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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 metalloproteinase 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.



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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

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 Spectrum-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 V16.0). 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 *SLC12A5* 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 SW1116 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

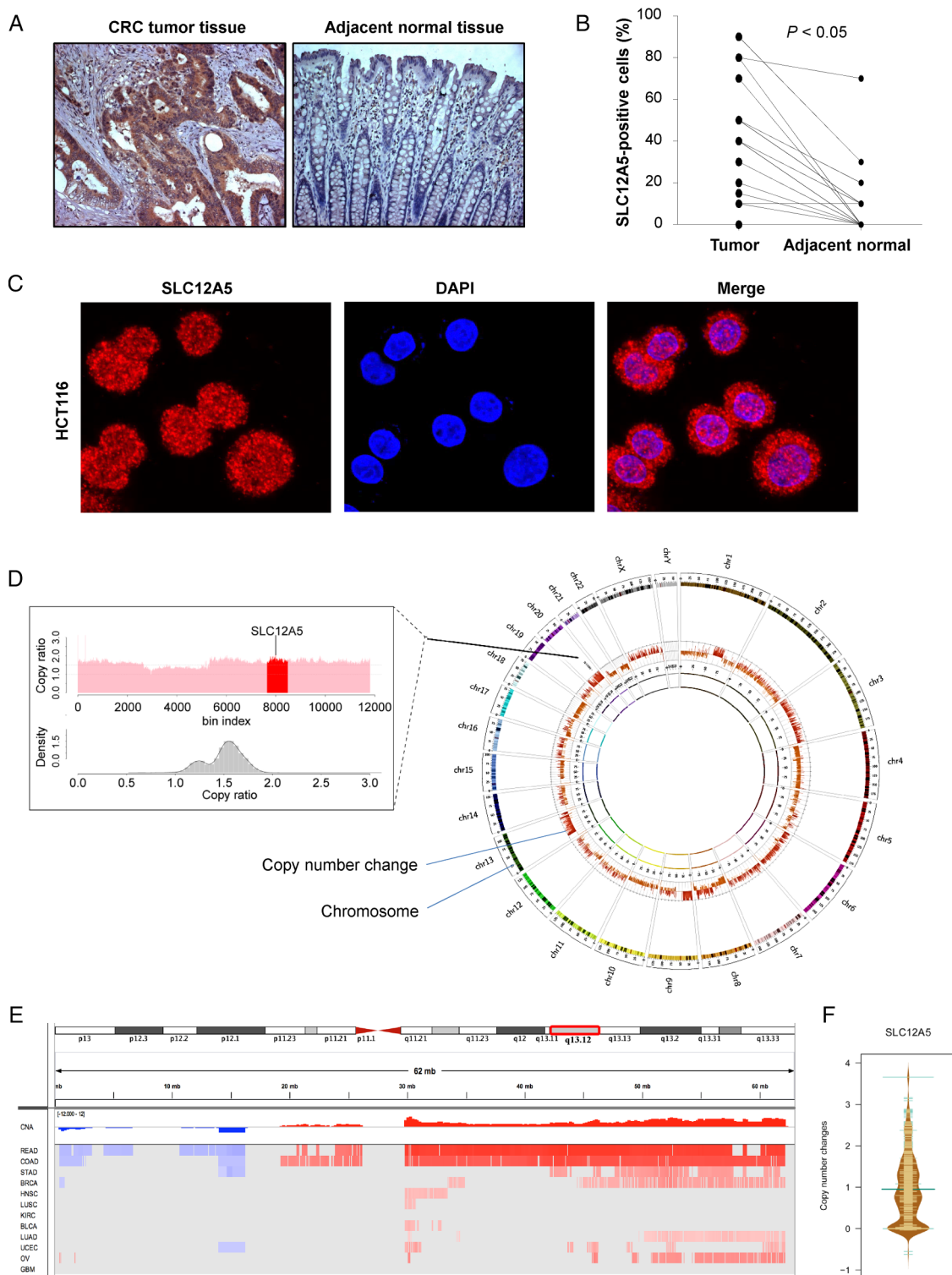


Figure 1 *SLC12A5* is amplified in colorectal cancer (CRC). (A) Representative images of *SLC12A5* protein expression in CRC tumour tissues and their adjacent tissues by immunohistochemistry. (B) The protein expression level of *SLC12A5* was significantly higher in primary CRC tumour as compared with 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.

