Original Article

Promoter Hypermethylation of *KiSS-1* Gene in Gastric Cancer

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ABSTRACT

Objective: To investigate the association between *KiSS-1* methylation and clinicopathological characteristics of gastric cancer and evaluate the role of peritoneal lavage fluid in detecting peritoneal metastases.

Methods: The methylation status of *KiSS-1* gene in 40 gastric cancer specimens, the corresponding adjacent normal mucosa, lymph nodes and peritoneal lavage fluid was investigated by methylation- spcific polymerase chain reaction(MS-PCR).

Results: Aberrant methylation of *KiSS-1* gene was detected in 55%(22/40) of the adjacent normal mucosa, 82.5% (33/40)of gastric cancer specimens, 80.95%(17/21) of the lymph nodes, and 42.5%(17/40) of peritoneal lavage fluid. Methylation in gastric carcinoma and lymphonode was more frequent than in non-neoplastic gastiric mucosa. Presence of *KiSS-1* methylation in peritoneal lavage fluid was significantly correlated with tumor invasion (P=0.043). The accuracy of *KiSS-1* methylation in peritoneal lavage fluid for diagnosing peritoneal metastasis was 70%, with a sensitivity of 77.8% and a specificity of 67.7%.

Conclusion: Aberrant methylation of *KiSS-1* gene is a common event in the occurrence and progression of gastric carcinoma, which may provide useful information for the early diagnosis of peritoneal metastases and a new therapy for gastric cancer.

Key words: Gastric cancer; Methylation; *KiSS-1*; Peritoneal lavage fluid

INTRODUCTION

Gastric cancer is the second most common cause of cancer death in the world[1]. As its molecular basis, deep involvement of aberrant DNA methylation has been indicated by the higher incidences of aberrant DNA methylation of known tumor suppressor genes than of mutations[2]. Aberrant methylation of DNA may happen in the promoter CpG island of tumor suppressor genes, where transcription of DNA into RNA begins. Transcription is the first major step in decoding DNA into a protein. An important aspect of the methylation mechanism is that it inactivates tumor suppressors genes[3]. Multiple recent reviews have shown that aberrant methylation of DNA in promoter CpG island and diminished expression are present in a number of tumor related gene in gastric cancer. For example, *RASSF1A*, an candidate tumor suppressor gene, is hypermethylated in gastric cancer[4,5]; *TIMP-3,* which has also been silenced, encodes a protease inhibitor that may help inhibit tissue invasion[6]. *KiSS-1*, a newly discovered metastasis suppressor gene, is silenced with aberrant CpG island hypermethylation in gastric cancer[7]. However, the relationship between methylaiton of *KiSS-1* gene and gastric cancer has not been elucidated yet.

In our study, *KiSS-1* gene methylation was detected in primary tumor, the corresponding adjacent normal mucosa, metastases lymph nodes and

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peritoneal irrigation fluid using methylation special PCR, to find out the association between the *KiSS-1* methylation and clinicopathological characteristics of gastric cancer and to demonstrate the role of testing *KiSS-1* methylation of peritoneal irrigation fluid in gastric cancer diagnosis.

MATERIALS AND METHODS

Clinicopathological Data

Specimens were collected from 40 cases with its adjacent normal mucosa of gastric carcinoma undergone a surgical resection in the Surgical Oncology Department of the First Affiliated Hospital of China Medical University during July 2008－January 2009. The enrolled patients included 28 males and 12 females with a mean age of 56 years (rang, 34-78 years). All of the patients did not undergo chemotherapy or radiotherapy before surgical resection.

Samples

Fifty ml of paysiological saline were injected into the Douglas cavity at the beginning of the operation and aspirated after gentle stirring, and then the peritoneal lavage fluid was collected from the cavity before a surgical resection. Half of the peritoneal lavage fluid was examined through conventional cytological methods with Papanicolaous staining, and the remaining half was centrifuged at 2000r/min. for 20min to collect the intact cells[8,9] and then kept in a liquid nitrogen tank. The tissue samples including primary tumor, corresponding paired adjacent normal mucosa and metastases lymph nodes were procured immediately after resection. Samples were stored at -70°C until DNA extraction. All the samples of the primary gastric cancer were evaluated by two experienced pathologists for diagnosis. Corresponding paired adjacent normal mucosa were obtained at least 3 cm from the distal negative surgical margin of 40 patients and the absence of malignancy was confirmed histopathologically. The lymph nodes were stained with HE method to confirm whether a true metastasis had occurred. Histological grades and tumor cell differentiation were confirmed by histopathological examination and the TNM stage was accorded to the Union International Contrele Cancer(UICC).

DNA Extraction and Bisulfite Treatment

Genome DNA was extracted by the hydroxyl-benzene-chloroform extraction method, and stored at -70°C until use. Treatment of DNA samples with bisulfite converts all unmethylated cytosines to uracils, while leaving methylcytosines unaltered. This allows the subsequent differentiation of methylated and unmethylated sequences by methylation-specific polymerase chain reaction (PCR).

