Hypermethylation of the Somatostatin Promoter Is a Common, Early Event in Human Esophageal Carcinogenesis

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BACKGROUND. The promoter of somatostatin (*SST*), a primary inhibitor of gastrin-stimulated gastric acid secretion, is hypermethylated in 80% of human colon cancers. The aim of the current study was to investigate whether and at what stage promoter hypermethylation of *SST* is involved in human esophageal carcinogenesis.

METHODS. *SST* promoter hypermethylation was examined by real-time methylation-specific polymerase chain reaction (PCR) (MSP) in 260 human esophageal tissue specimens. Real-time reverse-transcriptase PCR and MSP were also performed on esophageal cancer cell lines before and after treatment with 5-aza-2′-deoxycytidine (5-Aza-dC).

RESULTS. *SST* hypermethylation showed highly discriminative receiver-operator characteristic curve profiles, clearly distinguishing esophageal squamous cell carcinomas (ESCC) and esophageal adenocarcinomas (EAC) from normal esophagus (NE) ($P < .01$). Both *SST* methylation frequency and normalized methylation value (NMV) were significantly higher in Barrett metaplasia without dysplasia or EAC (BE), low-grade and high-grade (HGD) dysplasia occurring in BE, EAC, and ESCC than in NE ($P < .01$). *SST* hypermethylation frequency was significantly lower in NE (9%) than in BE (70%), HGD (71.4%), or EAC (71.6%), whereas 14 (53.8%) of 26 ESCCs exhibited *SST* hypermethylation. There was a significant relation between *SST* hypermethylation and BE segment length, a known clinical risk factor for neoplastic progression. Demethylation of KYSE220 ESCC and OE33 EAC cells with 5-Aza-dC reduced *SST* methylation and increased *SST* mRNA expression. *SST* mRNA levels in native unmethylated EACs were significantly higher than in native methylated EACs ($P < .05$).

CONCLUSIONS. *SST* promoter hypermethylation is a common event in human esophageal carcinomas and is related to early neoplastic progression in Barrett esophagus. ***Cancer* 2008;112:43–9.** *© 2007 American Cancer Society.*

KEYWORDS: somatostatin, hypermethylation, esophageal adenocarcinoma, esophageal squamous cell carcinoma, Barrett esophagus.

Esophageal cancer ranks sixth among frequent cancers worldwide, with 400,000 new cases diagnosed per year.[1] This malignancy exists in 2 main forms, each possessing distinct pathological characteristics: esophageal squamous cell carcinoma (ESCC), which occurs at high frequencies in many developing countries, especially in Asia; and esophageal adenocarcinoma (EAC), which is more prevalent in Western countries, with a rapid recent rate of increase in incidence.[1] Although significant advances have been made in treating esophageal cancers, these aggressive malignancies usually present as locally advanced disease with a very poor prognosis

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(approximately 14% 5-year survival).[2] Thus, it is important to discover novel early detection biomarkers and targets suitable for chemoprevention or therapy.

Somatostatin (SST) was first identified as a growth hormone release-inhibitory factor in ovine hypothalamus in 1973.[3] Since then, it has been shown that SST is not only present in the hypothalamus, but also produced in a variety of other endocrine and nonendocrine tissues. In the gastrointestinal tract, SST regulates endocrine and exocrine secretion, modulates motor activity, and is the primary inhibitor of gastrin-stimulated gastric acid secretion.[4] In recent years, several studies have suggested that SST functions as a tumor suppressor gene and possesses potent antitumor and antisecretory activity in several human cancers in vitro and in vivo.[5–8] Aberrant methylation of promoter CpG islands upstream of tumor suppressor genes is now well established as a major epigenetic mechanism of gene inactivation in tumorigenesis,[9] including ESCC and EAC.[10,11] More recently, data from our laboratory have shown that the *SST* promoter is methylated in 80% of human colon cancers, and that 5-aza-2′-deoxycytidine (5-Aza-dC) reverses *SST* promoter hypermethylation and restores *SST* mRNA expression in colon cancer cell lines.[12] Therefore, we hypothesized that *SST* might be inactivated via promoter hypermethylation in human esophageal cancers, and that hypermethylation of *SST* could function as an early event in the genesis of EAC.