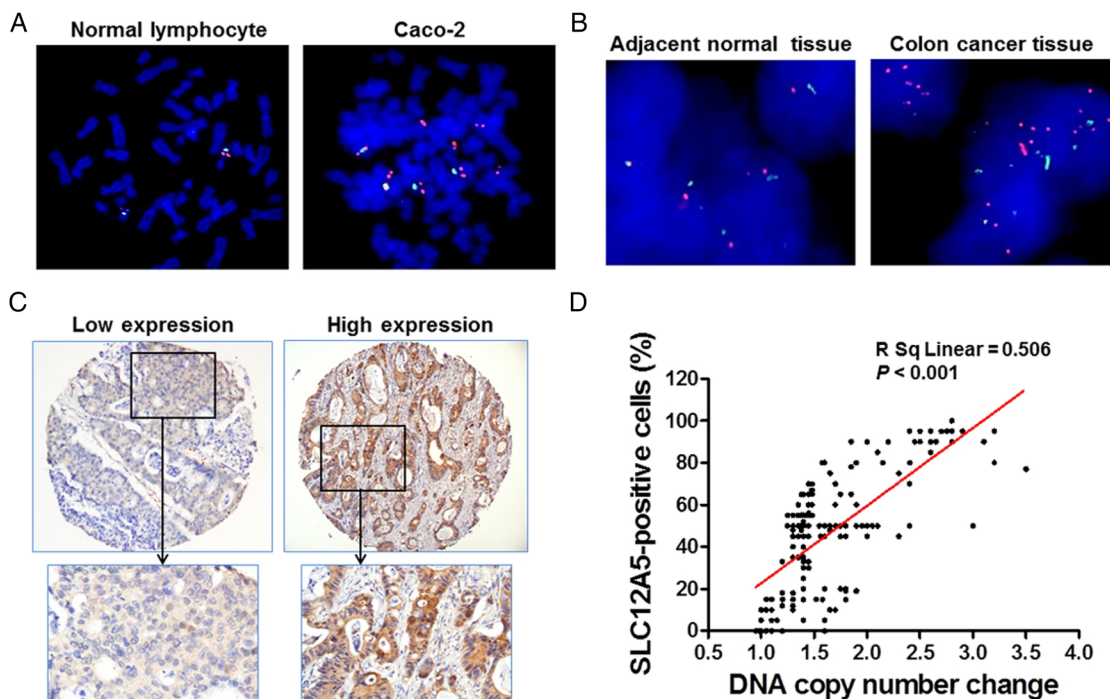


Figure 2 Amplification and overexpression of *SLC12A5* in colorectal cancers (CRCs). (A) Fluorescence in situ hybridisation (FISH) was performed in normal metaphase lymphocytes and colon cancer cell line Caco-2. The red signal indicates the location of *SLC12A5* and the green signal refers to centromere 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 *SLC12A5* 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 sh*SLC12A5*-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 sh*SLC12A5*-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

