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Somatostatin Receptor 1, a novel EBV-associated CpG hypermethylated gene, contributes to the pathogenesis of EBV-associated gastric cancer

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Background: *Somatostatin receptor 1* (*SSTR1*) was preferentially methylated in Epstein-Barr virus (EBV)-positive gastric cancer using promoter methylation array. We aimed to analyse the epigenetic alteration and biological function of *SSTR1* in EBV-associated gastric cancer (EBVaGC).

Methods: Promoter methylation was examined by combined bisulphite restriction analysis (COBRA) and pyrosequencing. The biological functions of *SSTR1* were evaluated by loss- and gain-of-function assays.

Results: Promoter hypermethylation of *SSTR1* was detected in EBV-positive gastric cancer cell lines (AGS-EBV) with *SSTR1* transcriptional silence, but not in EBV-negative gastric cancer cell lines with SSTR1 expression. Expression level of SSTR1 was restored in AGS-EBV by exposure to demethylating agent. Moreover, methylation level of *SSTR1* was significantly higher in EBV-positive primary gastric cancers compared with EBV-negative gastric cancers ($P = 0.004$). Knock-down of *SSTR1* in gastric cancer cell lines (AGS and BGC823) increased cell proliferation and colony formation ability, and promoted G1 to S-phase transition, enhanced cell migration and invasive ability. In contrast, ectopic expression of SSTR1 in gastric cancer cell lines (MKN28 and MGC803) significantly suppressed cell growth in culture conditions and reduced tumour size in nude mice. The tumour suppressive effect of *SSTR1* was associated with upregulation of cyclin-dependent kinase inhibitors (*p16*, *p15*, *p27* and *p21*); downregulation of oncogenes (*MYC* and *MDM2*), key cell proliferation and pro-survival regulators (*PI3KR1*, *AKT*, *BCL-XL* and *MET*); and inhibition of the migration/invasion-related genes (*integrins*, *MMP1* (*matrix metallopeptidase 1*), *PLAUR* (*plasminogen activator urokinase receptor*) and *IL8* (interleukin 8)).

Conclusion: *Somatostatin receptor 1* is a novel methylated gene driven by EBV infection in gastric cancer cells and acts as a potential tumour suppressor.

Epstein-Barr virus (EBV) was first reported to be detected in a subset of gastric cancers in 1992 by the uniform occurrence of EBV in all gastric cancer cells but not in the adjacent normal cells (Shibata and Weiss, 1992). Since then, many reports have showed this strong association and the role of EBV in gastric carcinogenesis has been recognised as new evidence (Herrmann and

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Niedobitek, 2003; Zur Hausen *et al*, 2004). EBV-associated gastric cancer (EBVaGC) harbours distinct chromosomal aberrations and is characterised by a unique transcription pattern that resembles but is not identical to that of nasopharyngeal carcinomas (NPC) (Imai *et al*, 1994). It shows distinct clinicopathological features compared with EBV-negative gastric carcinoma (Uozaki and Fukayama, 2008). These findings suggest that EBV has an important role in the development of EBV-positive gastric carcinomas. Gastric cancer is one of the most common malignance, and the worldwide occurrence of EBV-positive gastric carcinoma is estimated at $>90\,000$ patients annually (Fukayama *et al*, 2008). However, the oncogenic role of EBV in gastric cancer development remains largely unknown.

Recent studies indicated that epigenetic alterations, particularly promoter methylation of tumour suppressor genes were demonstrated more frequently in EBV-positive than in EBV-negative gastric cancer (Kaneda *et al*, 2012). Moreover, the mechanism of promoter methylation in association with EBV infection was also studied. DNA methyltransferase (DNMT), which is considered to have a key role in methylation in mammals, was reported to be activated by EBV infection in gastric cancer (Hino *et al*, 2009). Our previous study demonstrated that EBV infection induced the activation of DNMT3b and caused genome-wide methylation in a gastric cancer cell line (Zhao *et al*, 2012). Epithelial cells are refractory to EBV infection *in vitro*. This has hampered the study of the role of EBV in epithelial malignancies. The use of recombinant EBV-infected immortalised gastric epithelial cell model with stable EBV infection (AGS-EBV) has enabled this difficulty to be overcome. This cell model facilitated us for a genome-wide scan of promoter methylated genes to identify the EBV-associated methylation candidates by comparing AGS-EBV with its parental AGS cells using the methylated DNA immunoprecipitation microarray (MeDIP-chip) (Zhao *et al*, 2013). Of note, 886 candidates were detected with promoter hypermethylation, and one of them named as *Somatostatin receptor 1* (SSTR1) was validated to be a novel CpG hypermethylated gene in EBVaGC (Zhao *et al*, 2013), Methylation level of *SSTR1* is eight-fold higher in AGS-EBV cells as compared with AGS cells using MeDIP-chip assay (Zhao *et al*, 2013).

SSTR genes belong to G protein-coupled receptors family (Patel *et al*, 1995). They are crucial in regulating the growth inhibitory effect of somatostatin and reducing the tumour cells growth. *SSTR1* is located on chromosome 14q13 and its mRNA is widely distributed in human tissues including stomach (Patel, 1999). Loss of *SSTR1* has been found in pancreatic cancer, and overexpression of SSTR1 in pancreatic cancer cell lines induced cell-cycle arrest and inhibited tumour cell proliferation (Li *et al*, 2005, 2008). However, the role of *SSTR1* in gastric cancer remains elusive. In this study, the epigenetic regulation, biological function, molecular mechanism and clinical application of SSTR1 in EBVaGC were examined.