To test this hypothesis, we studied methylation of the *SST* gene promoter by real-time quantitative methylation-specific polymerase chain reaction (PCR) (qMSP) in 260 endoscopic esophageal biopsy specimens of differing histologies. In addition, the effect of a DNA methyltransferase inhibitor, 5-Aza-dC, on the reexpression of epigenetically silenced *SST* was studied in esophageal cancer cell lines. Our results revealed that *SST* promoter hypermethylation is a highly prevalent, early event in human esophageal carcinogenesis and is associated with other risks of neoplastic progression in Barrett esophagus.

MATERIALS AND METHODS

Tissue Samples

In the current study, 67 normal esophagi (NE), 60 Barrett metaplasias without dysplasia (BE, including 36 obtained from patients with Barrett only [Ba] and 24 BE obtained from patients with Barrett accompanied by EAC [Bt]), 19 low-grade dysplasia (LGD) and 21 high-grade dysplasia (HGD) cases occurring in BE, 67 EACs, and 26 ESCCs were examined. All patients provided written informed consent under a protocol approved by the Institutional Review Boards at the University of Maryland and Baltimore Veterans Affairs Medical Centers, where all esophagogastroduodenoscopies were performed. Biopsies were taken using a standardized biopsy protocol as previously described.[11] Research tissues were obtained from macroscopically apparent Barrett epithelium or from mass lesions in patients manifesting these changes at endoscopic examination, and histology was confirmed using parallel aliquots obtained at endoscopy. All biopsy specimens were stored in liquid nitrogen before DNA/RNA extraction. Clinicopathologic characteristics are summarized in Table 1.

Cell Lines

Three EAC (BIC, OE33, and SEG) and 9 ESCC (KYSE 110, 140, 180, 200, 220, 410, 450, 520, and 850) cell lines were obtained from collaborators at the University of Michigan (Dr. David Beer) and Kyoto University (Prof. Yutaka Shimada). These cells were cultured in 47.5% RPMI 1640, and 47.5% F-12 supplemented with 5% fetal bovine serum.

DNA and RNA Extraction

Genomic DNA was extracted from biopsies and cultured cells using a DNeasy Tissue Kit (Qiagen, Valencia, Calif). Total RNA was isolated from biopsies and cultured cells using TRIzol reagent (Invitrogen, Carlsbad, Calif). DNAs and RNAs were stored at −80°C before analysis.

Bisulfite Treatment and Real-Time Quantitative Methylation-specific PCR

DNA was treated with bisulfite to convert unmethylated cytosines to uracils before qMSP, as described previously.[12] Promoter methylation levels of *SST* were determined with the ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif), using primers and probes as described previously.[12] A standard curve was generated using serial dilutions of CpGenome Universal Methylated DNA (Chemicon, Temecula, Calif). The normalized methylation value (NMV) was defined as follows: NMV = (*SST-S*/*SST-FM*)/(*ACTB-S*/*ACTB-FM*), in which *SST-S* and *SST-FM* represent the methylation levels of *SST* in sample and universal methylated DNAs, respectively, whereas *ACTB-S* and *ACTB-FM* correspond to β-actin in sample and universal methylated DNAs, respectively.

Real-Time Quantitative Reverse-Transcriptase-PCR

To determine *SST* mRNA levels, 1-step real-time quantitative reverse-transcriptase PCR (RT-PCR) was performed using a Qiagen (Hilden, Germany) QuantiTect Probe RT-PCR Kit and the ABI 7700 Sequence

TABLE 1
Clinicopathologic Characteristics and Methylation Status of Somatostatin in Human Esophageal Tissues

