Site-specific Methylation of SST Gene May Serve as a Biomarker for Risk Prediction of Gastrointestinal Tract Cancers as Well as Promoting Malignant Behavior by Regulating Gene Expression

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Abstract

Background: DNA methylation plays an important role in the regulation of gene expression as well as the occurrence and development of cancer. Abnormal hypermethylation in the promoter region of tumor suppressor genes is a recognized molecular events related to carcinogenesis. Somatostatin (SST) is considered as a tumor suppressor gene. The study aims to explore the relationship between the promoter methylation status of SST and the risk of gastrointestinal tract cancers and further to evaluate its diagnostic value.

Methods: Tissue samples were used for DNA isolation and RNA isolation. SST methylation was detected by biosulfite sequencing PCR, and SST mRNA expression was detected by quantitative real-time reverse transcription-polymerase chain reaction. The receiver operating curve (ROC) was drawn to evaluate the efficacy of SST CpG methylation on the diagnosis of digestive tract tumors by calculating the sensitivity, specificity and Youden index.

Results: The methylation level of SST promoter in cancer tissue were significantly increased compared with adjacent control tissue (EC: 0.649 ± 0.078 vs. 0.569 ± 0.107, p <0.05, GC: 0.622 ± 0.09 vs. 0.588 ± 0.079, p <0.05, CC : 0.663 ± 0.083 vs. 0.617 ± 0.042 p <0.05). Significantly different methylation sites contained CpG +18, +25, +44, +94, +100, +127, and +129. SST methylation status was associated with clinical phenotypes such as lymph node metastasis and vascular tumor thrombus. CpG sites in two tumors (+85 in EC, +94 in CC) were positively correlated with the depth of infiltration. The expression level of SST in gastric and colorectal cancer tissue was significantly lower than that in adjacent control tissue (both p <0.001). However, no statistically significant difference was found in EC group (p = 0.32). Except CpG +25 and +85 sites in GC and the +25, +85 and +148 sites in CRC, the methylation rate of each CpG was negatively correlated with gene expression (all P <0.05). Combined detection of eight CpG sites methylation (+18,+42,+44,+85,+92,+94,+129 and+138) showed the best area under the curve of 0.817 with sensitivity of 76.5% and specificity of 75%.

Conclusions: Abnormal hypermethylation in SST promoter region increased the risk of gastrointestinal tract tumors, as well as promoting cancer progression by regulating gene expression. SST methylation status may serve as site-specific biomarker for risk prediction of gastrointestinal tract tumors.

Introduction

The gastrointestinal tract (GIT) including esophagus, stomach, and colorectum belong to tubular digestive organs. The tissue structures of GIT are highly similar, and also there are many similarities in the process of cancerization. Finding common molecular events involved in GIT cancers is helpful to interpret the cancerous pathway from the original source in GIT and also is helpful to identify tag-biomarker belong to GIT cancers. As we know that multiple genes and factors such as genetic variation and epigenetic changes participate in the occurrence and development of GIT cancers (1). DNA methylation is one of the most common epigenetic modification, mainly occurring at the fifth carbon atom of CpG island cytosine residue in the promoter region. Methylation in the promoter region of tumor suppressor genes leads to gene silencing by inhibiting gene transcription, thereby promoting the occurrence and development of tumors, affecting the risk and prognosis of tumors (2). DNA methylation is a reversible process (3), which commonly takes place in the early stage of tumorigenesis with the potential to be an early diagnostic cancer biomarker. Currently, the exploration of DNA methylation markers has become a hot spot in the epigenetics research of gastrointestinal tract tumors.

Growth hormone release inhibitory hormone (somatostatin, SST) is a member of the cyclic peptide family that has a broad inhibitory effect on the secretion of hormones such as growth hormone, insulin and glucagon. SST is located in human chromosome 3q27.3, and its protein somatostatin has two active forms, 14aa and 28aa, which are produced by the alternating cleavage of a single preproprotein encoded by the gene. SST has the effects of blocking the production of autocrine / paracrine growth-promoting hormone and growth factors, inhibiting growth factor-mediated mitotic signals and inducing apoptosis (4). It also inhibits the secretion of somatotropin, growth factors and has anti-angiogenic effects (5). These effects of directly or indirectly inhibiting tumorigenesis and development make SST have the potential to be a biomarker for detecting tumor risk. Our previous research has reported that SST is a core gene with high methylation and low expression in GC (6) and CRC (7). In addition, bioinformatics analysis results showed that SST in EC also had significant hypermethylation (data not shown). However, what is the correlation between the SST promoter methylation status and the risk of GIT cancers including esophageal cancer, gastric cancer, and colorectal cancer, and whether it plays a regulatory role by affecting gene expression levels, all of which remain to be further studied.

In this study, SST methylation status including average methylation, site-specific methylation, as well as mRNA expression level were detected in esophageal, gastric and colorectal cancers, simultaneously. On the basis, the efficacy of SST promoter methylation for diagnosis of GIT cancers was further evaluated. This research aimed at exploring comprehensively analyzed the effect of abnormal methylation in the promoter region of SST gene on the occurrence and development of GIT cancers by detecting methylation status and gene expression and clarify its potential as biomarker for GIT tumor risk. It is expected to provide valuable clues for revealing the possible mechanism of gastrointestinal tumors from the perspective of epigenetics, and to provide applicability signs for early diagnosis.