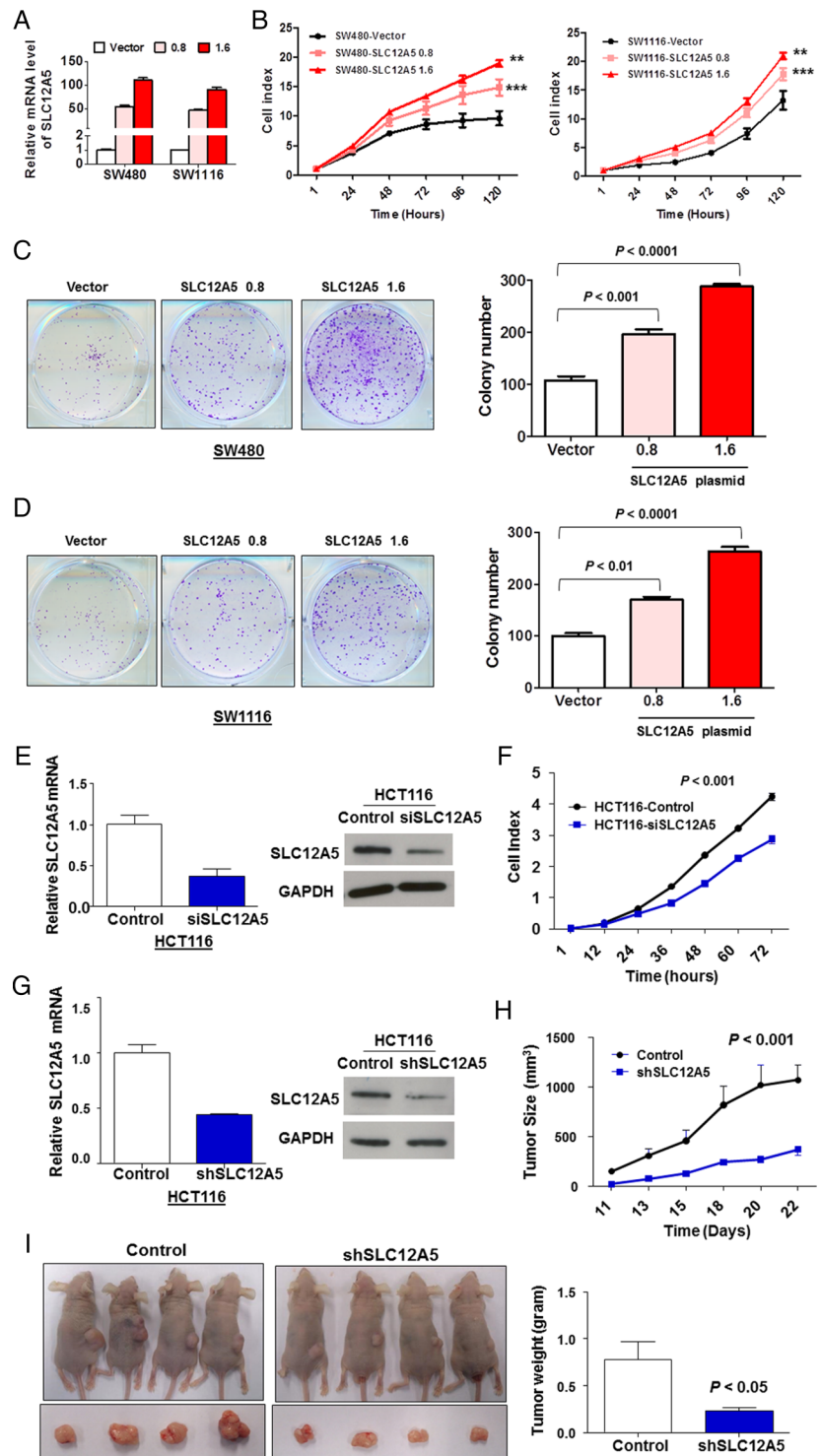
HCT116 cells by si*SLC12A5* arrested the cell cycle at the G1-S transition ($p<0.01$) (figure 4F). Western blot analysis showed that the protein expression of two master G1-S checkpoint regulators cyclin D1 and cyclin-dependent kinase (CDK4) was increased in *SLC12A5*-SW480 compared with the vector-transfected 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)- κ B 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, NF κ B 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 μ g) 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 μ g) 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 μ g 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 siSLC12A5 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 shSLC12A5 was demonstrated by real-time PCR (left panel) and western blot (right panel). (H) The tumour growth curve of HCT116 stably transduced with shSLC12A5 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

