Int. J. Cancer: **123,** 2331–2336 (2008)

Promoter hypermethylation of CDH13 is a common, early event in human esophageal adenocarcinogenesis and correlates with clinical risk factors

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Although the *CDH13* gene has been shown to undergo epigenetic silencing by promoter methylation in many types of tumors, hypermethylation of this gene in Barrett's-associated esophageal adenocarcinogenesis has not been studied. Two hundred fifty-nine human esophageal tissues were therefore examined for *CDH13* promoter hypermethylation by real-time methylation-specific PCR. *CDH13* hypermethylation showed discriminative receiver-operator characteristic curve profiles, sharply demarcating esophageal adenocarcinoma (EAC) from esophageal squamous cell carcinoma (ESCC) and normal esophagus (NE) ($p < 0.0001$). *CDH13* normalized methylation values (NMV) were significantly higher in Barrett's esophagus (BE), dysplastic BE (D) and EAC than in NE ($p < 0.0000001$). *CDH13* hypermethylation frequency was 0% in NE but increased early during neoplastic progression, rising to 70% in BE, 77.5% in D and 76.1% in EAC. Both *CDH13* hypermethylation frequency and its mean NMV were significantly higher in BE with than without accompanying EAC. In contrast, only 5 (19.2%) of 26 ESCCs exhibited *CDH13* hypermethylation. Furthermore, both *CDH13* hypermethylation frequency and its mean NMV were significantly higher in EAC than in ESCC, as well as in BE or D *vs.* ESCC. Interestingly, mean *CDH13* NMV was significantly lower in short-segment than in long-segment BE, a known clinical risk factor for neoplastic progression. Similarly, BE segment length was significantly lower in specimens with unmethylated than with methylated *CDH13* promoters. 5-aza-2′-deoxycytidine treatment of OE33 EAC and KYSE220 ESCC cells reduced *CDH13* methylation and increased *CDH13* mRNA expression. These findings suggest that hypermethylation of *CDH13* is a common, tissue-specific event in human EAC, occurs early during BE-associated neoplastic progression, and correlates with known clinical neoplastic progression risk factors.
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Key words: CDH13; hypermethylation; EAC; ESCC

CDH13 (also known as *H-cadherin* and *T-cadherin*), a member of the cadherin gene superfamily, was isolated and has been mapped to 16q24,[1] a locus that frequently undergoes deletion in human cancers, including esophageal carcinoma.[2,3] In contrast to other known cadherins such as E-cadherin, N-cadherin and P-cadherin, which are transmembrane proteins, CDH13 lacks conventional transmembrane and cytoplasmic domains and is attached to the plasma membrane through a glycosyl phosphatidyl inositol anchor.[1,4–6] Several studies have suggested that CDH13 functions as a tumor suppressor gene and possesses potent antitumor activity in several human cancers both *in vitro* and *in vivo*.[7–10] Overexpression of CDH13 in human breast carcinoma cells (MDAMB435) reduced their invasive potential *in vitro* and tumor formation *in vivo*, accompanied by reversion from invasive to normal cell morphology.[7] Loss of CDH13 protein expression is associated with tumorigenicity of human non-small cell lung cancer cells.[8,9] In cutaneous squamous cell carcinoma cells, overexpression of CDH13 induced a delay in the G_2/M phase of the cell cycle and reduced cell proliferation.[10] Downregulation of *CDH13* expression has been reported in various human cancers, including those arising in the breast, lung, ovary, stomach and colon.[1,8,9,11–14] It is now well-established that promoter hypermethylation correlates with silencing of gene transcription in many cancers,[15] including ESCC and EAC.[16–18] Furthermore, there is a growing body of evidence showing that abnormal methylation of DNA can be an early event in carcinogenesis and can serve as an early cancer detection biomarker,[15] including in EAC.[17–20] Hypermethylation of *CDH13* has been described in many human cancers,[8,14,21–29] including ESCC[24,29]; however, hypermethylation of *CDH13* in precancerous lesions such as Barrett's metaplasia (BE), as well as in BE-associated EAC, is an area that still remains to be explored. We investigated hypermethylation of the *CDH13* promoter by real-time quantitative methylation-specific PCR (qMSP) in 259 endoscopic esophageal biopsy specimens of differing histologies and correlated these data with clinicopathological features. Our results reveal that promoter hypermethylation of *CDH13* is a common event in EAC but not in ESCC and occurs early during BE-associated esophageal neoplastic progression, correlating with clinical criteria associated with neoplastic progression risk.

