

KISS1 methylation and expression as predictors of disease progression in colorectal cancer patients

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Abstract

AIM: To examine the effect of aberrant methylation of the *KISS1* promoter on the development of colorectal cancer (CRC) and to investigate reversing aberrant methylation of the *KISS1* promoter as a potential therapeutic target.

METHODS: *KISS1* promoter methylation, mRNA expression and protein expression were detected by methylation-specific polymerase chain reaction (PCR), real-time quantitative PCR and Western blotting, respectively, in 126 CRC tissues and 142 normal colorectal tissues. Human CRC cells with *KISS1* promoter hypermethylation and poor *KISS1* expression were treated *in vitro* with 5-aza-2'-deoxycytidine (5-Aza-CdR). After treatment, *KISS1* promoter methylation, *KISS1* mRNA and protein expression and cell migration and invasion were evaluated.

RESULTS: Hypermethylation of *KISS1* occurred fre-

quently in CRC samples (83.1%, 105/126), but was infrequent in normal colorectal tissues (6.34%, 9/142). Moreover, *KISS1* methylation was associated with tumor differentiation, the depth of invasion, lymph node metastasis and distant metastasis ($P < 0.001$). *KISS1* methylation was also associated with low *KISS1* expression ($P < 0.001$). Furthermore, we observed re-expression of the *KISS1* gene and decreased cell migration after 5-Aza-CdR treatment in a CRC cell line.

CONCLUSION: These data suggest that *KISS1* is down-regulated in cancer tissues *via* promoter hypermethylation and therefore may represent a candidate target for treating metastatic CRC.

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Key words: Colorectal cancer; *KISS1*; Methylation; 5-aza-2'-deoxycytidine; Metastasis

Core tip: *KISS1* promoter methylation and expression were measured in colorectal cancer (CRC) samples to determine the effect of aberrant methylation of the *KISS1* promoter during the development of CRC. We determined that *KISS1* hypermethylation occurred frequently in CRC and was associated with low *KISS1* expression. *KISS1* methylation was associated with tumor differentiation, the depth of invasion, lymph node metastasis and distant metastasis. We treated the human HCT116 colorectal carcinoma cell line, which exhibits *KISS1* promoter hypermethylation and poor *KISS1* expression, with 5-aza-2'-deoxycytidine *in vitro*. After treatment, we observed re-expression of the *KISS1* gene and decreased cell migration.

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INTRODUCTION

Metastasis is an important feature of colorectal cancer (CRC) and is a significant contributor to CRC-related morbidity and mortality. Despite the recent improvements in CRC treatment, such as enhanced surgical excision techniques, radiation therapy and chemotherapy, metastatic recurrence remains a significant clinical hurdle.

Early karyotypic analyses of melanomas by Lee *et al.*^[1] identified *KISS1* as a gene that suppresses the metastasis of many cancers, including CRC^[2-5]. The *KISS1* gene maps to chromosome region 1q32^[6] and is regulated by genes that are located on chromosome 6^[7,8]. The *KISS1* gene was predicted to encode a 154-amino acid protein that is processed to an amidated internal 54-amino acid peptide termed metastatin^[9], which strongly inhibits the hepatic, pulmonary and intraperitoneal metastases of several human solid tumors in xenograft models, while still allowing tumor growth at the orthotopic site^[1,10-12]. The mechanisms of metastatin-mediated inhibition of tumor metastasis remain unclear, but may be related to reduced cancer cell migration, diminished colony formation and other alterations in cell function^[13,14]. Moreover, numerous clinical reports have demonstrated that the loss of or reduction in *KISS1* expression in various human cancers is inversely correlated with tumor progression, metastasis and survival^[15-18]. The loss of *KISS1* expression has also been associated with tumor stage, tumor grade and survival in CRC, but the mechanisms by which *KISS1* expression is lost in CRC remain unknown. Aberrant methylation of a promoter region is associated with the silencing of numerous tumor suppressor genes in neoplasia^[19,20]. DNA CpG island methylation is an epigenetic mechanism of transcriptional silencing that occurs at various stages of colorectal tumorigenesis^[21-23]. To the best of our knowledge, epigenetic alterations in *KISS1* have not been reported in CRC. To elucidate the mechanism by which *KISS1* expression is lost in CRC, we determined the hypermethylation status of the CpG island in the *KISS1* promoter region and the epigenetic silencing of *KISS1* by comparing tissues from CRC patients and healthy volunteers. *In vitro* treatment of CRC cells with 5-aza-2'-deoxycytidine (5-Aza-CdR) restored *KISS1* gene expression and decreased cell migration and invasion. Our findings suggested that the re-expression of *KISS1* by active DNA demethylation may represent a novel opportunity for therapeutic intervention in CRC that could be exploited clinically to treat metastatic disease.

MATERIALS AND METHODS

Patients and tissue samples

Ethics approval for this study was obtained from the Fujian Province Health Service Ethics Committee and the Ethics Committees of all participating institutions. All patients consented to the molecular analyses. Tissue samples from 126 patients who had undergone surgical resection without preoperative chemotherapy or radiotherapy for colorectal tumors histologically diagnosed as

CRC were analyzed; 142 healthy volunteers with histologically proven normal colorectal mucosa were chosen for comparison. Tumor burden was determined using the American Joint Committee on Cancer TNM system^[24]. 65 males and 61 females with a mean age of 58.64 ± 10.75 years were included in the study. Patient data are summarized in Table 1. All tissue samples were collected at the First Affiliated Hospital of Fujian Medical University between April 2011 and March 2012, frozen in liquid nitrogen and stored at -80°C . Tumor tissue was obtained at the time of surgery from the central part of the lesion.

Cell lines and drug treatments

The HCT116, SW1116, SW480 and VOLO CRC cell lines were obtained from the cell bank at the Chinese Academy of Sciences. The cells were plated in culture dishes and allowed to grow to 70% confluence; at this point, fresh medium was added, and the incubation continued under either normoxic or hypoxic conditions. All the cell lines were grown in RPMI 1640 (Gibco, Life Technologies, Grand Island, NY, United States) supplemented with 10% fetal bovine serum (FBS; Gibco) and incubated in 5% CO₂ at 37 °C. 5-Aza-CdR (Sigma-Aldrich, St. Louis, MO, United States) was diluted in DMSO and added to the culture media at 0.1, 1, 5 or 10 μmol/L. The untreated plates (control group) were incubated with an equivalent volume of DMSO. Cells were collected 24 h, 3 d or 5 d after treatment.