MATERIALS AND METHODS

Cancer cell lines and culture condition. Gastric cancer cell lines (AGS, AGS-EBV, BGC823, MGC803, MKN28 and MKN45) were used in this study. AGS-EBV, an EBV-infected gastric cancer cell line (Feng *et al*, 2002), was a gift from Dr Shannon C. Kenney (Department of Oncology and Medicine, McArdle Laboratory for Cancer Research at the University of Wisconsin, Madison, Wisconsin). MKN28 and MKN45 were obtained from Riken Cell Bank (Tsukuba, Japan). BGC823 and MGC803 were gifts from Oncology Hospital, Beijing University, China. AGS was purchased from American Type Culture Collection (Manassas, VA, USA). The DNA samples isolated from five NPC cell lines (C666-1, CNE1, HK1, HONE1 and HNE1) and one immortalised normal nasopharyngeal epithelial cell line NP69 were gifts from Department of Clinical Oncology, the Chinese University of Hong Kong.

Human samples. Gastric cancer tissue samples including 12 EBV-positive gastric cancers and 14 EBV-negative gastric cancers, and 4 normal gastric biopsies were obtained from Prince Wales of Hospital, The Chinese University of Hong Kong, Hong Kong. All patients and controls gave informed consent for participation in this study and the study protocol was approved by the Ethics Committee of the Chinese University of Hong Kong.

Demethylation with 5-Aza-2′deoxycytidine agent treatment. Gastric cancer cells were treated with $2\,\mu$M DNA demethylation agent 5-Aza-2′deoxycytidine (5-Aza) (Sigma-Aldrich, St Louis, MO, USA) for 5 days and medium was refreshed every day.

Combined bisulphite restriction analysis. Combined bisulphite restriction analysis (COBRA) is performed to determine methylation levels at specific gene loci in genomic DNA after sodium bisulphite conversion by restriction enzyme digestion. Amplification of PCR was performed with $2\,\mu$l bisulphite-converted DNA. The PCR products were digested with *Bst*U I (New England Biolabs, Ipswich, MA, USA) and separated on 2% agarose gels.

Pyrosequencing. Pyrosequencing is a loci-specific quantitative method, which is based on sequencing-by-synthesis (Tost and Gut, 2007a). The methylation percentage of each CpG site was calculated by PyroMark CpG SW 1.0 software (Qiagen, Hilden, Germany). The peak showed the ratio of cytosine to thymine at each analysed CpG site, reflecting the proportion of methylated DNA (Tost and Gut, 2007b).

***In situ* hybridisation for EBV-encoded small RNA.** To examine the EBV infection in gastric cancer tissues, detection of EBV-encoded small RNA (EBER) was carried out as reported by us (Zhao *et al*, 2012).

Cell viability assay. Cell viability was assessed by the MTT assay (Promega, Madison, MI, USA) (Zhao *et al*, 2012). The experiments were performed in triplicate for three times.

Colony formation assay. Colony formation was performed as described previously (Zhao *et al*, 2012). The experiments were performed in triplicate for three times.

Cell-cycle analysis. About 50 000 propidium iodide-stained cells were counted and sorted by FACS Calibur Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and cell-cycle distributions were analysed using the ModFitLT software (BD Biosciences) (Zhao *et al*, 2012).

Cell migration assay. Cellular migration was evaluated using wound-healing assay in triplicate for three independent experiments.

Invasion assay. Invasion ability of gastric cells was determined using Matrigel Invasion chambers (BD Biosciences).

Human cancer pathway finder RT^2 profiler PCR array analysis. Gene expression profiles of gastric cancer cell lines stably transfected with sh-SSTR1 or sh-ctrl plasmid were analysed by the Human Cancer PathwayFinder RT^2 Profiler PCR Array (Qiagen), which contained 84 well-characterised genes with representative roles in tumorigenesis.

***In vivo* tumorigenicity.** Gastric cancer cell line MGC803 (1×10^6 cells in 0.1 ml PBS) stably transfected with SSTR1 expression vector or empty vector was injected subcutaneously into the dorsal flank of 4-week-old male Balb/c nude mice ($n=9$/group). Tumour diameter was measured every 4 days until 24 days. Animal experimental procedures were approved by the Animal Ethics Committee of the Chinese University of Hong Kong.

Statistical analysis. The results were expressed as mean ± standard deviation (s.d.). Mann–Whitney *U* test was performed to compare the variables of the two sample groups. Receiver Operating Characteristic (ROC) curve was used to estimate the cutoff value of the methylation percentage. The difference in tumour growth rate between the two groups of nude mice was determined by repeated-measures analysis of variance. Value of $P < 0.05$ was taken as statistical significance.

RESULTS

SSTR1 was a novel EBV-driven methylated gene in gastric cancer. *SSTR1* was reduced in EBV-positive cell lines AGS-EBV, while it was expressed in EBV-negative cell lines AGS, BGC823 and MKN45 as well as normal gastric tissues (Figure 1A). We validated the *SSTR1* methylation status in both EBV-positive and EBV-negative gastric cancer cells using COBRA. CpG hypermethylation was detected in EBV-positive AGS-EBV cells with *SSTR1* downregulation, whereas methylation was not found in EBV-negative gastric cancer cell lines including AGS, BGC823 and MKN45 cells which expressed SSTR1 (Figure 1A). We then treated AGS-EBV and AGS cell lines with DNA demethylation agent 5-Aza. The *SSTR1* mRNA expression was restored in AGS-EBV cells, but not in AGS cells by 5-Aza treatment (Figure 1B), indicating that the transcriptional silence of SSTR1 in AGS-EBV is mediated by its promoter methylation. The *SSTR1* methylation status was further evaluated and compared in AGS-EBV and AGS by pyrosequencing, as shown in Figure 1C, promoter methylation level of *SSTR1* was significantly higher in AGS-EBV than in AGS (31.25 ± 4.03% *vs* 1.25 ± 0.5%, $P < 0.0001$).