			NMV		Methylation status (Cutoff: 0.1)			
Clinical characteristics	No. of samples	Mean Age, Years	Mean	P*	Frequency	UM	M	P[†]
Barrett segment								
Short segment (<3 cm)	14	62.3	0.1063	<.05	28.6%	10	4	<.05[‡]
Long segment (≥3 cm)	16	62.8	0.3753		93.8%	1	15	
Histology								
Normal esophagus	67	64.4	0.0509		9.0%	61	6	
Barrett metaplasia	60	63.7	0.2564	<.05[§]	70.0%	18	42	
Barrett from non-EAC patients	36	62.5	0.2431	<.05[§]	61.1%	14	22	>.05[‡]
Barrett from EAC patients	24	65.5	0.2763	<.05[§]	83.3%	4	20	
Dysplasia in Barrett esophagus	40	65.3	0.2076	<.05[§]	67.5%	13	27	
Low-grade dysplasia	19	65.3	0.18	<.05[§]	63.2%	7	12	>.05[‖]
High-grade dysplasia	21	65.2	0.2325	<.05[§]	71.4%	6	15	
Esophageal adenocarcinoma	67	65.1	0.236	<.05[§]	71.6%	19	48	
Well differentiated	10	66.2	0.3271	<.05[§]	90.0%	1	9	>.05[‡]
Moderately differentiated	24	66.1	0.2441	<.05[§]	70.8%	7	17	
Poorly differentiated	22	65.5	0.2055	<.05[§]	68.2%	7	15	
Unknown	11	61	0.1963	<.05[§]	63.6%	4	7	
Esophageal squamous cell carcinoma	26	62.5	0.1948	<.05[§]	53.8%	12	14	
Well differentiated	3	61.7	0.1586	<.05[§]	66.7%	1	2	>.05[‡]
Moderately differentiated	11	62.7	0.2077	<.05[§]	45.5%	6	5	
Poorly differentiated	5	64.2	0.2625	<.05[§]	60.0%	2	3	
Unknown	7	61.1	0.1418	<.05[§]	57.1%	3	4	
Stage of EAC patients								
I	7	63	0.219		71.4%	2	5	>.05[‡]
II	15	65.2	0.2343		60.0%	6	9	
III	25	64.6	0.2388		72.0%	7	18	
IV	7	66.3	0.319		100%	0	7	
Lymph node metastasis in EAC patients								
Negative	25	64.9	0.2413	>.05	68.0%	8	17	>.05[‖]
Positive	25	64.6	0.2438		72.0%	7	18	
Smoking status of EAC patients								
Never	6	58.5	0.3063		83.3%	1	5	>.05[‡]
Former	24	68.5	0.2346		75.0%	6	18	
Current	13	60.8	0.2352		69.2%	4	9	
Alcohol consumption of EAC patients								
Never	16	65.3	0.2161		75.0%	4	12	>.05[‡]
Former	15	63	0.2966		80.0%	3	12	
Current	10	65.7	0.2242		70.0%	3	7	

NMV indicates normalized methylation value; UM, unmethylated; M, methylated; EAC, esophageal adenocarcinoma.
* Student *t* test.
† Excludes cases with unknown status.
‡ Fisher exact test.
§ Comparisons made with normal esophagus.
‖ Chi-square for independence test.

Detection System (Applied Biosystems). Primers and probes were the same as previously reported.[12] β-actin was used for normalization of data. A standard curve was generated using serial dilutions of qPCR Reference Total RNA (Clontech, Mountain View, Calif). The normalized mRNA value (NRV) was calculated according to the following formula for relative expression of target mRNA: NRV = *(TarS/TarC)/(ACTB-S/ACTB-C)*, where *TarS* and *TarC* represent levels of mRNA expression for the target gene in sample and control mRNAs, respectively, whereas *ACTB-S* and *ACTB-C* correspond to amplified β-actin levels in sample and control mRNAs, respectively.

5-Aza-dC Treatment of Esophageal Cancer Cell Lines

To determine whether *SST* inactivation was due to promoter hypermethylation in esophageal cancer, 2 esophageal cancer cell lines (KYSE220 and OE33)