Methylation-specific polymerase chain reaction

Total DNA was extracted from freshly thawed frozen tissue or cells using standard proteinase K and phenol-chloroform protocols. The DNA concentration and purity were evaluated by agarose gel electrophoresis and UV spectrophotometry. The methylation state of the *KISS1* gene was determined by bisulfite treatment of DNA followed by methylation-specific polymerase chain reaction (MS-PCR)^[25,26] with primers specific for either the methylated or the modified unmethylated forms (Table 2). Briefly, 2 μg of genomic DNA was denatured with 3 mol/L NaOH at 42 °C for 30 min and then incubated with freshly prepared 10 mmol/L hydroquinone and 3 mol/L sodium bisulfite (pH 5.0) at 50 °C in the dark for 16 h. After treatment, the DNA was purified using the Wizard DNA Clean-Up System (Promega, United States) according to the manufacturer's protocol. The purified DNA was re-extracted and resuspended in 20 μL of distilled water. The methylation status of the *KISS1* promoter was determined using the bisulfite-treated DNA as the PCR template and primers specific for the methylated and unmethylated alleles of the gene. The amplifications were performed in a 25 μL reaction (3 μL of bisulfite-treated DNA, 2 μL of 10X Taq Buffer, 0.5 μL of 10 mmol/L dNTP, 1.5 μL of 25 mmol/L MgCl₂, 1 μL of primers, 0.2 μL of TaqMan and 15.8 μL of ddH₂O) for 40 cycles (30 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C), followed by a final 7 min extension at 72 °C. Then, 6 μL of the amplified PCR product was electrophoresed on a

Table 1 Relationships between *KISS1* methylation and expression and clinicopathological characteristics in colorectal cancer patients

Clinicopathological traits	<i>n</i>	Methylated (<i>n</i>)	Rate	<i>P</i> value ¹	RQ ³	<i>P</i> value ²	Gray value ⁴	<i>P</i> value ²	
Age (yr)	≤ 60	78	65	83.33%	0.602	0.445 ± 0.062	0.367	0.534 ± 0.047	0.136
	> 60	48	40	83.33%		0.359 ± 0.075		0.420 ± 0.059	
Gender	Male	65	53	81.54%	0.577	0.345 ± 0.086	0.230	0.423 ± 0.047	0.071
	Female	61	52	85.25%		0.489 ± 0.083		0.560 ± 0.058	
Location	Proximal	67	57	85.07%	0.576	0.453 ± 0.073	0.261	0.466 ± 0.051	0.544
	Distal	59	48	81.36%		0.388 ± 0.092		0.512 ± 0.055	
Size (cm)	< 3	58	50	86.21%	0.424	0.442 ± 0.085	0.363	0.536 ± 0.049	0.130
	> 3	68	55	80.88%		0.343 ± 0.083		0.421 ± 0.056	
Histology well		46	31	67.39%	0.001 ^a	0.546 ± 0.095	0.016 ^a	0.575 ± 0.078	0.134
Moderate, poor		80	74	92.50%		0.243 ± 0.085		0.451 ± 0.041	
Depth of invasion	T1 + T2	44	27	61.35%	0.001 ^a	0.963 ± 0.112	0.001 ^a	0.875 ± 0.047	0.001 ^a
	T3 + T4	82	78	95.12%		0.223 ± 0.054		0.341 ± 0.029	
Lymph node metastasis	Yes	48	45	93.75%	0.014 ^a	0.168 ± 0.058	0.001 ^a	0.292 ± 0.034	0.001 ^a
	No	78	60	76.92%		0.682 ± 0.105		0.631 ± 0.050	
Distant metastasis	Yes	11	11	100.00%	0.019 ^a	0.024 ± 0.012	0.001 ^a	0.151 ± 0.031	0.014 ^a
	No	115	94	81.74%		0.486 ± 0.089		0.512 ± 0.038	

^a*P* < 0.05 vs relevant control group. ¹ χ^2 test; ²Student's *t* test; ³mean ± SD.

Table 2 Methylation-specific polymerase chain reaction primers and real-time polymerase chain reaction primers for *KISS1*

Primer name	Sequence	Annealing temp (°C)	Product (bp)
<i>KISS1</i> MSP primer	5'-AAAGTTTCGTTTCGGAGGGTTC-3'	58	172
	5'-CTTTTATAAAAACCCGAAATAACG-3'		
<i>KISS1</i> un-MSP primers	5'-AAAGTTTGTGTTTGGAGGGTTC-3'	58	173
	5'-AAAGTTTGTGTTTGGAGGGTTC-3'		
<i>KISS1</i> real-time PCR primers	5'-ACCTGGCTCTCTCACCAAG-3'	60	201
	5'-TAGCAGCTGGCTTCCTCTC-3'		
β -actin primers	5'-ATCTGGCACCACCTTCTACAATGAGCTGCG-3'	60	837
	5'-CGTCATACTCCTGCTGTGCTGATCCACATCTGC-3'		

PCR: Polymerase chain reaction.

2% acrylamide gel and visualized by ethidium bromide staining. Samples in which a band was present after amplification with the methylation-specific primers were considered positive for *KISS1* methylation. DNA from placental tissue was treated with M.SssI methyltransferase (New England Biolabs, Hitchin, Herts, United Kingdom) and used as a positive control. DNA from normal lymphocytes was used as a negative control for the methylated alleles of *KISS1*.