Materials And Methods

1.1 Patients and Specimens

The patients were recruited from the First Hospital of China Medical University and Cancer Hospital of China Medical University who underwent surgical resection without preoperative physical or chemical therapy from January 2013 to May 2018. Among them, 50 EC patients comprised of 46 males and 4 females with average age of 60 years (40-73); 99 GC patients comprised of 60 males and 31 females with average age of 60 years (25-83); 80 CRC patients comprised of 50 males and 30 females with average age of 61 years (30- 79). All tissue samples utilized in this study were coded and collected from tissues that remained diagnosis.

Tissue specimens including cancer and adjacent normal tissues were excised and directly put into RNAlater solution (RNAlater™ Stabilization Solution, Thermo Fishe Scientifific, Waltham, MA, USA), and stored at -80℃ after 24h. Detailed clinical data were collected from the medical records of inpatients, including lymph node metastasis, vascular tumor thrombus, depth of infiltration and differentiation.

The current study was approved by the Human Ethics Review Committee of the First Hospital of China Medical University (Shenyang, China) and Cancer Hospital of China Medical University (Shenyang, China). Informed consent has been obtained from patients.

1.2 DNA extraction

SDS-phenol extraction method was used to organize tissue DNA. Took 0.1-0.2g of tissue, shredded and ground, added 400μl TE, 25μl 10% SDS and 10μl 20mg / ml PK enzyme and mixed well. After 55℃ water bath for 2h, phenol, chloroform and isoamyl alcohol mixture were used to remove protein. DNA was precipitated with NaAc and absolute ethanol and finally dissolved with TE solution.

1.3 Biosulfite Sequence Polymerase chain reaction (BSP)

SST promoter methylation was detected by BSP (Biosulfite Sequence Polymerase chain reaction). Sulfite modification of genomic DNA was performed using Zymo DNA Methylation-Gold kit (ZYMO). The Predict CpG islands tool in MethPrimer 2.0 (http://www.urogene.org/methprimer2) was employed to find the CpG island in SST promoter region and design nested PCR primers for methylated island. Outer primer sequences were forward 5′-GTGTAATTGAGTGTGTATGTGTGGGAG-3' and reverse 5-ACAACAACCAAAAACTTCTACAAAAACTAAC-3′. Nested primer sequences were forward 5′-AATGTGTATGTTTATAGTATTGAGTGA-3′ and reverse 5′-AACACAACCCAAAACCAA-3′. Subsequently, PCR was carried out in a reaction condition of 94 ° C for 10min, (94 ° C 20S, 55 ° C 30S, 72 ° C 30S) for 40 cycles, 72 ° C for 10min, and finally stored at 4 ° C. The nested PCR product was subjected to Sanger sequencing, and the sequencing primer was 5′-AGAGGGAGAYGGTTGAGA-3′. C and T signal values of CpG site were read respectively. The methylation rate of each site was calculated according to Meth% = C / (C + T) * 100%.

1.4 Total RNA extraction and reverse transcription

Total RNA was extracted with TRIzol Reagent (TaKaRa, Dalian, China) from collected tissue. Rice-sized mucosa was digested with Trizol, and protein was removed with chloroform. RNA was precipitated with isopropanol and washed with 75% ethanol. After ethanol was evaporated, RNA was dissolved with DEPC water and the Nano Drop was used to detect total RNA concentration. Oligo(dT) primers were used in the reverse-transcription step. The PrimeScript RT Master Mix (Takara) kit was used for reverse transcription.

1.5 Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Realtime-PCR method was used to detect the relative expression of SST mRNA (TB Green Premix Ex Taq). And GAPDH was used as an internal reference to normalize SST expression. Primer sequences were listed as follows: SST, forward, 5′- CTGAACCCAACCAGACGGAG-3′, reverse, 5′-GCCATAGCCGGGTTTGAGTT-3′; GAPDH, forward, 5′-CCATCTTCCAGGAGCGAGATCCCT-3′, reverse, 5′-CCTGCAAATGAGCCCCAGCC-3′. The reaction conditions were 95℃ 30s, (94℃ 30s, 56℃ 20s, 72℃ 10s) for 40 cycles, and store at 4℃. △Ct and 2-△Ct values were calculated to evaluated the relative expression of SST.

1.6 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 23 software and R 3.5.3. Paired T test was used to compare the difference in methylation and mRNA expression between cancer and control tissue. Spearman rank correlation was used to analyze the correlation between SST promoter methylation rate and its expression. Receiver operating curve (ROC) was adopted to evaluate diagnostic efficacy and calculate sensitivity (SEN), specificity (SPE) and Youden index (YD). P <0.05 was considered as statistical significance.

Results

1.1 SST methylation rate in GIT cancers

We detected the methylation rates of 15 CpG sites, which were located in +18, +25, +34, +42, +44, +85, +92, +94, +97, +100, +116, +127, +129, +138, +148. **(Figure 1)**

The SST methylation rates in EC, GC, and CC were shown in **Table 1, Figure 2**.

In 42 cases of EC, the average methylation rate in cancer tissue of EC was significantly higher than that in control tissue (p<0.05), including the +18,+34,+42,+44,+92,+94,+100,+116,+127,+129,+138 and +148 CpG sites (p <0.05).

In 99 cases of GC, the average methylation rate in cancer tissue was significantly higher than that in control tissue (p <0.05), including the +18, +42,+44, +94,+100, +127 and +129 CpG sites (all p <0.05).

In 70 cases of CC, the average methylation rate in cancer tissue was significantly

higher than that in control tissue (p <0.05), including the +18,+34,+42,+44, +92, +94, +97, +100, +116, +127, +129, +138 and +148 CpG sites (all p <0.05).