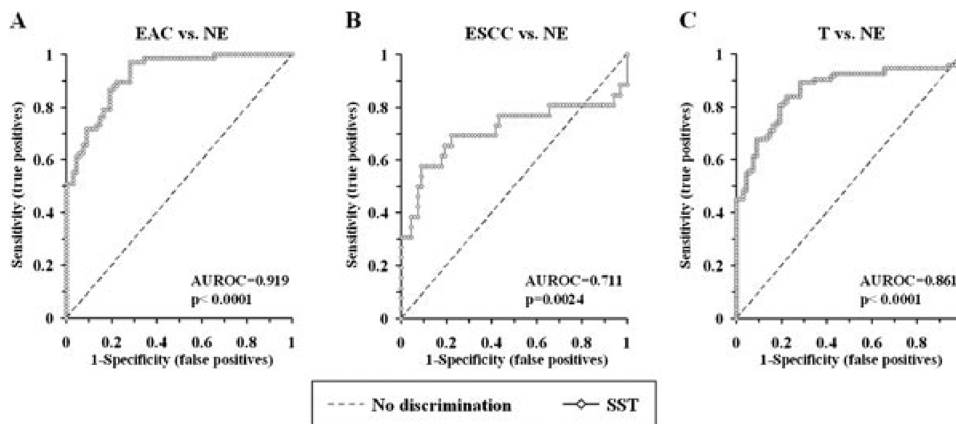

FIGURE 1. Receiver operator characteristic (ROC) curve analysis of normalized methylation values (NMVs). (A) ROC curve analysis of *SST* NMVs for normal esophagus (NE) versus esophageal adenocarcinoma (EAC). (B) NE versus esophageal squamous cell carcinoma (ESCC). (C) NE versus both types of esophageal carcinomas (T). The area under the ROC curve (AUROC) for the *SST* gene conveys the accuracy of this gene in distinguishing NE from EAC, ESCC, and T in terms of sensitivity and specificity.

were subjected to 5-Aza-dC (Sigma, St. Louis, Mo) treatment as previously described.[13,14] Briefly, 1×10^5 cells/mL were seeded onto a 100-mm dish and grown for 24 hours. Then, 1 μL of 5 mM 5-Aza-dC per ml of cells was added every 24 hours for 6 days. DNA and RNA were harvested on day 6.

Data Analysis and Statistics

Receiver-operator characteristic (ROC) curve analysis[15] was performed using NMVs for the 67 EAC, 26 ESCC, and 67 NE by Analyze-it software (Leeds, UK, v. 1.71). Using this approach the area under the ROC curve (AUROC) yielded optimal sensitivity and specificity to distinguish normal from malignant esophageal tissues, and corresponding NMV thresholds were calculated for *SST*. The cutoff value determined from this ROC curve was applied to determine the frequency of *SST* methylation in each tissue type included in the present study. For all other tests, Statistica software (version 6.1; StatSoft, Tulsa, Okla) was used. Differences with $P < .05$ were deemed statistically significant.

RESULTS

SST Promoter Hypermethylation in Different Esophageal Tissues

Promoter hypermethylation of the *SST* gene was analyzed in 67 NE, 60 BE (36 Ba and 24 Bt), 19 LGD, 21 HGD, 67 EAC, and 26 ESCC. *SST* promoter hypermethylation showed highly discriminative ROC curve profiles, which clearly distinguished both ESCC ($P = .0024$) and EAC ($P < .0001$) from NE. ROC curves with corresponding AUROCs for *SST* of ESCC versus NE, EAC versus NE, and combined esophageal cancer (T) versus NE are shown in Figure 1.

The cutoff NMV for *SST* (0.1) was chosen from the ROC curves to maximize sensitivity and specificity. Mean NMV and frequency of *SST* hypermethylation for each tissue type are shown in Table 1. Fourteen (53.8%) of 26 ESCCs exhibited hypermethylation of *SST*. The NMV of *SST* was significantly higher in ESCC, EAC, HGD, LGD, and BE than in NE ($P < .0000001$, Student t test). Incremental increases in the frequency of *SST* hypermethylation were observed during progression from NE (9%) to BE (70%), HGD (71.4%), and EAC (71.6%), whereas LGD (63.2%) demonstrated a slightly lower frequency of *SST* hypermethylation than did BE or HGD. Both *SST* hypermethylation frequency and mean NMV were higher in Bt than in Ba (83.3% vs 61.1% and 0.2763 vs 0.2431, respectively), with the difference in frequency approaching statistical significance ($P = .058$, Fisher exact test). Among 15 cases with corresponding NE, BE, and EAC, 1 (No. 2) was unmethylated, 1 (No. 13) was methylated only in EAC, 2 (Nos. 14, 16) were methylated only in BE, and the remaining 11 were methylated in both BE and EAC (Fig. 2A). Among 41 cases with corresponding NE and T, 5 (100%) of 5 cases (Nos. 22, 24, 34, 36, and 39) showing methylation in NE were also methylated in corresponding EAC, and the *SST* NMVs in T (mean = 0.231) were significantly higher than those in corresponding NE (mean = 0.055) ($P < .0000001$, Student t test for paired data) (Fig. 2B).