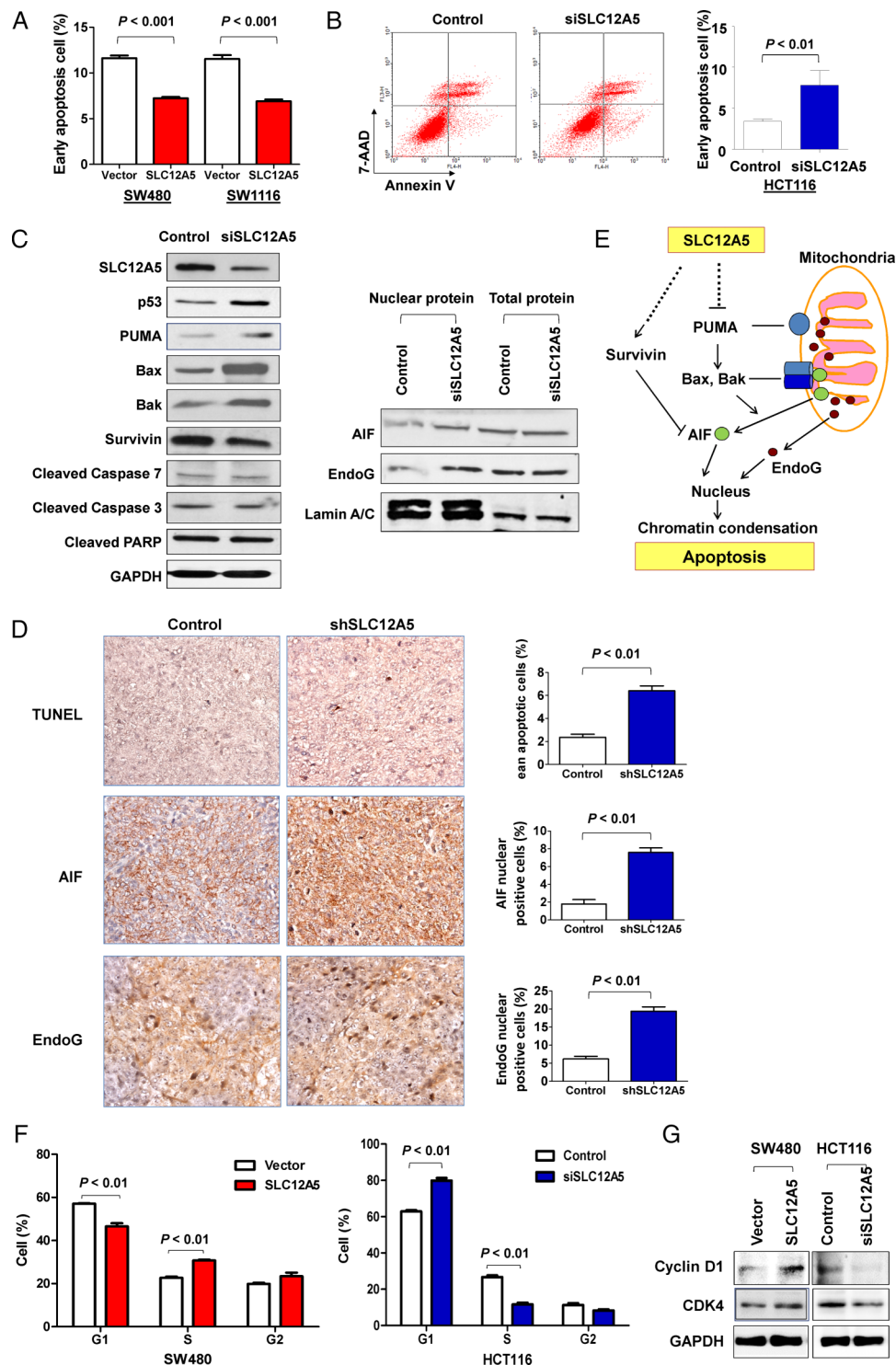
We also investigated the effects of SLC12A5 on metastasis using in vitro migration and Matrigel invasion assays and in vivo metastasis

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 \pm SD. (G) Protein expression of cyclin D1 and cyclin-dependent kinase (CDK4) was determined by western blot.