Joint analysis for the methylation difference of CpG sites in SST promoters for the three cancers showed that the methylation rates of CpG sites 7 sites including +18, +42, +44, +94, +100, +127, and +129 were meaningful significantly different from the adjacent control tissue. However, but the differences of CpG sites +25 and +85 were not statistically significant. The CpG sites of +34, +92 and +116 were significantly different in EC and CC, however, no significant difference was observed in GC. The CpG site +97 was significantly different in CC but not in EC and GC. The CpG sites +138 and +148 were significantly different in EC but not in the others.

Table 1 Methylation rates of CpG sites in GIT cancers

CpG site	Mean±Std						P Value		
	EC	EN	GC	GN	CRC	CRN	EC/EN	GC/GN	CRC/CRN
9	0.457±0.157	0.317±0.105	0.416±0.116	0.359±0.111	0.425±0.126	0.374±0.104	<0.001	<0.001	0.005
10	0.495±0.136	0.45±0.132	0.494±0.097	0.482±0.12	0.495±0.097	0.475±0.102	0.129	0.404	0.182
11	0.521±0.154	0.416±0.131	0.51±0.102	0.486±0.089	0.537±0.105	0.488±0.071	0.003	0.098	0.001
12	0.53±0.15	0.438±0.143	0.556±0.123	0.492±0.091	0.607±0.119	0.526±0.053	0.004	<0.001	<0.001
13	0.541±0.158	0.438±0.146	0.545±0.144	0.489±0.101	0.614±0.122	0.529±0.055	0.002	0.002	<0.001
14	0.483±0.121	0.469±0.117	0.479±0.122	0.474±0.091	0.518±0.101	0.52±0.073	0.565	0.734	0.9
15	0.72±0.098	0.668±0.117	0.678±0.11	0.669±0.085	0.74±0.099	0.708±0.067	0.032	0.551	0.019
16	0.664±0.125	0.57±0.136	0.62±0.132	0.576±0.099	0.698±0.116	0.62±0.081	0.002	0.009	<0.001
17	0.575±0.129	0.538±0.127	0.58±0.138	0.547±0.1	0.646±0.122	0.576±0.075	0.188	0.061	<0.001
18	0.624±0.111	0.568±0.121	0.602±0.132	0.568±0.106	0.691±0.111	0.623±0.067	0.017	0.041	<0.001
19	0.781±0.095	0.701±0.118	0.715±0.088	0.69±0.088	0.765±0.098	0.722±0.067	0.001	0.061	0.002
20	0.808±0.09	0.703±0.126	0.744±0.103	0.705±0.097	0.774±0.094	0.73±0.069	<0.001	0.009	0.001
21	0.827±0.091	0.708±0.131	0.765±0.104	0.711±0.105	0.799±0.095	0.753±0.063	<0.001	0.001	0.001
22	0.865±0.067	0.796±0.115	0.825±0.08	0.804±0.086	0.838±0.076	0.822±0.067	0.001	0.102	0.111
23	0.839±0.083	0.761±0.116	0.788±0.087	0.77±0.091	0.804±0.096	0.793±0.066	0.001	0.186	0.325
AVG	0.649±0.078	0.569±0.107	0.622±0.09	0.588±0.079	0.663±0.083	0.617±0.042	<0.001	0.008	<0.001

1.2 Correlation of methylation of SST and tumor biological behavior

We analyzed the methylation rate of SST promoter region in four tumor biological behaviors (Lymph node metastasis, Vascular tumor thrombus, Depth of infiltration and Differentiation). **(Table 2)**

In EC, two sites (+127, +129) were different in vascular tumor thrombus (0.861±0.071 vs. 0.794±0.090 p = 0.021, 0.878±0.068 vs. 0.813±0.093 p = 0.038). The methylation rates of the above two CpG sites in positive vascular cancer thrombus group was significantly higher than that in negative group. In the depth of infiltration, the muscular layer methylation rate at site +85 was the lowest, 0.422±0.105, serous layer was 0.507±0.117, and mucosa and submucosa were 0.534±0.172 (p = 0.041). At site +18, the methylation rate of poor differentiation was 0.616±0.137, and medium and high differentiation were 0.432±0.153 and 0.442±0.145 respectively (p = 0.014).

In GC, the methylation rate at site +25 was related to lymph node metastasis, and the positive group was significantly lower than the negative group (0.477±0.092 vs. 0.536±0.103 p = 0.013).

In CC, the methylation rate at position +94 was related to the depth of infiltration, and the serous layer was significantly higher than the muscular layer (0.718±0.098 vs. 0.649±0.129 p = 0.025). In this experiment, the infiltration depth of colorectal cancer did not have cases below the muscular layer.

1.3 SST mRNA expression in GIT cancers

The SST mRNA expressions in EC, GC, and CC were shown in **Figure 3.**

In 50 cases of EC, no statistically significant difference was found in SST mRNA expression between cancer and adjacent control tissue (0.0167 ± 0.0455 vs. 0.033 ± 0.1061, p = 0.32).

In 52 cases of GC, the expression of SST mRNA in cancer tissue was significantly lower than that in adjacent control tissue (0.0086 ± 0.0176 vs. 0.0318 ± 0.0404, p <0.001);

In 65 cases of CC, the expression of SST mRNA in cancer tissue was significantly lower than that in adjacent control tissue (0.0098 ± 0.0263 vs. 0.0819 ± 0.1372, p <0.001).