***KISS1* and CRC metastasis-associated gene transcript expression analysis by real-time quantitative PCR**

Total cellular RNA was extracted from CRC tissue and cell pellets with TRIZOL (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. The concentration and purity of total RNA diluted with diethyl pyrocarbonate (DEPC)-treated water was determined using a UV spectrophotometer, and the total RNA was frozen in liquid nitrogen and stored at -80 °C. Complementary DNA (cDNA) was synthesized using an M-MLV RTase cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, United States) with RNA as the template. *KISS1* transcript expression was evaluated using *KISS1*-specific primers (Table 2) in a 20 μ L reaction (2 μ L of template cDNA, 1 μ L of 10 μ mol/L primers, 10 μ L of 2X SYBR

Green Master Mix, 4 μ L of 25 mmol/L Mg²⁺ and 2 μ L of ddH₂O) on an ABI Prism 7500HT sequence detection system (Applied Biosystems) with the following cycling parameters: 3 min at 95 °C followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. *KISS1* transcript levels were calculated according to the comparative cycle threshold (CT) method using β -actin as an endogenous control. The final results were estimated as $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct$ *KISS1* for each sample was determined by subtracting the Ct value for *KISS1* from that for β -actin (control). Only triplicates with Ct values with a standard deviation < 0.20 were acceptable.

***KISS1*/metastin expression analysis by Western blot**

Tissues and cells were collected and lysed, and the DNA was sheared by ultrasonication. After quantification using the Bradford method, proteins were resolved by 10% sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and transferred onto nylon membranes. The membranes were blocked in 5% non-fat milk and 0.1% Tween 20 in Tris-buffered saline and probed with a mouse anti-*KISS1* antibody (Santa Cruz Biotechnology, Dallas, TX, United States) and an anti-GAPDH antibody (Santa Cruz Biotechnology); the signals were visualized using a chemiluminescence kit (SuperSignal; Pierce, Ther-

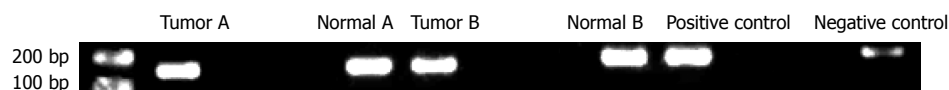


Figure 1 *KISS1* methylation analysis of colorectal cancer tumor samples by methylation-specific polymerase chain reaction. Positive control: Placental tissue DNA treated with M.SssI methyltransferase; Negative control: DNA from normal lymphocytes. Tumors A and B were scored as positive/methylated due to the presence of a methylation band, whereas normal samples A and B were scored as negative/unmethylated due to the absence of a methylation band. The methylated polymerase chain reaction (PCR) product is 172 bp, and the unmethylated PCR product is 173 bp.

mo Fisher Scientific, Rockford, IL, United States).

Cell invasion and migration analysis in a Transwell assay

We performed Transwell migration assays using tissue culture inserts to evaluate *in vitro* cell migration and invasion, as previously described^[27]. In these assays, we used a chemotactic gradient of FBS to evaluate the ability of cells to migrate through holes smaller than the diameter of a single cell. In the invasion assay, cells must digest the Matrigel before migrating through the pores. The cells were first pre-treated with 5-Aza-CdR according to the experimental design and then washed and seeded onto the inserts. The cells were not treated with 5-Aza-CdR for the duration of the assay. Cell invasion was analyzed in 24-well plates containing polyethylene terephthalate membrane cell culture inserts with an 8 μ m pore size (Corning, Tewksbury, MA, United States) that were coated with Matrigel (Sigma-Aldrich), mimicking the extracellular matrix. The upper compartment was seeded with 4×10^4 pre-treated cells, and the lower compartment was filled with culture medium supplemented with 10% FBS as a chemoattractant. After culturing for 24 h at 37 $^{\circ}$ C, the cells remaining on the upper side of the membrane were removed, and cells that had migrated and attached to the lower side of the membrane were stained with hematoxylin. All of the cells on the insert were counted using a light microscope at $200 \times$ magnification. *In vitro* cell migration was assessed using the same inserts and protocol as described above, except the membrane was not coated with Matrigel. All the analyses were performed in triplicate in 8 independent experiments. The migration and invasion assay results were normalized to the control condition for comparison purposes.

Statistical analysis

All the analyses were performed with SPSS version 13.0 (SPSS, Chicago, IL, United States). The frequency of *KISS1* gene methylation in groups based on clinicopathological variables was analyzed using the χ^2 and Fisher's exact tests. The relationships between *KISS1* expression and clinicopathological variables were compared using the independent samples *t*-test. The relationship between *KISS1* methylation status and expression was compared using the independent samples *t*-test. The changes in *KISS1* expression and cell invasion and migration with different doses of 5-Aza-CdR were compared using analysis of variance (ANOVA) after transformation with the least-significant difference (LSD) method. All the

reported *P* values are two sided, and *P* values < 0.05 were considered significant.

RESULTS

KISS1 methylation and correlation with clinicopathological factors in colorectal tissues

KISS1 methylation in CRC tissues was evaluated by MS-PCR (Figure 1), and *KISS1* hypermethylation was detected in 105 of 126 (83.33%) primary CRC tissues compared with 9 of 142 (6.30%) normal colorectal tissue samples. In the CRC samples, the *KISS1* methylation rate increased with the depth of local invasion (T1 + T2 *vs* T3 + T4, *P* = 0.001). *KISS1* methylation was also associated with tumor differentiation, lymph node metastasis and distant metastasis in CRC patients (Table 1); *KISS1* methylation occurred more frequently in tumors that metastasized to the lymph nodes (45/48, 93.75%) than in tumors without lymph node metastasis (60/78, 76.92%). Methylation was also more prevalent in patients with distant metastasis (9/9) than in patients without distant metastasis (94/115, 81.73%). No correlation was detected between *KISS1* methylation and patient sex, age, tumor location or tumor diameter.