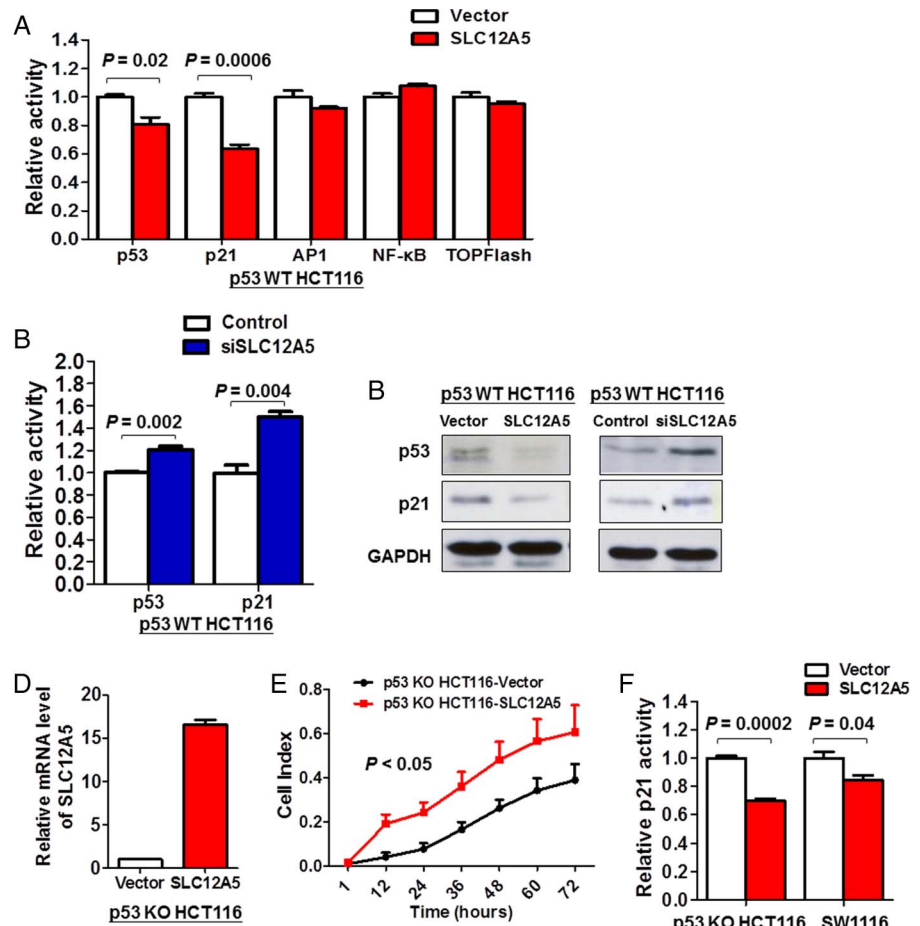
HCT116 cells than in mice injected with shSLC12A5-HCT116 cells (1/5, 20%) ($p < 0.01$) (figure 6C). Metastatic nodules in the lungs were confirmed histologically (figure 6C).

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 RT² 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 metalloproteinase 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 series of promoter-luciferase assays (p53-luc, p21-luc, activator protein 1 (AP1)-luc, nuclear factor κ B (NF κ B)-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 CRC²¹ 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

