

Analysis of the association of the expression of KiSS-1 in colorectal cancer tissues with the pathology and prognosis

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Received July 28, 2017; Accepted November 29, 2017

DOI: 10.3892/ol.2017.7630

Abstract. Colorectal cancer is a common malignant tumor of the digestive tract with high morbidity and mortality rates. The aim of the present study was to examine the expression level of KiSS-1 in tumor tissues of patients with colorectal cancer, and to explore the relationship with the clinicopathology and prognosis of patients with colorectal cancer. Frozen tumor tissue and corresponding cancer-adjacent normal tissue specimens were selected from 56 patients with colorectal cancer who were treated in the Department of Surgery of our hospital from May 2009 to December 2011. The expression levels of KiSS-1 messenger ribonucleic acid (mRNA) in tumor tissues and cancer-adjacent normal tissues were detected by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). The expression levels of KiSS-1 proteins in colorectal cancer tissues and cancer-adjacent normal tissues were further detected by immunohistochemistry. In addition, the association of the expression level of KiSS-1 proteins in tissues of colorectal cancer patients with pathological parameters and the prognosis of patients with colorectal cancer was analyzed combined with clinical data. The RT-qPCR results showed that the expression of KiSS-1 mRNA in colorectal cancer tissues was significantly lower than that in cancer-adjacent normal tissues ($P < 0.05$). Immunohistochemistry results indicated that the positive expression rate of KiSS-1 proteins in colorectal cancer tissues (26.79%) was significantly lower than that in cancer-adjacent normal tissues (80.36%). The low expression of KiSS-1 in colorectal cancer tissues was associated with the degree of differentiation, invasion and metastasis, as well as clinical staging. The 5-year overall survival rate of patients with colorectal cancer was 55.36% (31/56). The univariate survival

analysis showed that patients with lowly expressed KiSS-1 had worse prognosis. The low expression of KiSS-1 is closely associated with the occurrence and development of colorectal cancer, especially to the degree of differentiation, invasion and metastasis, as well as clinical staging. Thus, the expression of KiSS-1 in colorectal cancer tissues can be used as a reference for the prognosis of colorectal cancer, and KiSS-1 is a potential new target for the treatment of colorectal cancer.

Introduction

Colorectal cancer is a common malignant tumor of the digestive tract, with high morbidity and mortality rates. Its incidence ranks fourth in malignant tumors in China, and the mortality rate ranks fifth in tumors leading to death (1). In addition, the incidence and mortality rates of colorectal cancer in China are on the increase, which has deleterious effects on the lives and health of patients (2). Previous findings have shown that the 5-year survival rate of colorectal cancer patients with metastasis was significantly lower than that of colorectal cancer patients without metastasis (3,4). In the diagnosis of patients with colorectal cancer, metastasis occurs in approximately 25% of patients, which is the main reason leading to the poor curative effect in the clinical treatment for patients (5).

Metastasis suppressor and promoter genes play key roles in the process of tumor metastasis. An increasing number of scholars have been focused on the study of tumor metastasis suppressor genes. A previous study demonstrated that the effect of metastasis suppressor genes on tumor metastasis is more critical than that of metastasis promoter genes, and the reduction in the expression of metastasis suppressor genes or gene loss may induce the invasion and metastasis of tumor cells (6). The *KiSS-1* gene was initially identified in non-metastatic melanoma cells, and is an important tumor metastasis suppressor gene (7). *KiSS-1* genes are located at the long arm of human chromosome 1, and the gene encoding proteins can bind to the G protein-coupled receptor 54 (GPR54), resulting in physiological effects of inhibition of the proliferation, invasion and metastasis of tumor cells and induction of the differentiation and apoptosis of tumor cells (8). Further findings have shown that the expression level of *KiSS-1* genes is downregulated in a variety of tumors, including pancreatic (9), gastric (10), breast (11) and bladder cancer (12), and promotes tumor invasion and metastasis.

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Key words: colorectal cancer, KiSS-1, pathological parameters, prognosis

The findings of Siegel *et al* indicated that the expression level of KiSS-1 proteins in the tumor tissues of patients with colorectal cancer was significantly lower than that in the normal colorectal tissues. Additionally, KiSS-1 acts as a tumor suppressor gene in the occurrence and development of colorectal cancer (13).

