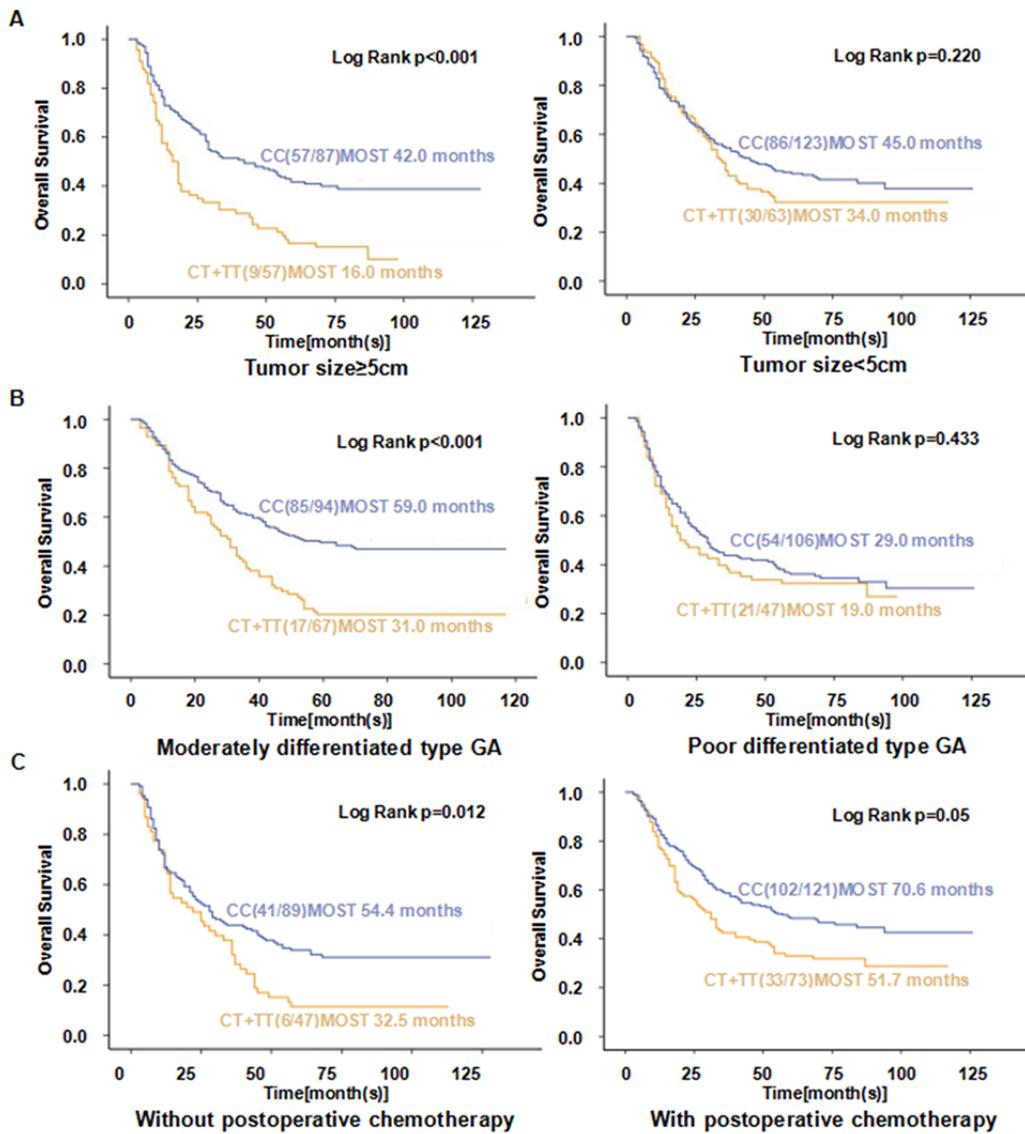


Figure 4

Subgroup Kaplan–Meier curves of median overall survival time (MOST) based on SLC39A6 rs1050631 genotype. Curves were calculated for subgroups stratified by (A) tumor size, (B) differentiation grade and (C) chemotherapy status. The log-rank test was used to calculate p values.

