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ORIGINAL ARTICLE

Increased expression of *Solute carrier family 12 member 5* via gene amplification contributes to tumour progression and metastasis and associates with poor survival in colorectal cancer

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ABSTRACT

Objective Using whole genome sequencing, we identified gene amplification of solute carrier family 12 member 5 (*SLC12A5*) located at 20q13.12 in colorectal cancer (CRC). We analysed its amplification, overexpression, biological effects and prognostic significance in CRC.

Design *SLC12A5* amplification status was evaluated by fluorescence in situ hybridisation (FISH). The effects of SLC12A5 re-expression or knockdown were determined in proliferation, apoptosis, invasion and metastasis assays. SLC12A5 target genes and related pathways were identified by reporter activity and cDNA microarray analyses. Clinical impact of SLC12A5 overexpression was assessed in 195 patients with CRC.

Results Amplification of *SLC12A5* was verified in 78 out of 191 (40.8%) patients with primary CRC by FISH, which was positively correlated with its protein overexpression ($p<0.001$). Biofunctional investigation of SLC12A5 revealed that SLC12A5 significantly increased cell proliferation, G1-S cell cycle transition, invasion/migration abilities, but suppressed apoptosis in vitro and promoted xenograft tumour growth as well as lung metastasis in vivo. The antiapoptosis effect by SLC12A5 was mediated through inhibiting apoptosis-inducing factor and endonuclease G-dependent apoptotic signalling pathway; and the pro-metastasis role was by regulating key elements of the matrix architecture, including matrix metallopeptidase and fibronectin. After a median follow-up of 50.16 months, multivariate analysis revealed that patients with SLC12A5 protein overexpression had a significant decrease in overall survival. Kaplan–Meier survival curves showed that SLC12A5 overexpression was significantly associated with shortened survival in patients with CRC.

Conclusions SLC12A5 plays a pivotal oncogenic role in colorectal carcinogenesis; its overexpression is an independent prognostic factor of patients with CRC.

Significance of this study

What is already known on this subject?

- *SLC12A5*, a gene located at 20q13.12, belongs to the SLC12 gene family and encodes the protein of solute carrier family 12, member 5.
- Gene amplification is one of the most common mechanisms which contributes to overexpression of genes favouring tumour development.

What are the new findings?

- *SLC12A5* amplification is identified for the first time in colorectal cancer (CRC) by whole genome sequencing. We have further verified *SLC12A5* amplification by fluorescence in situ hybridisation in 78 out of 191 (40.8%) patients with primary CRC.
- SLC12A5 plays strong tumourigenic role through inhibiting apoptosis and promoting metastasis evidenced by in vitro and in vivo tumorigenicity experiments.
- SLC12A5 inhibits apoptosis through suppressing apoptosis-inducing factor/endonuclease G-dependent apoptotic signalling and promotes metastasis through regulating key elements of the matrix architecture.
- SLC12A5 overexpression mediated by amplification is associated with poor prognosis of patients with CRC.

How might it impact on clinical practice in the foreseeable future?

- Detection of SLC12A5 overexpression may serve as a new biomarker for the prognosis of patients with CRC.

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in men and the second in women worldwide.[1] In Asian countries, the incidence of CRC has been rising rapidly.[2] Approximately 50%–60% of patients diagnosed with CRC develop metastases, which is the main cause of mortality in patients with CRC.[3 4] The pathogenic mechanisms underlying CRC development appear to be complex and heterogeneous. Emerging evidences indicate that CRC development is a multistep process with the accumulation of genetic and epigenetic alterations.[5] Copy number aberrations (CNAs), including chromosome

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gains and losses, or localised amplifications and deletions, are frequently found in human CRC and are major causes of aberrant activation of oncogenes and inactivation of tumour suppressor genes.[6–10] Some CNAs, such as copy number loss of chromosome 18q12.2, copy number gain at the chromosomal region between 11q13.3 and 11q22.3, gene amplification of MPL1/PZR, are closely related to clinical outcome or metastatic progression.[11–16] It is of great importance to identify and functionally characterise novel genes with CNAs that are associated with CRC.

It is well documented that CNAs on chromosome 20 are involved in human cancers. 20q13.12 is one of the most frequently amplified regions in CRC, as well as in other human cancers, which contains several putative oncogenes.[17–21] Our recent study using single-cell sequencing demonstrated an activating point mutation of solute carrier family 12 member 5 (*SLC12A5*), a gene located at 20q13.12, in CRC.[22] Targeted capture sequencing in a larger cohort of patients with CRC revealed that the mutation prevalence of *SLC12A5* is only 3.8% (7 out of 182 cases).[23] We found that *SLC12A5* was widely expressed in CRC tissues, while silenced or downregulated in their adjacent mucosal tissues. We, therefore, hypothesised that amplification of *SLC12A5* could be an important mechanism for its activation. Indeed, using whole-genome sequencing, we identified the frequent amplification of *SLC12A5* in human CRC. This was confirmed by an analysis of CRC genomic data of The Cancer Genome Atlas (TCGA), showing the frequent amplification of *SLC12A5* in CRC cases (https://www.synapse.org/#!Synapse:syn300013).

SLC12A5, also known as potassium chloride cotransporter 2, belongs to the *SLC12* gene family and encodes the protein of solute carrier family 12, member 5.[24] SLC12A5 protein is an integral membrane KCl cotransporter that maintains chloride homeostasis in neurons.[25 26] However, the role of *SLC12A5* amplification in CRC has not yet been explored. In this study, we evaluated the amplification status of *SLC12A5*, characterised the functional mechanisms and the clinical implication of SLC12A5 in colorectal carcinogenesis.

MATERIALS AND METHODS

Tissue samples

CRC tissue microarrays (TMAs) were constructed according to a method described previously.[27] The first set of TMA was generated from formalin-fixed, paraffin-embedded archive tissues of 191 patients with CRC, which were collected at The Prince of Wales Hospital, Hong Kong, from 2002 to 2010. The second set of TMAs was prepared from 195 CRC tissues, which were obtained from the Sun Yat-Sen University and Guangdong Provincial People's Hospital, Guangzhou, China, between January 2000 and November 2006. The patient's demographic and clinicopathological features were shown in table 1 and online supplementary table S1. The TNM status of CRCs was assessed according to the criteria of the sixth edition of the TNM classification of the International Union Against Cancer (2002).[28] Patients were being regularly followed up and the median follow-up duration since the time of diagnosis was 50.16 months (range 7–60 months). In addition, 15 paired primary tumour and adjacent non-tumour tissues from patients with CRC were obtained during operation prior to any therapeutic intervention at the Prince of Wales Hospital in Hong Kong. All subjects provided informed consent for obtaining the study specimens.

Fluorescence in situ hybridisation

The bacterial artificial chromosome clone RP11-465L10 at 20q13.12 containing *SLC12A5* gene was labelled with Sperctrum-Red (Vysis, Downers Grove, Illinois, USA). Chromosome 20 centromere probe labelled with Spectrum-Green (Vysis) was used as control. For cell line fluorescence in situ hybridisation (FISH), metaphase cells were collected after colcemid treatment, followed by hypotonic treatment and fixation, then were spread on slides. For tissue FISH, paraffin-embedding TMA sections were pretreated by de-waxing, gradient hydration and Proteinase K digestion. The Probes hybridisation procedure was performed according to the previous method.[29]

Statistical analysis

The results were expressed as mean±SD. Statistical analysis was performed using the SPSS statistical software package (standard V.16.0). The Pearson correlation coefficient was used to evaluate the correlation between *SLC12A5* gene amplification and expression in the clinical samples. The χ^2 test was used for comparison of patient characteristics and distributions of expression and covariates by vital status. Crude relative risks (RRs) of death

Table 1 Relationship between SLC12A5 expression and clinicopathological features in 195 colorectal cancer cases

Variable	Low expression (n=125)	Per cent	High expression (n=70)	Per cent	p Value
Mean age, years±SD	56.6±12.6		59.3±11.4		0.86
Gender					0.13
F	55	70.5	23	29.5	
M	70	59.8	47	40.2	
Location					0.39
Colon	99	62.7	59	37.3	
Rectal	26	70.3	11	29.7	
Grade					0.65
High	13	72.2	5	27.8	
Moderate	93	62.4	56	37.6	
Low	19	67.9	9	32.1	
TNM stage					0.03
I	46	63.0	27	37.0	
II	50	74.6	17	25.4	
III	25	58.1	18	41.9	
IV	4	33.3	8	66.7	

The TNM Staging System is based on the extent of the tumor (T), the extent of spread to the lymph nodes (N), and the presence of metastasis (M).

associated with SLC12A5 expression and other predictor variables were estimated by univariate Cox proportional hazards regression model first. Multivariate Cox model was constructed to estimate the adjusted RR for SLC12A5 expression. Overall survival in relation to expression was evaluated by the Kaplan–Meier survival curve and the log-rank test. Mann–Whitney U test or Student's t test was performed to compare the variables of two groups. The difference in cell viability and tumour growth rate between the two groups of nude mice was determined by repeated-measures analysis of variance. p Values <0.05 were taken as statistical significance. Additional detailed description of the materials and methods can be found in the online supplementary materials and methods section.