BE was defined as long-segment (LSBE) if it was $\geq$3 cm in length, or short-segment (SSBE) if $<$ 3 cm, according to generally accepted criteria.[16] The mean NMV of *SST* was significantly higher in LSBE (mean = 0.375) than in SSBE (mean = 0.106;

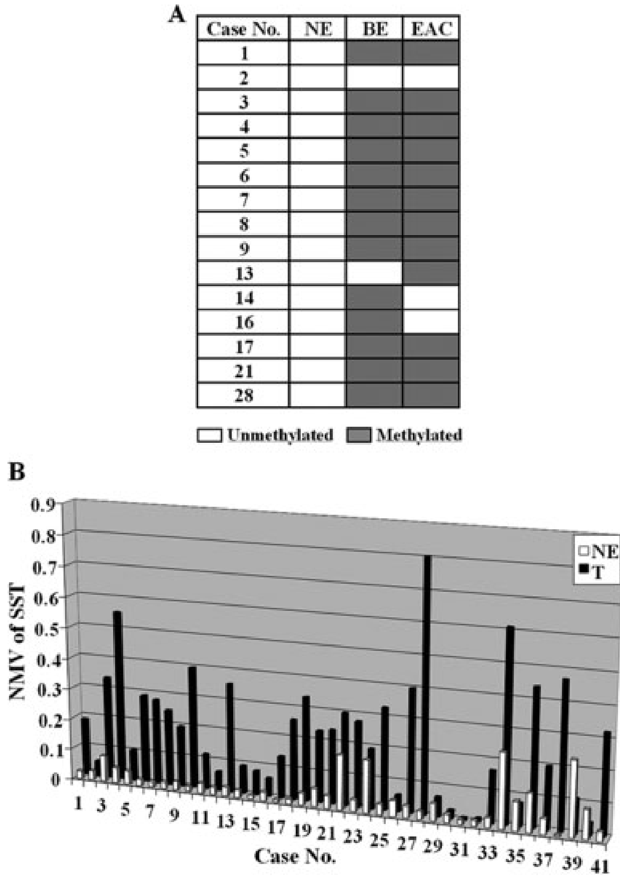

FIGURE 2. Methylation status of *SST* in corresponding esophageal samples. (A) Among 15 patients with matching normal esophagus (NE), Barrett esophagus (BE), and esophageal adenocarcinoma (EAC), 1 case (Patient 2) was unmethylated in all tissues, 1 case (Patient 13) was methylated only in EAC, 2 cases (Patients 14 and 16) were methylated only in BE, and the remaining 11 cases were methylated in both BE and EAC. (B) In 41 patients with corresponding NE and malignant esophageal carcinoma (T), 5 of 5 patients (Patients 22, 24, 34, 36, and 39) showing methylation in NE were also methylated in corresponding malignant esophageal tissues (T), and *SST* normalized methylation values for T (mean = 0.231) were significantly higher than those for matching NE (mean = 0.055; $P < .0000001$, Student *t* test for paired data).

$P = .00158$, Student *t* test). Similarly, the segment lengths of BEs with methylated *SST* promoters (mean = 5.68 cm) were significantly longer than the segment lengths of BEs with unmethylated *SST* promoters (mean = 1.73 cm; $P = .00073$, Student *t* test); and the frequency of *SST* hypermethylation was significantly higher in LSBE than in SSBE ($P = .0004$, Fisher exact test) (Table 1).