The present study aimed to investigate the expression of KiSS-1 in the tumor tissues of patients with colorectal cancer and its effects on pathological parameters and the prognosis of patients with colorectal cancer. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry were used to analyze the expression levels of KiSS-1 in the tumor tissues and cancer-adjacent normal tissues of patients with colorectal cancer. The effects of KiSS-1 expression on pathological parameters and the prognosis of patients with colorectal cancer were analyzed combined with clinical data.

Materials and methods

Data. For the clinical data, 56 patients with colorectal cancer treated in the Department of Surgery of our hospital from May 2009 to December 2011 were selected. The patients included 32 males and 24 females aged (55.7 ± 9.3) years who received surgical treatment for the first time, and had no history of chemotherapy and radiotherapy. Tumor tissues and cancer-adjacent normal tissues >10 cm away from the tumor were taken from the patients with colorectal cancer during surgery. A section of the tissues was immediately stored in liquid nitrogen, while the other section was fixed in 10% neutral formalin solution. The patients were diagnosed with colorectal cancer via pathological analysis. The study was approved by the Medical ethics committee of the Dezhou People's Hospital (Shandong, China). All the patients or their family members signed the informed consent.

The main reagents used were TRIzol, reverse transcription and RT-qPCR kits (Invitrogen, Carlsbad, CA, USA); primer synthesis [Takara Biomedical Technology (Dalian) Co., Ltd., Dalian, China]; rabbit anti-human KiSS-1 polyclonal antibody (18375-1-AP), general immuno histochemical test kit for anti-rat/rabbit (KIH-1) (Wuhan Sanying Biotechnology Co. Ltd., Wuhan, China), glyceraldehyde-3-phosphate dehydrogenase (GADPH) antibodies, horseradish peroxidase (HRP)-labeled antibodies (Wuhan Proteintech Group, Inc., Wuhan, China); and immunohistochemical staining kit SP-9001 (Beijing Zhongshan Golden Bridge Biological Technology Co., Ltd., Beijing, China).

Detection of the expression of KiSS-1 messenger ribonucleic acid (mRNA) in tissue specimens of patients by RT-qPCR. Total RNA in the frozen tissues of patients was extracted using TRIzol kits, and RNA concentration and purity were measured using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). When the ratio of A260/A280 was between 1.8 and 2.0, the samples were considered to be qualified. Reverse transcription was conducted according to the protocol. PCR was carried out according to the recommended method of RT-qPCR kits with the obtained complementary deoxyribonucleic acid (cDNA) as the template. Primer sequences are shown in Table I. Specific reaction conditions were: pre-denaturation at 94°C for

Table I. Primer sequences of RT-qPCR.

Gene name	Primer sequence
<i>KiSS-1</i>	F: 5'-ACCTGGCTCTTCTCACCAAG-3' R: 5'-TAGCAGCTGGCTTCCTCTC-3'
<i>GAPDH</i>	F: 5'-GCACCGTCAAGGCTGAGAAC-3' R: 5'-TGGTGAAGACGCCAGTGGA-3'

F, forward; R, reverse.

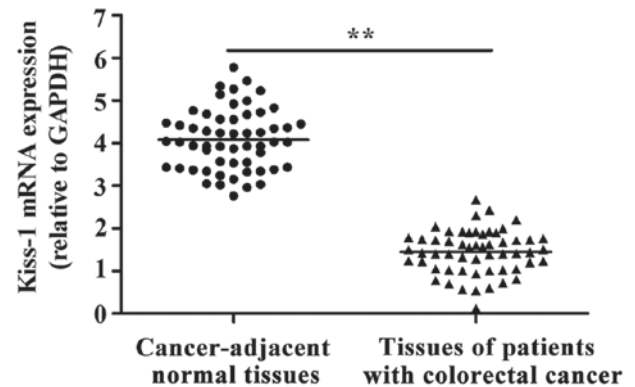


Figure 1. Detection of the expression of KiSS-1 in tissues of patients. The expression of KiSS-1 mRNA in tissues of patients with colorectal cancer is significantly decreased compared with that in cancer-adjacent normal tissues; ** $P < 0.01$.