RESULTS

Amplification of *SLC12A5* is identified in CRCs

We evaluated SLC12A5 protein expression in 15 pairs of colorectal tumours and adjacent normal tissues by immunohistochemistry. SLC12A5 protein expression was distributed in both nuclear and cytoplasm of CRC tumour cells in 93% of CRCs (14/15), but not in normal colon tissue cells (figure 1A). The protein expression level of SLC12A5 was significantly higher in primary CRCs as compared with their adjacent normal tissues (p<0.05) (figure 1B). We performed immunofluorescence and confocal microscopy of colon cancer cell line HCT116 and confirmed that the SLC12A5 protein expression was distributed in both nuclear and cytoplasm (figure 1C). As activating mutation of SLC12A5 was only detected in 3.8% (7 out of 182 cases) CRCs by targeted capture sequencing,[23] other mechanisms such as gene amplification may contribute to the enhanced expression of SLC12A5.

By whole-genome sequencing analyses, we identified *SCL12A5* amplification in CRC (figure 1D), which was confirmed by array comparative genomic hybridisation showing copy number gain of *SLC12A5* (see online supplementary table S2). To further confirm our identification, we evaluated the copy number variation from TCGA data (https://www.synapse.org/#!Synapse:syn300013). In TCGA cohort of 575 CRCs, *SLC12A5* was amplified in both colon cancer (q value=8.99E-07, frequency=72.6%) and rectal cancer (q value=0.003, frequency=88.9%), signifying its common occurrence in CRC (figure 1E, F).

Amplification of *SLC12A5* contributes to its overexpression in CRC tumour tissues

To examine the relationship between DNA amplification of *SLC12A5* and its overexpression, the DNA copy number change of *SLC12A5* was investigated by FISH in a TMA containing 191 CRC cases. Amplification of *SLC12A5* was not observed in normal metaphase lymphocytes, but detected in colon cancer cell line Caco-2 (figure 2A). Importantly, 40.8% (78/191) of primary CRCs exhibited amplification of *SLC12A5*, while there was no obvious copy number gain in adjacent normal tissues (figure 2B). The protein expression of SLC12A5 was investigated in the same TMA by immunohistochemistry (figure 2C). Pearson correlation analysis indicated that amplification of *SLC12A5* in CRC tissues was positively correlated with its protein overexpression (p<0.001) (figure 2D). These data suggest that upregulation of SLC12A5 protein in CRC is at least in part contributed by DNA amplification.

SLC12A5 promotes CRC cell growth in vitro and in vivo

To assess the potential function of SLC12A5, a series of in vitro biological experiments were performed with gain-of-function or loss-of-function of SLC12A5. SW480 and SW116 cells were stably transfected with different doses of SLC12A5 plasmids. Ectopic expression of SLC12A5 in these cells (figure 3A) caused a significant increase in the number of viable cells in a dose-dependent fashion in SW480 and SW1116 (p<0.01) compared with empty vector transfected cells, respectively (figure 3B). In keeping with this, the number of colonies increased significantly in a dose-dependent manner when transfected with SLC12A5 plasmids compared with empty vector-transfected SW480 and SW1116, respectively (figure 3C, D). On the other hand, we examined whether SLC12A5 is required for the tumourigenic phenotypes of CRC cells by silencing SLC12A5 expression with siRNA. The introduction of SLC12A5-specific siRNA (siSLC12A5) into HCT116 dramatically decreased the expression of SLC12A5 relative to control cells transfected with scrambled siRNA (figure 3E). SLC12A5 depletion by siRNA reversed the tumourigenic phenotype by inhibiting the cell growth rate compared with control cells (p<0.001) (figure 3F).

To further investigate the in vivo tumourigenic ability of SLC12A5, we constructed a lentiviral vector carrying shSLC12A5 to stably knock down the expression of SLC12A5 in HCT116 (figure 3G). The Control-HCT116 and shSLC12A5-HCT116 cells were injected subcutaneously into the dorsal flank of nude mice. Tumours induced by shSLC12A5-HCT116 cells showed significantly longer latency and smaller mean tumour volume than tumours induced by control cells (p<0.001) (figure 3H). At the end of experiments, xenograft tumours were isolated and the mean tumour weight of the shSLC12A5-HCT116 group was reduced by more than 70% as compared with controls (p<0.05) (figure 3I). Collectively, these data indicate that SLC12A5 possesses strong tumourigenicity in CRC both in vitro and in vivo.

SLC12A5 inhibits apoptosis in colon cancer cells

To explore the molecular basis involved in SLC12A5-enhanced tumour growth, the effect of SLC12A5 on apoptosis was assessed quantitatively by flow cytometry after staining with Annexin V and 7-amino-actinomycin. We found that overexpression of SLC12A5 significantly inhibited early apoptosis in SW480 and SW1116 (p<0.001) (figure 4A), as compared with control vector-transfected cells. To further clarify whether knockdown of SLC12A5 could reverse this phenotype, siRNAs against SLC12A5 were transfected into HCT116 cells. The percentage of early apoptotic cells in siSLC12A5 HCT116 cells was significantly increased, as compared with the control cells (p<0.01) (figure 4B).

SLC12A5 exerts an antiapoptotic function through the caspase-independent pathway

To define the molecular basis of cell death after SLC12A5 depletion, we assessed the key apoptosis regulators. Activation of caspases and the subsequent cleavage of poly (ADP-ribose) polymerase (PARP) are classic molecular hallmarks of apoptosis.[30] However, no changes of cleaved Caspase 3, 7 or cleaved PARP were observed after silencing SLC12A5 expression (figure 4C), indicating that SLC12A5 may inhibit apoptosis via a caspase-independent pathway. Apoptosis-inducing factor (AIF) and endonuclease G (EndoG) are known for mediating cell death independent of caspases by translocating from mitochondria into the nucleus where they induce DNA cleavage.[30] We, therefore, examined the nuclear protein expression of AIF and EndoG. We found that knockdown of SLC12A5 enhanced the nuclear protein levels of AIF and EndoG (figure 4C). The mitochondrial release of AIF and EndoG is mediated by pro-apoptotic Bax and Bak and their upstream regulators, p53

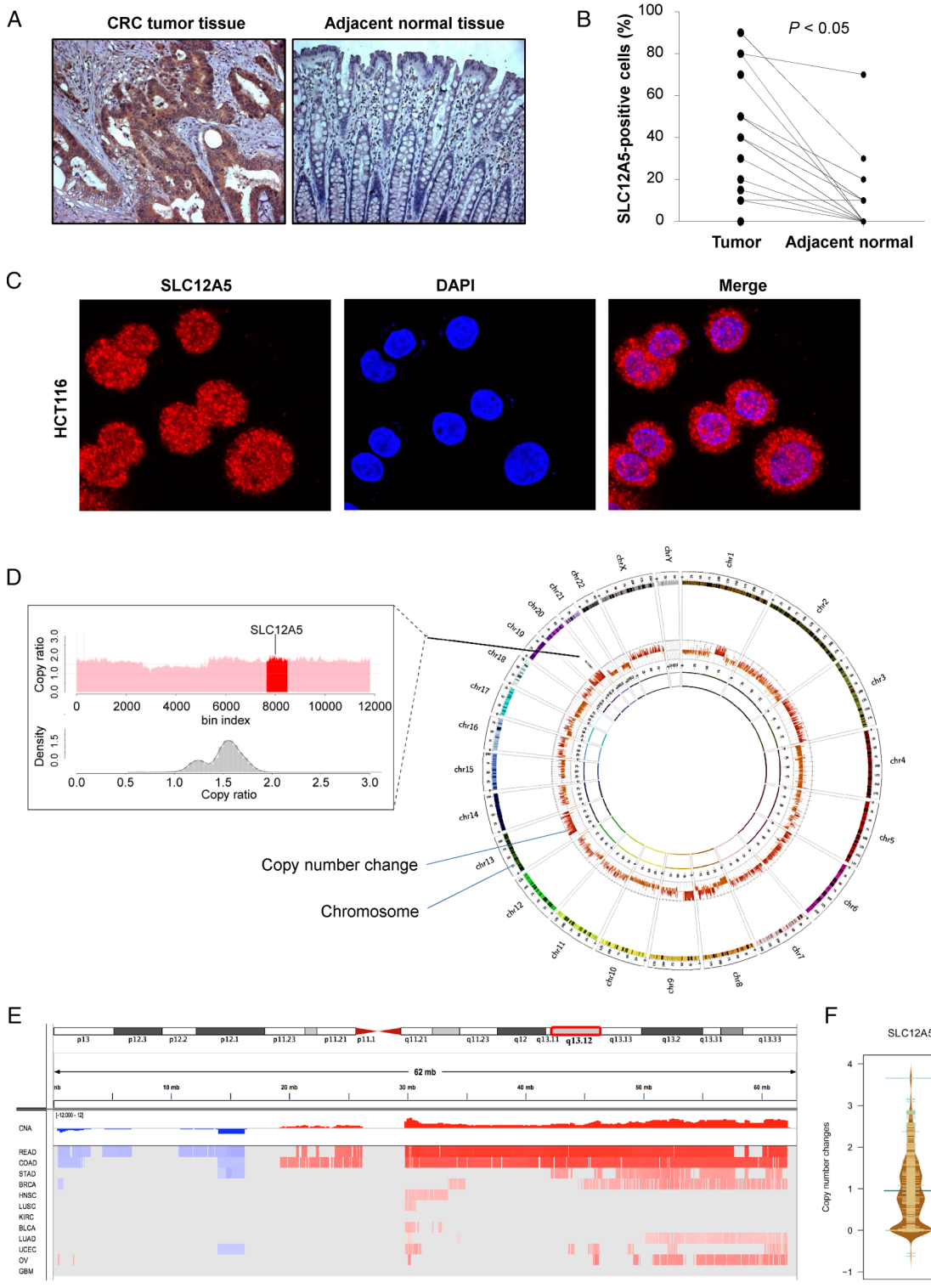

Figure 1 *SLC12A5* is amplified in colorectal cancer (CRC). (A) Representative images of SLC12A5 protein expression in CRC tumour tissues and their adjacent tissues by immunohistochemistry. (B) The protein expression level of SLC12A5 was significantly higher in primary CRC tumour as compared with their adjacent tissue (p<0.05). (C) SLC12A5 was expressed in both nuclear and cytoplasm in colon cancer cell line HCT116 by immunofluorescence and confocal microscopy. (D) The right panel shows the genomic profiles of CRC by whole genome sequencing, where the copy number alterations were indicated with red bars. The left panel shows the visualisation of copy ratio particularly for the whole chromosome 20. 20q13.12 is highlighted with red colour, which harbours the amplified gene *SLC12A5*. (E) Significant genomic alterations of 20q13.12 across 12 human cancer types in The Cancer Genome Atlas (TCGA) cohort. Red colour indicates copy number gain, while blue colour refers to copy number loss. BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; KIRC, kidney renal clear cell carcinoma; COAD, colon adenocarcinoma; DAPI, 4′,6-diamidino-2-phenylindole; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; LAML, acute myeloid leukaemia; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; READ, rectum adenocarcinoma; OV, ovarian serous cystadenocarcinoma; UCEC, uterine corpus endometrioid carcinoma. (F) Copy number changes of *SLC12A5* in 575 colorectal cancers from TCGA.