No significant associations were observed between *SST* promoter hypermethylation and patient age (data not shown), survival (data not shown), smoking or alcohol consumption status, tumor stage or lymph node metastasis, histologic tumor differentiation, or histologic type of esophageal carcinoma (EAC vs ESCC) (Table 1).

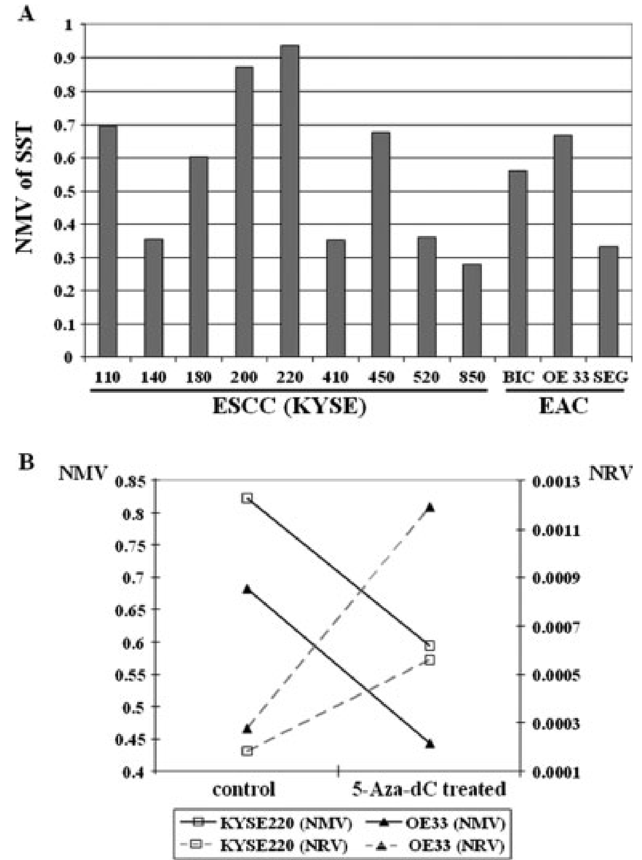

FIGURE 3. *SST* methylation status and levels of *SST* methylation and mRNA expression in esophageal cancer cell lines after treatment with 5-aza-2′-deoxycytidine (5-Aza-dC). (A) Twelve of 12 (3 esophageal adenocarcinoma [EAC] and 9 esophageal squamous cell carcinoma [ESCC]) esophageal cancer cell lines demonstrated high *SST* normalized methylation values (NMVs), exceeding the cutoff NMV level of 0.1. (B) KYSE 220 and OE33, which had the highest NMVs among the ESCC and EAC cell lines, respectively, were subjected to 5-Aza-dC treatment. After 5-Aza-dC treatment in both KYSE220 and OE33 cell lines, the NMV of *SST* was diminished, whereas the normalized mRNA value (NRV) of *SST* was increased.

SST Methylation and mRNA Levels in Esophageal Cancer Cell Lines After 5-Aza-dC Treatment

All 12 (3 EAC, 9 ESCC) esophageal cancer cell lines showed high *SST* NMV levels, greater than the cutoff level of 0.1 (Fig. 3A). KYSE 220 and OE33, which exhibited the highest NMVs among the ESCC and EAC cell lines, respectively, were subjected to 5-Aza-dC treatment. After 5-Aza-dC treatment the NMV of *SST* was diminished, whereas the mRNA level of *SST* was increased in both KYSE220 and OE33 cells (Fig. 3B).

Correlation Between Hypermethylation and mRNA Expression of *SST* in Esophageal Adenocarcinoma

To elucidate the relationship between DNA hypermethylation and mRNA expression of *SST*, we further determined *SST* mRNA levels in 22 EAC samples using real-time RT-PCR. The *SST* mRNA levels of EACs with unmethylated *SST* promoters (n = 6, mean ± SE = 0.01 ± 0.005) were significantly higher than those of EACs with methylated SST promoters (n = 16; mean ± SE = 0.003 ± 0.0009; $P = .047$, Student *t* test). There was a negative correlation between mRNA levels and NMV (Pearson correlation coefficient $r^2 = -0.29$; $P = .19$).