5 min, denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec and the amplification was conducted for 30 cycles. With GAPDH as the control, the expression level of KiSS-1 mRNA was calculated using the relative quantification method. The formula used was: ΔCt (target gene) = Ct (target gene) - Ct (control gene).

Detection of the expression of KiSS-1 proteins by immunohistochemistry. Tissues fixed in 10% neutral formalin solution were taken and embedded in paraffin, and then continuously cut into 4- μ m slices. Immunohistochemical staining was performed by streptavidin-peroxidase (SP) two-step method, and the procedures were carried out according to the protocol of the kits. Routine dewaxing was performed, citric acid solution was used for repairing protein, and goat serum was applied for sealing. KiSS primary antibodies (diluted at 1:200) were added drop by drop, and the tissues were incubated in a refrigerator at 4°C overnight. After washing by pre-cooling phosphate-buffered saline (PBS) solution three times, secondary antibodies (diluted at 1:1,000) were added drop by drop. The tissues were incubated at room temperature for 30 min and washed by pre-cooling PBS three times. Diaminobenzidine (DAB) development was then conducted, hematoxylin was used for re-dyeing, and finally gum was used for mounting.

Five $\times 400$ visual fields were randomly selected from each slice. Results of KiSS-1 protein-positive staining were that pale brown and chocolate-brown particles were precipitated in the cytoplasm. According to the percentage of stained cells and the staining intensity, scores were evaluated comprehensively:

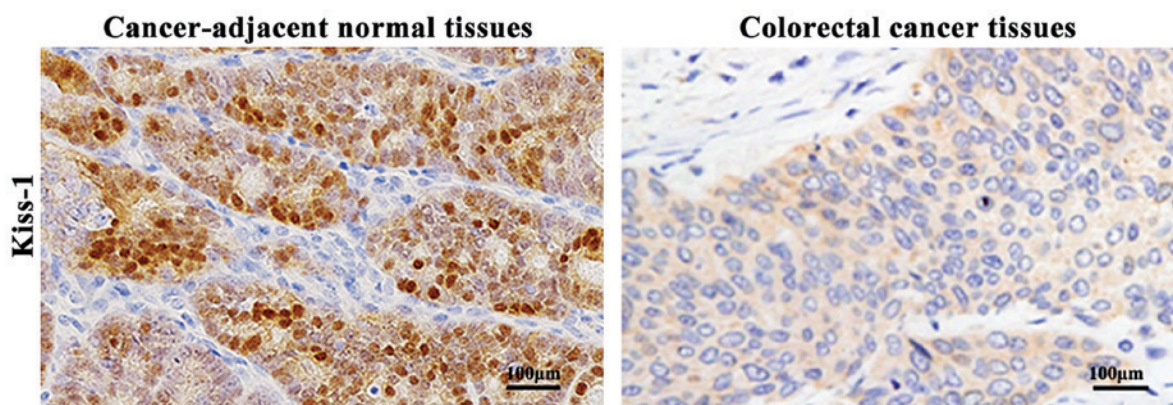


Figure 2. Detection of the expression of KiSS-1 proteins in tissues of patients by immunohistochemistry. The expression level of KiSS-1 in colorectal cancer tissues is significantly decreased compared with that in cancer-adjacent normal tissues.

0 points for positive cells <5%, 1 point for 5-25%, 2 points for 26-50% and 3 points for >51%; 0 points for basic colorlessness, 1 point for light yellow, 2 points for pale brown and 3 points for chocolate-brown. These scores were added, and ≤ 3 points represented negative expression, while >3 points represented positive expression.

Relationship of the expression of KiSS-1 in the tumor tissues of patients with colorectal cancer with pathological parameters and the prognosis of patients. According to the expression level of KiSS-1 in colorectal cancer tissues, 56 patients with colorectal cancer were divided into the KiSS-1 positive and KiSS-1 negative expression groups. Clinicopathological parameters of patients were examined and the association between the expression of KiSS-1 and pathological parameters of patients was analyzed. The 56 patients were followed up after surgery for 5 years. The survival time was calculated from the first day after surgery, and the follow-up was carried out once a month.