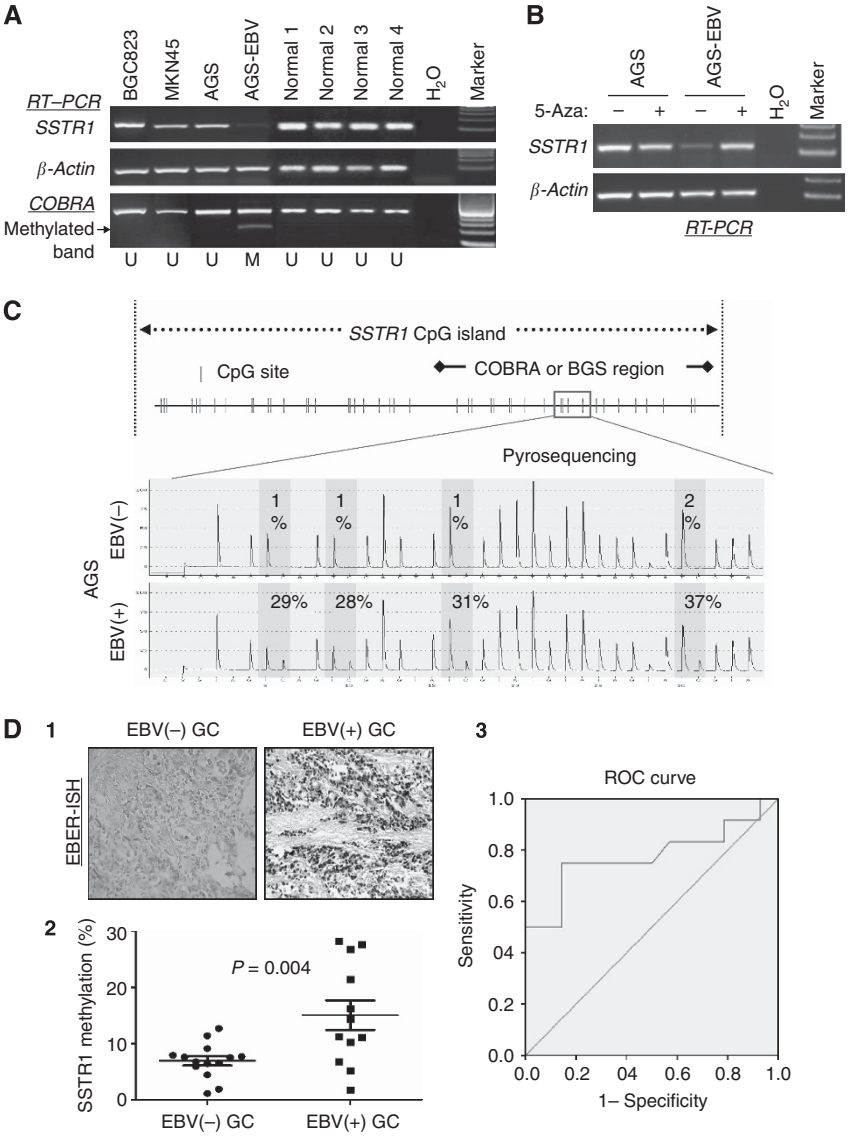

Figure 1. (**A**) SSTR1 mRNA expression was determined by RT–PCR. Promoter methylation of SSTR1 was detected by combined bisulphite restriction analysis (COBRA). U = unmethylation; M = methylation. (**B**) SSTR1 mRNA expression was restored after treatment with demethylation reagent 5-Aza in AGS-EBV. (**C**) (Upper panel) Diagram of SSTR1 CpG island. Each *vertical red bar* represents one CpG site. (Lower panel) Average methylaiton levels of SSTR1 in AGS-EBV and in AGS by pyrosequencing. (**D, 1**) Representative image of EBV infection in gastric cancer detected by *in situ* hybridisation for Epstein-Barr virus-encoded RNA (EBER). Dark brown nuclear staining indicates a positive hybridisation signal. (**2**) SSTR1 methylation was determined in primary EBV-positive and EBV-negative gastric cancers by pyrosequencing. (**3**) Receiver operating characteristics (ROC) curve analysis of the correlation between SSTR1 methylation and EBV infection in primary gastric cancers.

Table 1. Clinical pathologic features of *SSTR1* methylation in primary gastric cancer

Variable	Methylation ($n=11$)	(%)	Unmethylation ($n=15$)	(%)	*P*-value[a]
Age					0.724
Mean ± s.d.	51.5 ± 14.7		53.3 ± 9.8		
Gender					0.024
Male	11	55	9	45	
Female	0	0	6	100	
EBER					0.004
Positive	9	75	3	25	
Negative	2	14.3	12	85.7	
H.pylori infection					0.547
Positive	2	50	2	50	
Negative	3	25	9	75	
Lauren type					0.663
Intestinal	9	47.4	10	52.6	
Diffuse	1	25	3	75	
Mixed	1	33.3	2	66.7	
Differentiation					1.000
Poor	7	46.7	8	53.3	
Well/moderate	3	50	3	50	
TNM stage					0.798
II	1	50	1	50	
III	6	54.5	5	45.5	
IV	4	40	6	60	

Abbreviations: EBER = Epstein-Barr virus-encoded small RNA; s.d. = standard deviation; *SSTR1* = *Somatostatin receptor 1*; TNM = tumour, node, metastasis.
[a]*P*-values were obtained by Fisher's exact tests to compare the variables of each two groups except for age (unpaired *T* test).