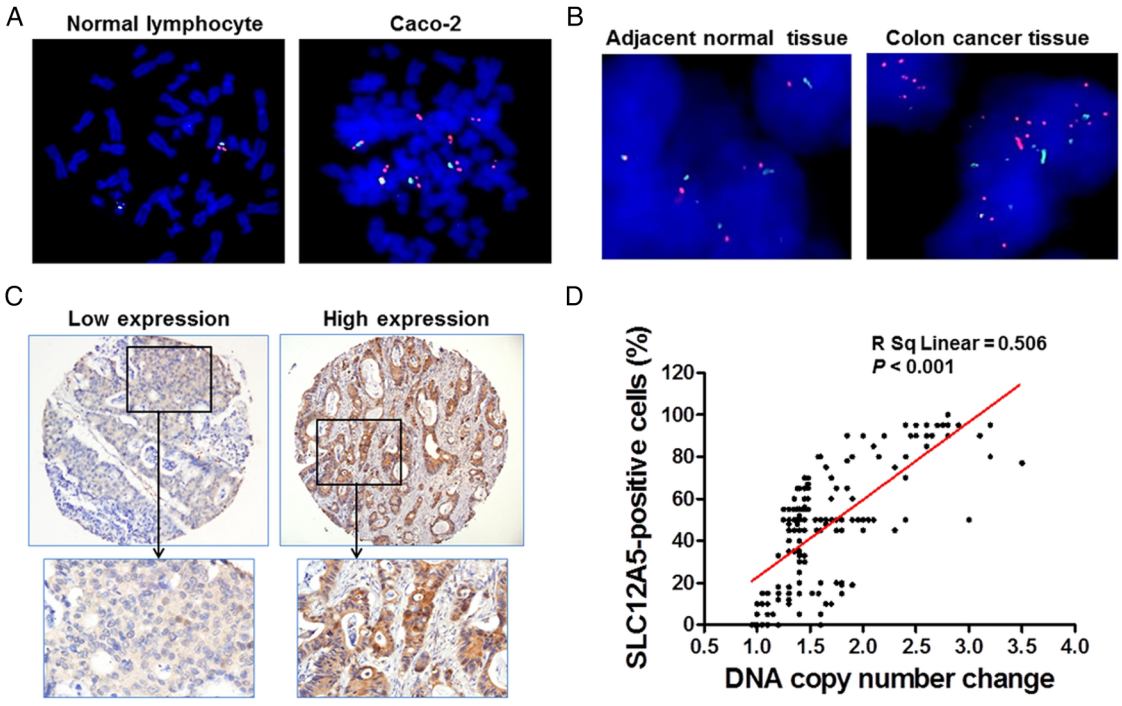

Figure 2 Amplification and overexpression of *SLC12A5* in colorectal cancers (CRCs). (A) Fluorescence in situ hybridisation (FISH) was performed in normal metaphase lymphocytes and colon cancer cell line Caco-2. The red signal indicates the location of *SLC12A5* and the green signal refers to centromere of chromosome 20. This result implied that the probe of *SLC12A5* was correctly located on chromosome 20. Amplification of *SLC12A5* was observed in Caco-2. (B) Representative image of *SLC12A5* gene amplification detected by FISH in primary CRC tissues, but not detected in their adjacent tissues. (C) Representative staining of high and low expression levels of SLC12A5 in CRC tissue microarray slides by immunohistochemistry. (D) The correlation between copy number changes of SLC12A5 and protein overexpression (R^2=0.506, p<0.001).

and p53 upregulated modulator of apoptosis (PUMA), which can influence the mitochondrial membrane permeabilisation.[31] Concordantly, SLC12A5 silencing increased the protein expression of pro-apoptotic factors p53, PUMA, Bax and Bak (figure 4C). Emerging evidence supported the fact that the inhibitor of apoptosis protein Survivin decreases the amount of AIF in the nucleus after apoptosis induction, thus protecting cells from death.[32 33] As shown in figure 4C, Survivin was significantly downregulated after knockdown of SLC12A5. To further confirm the antiapoptotic effect of SL12A5 in vivo, we performed TUNEL staining on the paraffin slides of mouse xenograft tumours. We found that TUNEL-positive apoptosis cells were significantly more in the shSLC12A5-HCT116 tumours compared with the control-HCT116 tumours (p<0.01) (figure 4D). Consistently, the nuclear protein expression of AIF and EndoG was significantly increased in the shSLC12A5-HCT116 tumours compared with the control-HCT116 tumours (figure 4D), which is consistent with the in vitro study by western blot (figure 4C). These results collectively suggest that SLC12A5 inhibits apoptosis by suppressing the activation of AIF–EndoG-dependent apoptotic pathway (figure 4E).

SLC12A5 promotes G1 to S phase transition in colon cancer cells

To characterise the oncogenic mechanism of SLC12A5 in colon cancer cell growth, we further investigated the role of SLC12A5 in cell cycle progression. We found that ectopic expression of SLC12A5 in SW480 (SLC12A5-SW480) significantly decreased the number of cells in G1 phase (p<0.01), but increased the number of cells in S phase (p<0.01) compared with control vector-transfected cells 12 h after serum stimulation (figure 4F). Conversely, knockdown SLC12A5 in HCT116 cells by siSLC12A5 arrested the cell cycle at the G1–S transition (p<0.01) (figure 4F). Western blot analysis showed that the protein expression of two master G1–S checkpoint regulators cyclin D1 and cyclin-dependent kinase (CDK4) was increased in SLC12A5-SW480 compared with the vector-transfected cells, while knockdown SLC12A5 in HCT116 had the opposite effect (figure 4G), confirming the role of SLC12A5 on promoting cell growth by regulating cell cycle progression in colon cancer cells.

SLC12A5 inhibits p53 and p21 reporter activity

To gain insights into the downstream signalling pathways modulated by SLC12A5 in CRC tumourigenesis, we examined the functional effect of SLC12A5 in several important cancer pathways, including p53, p21, activator protein-1 (AP-1), nuclear factor (NF)-κB and Wnt/β-catenin (TOPFlash), by luciferase reporter activity assay. Ectopic expression of SLC12A5 significantly suppressed p53 and p21 luciferase reporter activities in HCT116 (figure 5A), whereas no significant activity changes were observed in AP-1, NFκB and Wnt/β-catenin pathway reporters (figure 5A). To further confirm the importance of the p53 and p21 pathways mediated by SLC12A5 in colon cancer cells, we assessed the ability of abolishing SLC12A5 in the reporter activities of p53 and p21. Knockdown SLC12A5 significantly enhanced p53 and p21 reporter activities (figure 5B). Western blot results further validated that ectopic expression of SLC12A5 reduced the protein level of p53 and p21, while knockdown of SLC12A5 upregulated p53 and p21 in HCT116 cells (figure 5C).