Correlations between *KISS1* expression, *KISS1* methylation and clinicopathological factors

A correlation analysis of *KISS1* methylation and expression in CRC tissues revealed that there was lower *KISS1* mRNA expression in the methylated *KISS1* group than in the unmethylated group (0.231 ± 0.048 *vs* 1.214 ± 0.209 , *P* < 0.05) (Figure 2A). Moreover, metastin expression was lower in the *KISS1* methylated group than in the unmethylated group (0.388 ± 0.032 *vs* 0.945 ± 0.050 , *P* < 0.05) (Figure 2B and C). The comparisons between *KISS1* expression and the clinicopathological parameters of the CRC patients are summarized in Table 1. Low *KISS1* transcript expression was associated with various clinicopathological factors, including tumor differentiation, the depth of invasion, lymph node metastasis and distant metastasis (Figure 2D). Low metastin expression correlated with the depth of invasion, lymph node metastasis and distant metastasis (*P* < 0.05) (Figure 2E). *KISS1* transcript and metastin expression levels were independent of patient sex, age, tumor location, tumor differentiation and tumor diameter (*P* > 0.05).

KISS1 methylation and expression in human CRC cells

Four human CRC cell lines were initially screened using

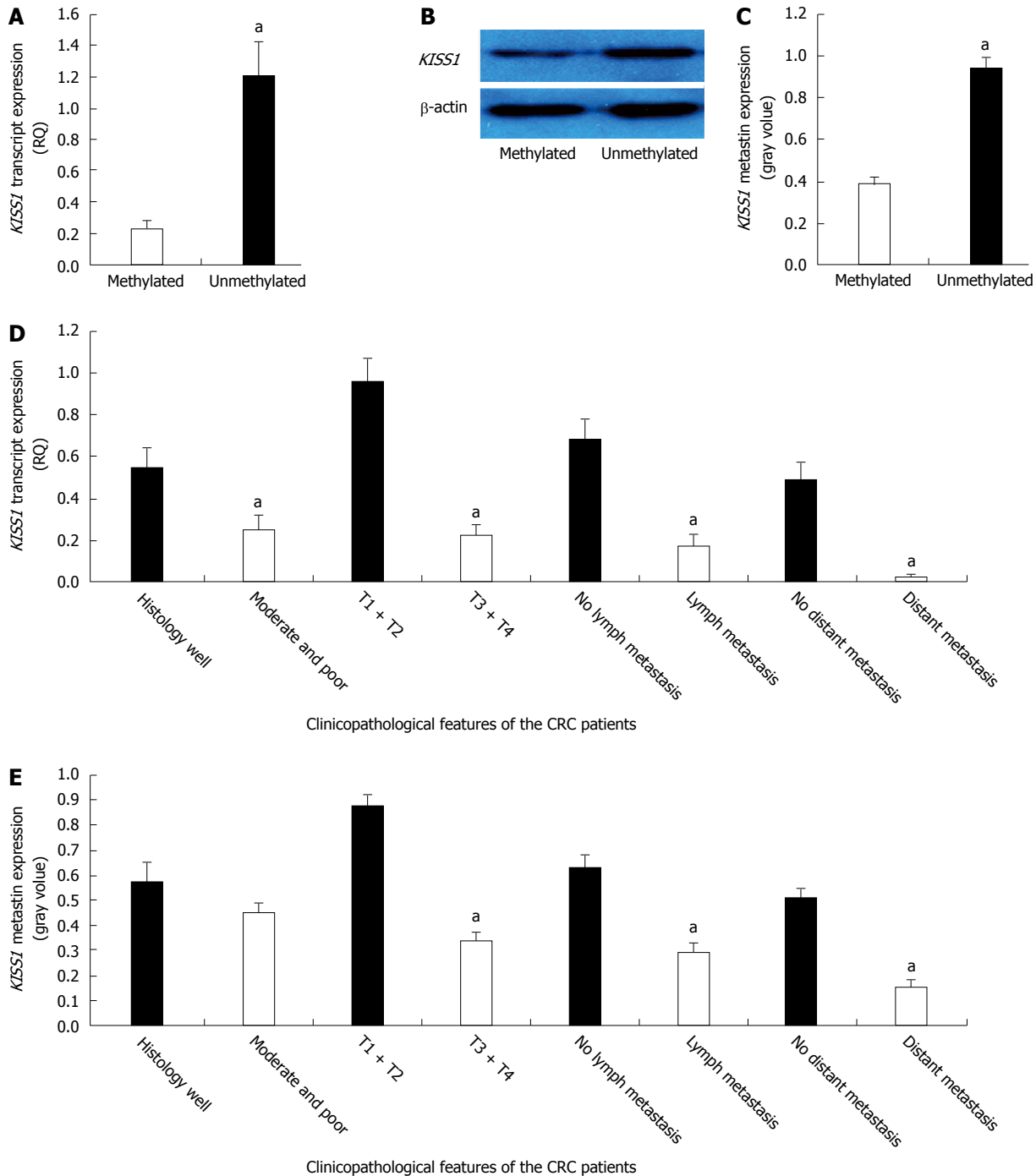


Figure 2 Correlations between *KISS1* expression and methylation and clinicopathological factors in colorectal cancer patients. A: *KISS1* transcript expression in 126 colorectal cancer (CRC) patients was determined by real-time quantitative polymerase chain reaction (PCR). The data are presented as the mean \pm SD. *KISS1* transcript expression was significantly lower in the methylated *KISS1* group than in the unmethylated *KISS1* group ($^aP < 0.05$ vs the methylated *KISS1* group); B: *KISS1*/metastatin protein levels in CRC tissues of the indicated genotypes were determined by Western blot. The same membranes were subsequently probed with an anti- β -actin antibody as a loading control; C: The *KISS1*/metastatin protein expression levels are presented as the mean \pm SEM. $^aP < 0.05$ vs the methylated *KISS1* group; D: The analysis of the relationship between *KISS1* transcript expression and patient clinicopathological factors revealed a negative correlation with tumor differentiation, depth of invasion, lymph node metastasis and distant metastasis ($^aP < 0.05$); E: Metastatin expression was also negatively correlated with the depth of invasion, lymph node metastasis and distant metastasis ($^aP < 0.05$).

bisulfite genomic sequencing and MS-PCR (Figure 3A). The methylation results were compared with the *KISS1* expression levels obtained by real-time quantitative PCR and Western blotting. The hypermethylated cell lines had low transcript levels (Figure 3B) and low metastatin expression (Figure 3C and D). The hypermethylated cell lines

exhibited increased invasion and migration compared with the unmethylated cell lines (Table 3). The HCT116 cell line, in which the *KISS1* gene is hypermethylated and silenced, was treated with the demethylating agent 5-Aza-CdR to further explore the link between *KISS1* hypermethylation and gene silencing.