SSTR1 hypermethylation was associated with EBV-positive primary gastric cancer. To verify the correlation between *SSTR1* methylation and EBV infection, *SSTR1* methylation status was compared in primary EBV-positive and EBV-negative gastric cancers by pyrosequencing. The EBV infection in gastric cancer tissues was confirmed by EBER *in situ* hybridisation (Figure 1D1). Promoter methylation level of *SSTR1* was significantly higher in EBV-positive gastric cancers (15.04 ± 8.69%) than in EBV-negative gastric cancers (6.93 ± 3.01%) ($P=0.004$) as determined by pyrosequencing (Figure 1D2).

The ROC curve analysis indicated that a cutoff value of 9.675% *SSTR1* methylation status in EBV-positive gastric cancers with a sensitivity and specificity of 75% and 85.7%, respectively (AUC = 0.777; 95% CI = 0.579 ~ 0.974) (Figure 1D3). Using the cutoff value of 9.675% *SSTR1* methylation, the association between clinicopathologic features of gastric cancers and the methylation levels of SSTR1 was evaluated. The SSTR1 methylation was associated with male gender ($P=0.024$) and EBER-positive staining ($P<0.005$) (Table 1), indicating a positive correlation between SSTR1 methylation and EBV infection.

SSTR1 knock-down induced cell proliferation in gastric cancer cell lines. To investigate the biological function of *SSTR1* in gastric cancer, we first examined the effect of SSTR1 knock-down on cell growth through RNA interference in AGS and BGC823 cells, which showed high expression of *SSTR1*. Successful SSTR1 knock-down by stable transfection with shRNA-SSTR1 plasmid was confirmed by real-time qRT–PCR and western blot (Figure 2A). Knock-down of *SSTR1* significantly promoted cell viability both in AGS ($P<0.001$) and in BGC823 ($P<0.05$) compared with the control cells (Figure 2B). The growth enhancive effect of SSTR1 knock-down was further proved by colony formation assay. The colonies formed by shRNA-SSTR1 transfected cells were significantly more in number and larger in size both in AGS (224.1 ± 54.8% increase, $P<0.01$) and in BGC823 (52.6 ± 9.1% increase, $P<0.05$) as compared with those formed by control shRNA-transfected cells (Figure 2C).

SSTR1 knock-down promoted cells to enter into S phase. We investigated the effect of SSTR1 on cell-cycle regulation by flow cytometry. Knock-down of SSTR1 led to significant decrease in the number of cells accumulating in the G1 phase of AGS ($P<0.01$) and BGC823 ($P<0.001$) compared with the control groups (Figure 3A). Concomitantly, there were significant increases in the number of the S-phase cells of AGS ($P<0.001$) and BGC823 ($P<0.001$) (Figure 3A). These results indicated that knock-down of SSTR1 induced cancer cell growth by promoting cells to enter into S phase, which was confirmed by the reduced protein expression of key G1 checkpoint regulators including cyclin-dependent inhibitors p21, p27 and p15 (Figure 3B).

SSTR1 knock-down increased migration and invasion ability of gastric cancer cells. Stable knock-down of SSTR1 markedly accelerated cell migration at the edges of scratch wound of AGS and BGC823 cells (Figure 4A). Quantitative analyses at 24 h showed significant increases in wound closure in AGS (4.3 ± 0.1-fold, $P<0.001$) and in BGC823 (1.2 ± 0.0-fold, $P<0.01$) cells transfected with sh-SSTR1 compared with the control cells (Figure 4A). Stable knock-down of SSTR1 also significantly increased the invasiveness of both AGS (2.5 ± 0.2-fold, $P<0.001$) and BGC823 (1.9 ± 0.4-fold, $P<0.01$) cells (Figure 4B).

Ectopic expression of SSTR1 inhibited proliferation and clonogenicity in gastric cancer cells. The effects of SSTR1 in gastric cancer were further examined by gain-of-function study, MKN28 and MGC803 cells were transfected with either an SSTR1 expression construct (pcDNA3.1-SSTR1) or an empty vector (pcDNA3.1). Ectopic expression of *SSTR1* in these cells was confirmed by RT–PCR and western blot (Figure 5A). The SSTR1 significantly decreased cell viability both in MKN28 ($P<0.001$) and in MGC803 ($P<0.001$) cells compared with the empty vector-transfected cells (Figure 5B). In addition, the colonies formed by SSTR1-transfected cells were dramatically fewer and smaller in MKN28 (74.5 ± 2.8% decrease, $P<0.01$) and in MGC803 (68.8 ± 10.9% decrease, $P<0.05$) (Figure 5C).

To assess whether the level of *SSTR1* gene expression affects EBV gene expression, we examined the expression of the immediate early lytic gene *BZLF1*, which is essential for the latent-lytic switch of EBV, and the representative latent gene *EBNA1* (Zhao *et al*, 2012) in SSTR1-overexpressed and control vector-transfected AGS-EBV cells by qRT–PCR. Results showed that expression of *BZLF1* and *EBNA1* was not changed by *SSTR1* re-expression (Supplementary Figure 1), demonstrating that the level of *SSTR1* gene expression does not affect EBV gene expression in EBVaGC.