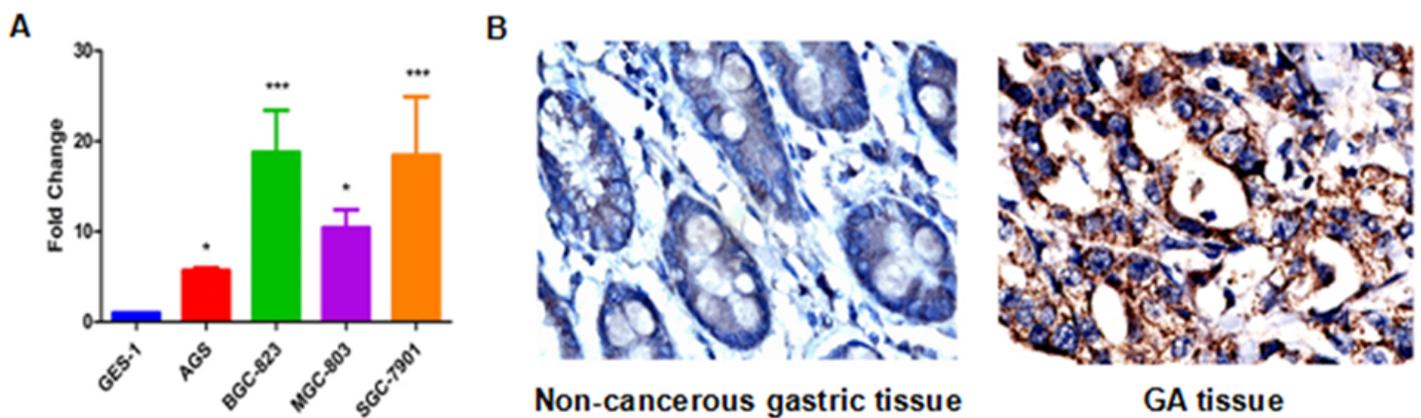
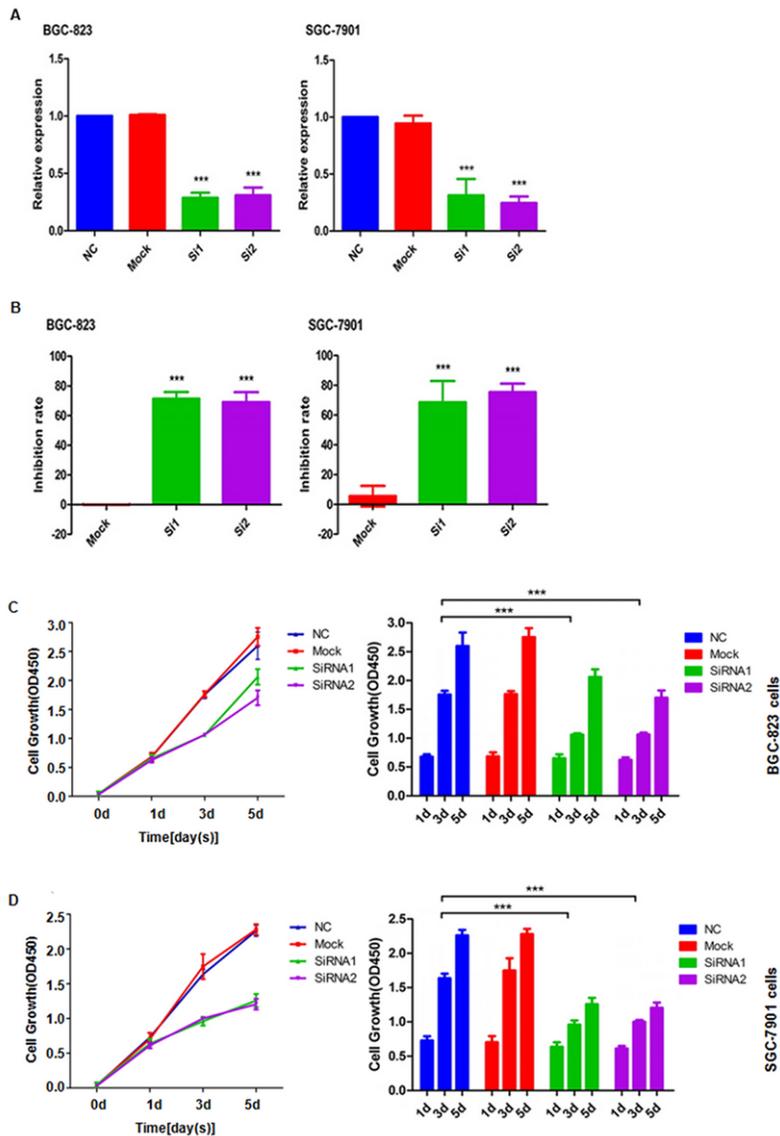