1.4 Correlation between SST promoter methylation and mRNA expression in GIT cancers

SST promoter methylation and SST mRNA expression had no significant correlation in EC group. In GC group, except CpG sites +25 and +85, the SST methylation and expression of other sites were negatively correlated (P <0.05). For CC group, except CpG sites of +25, +85 and +148, methylation rate was negatively correlated with expression (P <0.05). **(Table 3)**

Table 2 Correlation analysis of methylation of SST promoter region and tumor biological behavior of GIT cancers

Cancer	Parameter	Group	n	+18	+25	+34	+42	+44	+85	+92	+94	+97	+100	+116	+127
EC	Lymphatic metastasis	Positive	25	0.497	0.654	0.71	0.311	0.405	0.858	0.617	0.749	0.798	0.265	0.405	0.828
		Negetive	17												
	Vessel carcinoma embolus	Positive	9	0.718	0.976	0.786	0.587	0.414	0.249	0.526	0.651	0.303	0.171	0.303	**0.021**
		Negetive	33												
	Depth of infiltration	serosa	26	0.141	0.451	0.92	0.201	0.268	**0.041**	0.478	0.668	0.099	0.035	0.094	0.4
		muscular	13												
		mucosal and Submucosal	3												
	Differentiation	poorly	5	**0.014**	0.17	0.15	0.381	0.381	0.827	0.937	0.897	0.803	0.977	0.195	0.289
		moderately	21												
		high	16												
GC	Lymphatic metastasis	Positive	64	0.471	**0.013**	0.073	0.257	0.578	0.339	0.821	0.804	0.91	0.958	0.566	0.869
		Negetive	27												
	Vessel carcinoma embolus	Positive	57	0.961	0.993	0.085	0.608	0.426	0.348	0.724	0.931	0.879	0.583	0.688	0.737
		Negetive	34												
	Depth of infiltration	serosa	79	0.472	0.662	0.743	0.508	0.347	0.097	0.485	0.668	0.284	0.517	0.309	0.479
		muscular	9												
		mucosal and Submucosal	3												
	Differentiation	poorly	75	0.608	0.658	0.811	0.725	0.667	0.119	0.775	0.991	0.551	0.884	0.446	0.499
		moderately	12												
		high	4												
CRC	Lymphatic metastasis	Positive	38	0.658	0.56	0.939	0.81	0.923	0.758	0.721	0.973	0.758	0.651	0.21	0.272
		Negetive	42												
	Vessel carcinoma embolus	Positive	11	0.917	0.273	0.743	0.939	0.994	0.227	0.534	0.905	0.85	0.917	0.553	0.66
		Negetive	69												
	Depth of infiltration	serosa	59	0.581	0.891	0.267	0.184	0.102	0.9	0.13	**0.025**	0.184	0.083	0.119	0.058
		muscular	21												
		mucosal and Submucosal	0												
	Differentiation	poorly	25	0.76	0.36	0.53	0.289	0.151	0.519	0.24	0.081	0.351	0.271	0.426	0.139
		moderately	40												
		high	15												

Table 3 Correlation between SST promoter methylation and SST mRNA expression in GIT cancers

CpG site	r			P		
	EC	GC	CC	EC	GC	CC
+18	-0.063	-0.363	-0.23	0.57	< 0.001	0.008
+25	-0.13	-0.154	-0.17	0.239	0.118	0.053
+34	-0.071	-0.271	-0.23	0.518	0.005	0.008
+42	0.005	-0.393	-0.379	0.966	< 0.001	< 0.001
+44	0.026	-0.371	-0.396	0.816	< 0.001	< 0.001
+85	0.025	-0.107	0.083	0.818	0.28	0.346
+92	-0.001	-0.226	-0.193	0.989	0.021	0.028
+94	-0.003	-0.355	-0.406	0.975	< 0.001	< 0.001
+97	0.083	-0.252	-0.36	0.453	0.01	< 0.001
+100	0.048	-0.211	-0.361	0.663	0.031	< 0.001
+116	-0.089	-0.252	-0.273	0.419	0.01	0.002
+127	-0.034	-0.255	-0.272	0.758	0.009	0.002
+129	-0.047	-0.334	-0.306	0.668	0.001	<0.001
+138	0.074	-0.292	-0.179	0.501	0.003	0.041
+148	0.045	-0.212	-0.129	0.687	0.031	0.144
AVG	-0.026	-0.35	-0.374	0.813	< 0.001	< 0.001

1.5 The efficacy of SST promoter methylation for diagnosis of GIT cancers

Multivariate logistic regression was used to build appropriate diagnostic models. The diagnostic efficiency was assessed with sensitivity, specificity, Yorden Index and AUC of ROC. The combination of 8 CpG sites (+18, +42,+44,+85,+92,+94,+129,+138) had the largest area under the curve (AUC) of 0.817 with a sensitivity of 76.5% and a specificity of 75.0% GIT cancers. For GIT cancers, upper gastrointestinal tract, gastrointestinal, EC, GC, and CC models were established with AUC above 0.8. Co-differential sites that were significantly negatively correlated with expression in the first model were selected for modeling. In EC, the combination of 2 CpG sites (+18, +129) had the best diagnostic efficiency of AUC at 0.818 with a sensitivity of 80.0% and a specificity of 72.3%. (Table 4, Figure 4)