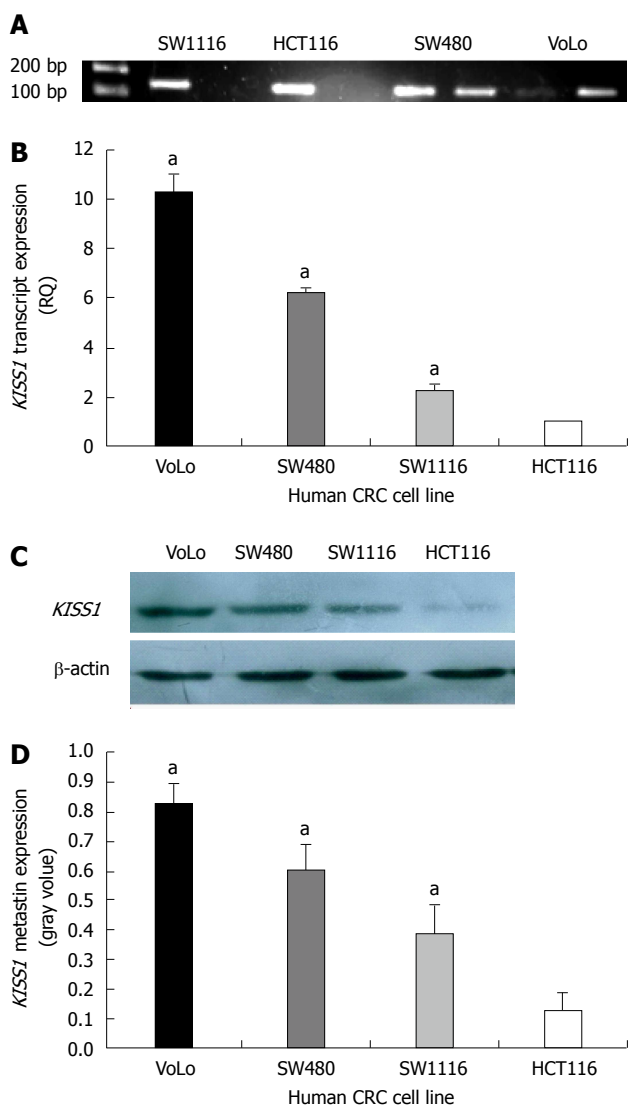


Figure 3 *KISS1* methylation and *KISS1* expression in various human colorectal cancer cell lines. A: MS-PCR for *KISS1* in human colorectal cancer (CRC) cell lines. A PCR band in lane M indicates a methylated *KISS1* gene, whereas a band in lane U indicates an unmethylated *KISS1* gene; B: *KISS1* gene expression in CRC cell lines was examined by real-time PCR, and the expression of *KISS1* was normalized to that of β -actin. The ratio of *KISS1* expression in other CRC cells to *KISS1* expression in HCT116 cells is shown on the Y-axis; C: Metastatin expression in CRC cells of the indicated genotypes was determined by Western blot. The same membranes were subsequently probed with an anti- β -actin antibody as a loading control; D: The metastatin expression levels are presented as the mean \pm SEM. ^a $P < 0.05$ vs HCT116 cells.

Changes in *KISS1* methylation after HCT116 cells were treated with 5-Aza-CdR

KISS1 methylation was detected in HCT116 cells by MS-PCR (Figure 4) after 24 h, 3 d or 5 d of treatment with different doses of 5-Aza-CdR. The results demonstrated that 5-Aza-CdR treatment decreased *KISS1* promoter hypermethylation.

Changes in *KISS1* expression after HCT116 cells were treated with 5-Aza-CdR

We evaluated *KISS1* gene expression after the cells were treated with the DNA methyltransferase inhibitor, 5-Aza-

CdR, to determine whether the observed loss of *KISS1* gene expression resulted from an epigenetic alteration. Guided by a preliminary dose study, we treated HCT116 cells with 5 μ mol/L 5-Aza-CdR and evaluated the changes in *KISS1* transcript and metastatin expression after 1, 3 and 5 d. Using real-time qualitative PCR, we determined that *KISS1* transcript expression was restored by 5-Aza-CdR treatment and that the expression level gradually increased in a time-dependent manner (Figure 5A). Metastatin expression also increased after treatment, as evidenced by Western blotting (Figure 5B and C).

We also determined the changes in *KISS1* transcript and metastatin expression in untreated HCT116 cells (negative control) and in cells treated with 0.1, 1, 5, or 10 μ mol/L 5-Aza-CdR for 5 d. *KISS1* expression (Figure 5D) and metastatin expression (Figure 5E and F) were restored by 5-Aza-CdR treatment. The *KISS1* and metastatin expression levels gradually increased in a time-dependent manner with increasing dose, although the difference between the 5 and 10 μ mol/L groups was not significant. In summary, these results indicated that *KISS1* is likely inactivated *in vitro* by methylation. In addition, our observations of enhanced *KISS1* expression in treated HCT116 cells provide support for the demethylation capability of 5-Aza-CdR.

Changes in the invasion and migration capabilities of HCT116 cells

Invasion and migration were evaluated by Transwell assay to further explore the link between these behaviors in cellular models of CRC and *KISS1* gene re-expression after 5-Aza-CdR treatment. Untreated HCT116 cells were highly invasive (Figure 6A) and migratory (Figure 6C) in these assays, whereas invasion (Figure 6B) and migration (Figure 6D) were significantly reduced after 5-Aza-CdR treatment. Furthermore, invasion and migration significantly decreased in a time-dependent manner compared with the control group after 1, 3 and 5 d of 5 μ mol/L 5-Aza-CdR treatment (Figure 6E). HCT116 cell migration and invasion significantly decreased in a dose-dependent manner after 5 d of treatment with 0.1, 1, 5, or 10 μ mol/L 5-Aza-CdR (Figure 6F) relative to untreated controls.