SSTR1 inhibited xenograft tumour growth in nude mice. We examined whether SSTR1 could suppress the growth of gastric cancer cells in nude mice *in vivo*. The tumour growth curve of MGC803 cells, stably transfected with *SSTR1* expression vector or vector control in nude mice was shown in Figure 5D. The tumour size was significantly smaller in SSTR1-transfected nude mice as compared with the vector control mice ($P<0.05$).

Identification of genes modulated by SSTR1. To gain insight into the molecular mechanism underlying the tumour suppressive effect of SSTR1, gene expression profiles of BGC823 cells with or without stable *SSTR1* knock-down were analysed by cDNA microarray. The SSTR1 was found to modulate the

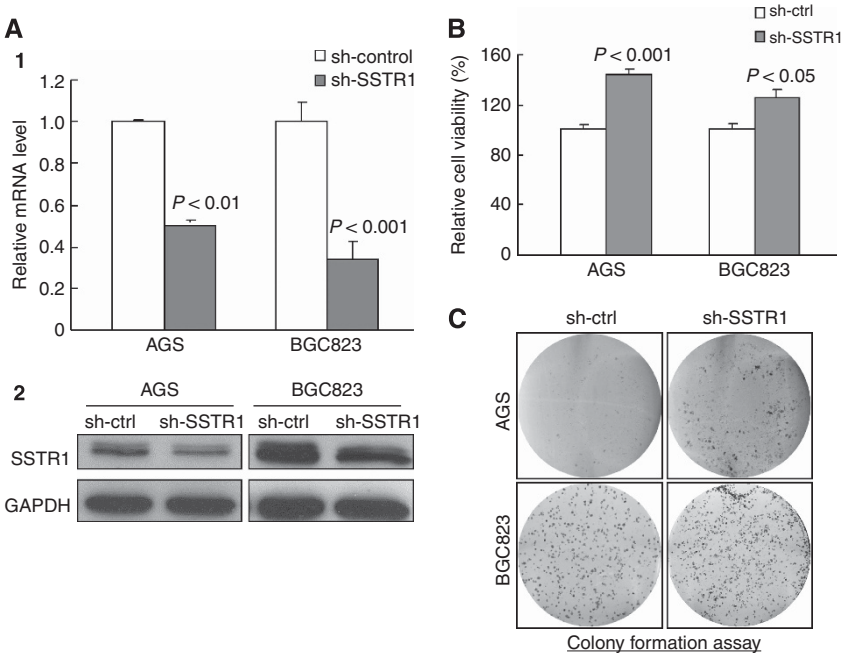

Figure 2. SSTR1 knock-down increased gastric cancer cell growth. (**A**) Knock-down of SSTR1 was evidenced at mRNA (**1**) and protein (**2**) level by RT–PCR and western blot, respectively. (**B**) SSTR1 knock-down increased cell proliferation in cancer cell lines. (**C**) Representative dishes of colony formation. Quantitative analyses of colony numbers indicated SSTR1 knock-down significantly increased clonogenicity in both AGS (224.1 ± 54.8% increase, $P<0.001$) and BGC823 (52.6 ± 9.1% increase, $P<0.05$).

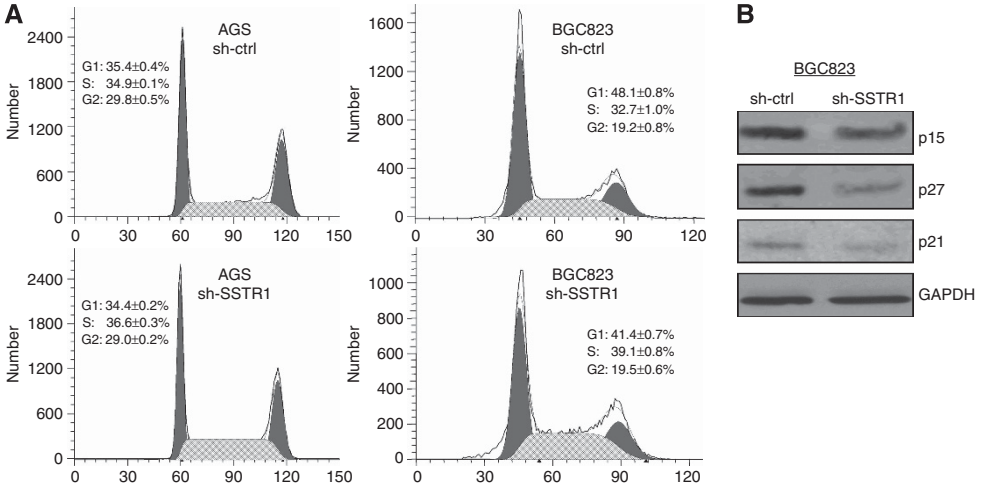

Figure 3. Knock-down of SSTR1 promoted cell-cycle progression. (**A**) The cell-cycle proportion was analysed using flow cytometry in AGS and BGC823 cells transfected with sh-control and sh-SSTR1, respectively. Quantitative analyses on the fraction of cells indicated significant differences between control and SSTR1 knock-down cells in cell-cycle phase distribution of G1 (AGS: $P<0.01$, BGC823: $P<0.0001$) and S (AGS: $P<0.001$, BGC823: $P<0.001$) phases. The data are mean ± s.d. of three independent experiments. (**B**) Effects of SSTR1 knock-down on protein expression of cell-cycle regulators p15, p21 and p27 by western blot.