As SLC12A5 promoted cell proliferation and inhibited cell apoptosis in p53-mutated cells SW480 and SW1116, which implies the tumour-promoting function of SLC12A5 could be independent of p53. To confirm this hypothesis, we transfected

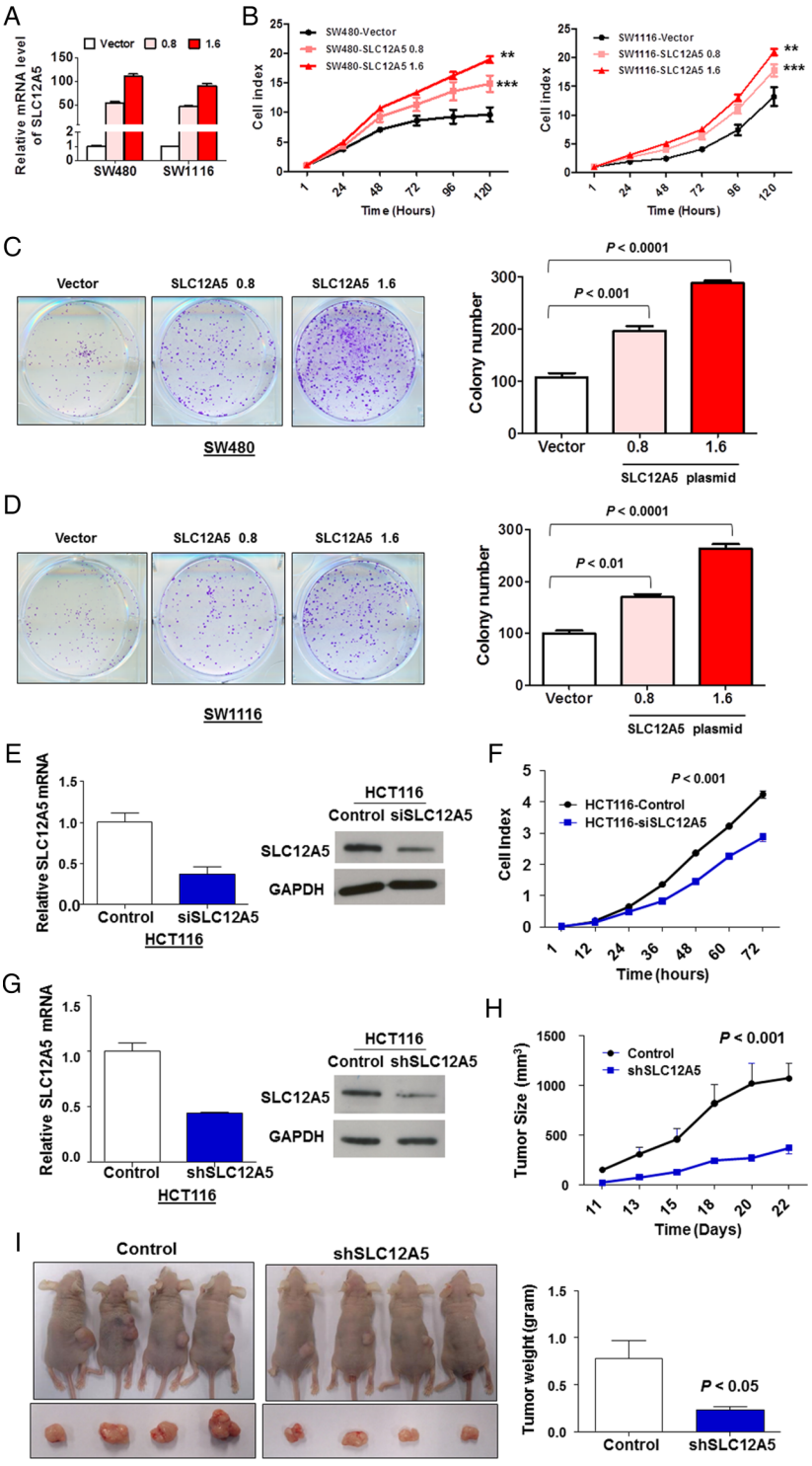

Figure 3 SLC12A5 promotes colorectal cancer cell growth. (A) Expression levels of *SLC12A5* were increased after transfection with different doses of *SLC12A5* plasmids (0.8 and 1.6 μg) in colon cancer cell lines SW480 and SW1116. (B) Ectopic expression of SLC12A5 significantly enhanced cell viability in a dose-dependent manner in both cell lines. (**p<0.01, ***p<0.001) (C) Empty vector and different doses of SLC12A5 plasmids (0.8 and 1.6 μg) were used to transfect cells in 24-well plates. Compared with empty vector-transfected cells, colony numbers significantly increased with transfection of 0.8 and 1.6 μg SLC12A5 plasmids in SW480 in a dose-dependent manner. (D) The number of colonies increased in a dose-dependent manner when transfected with SLC12A5 plasmids in SW1116. (E) Knockdown efficiency of SLC12A5 in HCT116 cells by transient transfection of si*SLC12A5* was examined by real-time PCR (left panel) and western blot (right panel), respectively. (F) Knockdown of SLC12A5 significantly inhibited cell viability in HCT116. (G) Knockdown efficiency of SLC12A5 in HCT116 cells by stable transfection of sh*SLC12A5* was demonstrated by real-time PCR (left panel) and western blot (right panel). (H) The tumour growth curve of HCT116 stably transduced with sh*SLC12A5* in nude mice was significantly dampened compared with HCT116 transduced with Control. (I) A representative picture of tumour formation in nude mice subcutaneously inoculated with shSLC12A5- or Control-HCT116 (left panel). Histogram represents mean of the tumour weight from the shSLC12A5 and Control groups (right panel).

SLC12A5 in p53 knockout (KO) HCT116 cells (figure 5D). Ectopic expression of SLC12A5 significantly enhanced cell growth in p53 KO HCT116 cells (figure 5E). We then determined the above five luciferase reporters in p53 KO HCT116 and SW1116. As shown in figure 5F, only p21 reporter activity was significantly downregulated. These data indicated that SLC12A5 suppressed p21 activity irrespective of p53 status.

SLC12A5 promotes tumour invasion and metastasis in vitro and in vivo

We also investigated the effects of SLC12A5 on metastasis using in vitro migration and Matrigel invasion assays and in vivo metastasis assay. The migration and Matrigel invasion assays showed that both the migration and the invasive capability of SLC12A5-SW1116 cells were greater than those of Vector-SW116 cells (figure 6A). By contrast, knockdown *SLC12A5* expression by siRNA in HCT116 cells eliminated the migratory and invasive ability of the HCT116 cells (figure 6B). These results indicate that SLC12A5 increases cell invasion and metastasis, which we further validated in vivo.

The in vivo metastasis assay was performed by injecting HCT116 cells transduced with shSLC12A5 or Control-shRNA into nude mice through the lateral tail vein to examine the cells' lung metastasis ability. Six weeks following injection, the incidence of lung metastases (5/5, 100%) was significantly higher in mice injected with Control-