Statistical treatment. Data were processed using Statistical Product and Service Solutions (SPSS) 17.0 software (IBM Corp., Armonk, NY, USA). Measurement data were expressed as mean \pm standard deviation, and t-test was used for comparisons between two groups. The χ^2 test was used for intergroup comparisons of countable data. The univariate survival analysis was performed using Kaplan-Meier method. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Detection of the expression of KiSS-1 in tumor tissues of patients with colorectal cancer by RT-qPCR. As shown in Fig. 1, the expression of KiSS-1 mRNA in colorectal cancer tissues was significantly decreased compared with that in cancer-adjacent normal tissues ($P < 0.01$).

Detection of the expression of KiSS-1 in tumor tissue specimens of patients with colorectal cancer by immunohistochemistry. As shown in Fig. 2, immunohistochemistry results indicated that the positiveness of KiSS-1 proteins was manifested as light brown or dark brown precipitates in the cytoplasm. The expression levels of KiSS-1 proteins in colorectal cancer

Table II. Association of the abnormal expression of KiSS-1 and clinicopathological parameters of patients with colorectal cancer.

Clinicopathological parameters	Case	KiSS-1	
		Negative expression (case, %)	χ^2 P-value
Sex			0.12 >0.05
Male	32	24 (75.00)	
Female	24	17 (70.83)	
Age (years)			0.05 >0.05
≥ 50	35	26 (74.29)	
<50	21	15 (71.43)	
Tumor size (cm)			0.51 >0.05
≥ 5	33	23 (69.70)	
<5	23	18 (78.26)	
Differentiation degree			14.24 <0.01
Low	22	10 (45.45)	
Moderate/high	34	31 (91.18)	
Invasion and metastasis			12.63 <0.05
Yes	36	32 (88.89)	
No	20	9 (45.00)	
Clinical staging			10.36 <0.01
I-II	25	13 (52.00)	
III-IV	31	28 (90.32)	

tissues were significantly decreased compared with that in the cancer-adjacent normal tissues ($P < 0.01$).

Results of the statistical analysis according to the standard of evaluation indicated that the positive expression rate of KiSS-1 proteins in colorectal cancer tissues was 26.79% (15/56), and the negative expression rate was 73.21% (41/56). The positive expression rate of KiSS-1 proteins in the cancer-adjacent normal tissues was 80.36% (45/56), and the negative expression rate was 19.64% (11/56).

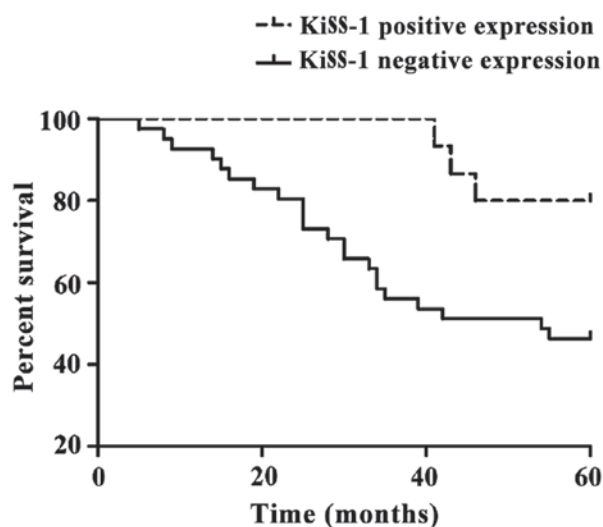


Figure 3. Expression of KiSS-1 and Kaplan-Meier survival curve of patients with colorectal cancer. Patients with negatively expressed KiSS-1 have worse prognosis than those with positively expressed KiSS-1.

Table III. Univariate survival analysis of the association between the expression of KiSS-1 and the overall survival rate of patients with colorectal cancer.

Group	Case	5-year survival case	5-year survival rate (%)	Wald (log-rank)	P-value
KiSS-1				5.36	<0.05
Positive expression	15	12	73.33		
Negative expression	41	19	48.78		

Association between the expression of KiSS-1 in tumor tissues of patients with colorectal cancer and clinicopathological parameters. According to the results of immunohistochemistry, patients with colorectal cancer were divided into the KiSS-1 positive and KiSS-1 negative expression groups, and the association between the expression of KiSS-1 in the tumor tissues of patients with colorectal cancer and clinicopathological parameters was examined. The results showed that the negative expression of KiSS-1 in tumor tissues of patients was correlated with tumor differentiation degree, invasion and metastasis as well as clinical staging (Table II).