Table 4 Diagnostic value of combinations of CpG sites

CpG sites	Tumor	AUC	Sen	Spe	YD	Cut off
+18,+42,+44,+85,+92, +94,+129,+138	Gastrointestinal tract tumor	0.817	0.765	0.75	0.515	50%
+18,+92,+94,+129,+138	Upper digestive tract tumor	0.816	0.681	0.849	0.53	57.20%
+42,+44,+85,+92,+94, +116,+129,+138	Gastrointestinal tumor	0.819	0.685	0.865	0.55	58.10%
+18,+25,+97,+129	Esophageal cancer	0.868	0.778	0.83	0.608	50.20%
+18,+92,+129,+148	Gastric cancer	0.812	0.657	0.838	0.495	57.40%
+44, +92, +94	Colorectal cancer	0.813	0.756	0.779	0.535	50.10%
+18, +129	Gastrointestinal tract tumor	0.713	0.65	0.681	0.331	49.70%
+18, +129	Upper digestive tract tumor	0.723	0.667	0.671	0.338	48.10%
+42	Gastrointestinal tumor	0.712	0.492	0.892	0.384	56.60%
+18, +129	Esophageal cancer	0.818	0.8	0.723	0.523	39.90%
+42, +129	Gastric cancer	0.682	0.576	0.727	0.303	52.80%
+44, +94	Colorectal cancer	0.796	0.732	0.814	0.546	49.40%

Discussion

The decrease of tumor suppressor gene expression is an important event of carcinogenesis process. Hypermethylation of DNA is related to promoter activity and can be secondary to trigger gene (tumor suppressor gene) silencing. Differences in gene expression caused by the methylation status of transcription factor binding sites in promoter region are important molecular mechanism for regulation of tumorigenesis and development (8). In this study, EC, GC, and CC tissue samples were used to detect the methylation status of 15 CpG sites in the SST promoter region in GIT cancers. We found that the hypermethylation status of SST promoter region was positively correlated with the risk of GIT cancers EC,GC and CC. The combination of 8 CpG sites (+18,+42,+44,+85,+92,+94,+129,+138) had the largest AUC of 0.817 with a SEN of 76.5% and a SPE of 75.0% for the three cancers, suggesting that SST methylation status may serve as a site-specific biomarker for risk prediction of GIT cancers. In addition, several common significant hypermethylation sites including CpG +85, +97, and +100 were significantly positively correlated with the depth of tumor invasion. Hypermethylation of SST promoter in GIT cancers was significantly negatively correlated with mRNA expression level, suggesting that SST abnormal hypermethylation can affect the occurrence and development of GIT cancers by inhibiting gene transcription. In addition, the combination of 8 CpG sites (+18,+42,+44,+85,+92,+94,+129,+138) had the largest AUC of 0.817 with a SEN of 76.5% and a SPE of 75.0% for the three cancers. This study provided applicability markers for the combined diagnosis of GIT cancers and also valuable clues for analyzing the possible mechanism of GIT cancers from an epigenetic perspective.

Previous studies have reported that abnormal methylation of APC and CDH1 genes is associated with gastric cancer and colorectal cancer risk (9). CDH4 gene exhibits hypermethylation in 78% colorectal cancer and 95% gastric cancer tissue (10). RUNX3 hypermethylation is significantly linked to the risk of esophageal cancer (11), gastric cancer (12)and colorectal cancer(13) . In the present study, we quantitatively detected the methylation rates of SST promoter region in GIT cancers and the results showed that the methylation rate of SST promoter region in EC.GC and CC were significantly higher than that of adjacent control tissue, with some common differential CpG sites. On the basis, we conducted a conjoint analysis for the efficacy of SST methylation-specific CpG sites in the diagnosis of GIT cancers. The results showed that multi-site combined diagnosis of esophageal and colorectal cancer had better SEN and SPE (83.3%, 69.0%, 63.1%, and 89.2%), CpG +42 has a higher SPE (91.2%) for gastric cancer, and SEN 50% and SET 87.9% for the combined diagnosis of triple cancer, suggesting that SST abnormal methylation has a potential role of diagnosing GIT cancers. Recent studies have suggested that abnormally high methylation of DNA promoters can be used as diagnostic biomarkers for lung cancer (14), colorectal cancer (15), pancreatic cancer(16) and gastric cancer (17) , playing an important role in the screening and diagnosis of digestive tumors (18) (19) (20). This study identified a new methylation marker SST that was significantly related to the risk of digestive tract GIT cancers. Future research can be used as a basis to analyze the role of methylation regulation and explore the value of clinical application.

Hypermethylation of promoter is one of the earliest somatic genome changes that occur during the development of various cancers (21).The abnormal methylation status at specific sites is more closely related to malignant biological behavior of tumors. This study found that three CpG sites of esophageal cancer increased with T stage. The methylation status of different CpG sites was related to the depth of vascular tumor thrombus and invasion. The methylation status of multiple CpG sites in gastric cancer was related to clinicopathological parameters of gender, infiltration depth, lymph node metastasis and Lauren classification. Colorectal cancer CpG sites methylation status was significantly related to age, gender, T stage, growth pattern, infiltration depth and other parameters. The +85, +97 and +100 CpG sites are the common sites related to the depth of gastrointestinal tumor invasion. The +85 CpG site methylation was related to multiple clinicopathological parameters while had no significant difference between cancer and normal control tissue, suggesting the limited role of +85 site in tumorigenesis but the significant role in tumor development. Previous studies have shown that although suppressor genes often show a high methylation status in the promoter region in tumors, not every CpG sites have abnormally high methylation status. Some sites even show low methylation status. Abnormal hypermethylation mainly affects cell biological function and DNA expression by inhibiting the binding of transcription factors to the promoter region, thereby promoting tumorigenesis. Zou B et al. found in the study of gastric cancer and colorectal cancer that the silencing of XAF1 gene 3

the hypermethylation CpG sites. They play a major role in transcription regulation (22). Ceccarelli V et al. confirmed that C/EBPδ was the specific tumor suppressor gene in leukemia. C/ EBPδ gene re-expression is regulated by a single CpG site demethylation. An in-depth functional study of the above SST-specific CpG sites is expected to analyze the molecular mechanism of SST abnormal methylation involved in the development of gastrointestinal tumors.