expressions of many important genes involved in multiple cellular process, including cell cycle, apoptosis, migration and invasion (Figure 6A). As shown in Figure 6A, SSTR1 induced cyclin-dependent inhibitors p16 (2.5-fold) and inhibited cell divison cycle 25 homologue A (*CDC25A*) (−6.0-fold) and *MYC* (−3.26-fold). The SSTR1 also reduced expression of apoptosis regulators, including *AKT* (−3.19-fold), BCL2-like 1 (*BCL-XL*) (−10.98-fold), p53 E3 ubiquitin protein ligase homologue (*MDM2*) (−1.61-fold), met proto-oncogene (*MET*) (−1.77-fold) and phosphoinositide-3-kinase, regulatory subunit 1 (alpha) (*PI3KR1*) (−1.51-fold). Morover, SSTR1 significantly down-regulated the expression of migration-related genes, including four integrins *ITGA1* (−1.51-fold), *ITGA2* (−2.51-fold), *ITGA3* (−2.20-fold) and *ITGB5* (−2.60-fold), interleukin 8 (*IL8*) (−2.03-fold), matrix metallopeptidase 1 (*MMP1*) (−4.49-fold) and plasminogen activator urokinase receptor (*PLAUR*) (−5.54-fold).

DISCUSSION

We found that *SSTR1* expression was significantly suppressed in AGS-EBV, while expressed in gastric cancer cells without EBV infection (Figure 1A). The decreased expression of *SSTR1* can be reversed by pharmacological demethylation treatment in AGS-EBV

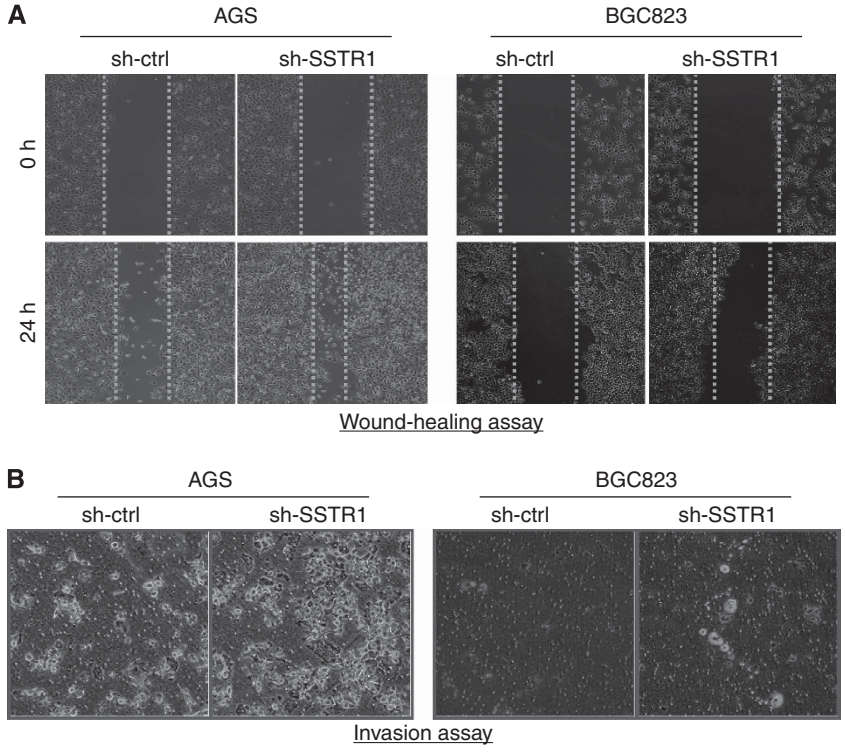

Figure 4. (**A**) Knock-down of SSTR1 increased the migration ability of AGS (4.3 ± 0.1-fold, $P<0.001$) and BGC823 (1.2 ± 0.0-fold, $P<0.01$) by wound-healing assay. (**B**) Knock-down of SSTR1 increased cell invasive ability of AGS (2.5 ± 0.2-fold, $P<0.001$) and BGC823 (1.9 ± 0.4-fold, $P<0.01$) by Matrigel invasion assay.

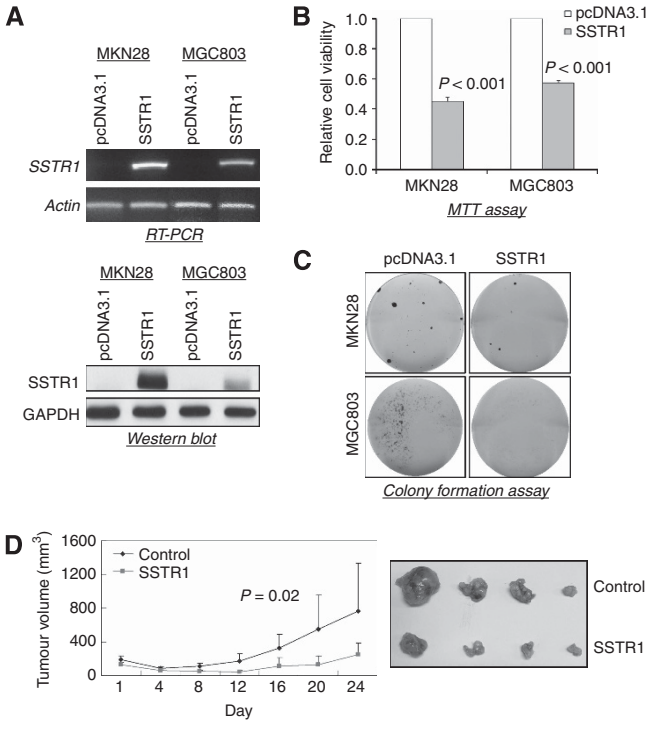

Figure 5. Ectopic expression of SSTR1 inhibited gastric cancer cell growth. (**A**) Ectopic expression of SSTR1 in MKN28 and MGC803 cell lines was evidenced by RT–PCR and western blot, respectively. (**B**) SSTR1 significantly suppressed the cell viability. (**C**) SSTR1 inhibited tumour cell clonogenicity of MKN28 (74.5 ± 2.8% decrease, $P<0.01$) and MGC803 (68.8 ± 10.9% decrease, $P<0.01$) by colony formation assay. (**D**) SSTR1 inhibits growth of xenograft tumours derived from MGC803 *in vivo* ($n=9$/group).