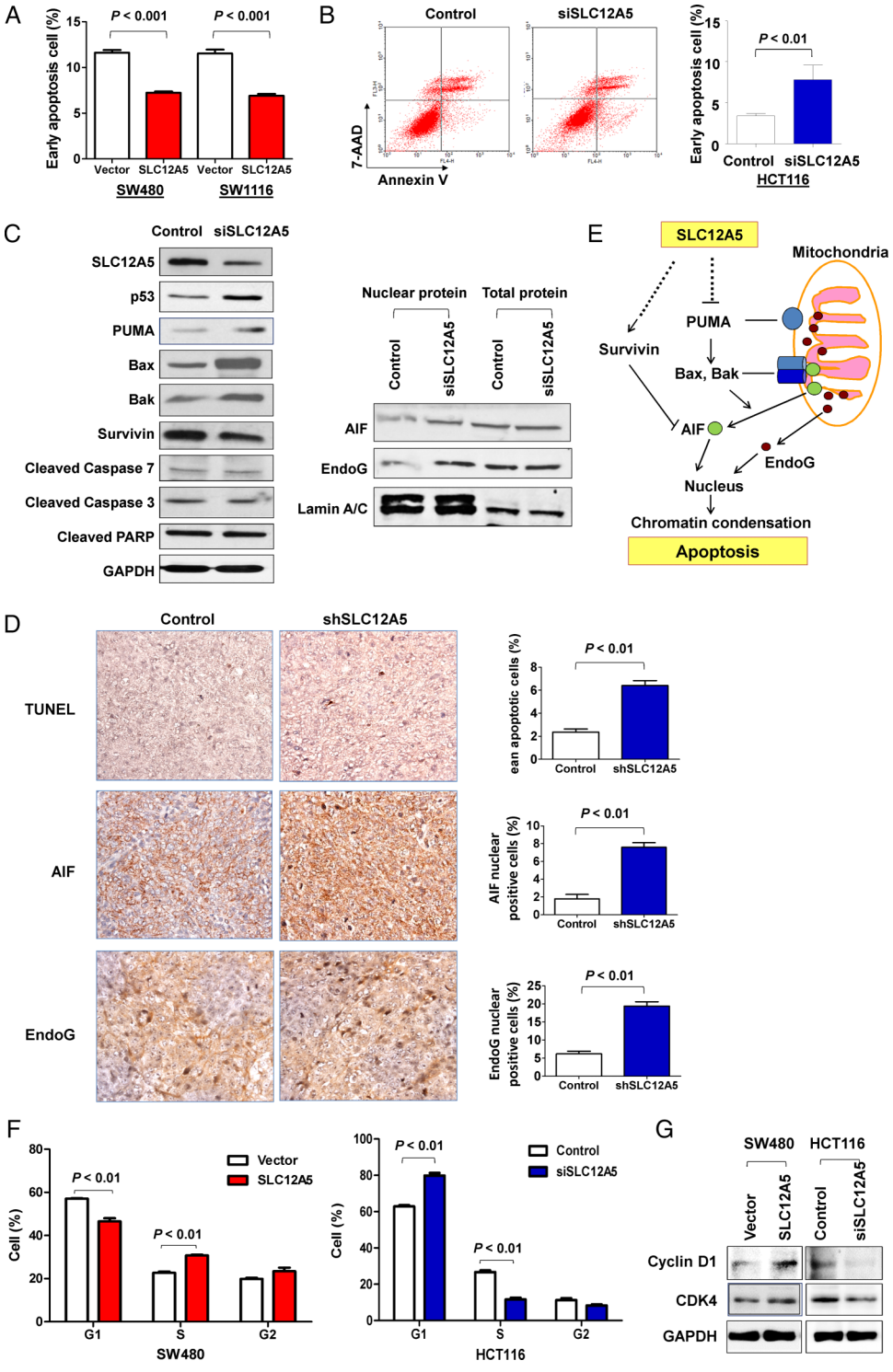

Figure 4 SLC12A5 reduces apoptosis in colorectal cancer cell lines. (A) Overexpression of SLC12A5 significantly inhibited early apoptosis in SW480 and SW1116 (B) Knockdown of SLC12A5 significantly increased the proportion of apoptotic cells in HCT116 cells by flow cytometry analyses following Annexin V and 7-amino-actinomycin (7-AAD) staining ($p<0.01$). (C) Protein expression of key apoptosis-related genes was evaluated by western blot in HCT116 cells transfected with siSLC12A5 or Control. After silencing SLC12A5 expression, no changes of cleaved Caspase 3, 7 nor cleaved poly(ADP-ribose) polymerase (PARP) were observed. SLC12A5 silencing increased the protein expression of p53, p53 upregulated modulator of apoptosis (PUMA), Bax and Bak and downregulated the inhibitor of apoptosis protein Survivin. Knockdown of SLC12A5 enhanced the nuclear protein levels of apoptosis-inducing factor (AIF) and endonuclease G (EndoG). (D) TUNEL-stained sections of mouse xenograft tumours displayed apoptotic cells; representative images of AIF and EndoG immunohistochemistry-stained sections of mouse xenograft tumours. (E) Schematic diagram for the proposed mechanisms of SLC12A5 exerting antiapoptotic function via caspase-independent signalling pathway. (F) The number of cell distribution was determined by flow cytometry. Values are mean±SD. (G) Protein expression of cyclin D1 and cyclin-dependent kinase (CDK4) was determined by western blot.

HCT116 cells than in mice injected with shSLC12A5-HCT116 cells (1/5, 20%) ($p<0.01$) (figure 6C). Metastatic nodules in the lungs were confirmed histologically (figure 6C).

SLC12A5 regulates extracellular matrix remodelling genes

To gain further insight into the mechanisms by which SLC12A5 promotes CRC cell invasion and metastasis, SLC12A5-modulated downstream target genes were characterised by Human Tumour Metastasis RT[2] Profiler PCR array in HCT116 cells transfected with siSLC12A5 or control siRNA. The results showed that knockdown of *SLC12A5* in HCT116 cells upregulated nine antimetastasis genes, including *metastasis suppressor (MTSS1), p53, NME/NM23 nucleoside diphosphate kinase (NME1), fibronectin 1 (FN1), neurofibromin 2, FAT tumour suppressor homologue 1 (FAT1), breast cancer metastasis suppressor 1 (BRMS1), tissue inhibitor of metalloproteinase 2 (TIMP2), cathepsin K (CTSK)* and downregulated four pro-metastasis genes, namely matrix metallopeptidase 2 (*MMP2*), *hepatocyte growth factor (HGF), MMP 13 and MMP10*, as compared with Control-HCT116 cells, with more than a twofold change at mRNA levels (see online supplementary table S3). Selected downstream effectors of *SLC12A5* were validated in two more colon cancer cell lines by overexpression of *SLC12A5*. Consistent with the array data, ectopic expression of SLC12A5 in

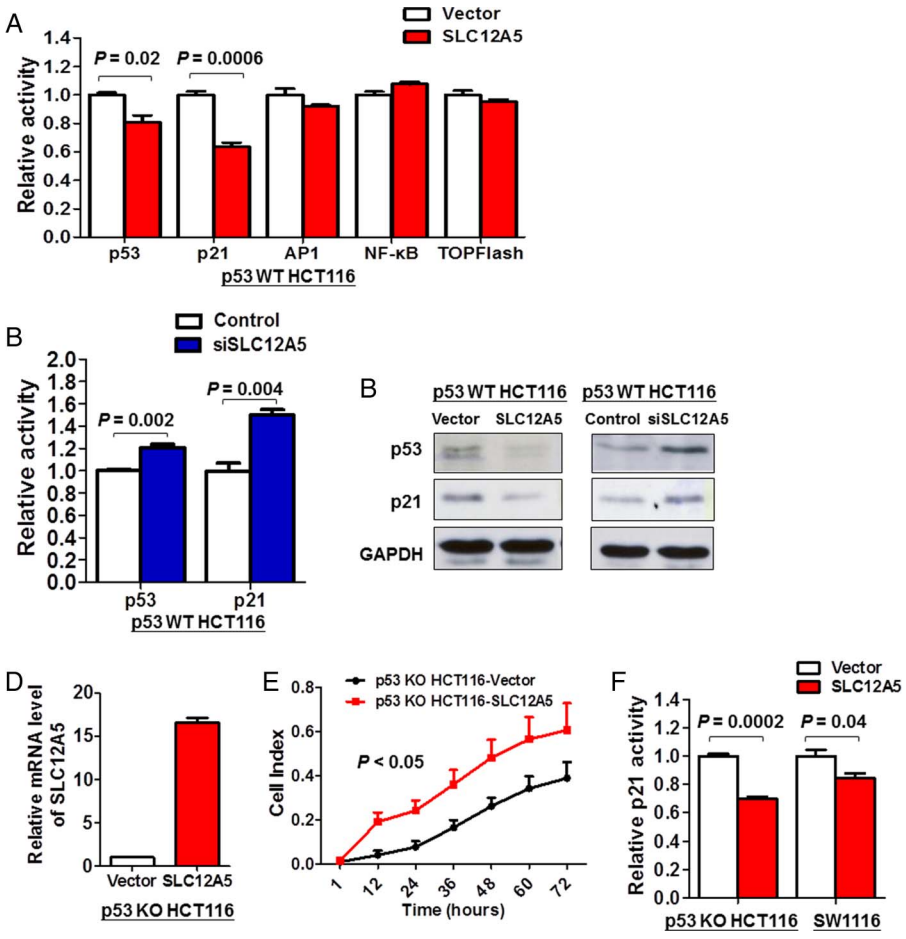

Figure 5 SLC12A5 suppressed p53 and p21 luciferase reporter activity. (A) To screen for SLC12A5 target signalling pathways, a serial of promoter-luciferase assays (p53-luc, p21-luc, activator protein 1 (AP1)-luc, nuclear factor κB (NF κB)-luc, and Wnt/β-catenin) were performed in *SLC12A5* stably transfected p53 wildtype (WT) HCT116 cells compared with vector control cells. Luciferase activities were determined by dual luciferase assay system at 48 h post-transfection. Ectopic expression of SLC12A5 suppressed p53 and p21 luciferase reporter activity in p53 WT HCT116. (B) Knockdown of SLC12A5 increased p53 and p21 luciferase reporter activity in p53 WT HCT116. (C) The western blot results confirmed the findings of luciferase reporter activity assays in p53 WT HCT116. (D) Ectopic expression of SLC12A5 was determined in p53 knockout (KO) HCT116 cells by real-time PCR. (E) Ectopic expression of SLC12A5 significantly promoted cell growth in p53 KO HCT116 cells. (F) SLC12A5 in HCT116 cells suppressed p21-luciferase reporter activity in both p53 KO HCT116 and SW116 cells.

SW480 and SW1116 downregulated MTSS1 and FN1, but upregulated MMP2, HGF and MMP13 as determined by RT-PCR and western blots (figure 6D, E). Taken together, our findings strongly suggest that SLC12A5 promote invasion and metastasis by regulating extracellular matrix-remodelling genes.

Overexpression of SLC12A5 is associated with poor prognosis of patients with CRC

We further evaluated the clinicopathological and prognostic significance of SLC12A5 in patients with CRC. As shown in table 1, overexpression of SLC12A5 was positively associated with a more advanced TNM stage ($p<0.05$), which is consistent with the role of SLC12A5 on tumour metastasis. Kaplan–Meier survival analysis showed that patients with CRC with SLC12A5 overexpression had significantly shorter survival compared with those with SLC12A5 low expression ($p=0.009$, log-rank test) (figure 7). In univariate Cox regression analysis, SLC12A5 overexpression was associated with an increased risk of cancer-related death (RR, 2.38; 95% CI 1.21 to 4.66; $p=0.012$) (table 2). After the adjustment for potential confounding factors, multivariate Cox regression analysis showed that SLC12A5 overexpression was an independent predictor of poorer survival of patients with CRC (RR, 2.30; 95% CI 1.16 to 4.60; $p=0.018$) (table 2).