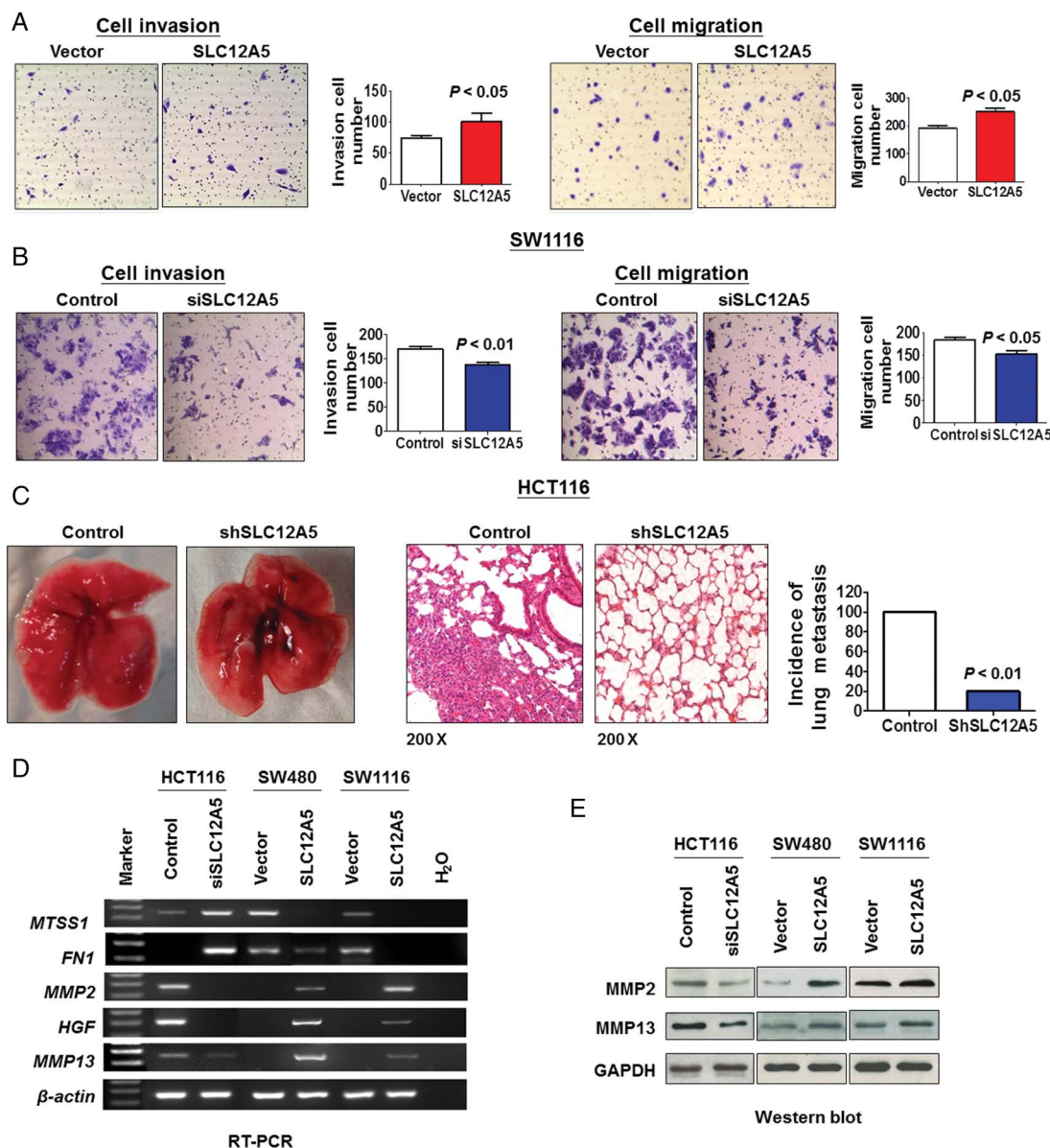


Figure 6 SLC12A5 promotes tumour metastasis in vitro and in vivo. (A) Ectopic expression of SLC12A5 promoted cell invasion and migration in SW1116 cells. (B) Knockdown of SLC12A5 suppressed cell invasion and migration in HCT116 cells. (C) Representative images of lungs with or without metastasis (left 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 metalloproteinase.

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 retracts 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

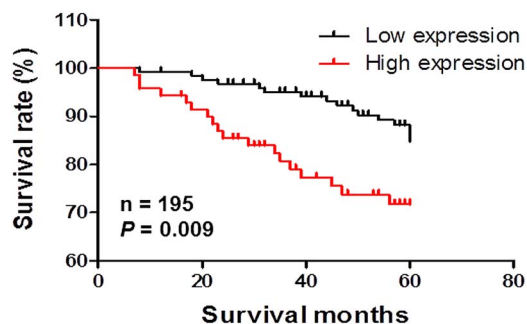


Figure 7 Clinical significance of SLC12A5 in colorectal cancers (CRCs). Kaplan–Meier survival analysis according to SLC12A5 expression in 195 patients with CRC. Patients with CRC with SLC12A5 overexpression had poorer survival than others. The difference is statistically significant based on the log-rank 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

Variable	Univariate analysis		Multivariate analysis	
	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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REFERENCES