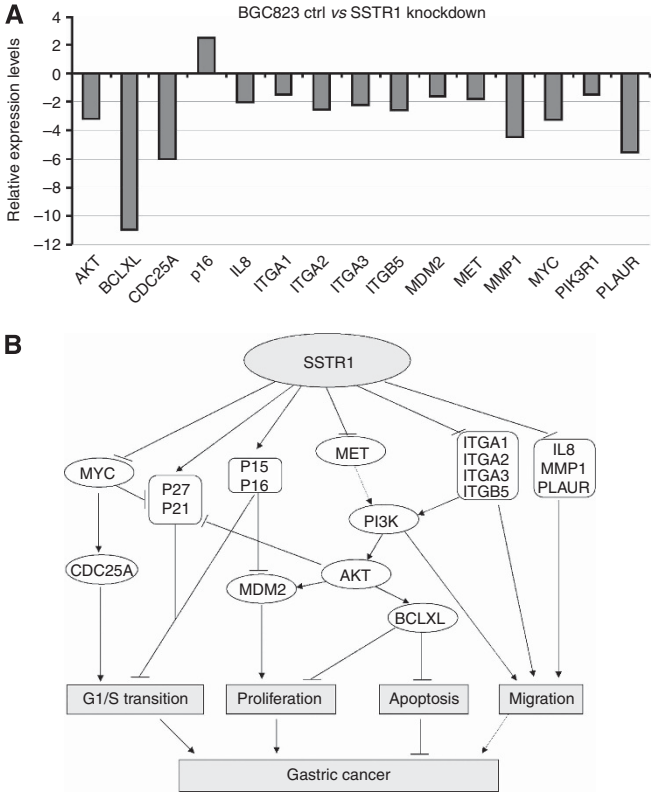

Figure 6. (**A**) Human cancer pathway cDNA microarray was applied to analyse the molecular basis of SSTR1 as a potential tumour suppressor gene. Downregulated or upregulated genes by SSTR1 are shown. (**B**) Schematic diagram of the molecular events for SSTR1 function as a tumour suppressor through regulating cell-cycle progression, inhibiting proliferation, inducing apoptosis and suppressing migration/invasion.

(Figure 1B2). Consistent with the high methylation level identified in the promoter region of *SSTR1* in AGS-EBV (eight-fold increase) by MeDIP-chip, dense promoter methylation of *SSTR1* in AGS-EBV cells was confirmed in this study by COBRA (Figure 1B2). Collectively, these results suggest that promoter hypermethylation is the principle mechanism for inactivation of SSTR1 in AGS-EBV cells.

To determine the methylation status of *SSTR1* in EBVaGC *in vivo*, we compared the promoter methylation levels of *SSTR1* by pyrosequencing in EBV-positive and EBV-negative gastric cancers. We found that methylation of *SSTR1* was significantly higher in EBV-positive gastric cancer tissues compared with EBV-negative gastric cancer tissues ($P = 0.004$, Figure 1D). In this regard, we examined the association between *SSTR1* methylation and the clinical outcome of EBV-positive gastric cancer patients. Our results showed that *SSTR1* methylation was significantly associated with male gender (Table 1), which is in line with other observation that EBV-positive gastric cancer was more prevalent in male than in female gastric cancer patients (Koriyama *et al*, 2005). Moreover, *SSTR1* methylation was demonstrated to be correlated with EBER-positive staining in gastric cancer tissues ($P = 0.004$) (Table 1). These results provide important evidence that the promoter methylation of *SSTR1* is associated with EBV infection, which may have a crucial role in the development of EBVaGC.

In addition to gastric cancer, there are several other types of EBV-associated cancers, such as NPC and Hodgkin's lymphoma. Hypermethylation of multiple genes (*RASSFIA*, *SHP1*, *DAPK*, *P16*, *RARβ2*, etc.) was reported to be common alterations in NPC, thus epigenetic change may have a role in NPC (Challouf *et al*, 2012). The genome-wide host gene methylation changes caused by EBV infection in germinal centre B cells indicated that EBV-caused aberrant methylation involves in the pathogenesis of Hodgkin's lymphoma (Leonard *et al*, 2011). To evaluate if *SSTR1* methylation can also be observed in other EBV-associated cancer, we examined the methylation status of *SSTR1* in five NPC cell lines (C666-1, CNE1, HK1, HONE1 and HNE1), and one immortalised normal nasopharyngeal epithelial cell line NP69 by COBRA. We demonstrated that *SSTR1* was completely methylated in the EBV-positive NPC cell line C666-1, partially methylated in four other EBV-negative NPC cell lines (CNE1, HK1, HONE1 and HNE1), and unmethylated in NP69 (Supplementary Figure 2). This result suggested that *SSTR1* promoter methylation may be a common event in EBV-associated cancer.