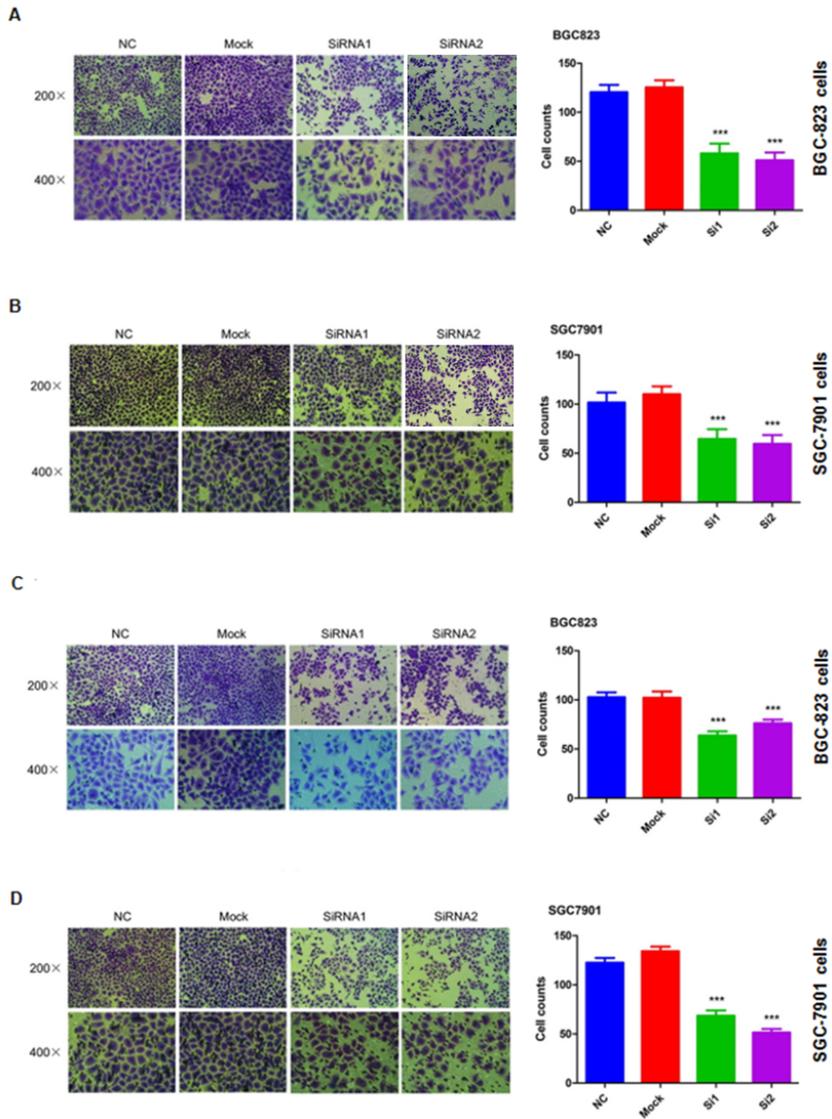
Figure 5

SLC39A6 expression in gastric cancer cell lines and tissues. (A) Quantitative RT-PCR showed that SLC39A6 mRNA levels were significantly higher in the gastric cancer cell lines AGS, BGC-823, SGC-7901 and MGC-803 than in normal gastric cell line GES-1 (all  $p < 0.05$ ). (B) Immunohistochemistry of tissue slices showing up-regulation of SLC39A6 protein in gastric adenocarcinoma (GA) tissues relative to non-cancerous gastric tissue. Magnification,  $\times 400$ .



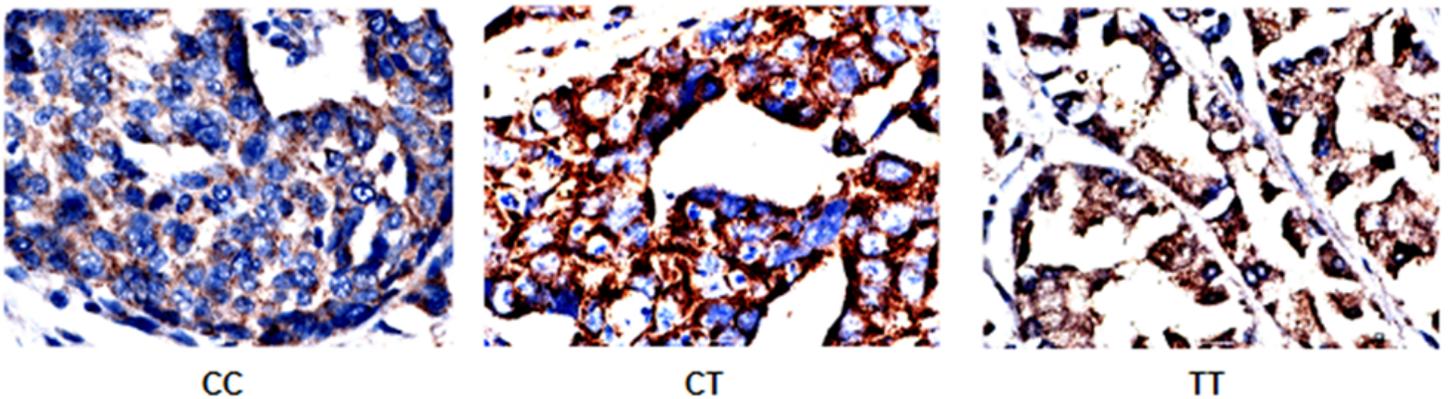
**Figure 6**

SLC39A6 silencing significantly decreases gastric cancer cell proliferation. (A-B) Quantitative RT-PCR shows that transfection of gastric cancer cell lines BGC-823 and SGC-7901 with SLC39A6 siRNA1 and siRNA2 significantly suppressed SLC39A6 expression. (A) Levels of SLC39A6 mRNA. (B) Extent of SLC39A6 knockdown achieved with each siRNA. (C-D) SLC39A6 knockdown inhibited proliferation of (C) BGC-823 and (D) SGC-7901 cells ( $p < 0.05$  vs. control cells untransfected (Mock) or transfected with scrambled siRNA (NC)).



**Figure 7**

SLC39A6 silencing significantly decreases gastric cancer cell migration and invasion. (A-B) SLC39A6 knockdown inhibited migration by (A) BGC-823 and (B) SGC-7901 cells ( $p < 0.05$  vs. Mock or NC controls). (C-D) SLC39A6 knockdown inhibited invasion by (C) BGC-823 and (D) SGC-7901 cells.



**Figure 8**

Representative immunohistochemistry results of SLC39A6 protein in gastric cancer tissue from patients with different genotypes (CC, CT, TT) at SLC39A6 rs1050631. Magnification, ×400.