DISCUSSION

In this study, amplification of *SLC12A5* was detected in 40.8% (78/191) of primary CRCs by FISH, but not in their adjacent tissues. Compared with the low frequency of *SLC12A5* point mutation (3.8%) reported in our recent study based on targeted capture sequencing,[23] amplification of *SLC12A5* is a common event in colorectal tumourigenesis. *SLC12A5* was located on chromosome 20q13.12, which is one of the most frequently amplified regions in CRC[21] and in other cancer types such as breast, ovarian and gastric cancers.[19 20] The 20q13.12 amplicon encodes 11 genes, none of which have been unequivocally described with oncogenic function in CRC yet.[21] Gene amplification is thought to promote overexpression of genes favouring tumour development.[34 35] Importantly, we revealed that the amplification of *SLC12A5* was positively associated with its protein overexpression in the 191 CRC cases ($p<0.001$), suggesting that increased *SLC12A5* copy number contributes to the up-regulation of SLC12A5, which might play an important oncogenic role in CRC. In keeping with our finding, SLC12A5 was upregulated in cervical cancer.[36]

A series of in vitro and in vivo functional experiments revealed that SLC12A5 possessed a strong tumourigenic function in CRC. SLC12A5 enhanced tumour cell survival, which is attributable to its antiapoptotic and pro-proliferative ability. Defective apoptosis is one of the hallmarks of cancer. Although caspase activation is considered a cardinal feature of apoptosis,[37] an alternative pathway of apoptosis has been described that does not require caspase activation, which is characterised by the nuclear translocation of AIF and EndoG.[30 38 39] In this study, knockdown of SLC12A5 induced apoptosis without caspase activation, suggesting that SLC12A5 modulated apoptosis via a caspase-independent pathway. In line with this hypothesis, we found that silencing SLC12A5 enhanced the nuclear protein levels of AIF and EndoG (figure 4). The release of AIF and EndoG is regulated by mitochondrial outer membrane

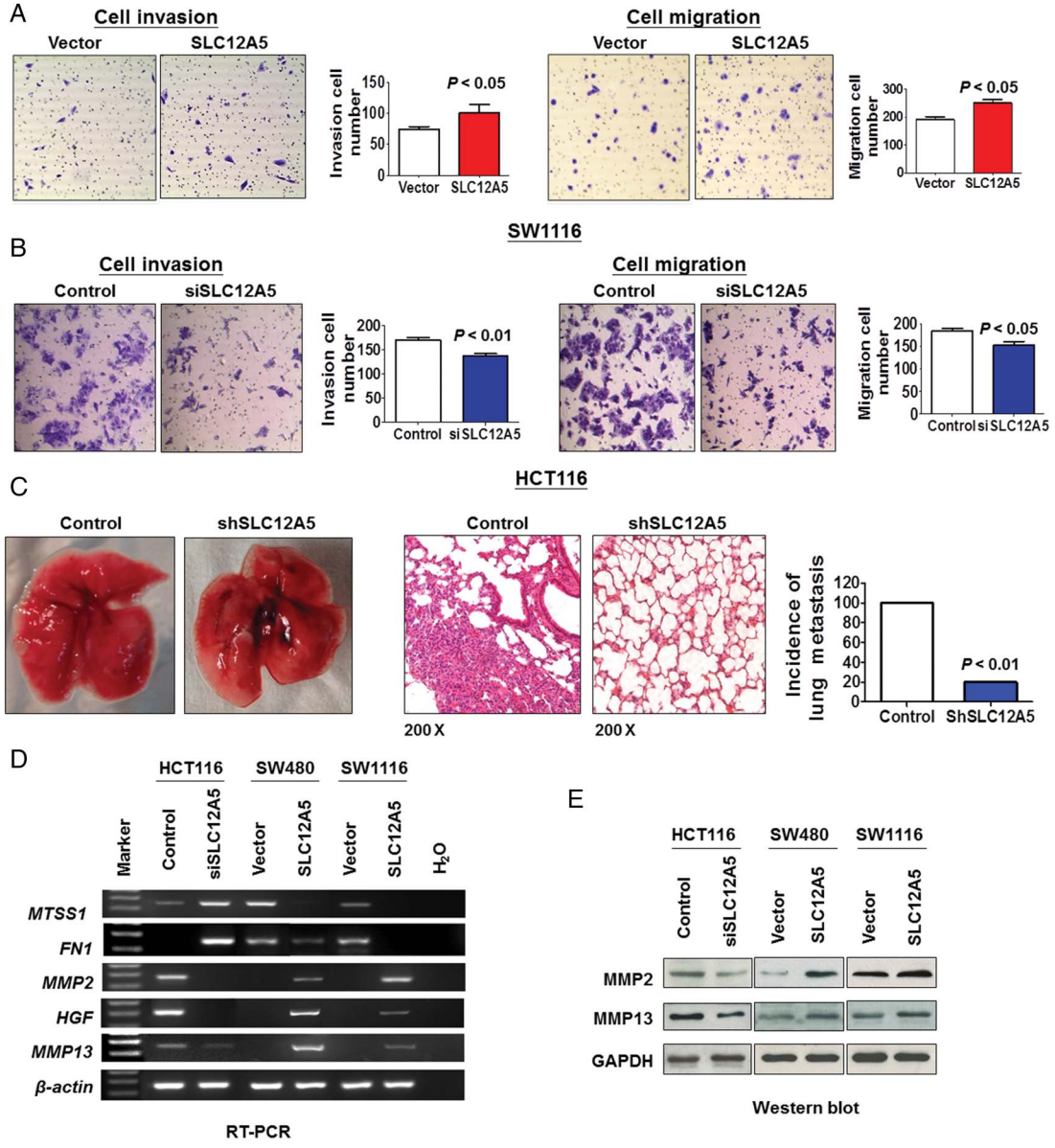

Figure 6 SLC12A5 promotes tumour metastasis in vitro and in vivo. (A) Ectopic expression of SLC12A5 promoted cell invasion and migration in SW1116 cells. (B) Knockdown of SLC12A5 suppressed cell invasion and migration in HCT116 cells. (C) Representative images of lungs with or without metastasis (left panel). H&E staining of lung tissues from nude mice injected with Control-HCT116 or shSLC12A5-HCT116 cells (200x magnification, middle panel). Incidence of lung metastasis (right panel). (D) Validation of the key metastasis-related genes identified by cDNA expression array by RT-PCR. (E) Western blot further confirmed the deregulation of metastasis-related genes. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HGF, hepatocyte growth factor; MMP, matrix metallopeptidase.

permeability, which is modulated by the relative abundance of the pro-apoptotic Bax and Bak.[31] After being activated by p53, PUMA interacts with antiapoptotic Bcl-2 family members, thereby freeing pro-apoptotic molecules, Bax and Bak.[30] Consistent with the antiapoptotic role of SLC12A5, nuclear translocation of AIF and EndoG by silencing SLC12A5 was paralleled with the upregulation of p53, PUMA, Bax and Bak. Bax and Bak can form complexes at the mitochondrial outer membrane to facilitate the release of AIF and EndoG into nucleus.[40] On the other hand, the inhibitor of apoptosis protein Survivin retrains AIF in mitochondria and suppresses AIF activity, thus further enhancing apoptotic resistance.[32] Collectively, our data support that SLC12A5 inhibits caspase-independent apoptosis by deregulating AIF and EndoG in CRC (figure 4E). Cell cycle analysis revealed a decrease in G1 phase cells and an increase in S phase cells in SLC12A5-transfected cells, and a concomitant increase of protein expression of the master G1–S transition regulators cyclin D1 and CDK4. These results indicated that SLC12A5 could facilitate DNA synthesis and promote G1–S transition, contributing to its oncogenic effect in CRC.

Moreover, SLC12A5 inhibited p53 and p21 luciferase reporter activities in HCT116, which is p53 wild type. p53 and p21 were implicated in apoptosis,[30] and the inhibition of these factors may contribute to the antiapoptotic effect of SLC12A5. Consistent with this effect, it has been reported that supplementation of KCl in extracellular medium was most effective in preventing hypoxia-induced apoptosis.[41] It is further revealed that KCl supplementation downregulated a distinct p53-regulated cellular sub-network of genes involved in regulation of DNA replication.[41] On the other hand, SLC12A5 also exhibited

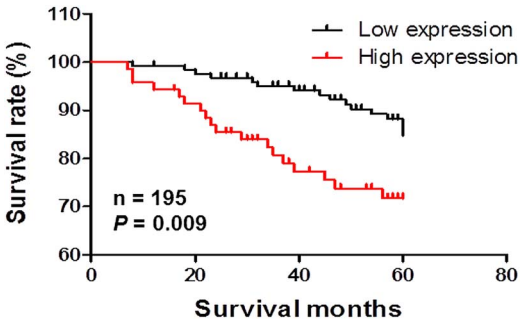

Figure 7 Clinical significance of SLC12A5 in colorectal cancers (CRCs). Kaplan–Meier survival analysis according to SLC12A5 expression in195 patients with CRC. Patients with CRC with SLC12A5 overexpression had poorer survival than others. The difference is statistically significant based on the log-rank test (p=0.009).

tumour-promoting effect and suppressed p21 reporter activity in p53 KO and mutated CRC cells, indicating that SLC12A5 promotes tumourigenesis by inhibiting p21 reporter activity regardless of p53 status. In line with this, the carboxyl terminus of p21 was reported to activate cell apoptosis without caspase involvement in a p53-independent manner, which is associated with the release of AIF and EndoG from mitochondria.[42]

Consistent with its tumourigenic function, SLC12A5 also induces tumour invasion and metastasis. Ectopic expression of SLC12A5 in colon cancer cell lines promoted cell migration and invasion in vitro. Moreover, in a cancer metastasis mouse model, depletion of SLC12A5 by shRNA in HCT116 cells significantly inhibited lung metastasis in vivo. The molecular mechanisms by which SLC12A5 exerts its pro-invasive and pro-metastasis functions in CRC were evaluated by cDNA microarray and immunoblotting. We revealed that the enhanced cell invasion and migration by SLC12A5 were mediated via downregulation of key antimetastasis genes, including *MTSS1*, *p53*, *NME1*, *FN1*, *NF2*, *FAT1*, *BRMS1*, *TIMP2* and *CTSK*, and upregulation of the important pro-metastasis genes *HGF*, *MMP10*, *MMP13* and *MMP2* (see online supplementary table S3). Of which, MTSS1 plays a role in governing the metastatic nature of breast cancer cells, as well as a prognostic indicator of disease-free survival in patients with breast cancer.[43] FN1 is a glycoprotein and is known to be involved in matrix remodelling, cell adhesion and migration processes, which affect cell motility by regulating actin polymerisation. The inhibitory effects of FN1 on cancer cell migration and metastasis have been well documented.[44 45] MMP10, MMP13 and MMP2 are members of extracellular proteinases with key functions in the formation and remodelling of tumour invasion.[46 47] HGF stimulates tumour cell migration by binding specifically to its receptor c-met in CRC.[44 46–48] Taken together, SLC12A5 promoted tumour metastasis through mediating key elements of the matrix architecture such as MMP and fibronectin.