Analysis of the survival condition and prognosis of patients. The 5-year follow up was statistically analyzed, and showed that of the 56 patients with colorectal cancer, 31 survived and 25 succumbed to the disease. The 5-year overall survival rate was 55.36% (31/56). Results of the Kaplan-Meier analysis of survival curves for colorectal cancer patients are shown in Fig. 3. Patients with negatively expressed KiSS-1 had worse prognosis. Results of the univariate survival analysis are shown in Table III. KiSS-1 significantly affected the overall survival rate of colorectal cancer, which was an independent factor affecting the survival of patients with colorectal cancer ($P < 0.05$).

Discussion

Colorectal cancer is a common malignant tumor of the digestive tract with a high metastatic rate, whose incidence ranks second in female tumors and third in male tumors worldwide (1).

In recent years, the *KiSS-1* gene has become a hot spot in tumor research. Previous findings have shown that KiSS-1 proteins can inhibit tumor cell proliferation and invasion and induce tumor cell apoptosis (14). The coding product of *KiSS-1* genes is polypeptide with the same length as that of 54 amino acids, which can bind specifically to GPR54 or AXOR12. After binding to GPR54, polypeptide induces phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis to elevate the intracellular calcium concentration, thus activating intracellular signaling pathways to play roles in inhibiting cell proliferation and differentiation (15,16). Stafford *et al* showed that proteins encoded by KiSS-1 bind to GPR54 to activate phospholipase C, producing the second messenger inositol trisphosphate (IP3) and diglycerides in cells to inhibit cell proliferation and promote cell apoptosis (8). Other findings have shown that KiSS-1 can suppress the invasion and metastasis of tumor cells, and its mechanism is mainly to downregulate the expression of matrix metalloproteinase-9 (MMP-9) in order to carry out its roles (17).

The present study aimed to study the expression of KiSS-1 in tissues of colorectal cancer patients and its effects on pathological parameters and the prognosis of patients with colorectal cancer. In the present study, RT-qPCR was used to detect the expression of KiSS-1 mRNA in tumor tissues and cancer-adjacent normal tissues of patients with colorectal cancer. The results showed that the transcription level of KiSS-1 mRNA in the tissues of patients with colorectal cancer was significantly lower than that in the cancer-adjacent normal tissues. The results of immunohistochemistry also confirmed that the expression level of KiSS-1 proteins in tissues of patients with colorectal cancer was significantly decreased compared to that in cancer-adjacent normal tissues. In order to determine the association between its expression and the pathological parameters of patients, statistical analysis was conducted, which indicated that the negative expression of KiSS-1 in the tumor tissues of patients was correlated with the degree of tumor differentiation, invasion and metastasis as well as clinical staging. The univariate Kaplan-Meier survival analysis was used to assess the effect of KiSS-1 expression on the 5-year overall survival rate of patients. The results showed that KiSS-1 significantly affected the overall survival of colorectal cancer, and patients with lowly expressed KiSS-1 have lower 5-year survival rate and worse prognosis.

Dhar *et al* found the presence of lowly expressed *KiSS-1* genes in tissues of patients with gastric cancer, and KiSS-1 is closely associated with the metastasis of gastric cancer (18). Studies of Masui *et al* suggested that the expression of *KiSS-1* genes in pancreatic cancer tissues was significantly lower than that in normal tissues, and the low expression of *KiSS-1* genes may be associated with the metastasis and staging of pancreatic cancer (19). Results of the aforementioned studies are consistent with those of the current study. In the present study, the expression of KiSS-1 was confirmed to be downregulated in the tumor tissues of patients with colorectal cancer. It was

also shown that the expression of KiSS-1 was correlated with the degree of tumor differentiation, invasion and metastasis, clinical staging and prognosis.

In summary, KiSS-1 is lowly expressed in tumor tissues of colorectal cancer patients, which is closely associated with the occurrence and development of colorectal cancer, the degree of differentiation, invasion and metastasis, as well as clinical staging. KiSS-1 also affects the 5-year survival rate of patients. Patients with lowly expressed KiSS-1 have a lower 5-year survival rate. Consequently, the expression of KiSS-1 of tumor tissues of patients can be used as a reference factor for the prognosis estimation for colorectal cancer patients.

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