The putative tumour suppressor function of SSTR1 in gastric cancer was further examined by both *in vitro* and *in vivo* assays. Knock-down of SSTR1 in gastric cancer cell lines significantly induced cell growth by increasing cell viability and clonogenicity (Figure 2B and C). While, ectopic expression of SSTR1 exhibited marked growth-suppressing effect in culture conditions and also reduced tumour size in nude mice (Figure 5). Collectively, these results indicate that SSTR1 functions as a tumour suppressor in gastric cancer. We further revealed the molecular basis of how SSTR1 exerts the tumour suppressor effect in gastric cancer (Figure 6). Using a cDNA microarray, we identified that SSTR1 exerted its tumour suppressive function through inhibiting the key cell proliferation and apoptosis regulators, inducing *MDM2*, *AKT*, *PI3KR1*, *BCL-XL* and *MET*. The MDM2 functions as an oncogene and controls the activity of p53 in many human tumours (Momand *et al*, 2000). After phosphorylation by AKT, MDM2 is activated and localised in the nucleus to induce p53 degradation and cell survival (Jeong *et al*, 2005). Akt can block apoptosis and thereby promote cell survival. The PI3KR1 recruits the catalytic subunit of PI3K (Hixon *et al*, 2010). The PI3K-Akt signalling pathway has been implicated as a major pro-survival factor in many types of cancer (Hixon *et al*, 2010). BCLXL is a member of the anti-apoptotic BCL2 family (Plas and Thompson, 2002). AKT also confers survival signals through phosphorylation of the effector sequestered from BCLXL, leading to cell survival (Flusberg *et al*, 2001). The MET proto-oncogene has been found in a number of human cancers and has an important role in cancer development through activation of key oncogenic pathways such as PI3K-Akt signalling pathway (Gherardi *et al*, 2012).

To evaluate the cell-cycle regulation in contributing to the diminution of tumour growth by SSTR1, we performed Flow cytometry analysis of sh-SSTR1-transfected AGS and BGC823. Cell-cycle distribution analysis revealed that knock-down of SSTR1 significantly reduced proportion of cells in the G1 phase, with a concomitant increase of cells in S phase, inferring that SSTR1 knock-down promoted cell-cycle transition from G1 to S phase. To disclose the molecular basis underlying G1-S phase transition by SSTR1, we evaluated expression of the key regulatory factors that control G1-S checkpoint. The increased G1-S phase transition by SSTR1 knock-down was identified to be associated with down-regulation of cyclin-dependent kinase inhibitors p15, p16, p21 and p27 and upregulation of MYC and CDC25A (Figure 3C and 6) by western blot and cDNA expression array. The role of p15, p16, p21 and p27 as major players in G1 arrest has been well documented (Sherr, 1996). p21 and p27 are potent inhibitors of cyclin D/Cdk4 and cyclin E/Cdk2 activities; p15 is an important inhibitor of cyclin D/CDK4/6 activity (Sherr, 1996). *MYC* is an oncogene in human cancers (Alitalo *et al*, 1987). *MYC* expression is critical for entry of cells into S phase of cell cycle (Amati and Land, 1994). The *CDC25A* encodes an important G1-specific protein phosphatase and is required for the cell-cycle initiation and the progression through G1 phase (Zornig and Evan, 1996). The *CDC25A* has been identified as a direct transcriptional target of MYC and has a pivotal role in regulating MYC and cell cycle (Galaktionov *et al*, 1996). In addition, AKT negatively regulates p21 and p27 by direct phosphorylation (Rossig *et al*, 2001; Shin *et al*, 2002). It has been reported that SSTR1 was silenced in human pancreatic cancer and ectopic expression of SSTR1 caused cell-cycle arrest at the G0/G1 phase and inhibited pancreatic cancer cell proliferation (Li *et al*, 2005, 2008). Thus, the mechanism of SSTR1 mediated cell-cycle progression is most likely associated with deregulation of cell-cycle regulators in gastric cancer, thereby promoting cell proliferation.

Moreover, ablation of SSTR1 in gastric cancer cells increased migration and invasion (Figure 4). The suppressive effect of SSTR1 on migration and invasion ability is at least due to the downregulation of integrin family member (*ITGA1, ITGA2, ITGA3* and *ITGB5*), and other important migration/invasion-related genes *MMP1*, *PLAUR* and *IL8* (Figure 6). Integrins take part in cell migration and invasion during tumorigenesis through directly binding to extracellular matrix (ECM) (Guo and Giancotti, 2004). Integrins also regulate cell proliferation and enhance cell survival through activation of PI3K/AKT signalling pathway (Assoian and Klein, 2008). The anti-migration effect by SSTR1 is also attributed to the downregulation of MMP1, PLAUR and IL8. Matrix metallopeptidase 1 belongs to MMPs family of proteolytic enzymes, which degrade the components of ECM (Brinckerhoff and Matrisian, 2002). Matrix metallopeptidases have a crucial role in inducing invasion and migration of tumour cells (Egeblad and Werb, 2002). The PLAUR has the ability to degradate ECM and is associated with wound healing, invasion and metastasis (Ploug *et al*, 2002). Interleukin 8 is a potential stimulator of neutrophil transendothelial migration (Koch *et al*, 1992). Thus, the induction of cell invasion and migration by SSTR1 knock-down could be mediated by these downstream effectors of SSTR1 (Figure 6).

In conclusion, we have identified that *SSTR1* is a novel EBV-associated promoter hypermethylation gene in gastric cancer. *SSTR1* has an important tumour suppressive role in gastric cancer through modulating the expression of the important effectors involved in the regulation of cell proliferation, apoptosis, cell cycle and invasion. Epigenetic silencing of *SSTR1* by EBV infection may contribute to the pathogenesis of EBV-associated gastric cancer.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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