At present, among the studies about SST expression in gastrointestinal tract tumors, Jackson K et al. found that 93% gastric cancer samples had down-regulated SST transcription(19). Leiszter K et al. pointed out that the expression level of SST in colorectal cancer tissue decreased significantly compared with normal control tissue. The expression of SST in esophagus has not been reported. SST is mainly secreted by D cells in brain and gastric antrum, and is expressed throughout the body, with higher levels expressed in stomach, intestine and brain tissue. In this study, the expression levels of SST in the three cancers showed different levels of down-regulation, among which gastric cancer and colorectal cancer were significantly down-regulated, while the difference in esophageal cancer tissue did not have statistical significance (P = 0.32). We speculated that it might be due to the low expression level of SST in esophageal tissue. Therefore, future research on SST and esophageal cancer risk needs to be further studied. As a neurotransmitter, SST is an inhibitor of hormone and growth factor secretion, which can inhibit the release of gastric acid, pepsin, gastrin, etc., reduce gastrointestinal secretion and inhibit the growth of digestive tract cells. It also inhibits growth hormone, thyroid stimulating hormone and the secretion of insulin and glucagon. SST maintains the stability of the above hormone levels in the body. Recent studies have found that SST can also be used as an immunomodulator to inhibit cell proliferation under specific conditions (4). SST blocks the production of autocrine / paracrine growth-promoting hormones and growth factors. On the one hand, it can inhibit cell proliferation by inhibiting growth factor-mediated mitotic signals and inducing apoptosis (23). On the other hand, it can also inhibit the production and growth of tumor cells by the effect of inhibiting the secretion of growth hormone and growth factor and anti-angiogenesis indirectly (24). Therefore, SST production is suppressed and expression is reduced, causing abnormal increase in secretion of growth hormone and growth factor. Then the secretion of digest tract hormone increases and promotes the growth, mitosis and proliferation of cells in the digestive tract. Thus SST has an inhibitory effect on the occurrence and development of gastrointestinal tract tumors. The results of this study confirmed that the down-regulation of SST expression was an important factor in the development of gastrointestinal tumors. It remains to be clarified whether it is related to the risk of esophageal cancer.

The correlation analysis between the methylation status of SST promoter region and mRNA expression showed that multiple CpG sites and average methylation rate in gastric cancer and colorectal cancer were significantly negatively correlated with SST expression. Usually, the CpG site of gene promoter region is methylated, and the addition of a new methyl group to the cytosine 5 carbon atom causes the chromatin conformation of this region to change, which in turn blocks the interaction of transcription factors with this region, inhibiting gene transcription, reducing protein expression and affecting the normal biological function of cells (25).It is an important epigenetic regulatory factor for tumorigenesis and development. We took advantage of bioinformatics to predict and analyze that the common differences methylated CpG sites +42 and +44 of three tumors were the binding sites of transcription factors RHOXF1, ETS1, GSC, GSC2, DPRX, OTX1 and OTX2. The CpG sites +127 and +129 were the binding sites of transcription factors EBF1 and NR2C2.We speculated that during the malignant transformation of digestive tract epithelial cells, the abnormally high methylation status of the specific CpG site in the promoter region of SST gene may inhibit the transcription of SST gene and reduce the expression of mRNA by inhibiting the binding of a transcription factor to the transcription binding site, thereby promoting the occurrence and development of tumors. The above hypothesis needs to be confirmed by in-depth molecular biology experiments.

In summary, our study suggested that the abnormal hypermethylation in SST promoter region increased the risk of GIT cancers. gastrointestinal tract tumors. It may promote the cancergenesis and cancer progression by inhibiting gene transcription.The site-specific methylation status in SST may serve as a potential biomarker for risk prediction of GIT cancers. gastrointestinal tract tumors. The abnormal methylation status at specific sites is more closely related to malignant biological behavior of GIT cancers.

Abbreviations

SST somatostatin

ROC The receiver operating curve

CpG cytosines followed by guanine residues

EC esophageal cancer

GC gastric cancer

CC colorectal cancer

GIT gastrointestinal tract

TE Tris-EDTA buffer solution

BSP Biosulfite Sequence Polymerase chain reaction

SEN sensitivity

SPE specificity

YD Youden index

Declarations

Ethics approval and consent to participate: The study was approved by the Ethics Review Committee of China Medical University (Shenyang, China).

Consent for publication: Not applicable

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare no conflicts of interest.

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Authors' contributions: DXT and SLP designed the study; DXT, SLP, LZ, and YZ performed the experiments and prepared the figures; SLP, YY, and LZ contributed to drafting the manuscript. All authors read and approved the final manuscript.