A clinical association evaluation showed that overexpression of SLC12A5 was associated significantly with advanced tumour stage ($p<0.001$). Patients with CRC vary greatly in clinical outcome, depending on the growth status and aggressiveness of tumours. At present, the most important clinical prognostic indicator of disease outcome is TNM staging. Nevertheless, some patients with CRC with low TNM stages are still afflicted with disease recurrence. Therefore, additional prognostic biomarkers are needed to provide better risk assessment. In this regard, we further examined the influence of SLC12A5 overexpression on clinical outcomes of 195 patients with CRC. Both univariate and multivariate cox regression analyses showing high expression levels of SLC12A5 were correlated significantly with shortened overall survival, suggesting that SLC12A5 overexpression could be regarded as an independent new prognostic marker for CRC.

In conclusion, amplification of SLC12A5 is a common event in CRC. Overexpression of SLC12A5 plays a pivotal oncogenic role in colorectal carcinogenesis by inhibiting apoptosis through mediating AIF-dependent and EndoG-dependent apoptotic signalling pathway and promoting metastasis by regulating key elements of the matrix architecture. SLC12A5 may serve as an independent prognostic biomarker for patients with CRC.

Table 2 Cox regression analysis of potential poor prognostic factors for patients with colorectal cancer

	Univariate analysis		Multivariate analysis	
Variable	**RR (95% CI)**	**p Value**	**RR (95% CI)**	**p Value**
Age	1.02 (0.99 to 1.05)	0.13	1.02 (0.99 to 1.05)	0.24
Gender				
Male	0.88 (0.45 to 1.73)	0.70	0.78 (0.39 to 1.54)	0.45
Female	1.00		1.00	
Location				
Colon	0.57 (0.20 to 1.60)	0.28		
Rectal	1.00			
Grade				
High or moderate	1.33 (0.66 to 2.68)	0.43		
Low	1.00			
TNM stage				
I	0.06 (0.01 to 0.22)	<0.001	0.07 (0.16 to 0.27)	<0.001
II	0.19 (0.07 to 0.53)	0.001	0.28 (0.09 to 0.80)	0.017
III	0.53 (0.21 to 1.38)	0.19	0.69 (0.26 to 1.81)	0.45
IV	1.00		1.00	
SLC12A5 expression in tumour tissues				
High	2.38 (1.21 to 4.66)	0.01	2.30 (1.16 to 4.60)	0.018
Low	1.00		1.00	

RR, relative risk; The TNM Staging System is based on the extent of the tumor (T), the extent of spread to the lymph nodes (N), and the presence of metastasis (M).

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SUPPLEMENTARY MATERIAL AND METHODS

Cell lines and cell culture

The human colon cancer cell lines Caco-2, HCT116, SW480 and SW1116 were obtained from the American Type Culture Collection (Manassas, VA, USA). HCT116 p53 knockout (KO) cell line was kindly provided by Professor Bert Vogelstein (Johns Hopkins University, Baltimore, MD). HCT116 p53 WT and HCT116 p53 KO cell lines were grown in McCoy's 5A medium (Invitrogen, Carlsbad, CA). Other cell lines were cultured in RPMI 1640 medium (Gibco BRL, Rockville, MD).

Whole genome sequencing

Genomic DNA from primary CRC tumour and peripheral blood was fragmented to an average size of 500 nucleotides. Standard Illumina protocols and Illumina paired-end adapters were then used for library preparation. DNA library sequencing was then performed on an Illumina Solexa sequencing platform as per manufacturer's instructions.

Array CGH assays

Human 244K CGH microarray (Agilent Technologies, Santa Clara, CA) was employed in a 'two-color' process to measure DNA copy number changes in the same case. Labelling reactions were performed with 1 μg genomic DNA with Agilent Genomic DNA Labeling Kit PLUS (Agilent Technologies). The microarray chip was scanned by the Agilent Microarray Scanner. Data analysis was done by the Agilent Feature Extraction 9.1 and CGH Analytics 3.4. In brief, a log2 expression ratio was

computed and normalized for forward and reverse fluor experiments using the CGH Analytics 3.4 software.

RNA extraction, semi-quantitative RT-PCR and real-time PCR analyses

Total RNA was extracted from cell pellets or tissues using Quizol reagent (Qiagen, Valencia, CA) and cDNA was synthesized (Roche, Indianapolis, IN). Semi-quantitative RT-PCR was performed using Hot-star DNA polymerase (Invitrogen). Real-time PCR was performed using SYBR Green master mixture on HT7900 system (Applied Biosystems, Foster City, CA). Primer sequences are listed in supplementary Table 4.

Immunofluorescence and confocal microscopy

HCT116 cells were seeded in 12-well plates and fixed in 4% paraformaldehyde (Sigma) for 30 min. Cells were then permeabilized with PBS containing 0.1% Triton X-100 for 3 min and incubated with a 5 mg/ ml BSA blocking solution. Cells were incubated with SLC12A5 antibody (1 : 100, Abcam, Cambridge, UK), followed by incubation with a secondary antibody. Chromosomes were stained with DAPI in PBS for 5 min. The immunofluorescence images were taken with a confocal microscope (FV1000, Olympus, Tokyo, Japan).

Immunohistochemistry

Immunohistochemistry for SLC12A5 was performed on paraffin sections of paired CRC slides and TMAs using anti-SLC12A5 antibody (Millipore, Billerica, MA). The extent of *SLC12A5* staining was scored by assigning the percentage of positive tumour cells (0, none; 1, < 20% of positive staining cells; 2, 20-50% of positive

staining cells; 3, >50% of positive staining cells). The case with score 0, 1 or 2 was defined as low expression, while the case with score 3 was regarded as high expression. Immunohistochemistry for AIF and EndoG was also performed on paraffin slides of mouse xenograft tumours using anti-AIF and anti-EndoG antibodies (Cell Signalling Technology, Cambridge, UK).

Transfections

Cells were transfected with pCMV6-AC-GFP (PS100010, OriGene, Rockville, MD), or pCMV6-*SLC12A5* (RG223680, OriGene) using Lipofectamine 2000 (Invitrogen), respectively. RNA and proteins were harvested at 48 or 72 h after transfection.

RNA interference

The *SLC12A5* siRNA pool of three duplexes (5'-GGCUCAAUCCGGAGAAAGAdTdT-3' (sense) and 3'-dTdT CCGAGUUAGGCCUCUUUCU-5' (antisense); 5'-CAACCGCAAUGGUGAUGAAdTdT-3' (sense) and 3'-dTdT GUUGGCGUUACCACUACUU-5' (antisense) and 5'-CCUUAUGUCUUCAGUGAUAdTdT-3' (sense) and 3'-dTdT GGAAUACAGAAGUCACUAU-5' (antisense)) targeting human *SLC12A5* were synthesized by Ribo Company (RiboBio, Guangzhou, China). Scrambled siRNA was used as a negative control (siControl). 50 nM of si*SLC12A5* or siControl were transfected into cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Cell viability assay

Cell viability was monitored by the xCELLigence Real Time Cell Analyzer (Roche Applied Science, Mannheim, Germany). The cell index was derived from measured cell-electrode impedance that correlates with number of cells and cell viability. The experiment was performed in triplicate wells for three independent experiments.

Colony formation assay

Empty vector and different doses of *SLC12A5* plasmids (0.8 μg and 1.6 μg) were used to transfect cells in 24-well plates. After 48 h of transfection, cells were collected and seeded (2×10^3/well) in 6-well plate and selected with G418 at 0.5 mg/mL (Calbiochem, Darmstadt, Germany) for 10-14 days. Colonies (≥ 50 cells/colony) were counted after staining with 5% crystal violet. The assay was carried out in triplicate wells for three independent experiments.

Annexin V apoptosis assay

Apoptosis was assessed by flow cytometry after staining with Annexin V (FITC-conjugated) (BD Biosciences, Erembodegem, Belgium) and 7-amino-actinomycin (7-AAD) (BD Biosciences). Cell populations were counted as viable (Annexin V-negative, 7-AAD-negative), early apoptotic (Annexin V-positive, 7-AAD-negative), and late apoptotic (Annexin V-positive, 7-AAD-positive), and necrotic cells (Annexin V-negative, 7-AAD-positive). The experiments were performed in triplicate for three times independently.

In Situ DNA Nick End Labeling

Terminal deoxynucleotidyl transferase-mediated nick-end labelling (TUNEL) was performed following the Manufacturer's protocol (single-stranded DNA apoptosis

TUNEL kit, Roche, Indianapolis, IN). Nuclei with clear brown staining were regarded as apoptotic cells.

Cell cycle analysis

Cells were seeded to 6-well plates and transfected with empty vector, SLC12A5 plasmid, siSLC12A5 or siControl. Cells were starved by adding serum-free medium for G1 synchronization. After 24 hours, medium containing 10% fetal bovine serum (FBS) was added for an additional 12 hours. Cells were fixed in 75% ethanol, stained with propidium iodide (PI), and analyzed by flow cytometry (Becton Dickinson Biosciences, Bedford, MA). The results were analyzed with ModFit LT2.0 software (Coulter Electronics, Hialeah, FL).