DISCUSSION

KISS1 has been identified as a gene that suppresses metastasis in numerous types of cancer, including gastric carcinoma, breast carcinoma and colorectal carcinoma^[2-5]; furthermore, the protein encoded by *KISS1*, metastatin, has been reported to be a suppressor of tumor metastasis^[17,28,29]. Moreover, metastatin expression is lost in various carcinoma cell lines and primary tumors. Kostakis *et al.*^[30] also found that the expression of *KISS1* was much higher in normal than in malignant colonic mucosa, *KISS1* expression was higher in larger tumors (> 4 cm) than in smaller ones (\leq 4 cm) and in stages III and IV than in stages I and II. In addition, it was higher in patients with lymph node metastases. In our study, decreased *KISS1* mRNA and metastatin expression levels were significantly associated with an increased depth of invasion and an

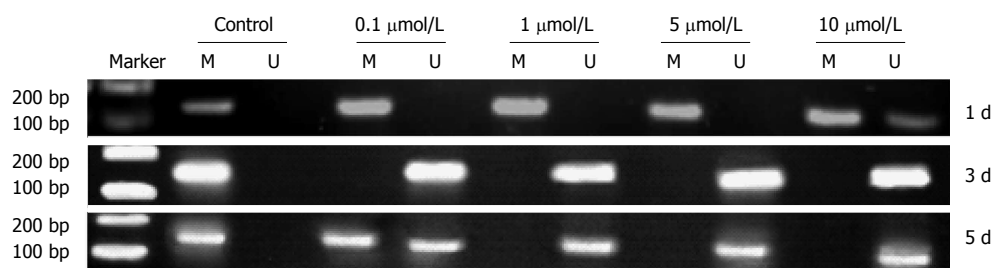


Figure 4 Changes in *KISS1* methylation after treatment of HCT116 cells with different doses of 5-Aza-CdR. MS-polymerase chain reaction (PCR) for *KISS1* in HCT116 cells after 5-Aza-CdR treatment. HCT116 cells were treated for 1 d, 3 d or 5 d with 0 $\mu\text{mol/L}$ (control), 0.1, 1, 5 or 10 $\mu\text{mol/L}$ 5-Aza-CdR. A PCR band in lane M indicates a methylated *KISS1* gene; a band in lane U indicates an unmethylated *KISS1* gene. Marker: 100-bp DNA ladder I; M: Methylated primer product; U: Unmethylated primer product.