DISCUSSION

In the current study, we systematically investigated hypermethylation of the *SST* gene promoter in primary human esophageal lesions of differing histologic types and grades, as well as in esophageal carcinoma cell lines. We found that hypermethylation of the *SST* promoter was significantly more frequent in the premalignant lesions, metaplastic BE and BE with high-grade dysplasia, as well as in frank EAC and ESCC, than in normal esophageal epithelium. Thus, we conclude that hypermethylation of the *SST* promoter occurs early in some subjects, that the frequency of this event increases early during esophageal carcinogenesis, especially in EAC, and that this epigenetic alteration is a common event in human esophageal cancer.

The length of the BE segment as a predictive factor for esophageal neoplastic progression remains controversial, due to conflicting results among different studies. Although several previous studies reported that patients with SSBE can develop dysplasia[17] or EAC,[16,18] some prospective studies have shown an increased risk of EAC development with LSBE.[19–21] In a prospective cohort study of 309 Barrett patients followed in the Seattle Barrett Esophagus Project, segment length was not related to cancer risk ($P > .2$); however, when patients with HGD at the time of study entrance were excluded, a trend was observed, with a 5-cm difference in length associated with a 1.7-fold (95% confidence interval, 0.8–3.8-fold) increase in cancer risk.[16] Weston et al.[19] reported significant differences in the frequency of both dysplasia and EAC between SSBE and LSBE, at 8.1% versus 24.4% for dysplasia ($P < .0001$) and 0% versus 15.4% for EAC ($P < .0005$). Hirota et al.[20] reported that the prevalence of dysplasia and cancer differed significantly in patients with SSBE versus patients with LSBE in a comprehensive prospective study of 889 consecutive patients. More recently, Hage et al.[21] reported a significantly increased risk of progression to HGD or EAC with LSBE after a mean follow-up of 12.7 years. Thus, it is likely that length of Barrett epithelium is an important risk factor for both the prevalence and the incidence (future development) of dysplasia and cancer. In the current study, *SST* promoter hypermethylation was significantly more frequent in LSBE, which has a higher malignant potential than does SSBE. Thus, *SST* methylation represents a molecular correlate of BE segment length, as well as a potential biomarker for the prediction of BE progression. This finding further supports *SST* hypermethylation as a very early harbinger of esophageal neoplastic transformation.

The diagnosis of LGD occurring in BE is plagued by high interobserver variability, even among gastrointestinal pathologists, and is a poorly defined entity.[22,23] Montgomery et al.[24] reported that only 4 (15%) of 25 LGD cases progressed to EAC, with 60 months' median progression-free survival, whereas fully 20 (61%) of 33 HGD cases progressed to EAC, with 8 months' median progression-free survival. Data reviewed by the American College of Gastroenterology, including 783 BE patients followed for 2.9 to 7.3 years, showed that EAC developed in 2% of patients without dysplasia, 7% with LGD, and 22% with HGD.[18] Furthermore, Skacel et al.[22] reported that 16 (64%) of 25 patients with LGD at initial pathologic examination no longer demonstrated dysplasia on subsequent surveillance endoscopies, and a similar finding was reported by Conio et al.[25] In the current study the frequency of *SST* hypermethylation was actually lower in LGD (63.2%) than in BE (70%); thus, LGD did not differ significantly from BE in terms of this biomarker. More research is needed to clarify the significance of LGD in EAC development.

In this study, reversion of methylation and restoration of *SST* expression were induced in both KYSE220 and OE33 esophageal cancer cells by 5-Aza-dC treatment. Restoration of *SST* mRNA expression due to 5-Aza-dC treatment is consistent with the interpretation that DNA hypermethylation silenced the *SST* gene. The involvement of CpG island hypermethylation in silencing of the *SST* gene is also supported by observations that *SST* mRNA levels in EACs with unmethylated *SST* promoters were significantly higher than those in EACs with methylated *SST* promoters.

The current study indicates that hypermethylation of the *SST* promoter, leading to gene silencing, is a common event in human esophageal carcinomas and occurs early in Barrett-associated esophageal adenocarcinogenesis. These results provide a basis for further research on *SST* as a potential biomarker

for the early diagnosis, classification, stratification, and prognostication of esophageal cancers.

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