Migration and Matrigel invasion assays

Migration and Matrigel invasion assays were performed as previously described[1]. For migration assay, cells were seeded into the upper chamber of a Transwell insert (pore size, 8 μm; Corning Falcon) and then placed into the transwell containing medium with 10% FBS in the lower chamber. For invasion assay, cells were seeded seeded in a Matrigel-coated chamber (Becton Dickinson, Waltham, MA, USA). After 48 h, cells that remained in the lower surface of the insert were stained with crystal violet. Experiments were conducted in triplicate.

Lentivirus packaging and transduction

Two short hairpin RNAs (shRNA-1, 5'-gatcgGCAGCACAACACTGTGCTTGTTTCAAGAGAACAAGCACAGTGTTGTGCTGCTTTTTTc-3' and shRNA-2, 5'-

gatcgGCGAGGTCATCACCATCTACTTTCAAGAGAAGTAGATGGTGATGACC TCGCTTTTTTc-3') targeting the SLC12A ORF (Genbank no. NM_020708) and a non-targeting RNA sequence serving as a negative control (shControl) were cloned into the pGMLV-SC1 vector (Genomeditech, Shanghai, China). Virus packaging was performed in HEK293T cells using the GM easyTM Lentiviral Packaging Kit (Genomeditech, Shanghai, China). HEK293T cells were cultured in DMEM with 10% FBS. Forty-eight hours after transfection, the supernatant was harvested and cleared by centrifugation. The HCT116 cells were transduced with the lentivirus containing sh*SLC12A5* or shControl. Forty-eight hours after infection, 1 μg/mL of puromycin was added to the media for 2 weeks to select the cells infected with the lentivirus.

Tumour xenograft mouse model

Male athymic 4-week-old Balb/c nude mice were housed under standard conditions and cared for according to the institutional guidelines for animal care. All animal experimental procedures were approved by the Animal Ethics Committee of the Chinese University of Hong Kong. To determine the tumourigenicity of *SLC12A5 in vivo*, HCT116 cells (2×10^6 cells in 0.1 mL PBS) transduced with the lentivirus containing *SLC12A5*-shRNA or control shRNA were injected subcutaneously into the dorsal left flank of 4-week-old male Balb/c nude mice (4/group). Tumour diameter was measured every 2 days until 3 weeks. Tumour volume (mm^3) was estimated by measuring the longest and shortest diameter of the tumour and calculating as previously described.[2] The mice were euthanized on the fourth week, and the tumours were excised and embedded in paraffin. Sections (5 μm) of tumours were stained with H&E to visualize the tumour structure.

Standard tail vein metastatic assay

To investigate experimental lung metastasis, male 4-week-old Balb/c nude mice were used in standard tail vein metastatic assay as previously described[3] HCT116 cells (5×10^6 cells in 0.1 mL PBS) with *SLC12A5*-shRNA or control shRNA were injected into the lateral tail veils of each nude mouse (5/group). Six weeks after injection, the mice were sacrificed and examined. The lungs and livers were dissected and paraffin embedded, and the sections were stained with hematoxylin and esosin. The metastases were counted under microscopy for occurrence of metastases in a double-blind manner.

Western blot analysis

Total protein or nuclear protein was extracted and protein concentration was measured by the DC protein assay method of Bradford (Bio-Rad, Hercules, CA). Thirty micrograms of protein from each sample were separated on 12% SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare, Piscataway, NJ). Blots were immunostained with primary antibody and secondary antibody, respectively.

Luciferase reporter assay

To investigate the signaling pathways modulated by *SLC12A5*, five well-known signaling pathway luciferase reporters were examined in HCT116 and SW1116 cells, including p53-luc (14xp53 binding sites), p21-luc (2.1 kb p21 promoter), AP1-luc (7xAP1 binding sites), NFκB-luc (5xNFκB binding sites), and TOPFlash (4xTCF binding sites). Cells (1×10^5 cells/well) were co-transfected with luciferase report plasmid (195 ng/well), pRL-CMV vector (5 ng/well) and *SLC12A5* plasimd or control

empty vector for overexpression study, or 50nM si*SLC12A5* or Control siRNA for knockdown study using lipofectamine 2000 (Invitrogen). Cells were harvested 48 hours post-transfection and luciferase activities were analyzed by the dual-luciferase reporter assay system (Promega, Madison, WI).

Human tumour metastasis PCR array

Gene expression profiles of HCT116 cells transfected with si*SLC12A5* or siControl were analyzed by Human Tumour Metastasis RT^2 Profiler PCR array (Super Array Bioscience, Frederick, MD). This array contains 84 functionally well characterized genes involved in tumour metastasis (http://www.sabiosciences.com). Genes expression with fold-changes more than or less than 2.0 were considered to be of biological significance.

Reference

1. Zhang H, Hao Y, Yang J, *et al.* Genome-wide functional screening of miR-23b as a pleiotropic modulator suppressing cancer metastasis. *Nat Commun* 2011;2:554.
2. Xu L, Li X, Chu ES, *et al.* Epigenetic inactivation of BCL6B, a novel functional tumour suppressor for gastric cancer, is associated with poor survival. *Gut* 2012;61(7):977-85.
3. Saur D, Seidler B, Schneider G, *et al.* CXCR4 expression increases liver and lung metastasis in a mouse model of pancreatic cancer. *Gastroenterology* 2005;129(4):1237-50.

Supplementary Table 1. Clinicopathological features of CRC patients

Variable	No.	%
Mean age, y ± SD	64.3±12.8	
Gender		
M	104	55.5%
F	87	45.5%
Location		
Colon	40	24.5%
Rectum	123	75.5%
Grade		
High	5	2.6%
Moderate	177	92.7%
Low	9	4.7%
TNM		
I	28	14.7%
II	81	42.4%
III	56	29.3%
IV	26	13.6%

Supplementary Table 2. Amplification of *SLC12A5* was verified by comparative genomic hybridization (array CGH)

Gene Name	Probe Name	Log_2 Ratio	Copy Ratio
SLC12A5	A_16_P03524624	0.355	1.279
SLC12A5	A_16_P03524631	0.739	1.669
SLC12A5	A_16_P21143897	0.778	1.715
SLC12A5	A_16_P03524640	0.714	1.640
SLC12A5	A_18_P13807773	0.695	1.619
SLC12A5	A_16_P21143916	0.841	1.791
SLC12A5	A_16_P41338750	0.443	1.359
SLC12A5	A_16_P34722776	0.572	1.487
SLC12A5	A_16_P03524656	0.142	1.103
SLC12A5	A_16_P03524659	0.632	1.550
SLC12A5	A_16_P21143939	0.874	1.833
SLC12A5	A_16_P03524668	0.523	1.437
SLC12A5	A_16_P03524673	0.702	1.627
SLC12A5	A_16_P03524678	0.328	1.255
SLC12A5	A_16_P03524681	0.889	1.852
SLC12A5	A_16_P34723117	0.216	1.162
SLC12A5	A_14_P138449	0.669	1.590
Average		**0.595**	**1.528**

Supplementary Table 3. Changes in gene expression after knockdown of *SLC12A5* in HCT116 cells

Gene name	Symbol	Fold-change	Gene location	Gene function
Metastasis Suppressor	MTSS1	12.69	8p22	Inhibits metastasis and proliferation
Tumor Protein P53	p53	10.7	17p13.1	Induces apoptosis, inhibits proliferation and metastasis
NME/NM23 Nucleoside Diphosphate Kinase	NME1	8.07	17q21.3	Negatively regulates proliferation and participates in cell adhesion
Fibronectin 1	FN1	7.22	2q34	Inhibits tumor growth, angiogenesis and metastasis
Neurofibromin 2	NF2	6.88	22q12.2	Suppresses cell proliferation and tumorigenesis
FAT Tumor Suppressor Homolog 1	FAT1	5	4q35	Inhibits cell migration
Breast Cancer Metastasis Suppressor 1	BRMS1	3.59	11q13	Inhibits metastasis and promotes apoptosis
Tissue Inhibitor Of Metalloproteinase 2	TIMP2	2.69	17q25	Inhibits metastasis
Cathepsin K	CTSK	2.58	1q21	Participates in tumor progression and metastasis
Matrix Metallopeptidase 10	MMP10	-2.02	11q22.3	Protein hydrolysate, promotes metastasis
Matrix Metallopeptidase 13	MMP13	-2.06	11q22.3	Decomposes, protein hydrolysate and promotes metastasis
Hepatocyte Growth Factor	HGF	-12.67	7q21.1	Participates in proteolysis,
Matrix Metallopeptidase 2	MMP2	-14.19	16q13-q21	Decomposes, protein hydrolysate and promotes metastasis

Supplementary Table 4. DNA sequences of primers used in this study

Primer name	Sequence (5'-3')
RT-PCR	
SLC12A5-F	GCAGGAGCCATGTACATCCT
SLC12A5-R	CCATGCAGGTGAGCACACA
β-actin-F	GTCTTCCCCTCCATCGTG
β-actin-R	AGGGTGAGGATGCCTCTCTT
FN1-F	ACAACACCGAGGTGACTGAGAC
FN1-R	GGACACAACGATGCTTCCTGAG
HGF-F	GAGAGTTGGGTTCTTACTGCACG
HGF-R	CTCATCTCCTCTTCCGTGGACA
MMP2-F	AGCGAGTGGATGCCGCCTTTAA
MMP2-R	CATTCCAGGCATCTGCGATGAG
MMP13-F	CCTTGATGCCATTACCAGTCTCC
MMP13-R	AAACAGCTCCGCATCAACCTGC
MTSS1-F	TTCAGTGCTCCAGCGGCTACAG
MTSS1-R	GGAATGGTGGAGGACTTGTCGA