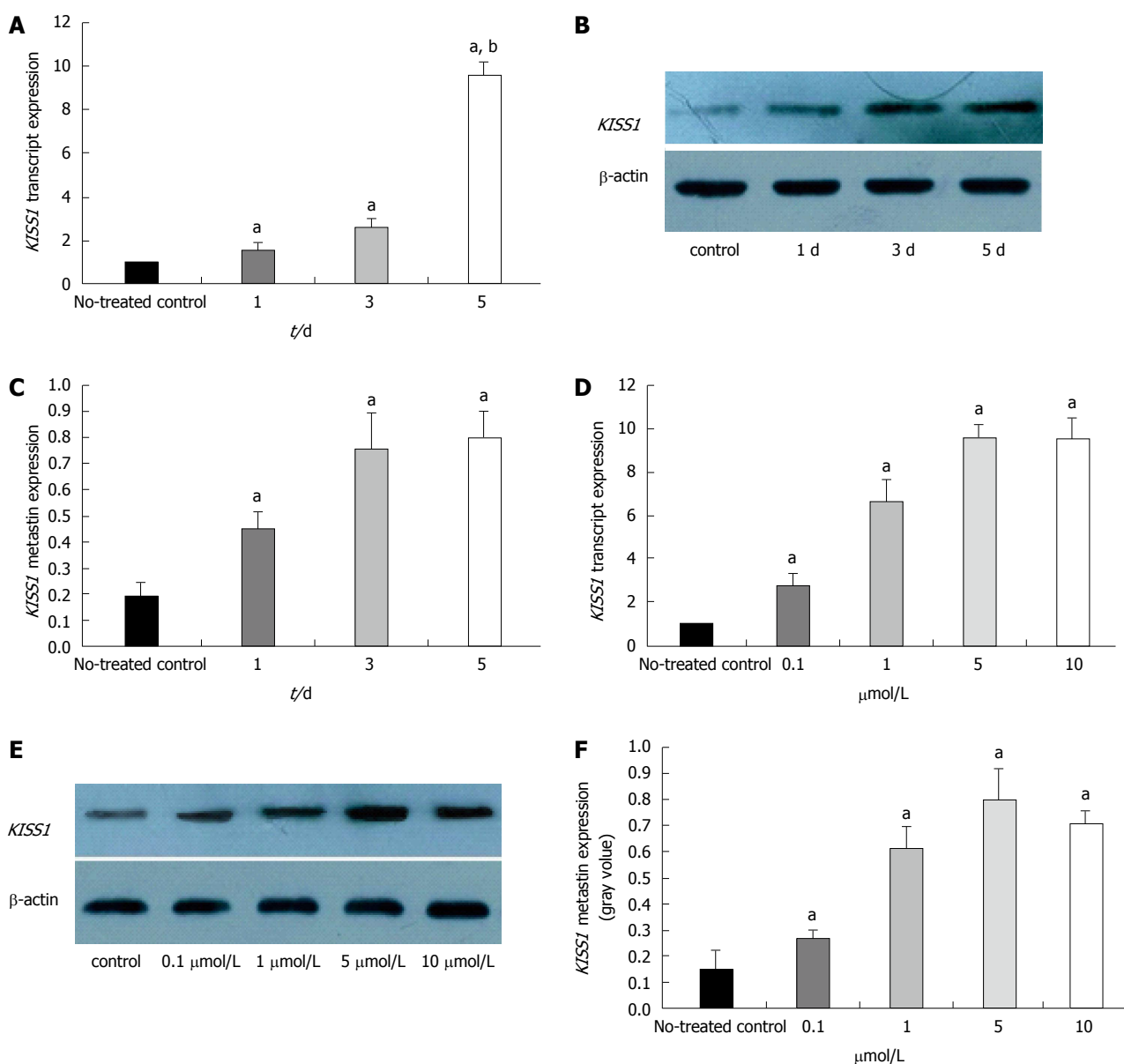


Figure 5 Changes in *KISS1* expression after treatment of HCT116 cells with 5-Aza-CdR *in vitro*. A: *KISS1* transcript expression in HCT116 cells was examined by real-time polymerase chain reaction after 5-Aza-CdR treatment for the indicated times. *KISS1* expression was normalized to that of β -actin. The ratio of *KISS1* expression in treated cells to that in untreated control cells is plotted on the Y-axis. *KISS1* transcript expression gradually increased over time; B: Metastin expression levels after treatment with 5 $\mu\text{mol/L}$ 5-Aza-CdR for different times were determined by Western blot. The same membranes were subsequently probed with an anti- β -actin antibody as a loading control; C: Metastin expression levels gradually increased over time; D: *KISS1* transcript expression gradually increased with increasing concentrations of 5-Aza-CdR after 5 d; E: Metastin protein levels were determined by Western blotting after a 5-d treatment with different concentrations of 5-Aza-CdR; F: Metastin expression gradually increased with increasing concentrations of 5-Aza-CdR. The data are presented as the mean \pm SE. ^a $P < 0.05$ vs untreated control.

Table 3 Invasion and migration of various human colorectal cancer cell lines

Human CRC cells	HCT116	SW1116	SW480	VoLo	F	P value
Invasion	38.93 ± 2.89	20.29 ± 1.79 ^a	11.14 ± 2.19 ^a	6.77 ± 0.79 ^a	143.486	0.001
Migration	48.12 ± 2.54	37.21 ± 2.92 ^a	34.35 ± 2.08 ^a	28.41 ± 1.85 ^a	36.058	0.001

^aP < 0.05 vs HCT116 cells. CRC: Colorectal cancer.

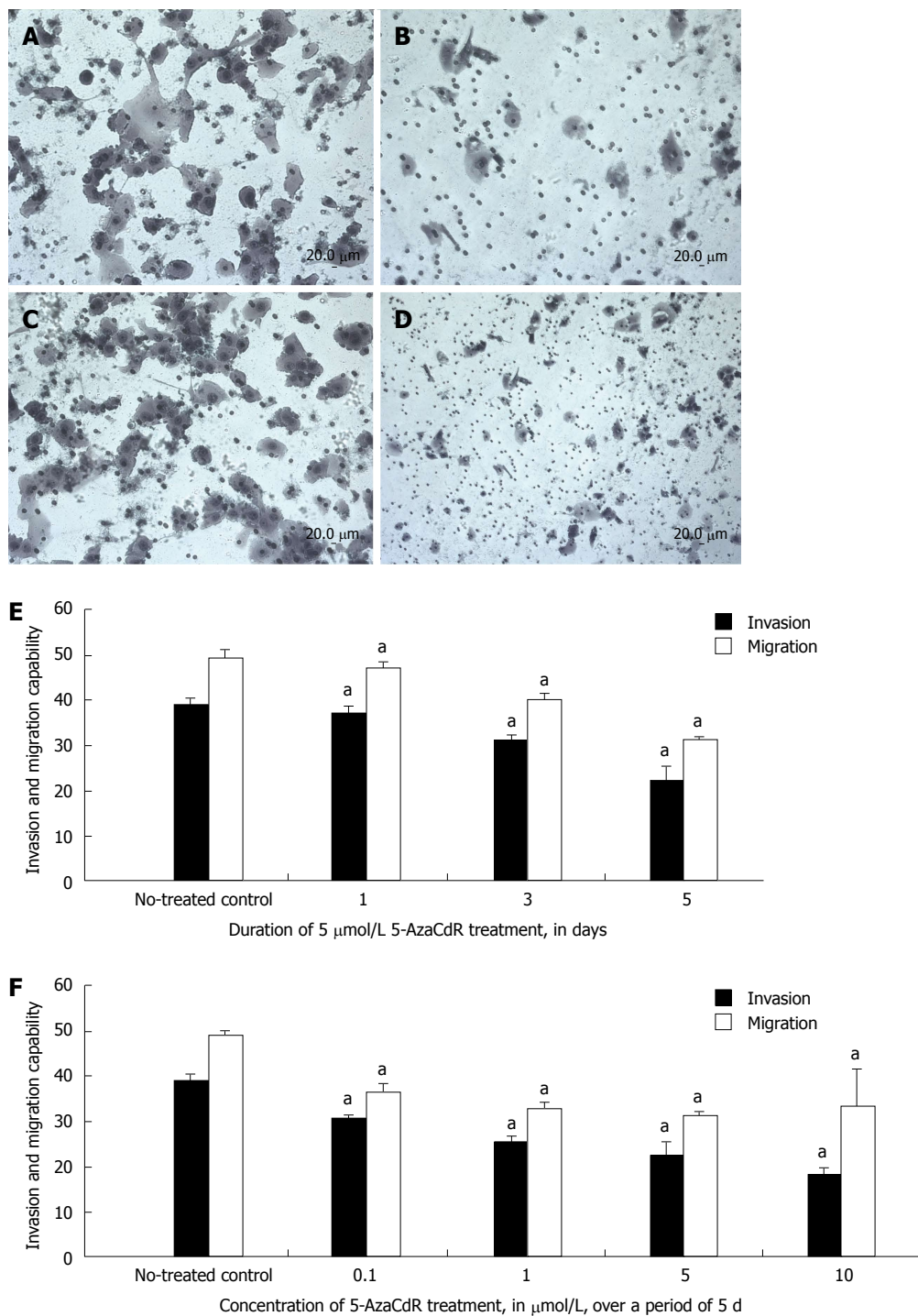


Figure 6 Changes in HCT116 cell invasion and migration after 5-Aza-CdR treatment. A: The invasion of untreated HCT116 cells was determined using a Matrigel invasion assay; B: HCT116 cell invasion in one well after treatment with 5-Aza-CdR; C: Cell migration was determined in untreated cells using a migration assay; D: Cell migration after treatment with 5-Aza-CdR; E: HCT116 cell invasion and migration gradually decreased in a time-dependent manner after treatment; F: HCT116 cell invasion and migration gradually declined with increasing concentrations of 5-Aza-CdR. Each bar represents the mean of 3 independent wells. Scale bar, 20 μm. ^aP < 0.05 vs non-treated control.

increased incidence of lymph node metastasis and distant metastasis in CRC patients ($n = 126$). These results suggested that the *KISS1* gene may be a suppressor of metastasis in CRC, as well as in other malignant tumors, but it remains unknown how and why *KISS1* expression was reduced or silenced.