- Jemal A, Bray F, Center MM, *et al.* Global cancer statistics. *CA Cancer J Clin* 2011;61:69–90.
- Sung JJ, Lau JY, Young GP, *et al.* Asia Pacific consensus recommendations for colorectal cancer screening. *Gut* 2008;57:1166–76.
- Lubbe WJ, Zuzga DS, Zhou Z, *et al.* Guanylyl cyclase C prevents colon cancer metastasis by regulating tumor epithelial cell matrix metalloproteinase-9. *Cancer Res* 2009;69:3529–36.
- Rokavec M, Oner MG, Li H, *et al.* IL-6R/STAT3/miR-34a feedback loop promotes EMT-mediated colorectal cancer invasion and metastasis. *J Clin Invest* 2014;124:1853–67.
- Fearon ER. Molecular genetics of colorectal cancer. *Annu Rev Pathol* 2011;6:479–507.
- Beroukhim R, Mermel CH, Porter D, *et al.* The landscape of somatic copy-number alteration across human cancers. *Nature* 2010;463:899–905.
- Brosens RP, Haan JC, Carvalho B, *et al.* Candidate driver genes in focal chromosomal aberrations of stage II colon cancer. *J Pathol* 2010;221:411–24.
- Ji H, Kumm J, Zhang M, *et al.* Molecular inversion probe analysis of gene copy alterations reveals distinct categories of colorectal carcinoma. *Cancer Res* 2006;66:7910–19.
- Nakao K, Mehta KR, Fridlyand J, *et al.* High-resolution analysis of DNA copy number alterations in colorectal cancer by array-based comparative genomic hybridization. *Carcinogenesis* 2004;25:1345–57.
- Ried T, Knutzen R, Steinbeck R, *et al.* Comparative genomic hybridization reveals a specific pattern of chromosomal gains and losses during the genesis of colorectal tumors. *Genes Chromosomes Cancer* 1996;15:234–45.
- Nakao M, Kawauchi S, Furuya T, *et al.* Identification of DNA copy number aberrations associated with metastases of colorectal cancer using array CGH profiles. *Cancer Genet Cytogenet* 2009;188:70–6.
- Postma C, Koopman M, Buffart TE, *et al.* DNA copy number profiles of primary tumors as predictors of response to chemotherapy in advanced colorectal cancer. *Ann Oncol* 2009;20:1048–56.
- Poulogiannis G, Ichimura K, Hamoudi RA, *et al.* Prognostic relevance of DNA copy number changes in colorectal cancer. *J Pathol* 2010;220:338–47.
- Sheffer M, Bacolod MD, Zuk O, *et al.* Association of survival and disease progression with chromosomal instability: a genomic exploration of colorectal cancer. *Proc Natl Acad Sci USA* 2009;106:7131–6.
- Venkatachalam R, Ligtenberg MJ, Hoogerbrugge N, *et al.* Predisposition to colorectal cancer: exploiting copy number variation to identify novel predisposing genes and mechanisms. *Cytogenet Genome Res* 2008;123:188–94.
- Jia D, Jing Y, Zhang Z, *et al.* Amplification of MPZL1/PZR promotes tumor cell migration through Src-mediated phosphorylation of cortactin in hepatocellular carcinoma. *Cell Res* 2014;24:204–17.
- Hobaus J, Hummel DM, Thiem U, *et al.* Increased copy-number and not DNA hypomethylation causes overexpression of the candidate proto-oncogene CYP24A1 in colorectal cancer. *Int J Cancer* 2013;133:1380–8.
- Jiang MC, Yeh CM, Tai CJ, *et al.* CSE1L modulates Ras-induced cancer cell invasion: correlation of K-Ras mutation and CSE1L expression in colorectal cancer progression. *Am J Surg* 2013;206:418–27.
- Rooney PH, Murray GI, Stevenson DA, *et al.* Comparative genomic hybridization and chromosomal instability in solid tumours. *Br J Cancer* 1999;80:862–73.
- Weiss MM, Snijders AM, Kuipers EJ, *et al.* Determination of amplicon boundaries at 20q13.2 in tissue samples of human gastric adenocarcinomas by high-resolution microarray comparative genomic hybridization. *J Pathol* 2003;200:320–6.
- Xie T, Ario G, Lamb JR, *et al.* A comprehensive characterization of genome-wide copy number aberrations in colorectal cancer reveals novel oncogenes and patterns of alterations. *PLoS ONE* 2012;7:e42001.
- Yu C, Yu J, Yao X, *et al.* Discovery of biclonal origin and a novel oncogene SLC12A5 in colon cancer by single-cell sequencing. *Cell Res* 2014;24:701–12.
- Yu J, Wu WK, Li X, *et al.* Novel recurrently mutated genes and a prognostic mutation signature in colorectal cancer. *Gut* 2015;64:636–45.
- Hebert SC, Mount DB, Gamba G. Molecular physiology of cation-coupled Cl-cotransport: the SLC12 family. *Physiol Rev* 2004;84:580–93.
- Payne JA, Stevenson TJ, Donaldson LF. Molecular characterization of a putative K-Cl cotransporter in rat brain. A neuronal-specific isoform. *J Biol Chem* 1996;271:16245–52.
- Song L, Mercado A, Vazquez N, *et al.* Molecular, functional, and genomic characterization of human KCC2, the neuronal K-Cl cotransporter. *Brain Res Mol Brain Res* 2002;103:91–105.
- Xie D, Sham JS, Zeng WF, *et al.* Heterogeneous expression and association of beta-catenin, p16 and c-myc in multistage colorectal tumorigenesis and progression detected by tissue microarray. *Int J Cancer* 2003;107:896–902.
- Zhu W, Cai MY, Tong ZT, *et al.* Overexpression of EIF5A2 promotes colorectal carcinoma cell aggressiveness by upregulating MTA1 through C-myc to induce epithelial-mesenchymal transition. *Gut* 2012;61:562–75.
- Guan XY, Sham JS, Tang TC, *et al.* Isolation of a novel candidate oncogene within a frequently amplified region at 3q26 in ovarian cancer. *Cancer Res* 2001;61:3806–9.
- Galluzzi L, Joza N, Tasdemir E, *et al.* No death without life: vital functions of apoptotic effectors. *Cell Death Differ* 2008;15:1113–23.
- Arnould D, Gaume B, Karbowski M, *et al.* Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. *EMBO J* 2003;22:4385–99.
- Liu T, Brouha B, Grossman D. Rapid induction of mitochondrial events and caspase-independent apoptosis in Survivin-targeted melanoma cells. *Oncogene* 2004;23:39–48.
- Pavlyukov MS, Antipova NV, Balashova MV, *et al.* Survivin monomer plays an essential role in apoptosis regulation. *J Biol Chem* 2011;286:23296–307.
- Starzynowski DT, Lockwood WW, Delehouzee S, *et al.* TRAF6 is an amplified oncogene bridging the RAS and NF-kappaB pathways in human lung cancer. *J Clin Invest* 2011;121:4095–105.
- Tsafir D, Bacolod M, Selvanayagam Z, *et al.* Relationship of gene expression and chromosomal abnormalities in colorectal cancer. *Cancer Res* 2006;66:2129–37.
- Wei WC, Akerman CJ, Newey SE, *et al.* The potassium-chloride cotransporter 2 promotes cervical cancer cell migration and invasion by an ion transport-independent mechanism. *J Physiol* 2011;589:5349–59.
- Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 2002;108:153–64.
- Broker LE, Krut FA, Giaccone G. Cell death independent of caspases: a review. *Clin Cancer Res* 2005;11:3155–62.
- Ren SX, Cheng AS, To KF, *et al.* Host immune defense peptide LL-37 activates caspase-independent apoptosis and suppresses colon cancer. *Cancer Res* 2012;72:6512–23.
- Ishtutsuka K, Hideshima T, Hamasaki M, *et al.* Novel inosine monophosphate dehydrogenase inhibitor VX-944 induces apoptosis in multiple myeloma cells primarily via caspase-independent AIF/Endo G pathway. *Oncogene* 2005;24:5888–96.
- Gopalani NK, Meena RN, Prasad DN, *et al.* Cooperativity between inhibition of cytosolic K⁺ efflux and AMPK activation during suppression of hypoxia-induced cellular apoptosis. *Int J Biochem Cell Biol* 2012;44:211–23.
- Dong C, Li Q, Lyu SC, *et al.* A novel apoptosis pathway activated by the carboxyl terminus of p21. *Blood* 2005;105:1187–94.