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Figures

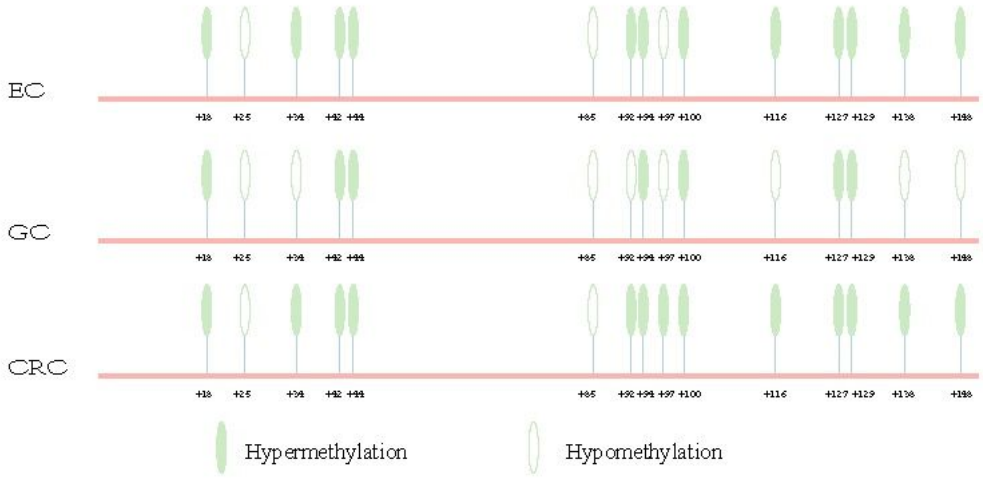

Figure 1

CpG sites of SST in gastrointestinal tract cancers.

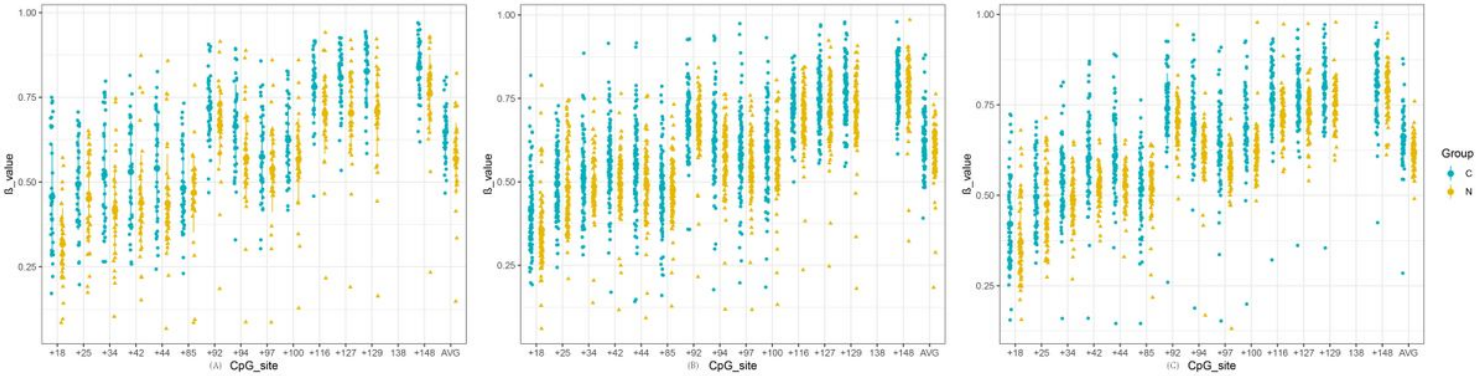

Figure 2

Methylation status of CpG sites in gastrointestinal tract cancers. (A) EC vs. Control (B) GC vs. Control (C) CC vs. Control.