Methylation-specific PCR Reaction

DNA was purified using a Wizard DNA clean-up system (Promega) according to the manipulation instrument. The volume of total reaction mixture was set at 20μl, including 3μl DNA, 2μl 10×PCR buffer, 1.6μl dNTP, 0.8μl primers (the sense and antisense chain), 0.15μl Taq enzyme, and 11.65μl double-distilled water. The reaction consisted of Pre-denaturing at 94°C for 5min, followed by 35 cycles, consisting of 94°C for 30 s, 58°C for 30 s, 72°C for 20 s, and with a final extension at 72°C for 5 min. The following primers were used for the detection of human *KiSS-1* promoter methylation:M-sense, 5'-AAAGTTTCGTTTCGGAGGGTTC-3' and M-anti-sense, 5'-CTTTTATAAAACCCGAAATAACG-3' for the methylated sequence of the human *KiSS-1* promoter; U-sense, 5'-AAAGTTTTGTTTTGGAGG GTTT-3' and U-antisense, 5'-AAAGTTTTGTTTT GGAGGGTTT-3' for the unmethylated sequence of the human *KiSS-1* promoter. The predicted products for methylated and unmethylated DNA were 172 and 173 bp, respectively. The products were subjected to 2.5% agarose gel electrophoresis at 120V for 40 min and quantitated with the FluorChen 2.0 system. DNA from peripheral lymphocytes of healthy individual and water were used as negative controls.

Statiscal Analysis

Statistical analysis was performed using the SPSS13.0 package. Chi-square test was used to analyze the relationship between *KiSS-1* methylation and clinicopathological characteristics in primary tumor and its matched adjacent normol mucosa, metastases lymphnodes, peritoneal lavage fluid. Fisher's exact test was used to analyze the association between *KiSS-1* methylation in peritoneal lavage fluid and peritoneal metastasis. $P<0.05$ was regarded to be statistically significant.

RESULTS

Methylation and Clinicopathological Characteristics

The *KiSS-1* methylation of the tumro samples and

its matched adjacent normal mucosa, metastases lymphnodes was detected by methylation special-PCR (Figure 1). Our data demonstrated that methylation was found in 82.5% (33/40) of primary tumor, in 80.95% (17/21) of metastases lymphnodes and in 55% (22/40) of matched adjacent normal mucosa. Methylation of in gastric carcinoma and lymphnode was more frequent than in non-neoplastic gastiric mucosa, and there were significant differences between the former two and the latter. *KiSS-1* methylation had no significant relation with the clinicopathological characteristics(Table 1).

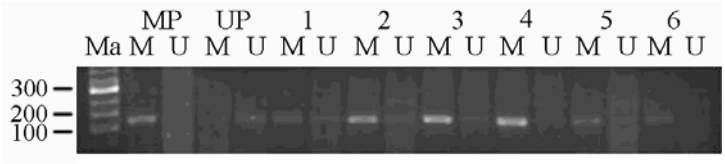

Figure 1. MSP results: M: methylation result; U: unmethylation result; Ma: 50bp DNA Ladder Marker; MP: methylation positive control; UP: unmethylation positive control.

Table 1. *The association between KiSS-1 methylation and clinicopathological characteristics in gastric cancer*

Variable	No.of patients	*KiSS-1* methylation in primary tumor	*P* value
Age			0.136
=60 year	29	25	
>60year	11	8	
Tumor size			0.062
=5cm	24	22	
>5cm	16	11	
Borrmann			0.163
I+II	19	14	
III+IV	21	19	
Tumor difference			0.27
Well/moderate	19	17	
Poor	21	16	
Tumor invasion			0.206
T^1+T^2	26	20	
T^3+T^4	14	13	
Nodal status			0.787
N^-	19	16	
N^+	21	17	

Relationship between Peritoneal Metastasis and Methylation of Peritoneal Lavage Fluid

In the study, we observed that the promoter of *KiSS-1* gene was hypermethylated, at a ratio of 42.5%(17/40). Among the 19 cases, 9 cases had peritoneal metastasis. The accuracy of *KiSS-1* methylation in peritoneal lavage fluid for diagnosing peritoneal metastasis was 70%, with a sensitivity of 77.8%, a specificity of 67.7%, PPV 41.2%, and NPV 91.3% (Table 2).

Table 2. *sociation between KiSS-1 methylation in peritobeal lavage and peritoneal metastasis*

PLM	Peritoneal metastasis	
	+	—
+	7	10
—	2	21

PLM: methylation in peritoneal lavage

As shown in Table 3, the presence of *KiSS-1* methylation in peritoneal lavage fluid was significantly correlated with tumor invasion

(P=0.043). The results of the experiment also showed that *KiSS-1* methylation in peritoneal lavage fluid was found more frequently in tumor with lymph node metastasis than in tumor without lymph node metastasis.