A previous study suggested that *KISS1* is inactivated by epigenetic mechanisms in bladder cancer and demonstrated the clinical relevance of *KISS1* hypermethylation in uroepithelial tumors^[31]. DNA methylation is a common mechanism of epigenetic gene silencing. In the current study, *KISS1* hypermethylation was detected in 83.33% (105/126) of the primary CRC cases, which was significantly higher than the rate observed in normal colorectal tissues (6.30%, 9/142). We also identified significant correlations between methylation and numerous clinicopathological characteristics of CRC patients, such as the depth of invasion, the incidence of lymph node metastasis and the incidence of distant metastasis. Moya *et al.*^[32] found that the loss of *KISS1* expression correlated with tumor stage, grade, recurrence, and disease-specific survival. He also suggested that *KISS1* was epigenetically modified in CRC. Additionally, several approaches confirmed that *KISS1* expression levels were related to methylation levels; *KISS1* expression was decreased or undetectable in tissues and cell lines with *KISS1* hypermethylation compared to those with unmethylated *KISS1*. Increased methylation levels, together with the loss of *KISS1* expression, was also associated with advanced CRC tumor stage and poor clinical outcome. Okugawa *et al.*^[33] suggested that the loss of *KISS1* appears to correlate with the progression of lymph node metastasis. An assessment of *KISS1* expression may assist in the accurate colorectal cancer diagnosis and may contribute to the prediction of clinical outcomes.

HCT116 cells harboring hypermethylation and low expression of *KISS1* were exposed to different concentrations of 5-Aza-CdR *in vitro* to specifically restore *KISS1* transcript expression and therefore assess the biological effect of methylation on *KISS1* expression. Our results substantiated those reported for gastric cancer^[26]; overall, these assays revealed that *KISS1* was aberrantly silenced by CpG island hypermethylation and that *KISS1* expression was restored by 5-Aza-CdR treatment in a dose- and time-dependent manner. The protein product of the *KISS1* gene, metastin, may reduce cancer cell migration and inhibit colony formation^[9,13,14]. In our assays, HCT116 cell migration and invasion were significantly reduced following 5-Aza-CdR-mediated restoration of *KISS1* expression. A statistically significant association was identified between reduced invasion and migration and 5-Aza-CdR treatment in a dose- and time-dependent manner.

It has been suggested that *KISS1* methylation and transcriptional silencing are associated with increased tumor stage and grade. *KISS1* is a potential suppressor of tumor metastasis, and the transcriptional silencing of this gene results partly from *KISS1* promoter methylation in CRC. Thus, DNA methyltransferase inhibitors, such as 5-Aza-CdR, can restore *KISS1* expression and reduce

the invasion and migration of certain human CRC cells, thereby providing a potential target and novel therapeutic strategy for metastatic CRC.

Although the present study did not elucidate the role of *KISS1* hypermethylation in CRC progression, the epigenetic silencing of *KISS1* might enable a better understanding of how *KISS1* contributes to CRC. Further studies are warranted to ascertain the influence of *KISS1* hypermethylation on the expression of its mRNA and protein products, on CRC progression and on the development and/or progression of other malignancies. We also hope to explore additional mechanisms involved in *KISS1* gene silencing.

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COMMENTS

Background

Colorectal cancer (CRC) is one of the most common types of gastrointestinal cancer, and even patients undergoing aggressive therapies have a poor prognosis due to the metastatic nature of CRC. Metastasis is a critical aspect of CRC that contributes significantly to CRC-related morbidity and mortality. Despite recent improvements in CRC treatments, such as enhanced surgical excision techniques, radiation therapy and chemotherapy, metastatic recurrence remains a significant clinical hurdle for patients with CRC.

Research frontiers

KISS1 was identified as a gene that suppresses the metastasis of various cancers, including CRC. The *KISS1* gene plays an important role in CRC metastasis and has gradually become a popular research subject.

Innovations and breakthroughs

Prior research has illustrated that the loss of *KISS1* expression is associated with tumor stage, tumor grade and survival in CRC, but the mechanisms by which *KISS1* expression is lost in CRC remain unknown. Herein, authors determined the hypermethylation status of the CpG island in the *KISS1* promoter region and investigated the epigenetic silencing of *KISS1* by comparing tissues from CRC patients and healthy volunteers. The results indicated that *KISS1* methylation was associated with tumor differentiation, the depth of invasion, lymph node metastasis and distant metastasis. Furthermore, treatment of a CRC cell line with 5-Aza-CdR resulted in re-expression of the *KISS1* gene and decreased cell migration. These results suggested that the re-expression of *KISS1* by active DNA demethylation may represent a novel opportunity for therapeutic intervention in CRC that could be exploited clinically to treat metastatic disease.

Applications

The study results suggest that *KISS1* may be down-regulated in cancer tissues through promoter hypermethylation and may therefore represent a candidate target for treating CRC metastasis.

Terminology

The *KISS1* gene is a newly discovered suppressor of metastasis that maps to the 1q32 chromosome region and is regulated by genes located on chromosome 6. The *KISS1* gene was discovered by Lee in early karyotypic analyses of melanomas and was predicted to encode a 154-amino acid protein that is processed to an amidated internal 54-amino acid peptide termed metastin. *KISS1* was identified as a gene that suppressed the metastasis of various types of cancer.

Peer review

This study investigated the effect of aberrant methylation of the *KISS1* promoter on the development of CRC and proposed reversing the aberrant methylation of the *KISS1* gene promoter as a potential therapeutic strategy in this disease. This topic is relatively novel. The paper is well written, and the results are interesting.

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