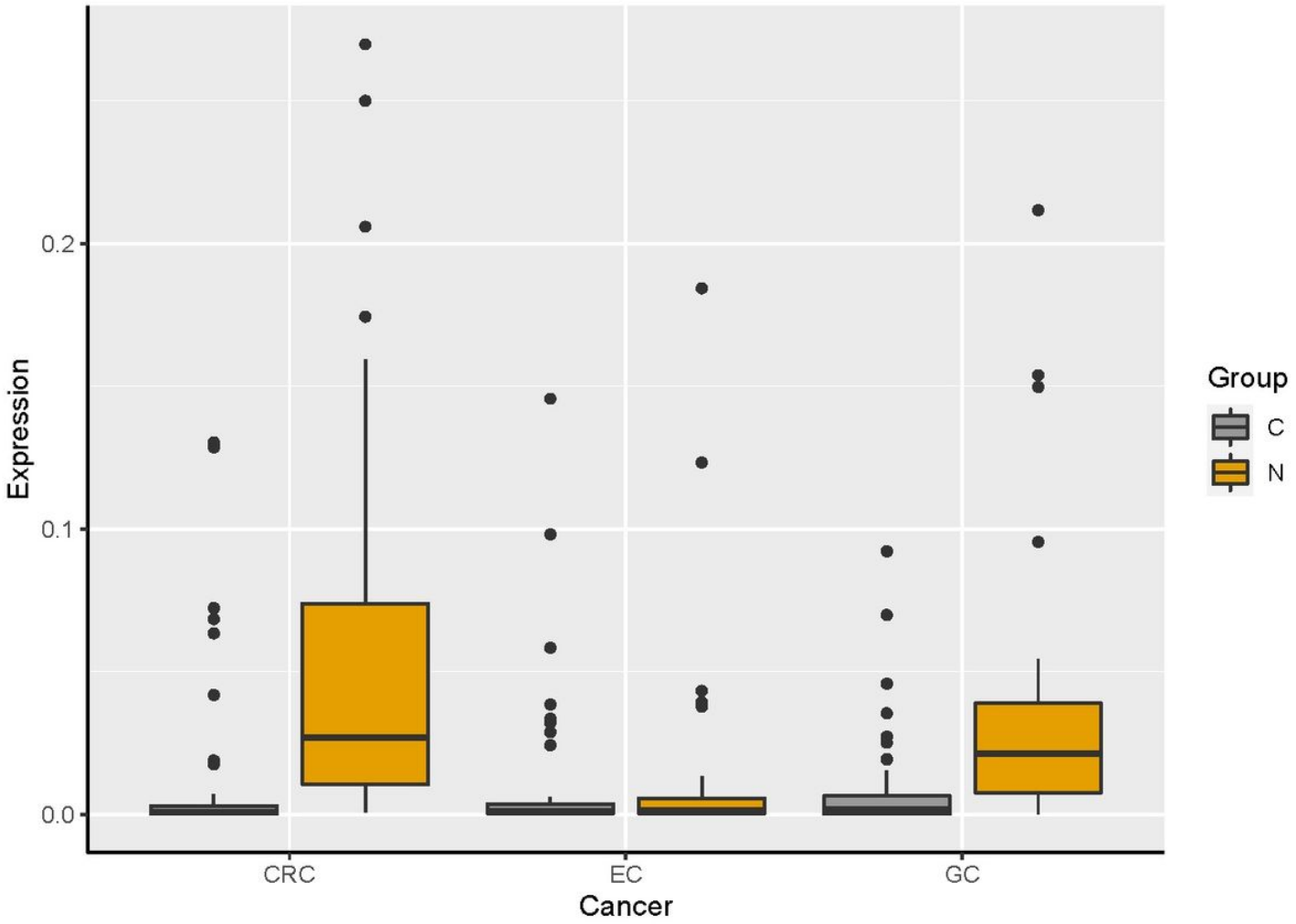

Figure 3

Difference analysis of SST mRNA expression in GIT cancers.

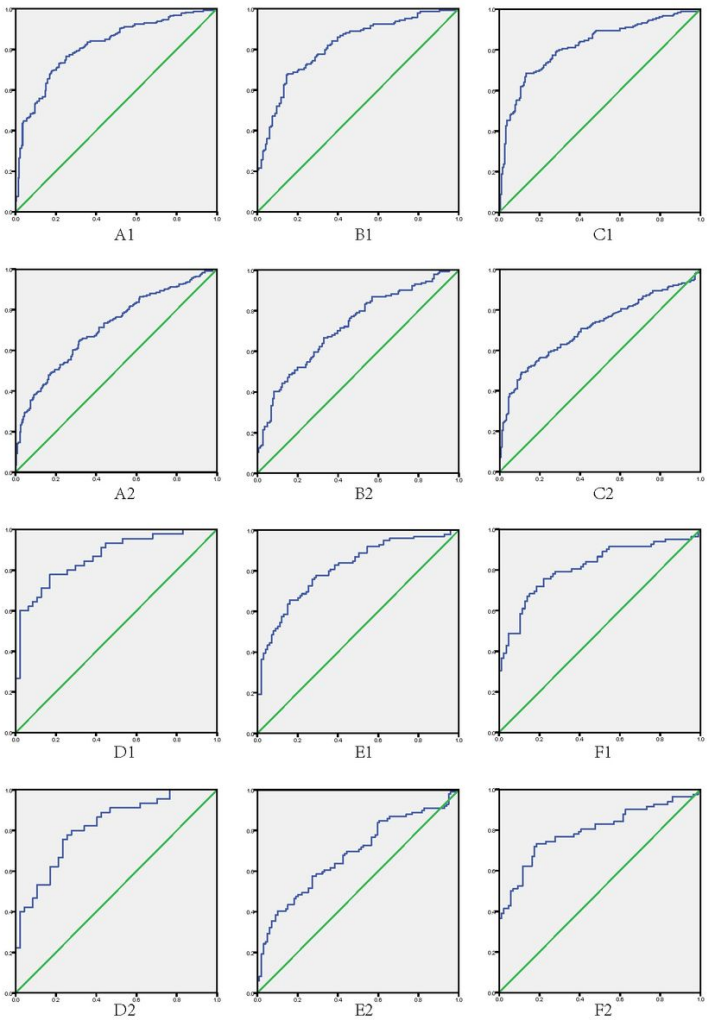

Figure 4

ROC of diagnostic model for GIT cancers. (A1): ROC of +18,+42,+44,+85,+92,+94,+129,+138 CpG sites for Gastrointestinal tract tumor; (A2): ROC of +18, +129 CpG sites for Gastrointestinal tract tumor; (B1): ROC of +18,+92,+94,+129,+138 CpG sites for Upper digestive tract tumor; (B2): ROC of +18, +129 CpG sites for Upper digestive tract tumor; (C1): ROC of +42,+44,+85,+92,+94,+116,+129,+138 CpG sites for Gastrointestinal tumor; (C2): ROC of +42 CpG site for Gastrointestinal tumor; (D1): ROC of +18,+25,+97,+129 CpG sites for Esophageal cancer; (D2): ROC of +18, +129 CpG sites for Esophageal cancer; (E1): ROC of +18,+92,+129,+148 CpG sites for Gastric cancer; (E2): ROC of +42, +129 CpG sites for Gastric cancer; (F1): ROC of +44, +92, +94CpG sites for Colorectal cancer; (F2): ROC of +44,+94 CpG sites for Colorectal cancer.