- 43 Parr C, Jiang WG. Metastasis suppressor 1 (MTSS1) demonstrates prognostic value and anti-metastatic properties in breast cancer. *Eur J Cancer* 2009;45:1673–83.
- 44 Soikkeli J, Podlasz P, Yin M, *et al.* Metastatic outgrowth encompasses COL-I, FN1, and POSTN up-regulation and assembly to fibrillar networks regulating cell adhesion, migration, and growth. *Am J Pathol* 2010;177:387–403.
- 45 Waalkes S, Atschekzei F, Kramer MW, *et al.* Fibronectin 1 mRNA expression correlates with advanced disease in renal cancer. *BMC Cancer* 2010;10:503.
- 46 Mendes O, Kim HT, Lungu G, *et al.* MMP2 role in breast cancer brain metastasis development and its regulation by TIMP2 and ERK1/2. *Clin Exp Metastasis* 2007;24:341–51.
- 47 Yamada T, Oshima T, Yoshihara K, *et al.* Overexpression of MMP-13 gene in colorectal cancer with liver metastasis. *Anticancer Res* 2010;30:2693–9.
- 48 Otte JM, Schmitz F, Kiehne K, *et al.* Functional expression of HGF and its receptor in human colorectal cancer. *Digestion* 2000;61:237–46.