Table 3. *Association between KiSS-1 methylation in peritoneal lavage fluid and clinicopathologica factors*

Variable	No.of patients	*KiSS-1* methylation in peritoneal lavage fluid	*P* value
Tumor invasion			0.043
T_1+T_2	28	9	
T_3+T_4	12	8	
Nodal status			0.184
N-	19	6	
N+	21	11	

DISCUSSION

Epigenetic alteration, conducted by the DNA methyltransferases (DNMTs) catalyzing the methylation of the 5 position of the cytosine ring using S-asenosylmethionine as the donor molecule for the methyl group, has been shown to play an important role in tumorigenesis and progression[3]. Methylation of promoter CpG islands leads to transcriptional silencing of their downstream genes. In various human cancers, silencing of tumor suppressor genes, such as *CDKN2A(p16), CDH1* (Ecadherin) and *MLH1,* is known to be one of the major mechanisms for their inactivation, along with mutations and LOH. *KiSS-1* is a metastasis-suppressing gene found in human melanoma cell lines in 1996 and peptides derived from its product are natural ligands of orphan protein-coupled receptor GPR54. Lots of researches have suggested that *KiSS-1* gene is associated closely with metastases of melanoma[10], breast cancer[11,12], thyroid cancer[13], esophageal cancer[14], gastric cancer[15] and pancreatic cancer[16]. The mechanisms of the decreased expression of *KiSS-1* appear to be multifactorial, and more studies need to be elucidated. To expore the novel methylation-silenced genes in gastric cancer, Yamashita *et al*[7] carried out a chemical genomic screening, a genome-wide search for genes up-regulated by treatment with a demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC). After 5-aza-dC treatment of a gastric cancer cell line (AGS) 579 genes were upregulated 16-fold or more, using an oligonucleotide microarray with 39000 genes. The *KiSS-1* was upregulated 54.8-fold, and its methylation was found in 80%(8/10) of primary gastric cancers. This result is approximates to our results.

In this study, we studied the association between *KiSS-1* methylation and primary tumor, corresponding paired adjacent normal mucosa and metastases lymph nodes in gastric cancer. The results of this experiment indicate that the methlytion in primary tumor (82.5%) and metastases lymph nodes (80.95%) was much higher than corresponding paired adjacent normal mucosa (55%), and there were significant differences between the former two and the latter, respectively. *KiSS-1* methylation was not correlated with the clinico- pathological characteristics.

Methylation alteration can be found not only in solid cancer tissues but also in various remote samples derived from patients with cancer, such as sputum for lung cancer[17,18], urine for urologic tumors[19], saliva for head and neck squamous cell carcinoma[20], breast fluid[21] and serum or plasma for almost all types of cancer[22-26]. More recent studies have been carried out to detect differences in various remote samples and demonstrated that DNA methylation could be act as a promising biomarker in early detection and prognosis[27]. In our study, the association between *KiSS-1* methylation in peritoneal lavage fluid and peritoneal metastasis was examined. We observed that the promoter of *KiSS-1* gene was hypermethylated at a ratio of 42.5% (17/40). The presence of *KiSS-1* methylation in peritoneal lavage fluid was significantly correlated with tumor invasion (P= 0.043). The reason may be that the incidence of free cancer cells fall off into peritoneal cavity increase according to the depth of tumor invasion. Among the 17 cases with promoter hypermethylation, 9 cases had peritoneal metastasis including cytologically positive peritoneal lavage. *KiSS-1* promoter hypermethylation in peritoneal lavage fluid showed a higher sensitivity (77.8%) for the diagnosis of peritoneal dissemination than cytological examination of peritoneal lavage fluid only. As known, the methylation alteration attractively acts as a biomarker for some reasons. Firstly, the methylation signal can be detected at low concentrations. Secondly, the methylation pattern and the underlying DNA are more stable than RNA[28]. However, methylation alteration in peritoneal lavage fluid showed a lower specificity for diagnosis of peritoneal dissemination. Possible explanations are as follows. Firstly, most of the cells in peritoneal lavage are peritoneal mesothelial cells which may cause false positive of the *KiSS-1* methylation. Secondly, the discrepancy of the methlytion profile exists sometimes between the peritoneal lavage samples and the cancer tissue. In order to solve these problems, a serial tests of *KiSS-1*

methylation and other examinations, for example, carcino-embryonic antigen in peritoneal lavage, could be used for diagnosing peritoneal dissemination to enhance specificity. The results of the experiment also show that *KiSS-1* methylation in peritoneal lavage fluid is more frequently found in tumor with lymph node metastasis than in tumor without lymph node metastasis, although the difference between them did not reach a statistical significance. These findings suggest that the *KiSS-1* methylation in peritoneal lavage fluid can be considered to be a biomarker for predicting peritoneal metastasis.

In summary, hypermethylation of *KiSS-1* promoter is a common event in the development and progress of human gastric cancer, it may provide useful information for the diagnosis and assessment of occult metastasis in lymphonode. *KiSS-1* methylation in peritoneal lavage fluid could act as a biomarker to evaluate peritoneal metastasis. However, more research should be carried out to further explore the mechanism of *KiSS-1* hypermethylation in gastric cancer, and the promoter hypermethylation of *KiSS-1* gene provides a new idea for prevention and treatment of gastric cancer.

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