Material and methods

Tissue samples

The 259 specimens examined in the current study comprised 66 from normal esophagus (NE), 60 of non-dysplastic Barrett's metaplasia {BE, including 36 obtained from patients with BE alone (Ba) and 24 from patients with BE accompanied by EAC (Bt)}, 40 from dysplastic BE {D, including 19 low-grade (LGD) and 21 high-grade (HGD)}, 67 EACs and 26 ESCCs. All patients provided prior written informed consent under a protocol approved by the Institutional Review Boards at the University of Maryland School of Medicine, the Baltimore Veterans Affairs Medical Center and the Johns Hopkins University School of Medicine. Biopsies were obtained using a standardized biopsy protocol as previously described.[30] Research tissues were taken from grossly apparent BE epithelium or from mass lesions in patients manifesting these changes at endoscopic examination, and histology was confirmed using parallel aliquots culled from identical locations at

Abbreviations: AUROC, area under the ROC curve; Ba, BE from Barrett's patient; BE, Barrett's metaplasia; Bt, BE from EAC patient; EAC, esophageal adenocarcinoma; ESCC, esophageal squamous cell carcinoma; HGD, high-grade dysplasia in BE; LGD, low-grade dysplasia in BE; LSBE, long-segment BE; MSP, methylation-specific PCR; NE, normal esophageal epithelium; NMV, normalized methylation value; SSBE, short-segment BE; 5-Aza-C, 5-aza-2′-deoxycytidine; ROC curve, receiver-operator characteristic curve; RT-PCR, reverse-transcription polymerase chain reaction.

Grant sponsor: NIH; Grant numbers: CA085069, CA 084986, CA001808, CA106763.

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Received 14 March 2008; Accepted after revision 16 June 2008
DOI 10.1002/ijc.23804
Published online 26 August 2008 in Wiley InterScience (www.interscience.wiley.com).

TABLE I – CLINICOPATHOLOGIC CHARACTERISTICS AND METHYLATION STATUS OF *CDH13* IN HUMAN ESOPHAGEAL TISSUES

Clinical characteristics[1]	Number of samples	Age (year) mean	NMV[2]		Methylation Status (cutoff 0.06)[3]			
			mean	*p*	Frequency	UM	M	*p*
Histology								
Normal esophagus	66	64.3	0.0054		0%	66	0	
BE	60	63.7	0.3122	$< 0.00001*/#	70%	18	42	
Ba	36	62.5	0.2623		58.3%	15	21	<0.05†
Bt	24	65.5	0.3871	$< 0.05	87.5%	3	21	
Dysplasia in Barrett's esophagus	40	65.3	0.3383	$<0.00001*/#	77.5%	9	31	
Low-grade dysplasia	19	65.3	0.2833	$< 0.000001*	78.9%	4	15	NS†
High-grade dysplasia	21	65.2	0.388	$< 0.000001*	76.2%	5	16	
EAC	67	65.1	0.2392	$< 0.000001*/#	76.1%	16	51	<0.0001†
ESCC	26	62.5	0.0458	$<0.01*	19.2%	21	5	
Barrett's segment of Ba								
Short-segment (<3cm)	14	62.3	0.131	$< 0.01	28.6%	10	4	<0.01†
Long-segment (>=3cm)	16	62.8	0.4071		87.5%	2	14	
Stage of EAC patients								
I	7	63	0.3081	¶NS	85.7%	1	6	NS†
II	15	65.2	0.2408		73.3%	4	11	
III	25	64.6	0.2111		72%	7	18	
IV	7	66.3	0.2921		100%	0	7	
Lymph node metastasis in EAC patients								
Negative	25	64.9	0.2751	$NS	75%	5	20	NS‡
Positive	25	64.6	0.2277		76%	6	19	
Smoking status of EAC patients								
Never	6	58.5	0.2984	¶NS	100%	0	6	NS†
Former	24	68.5	0.2143		79.2%	5	19	
Current	13	60.8	0.2561		76.9%	3	10	
Alcohol drinking status of EAC patients								
Never	16	65.3	0.2209	¶NS	75%	4	12	NS†
Former	15	63	0.2524		86.7%	2	13	
Current	10	65.7	0.2427		80%	2	8	

[1]BE, Barrett's metaplasia; Ba, BE from patients with Barrett's alone; Bt, BE from patients with Barrett's accompanied by EA; EAC, esophageal adenocarcinoma; ESCC, esophageal s qua mo us cell carcinoma.–[2]NMV: normalized methylation value; $Mann-Whitney U test; *comparisons made to normal esophagus; #comparisons made to ESCC; ¶Kruskal-Wallis test.–[3]UM, unmethylated; M, methylated; †Fisher's exact test; ‡Chi-square for Independence test.

NS, not significant.

endoscopy. All research biopsy specimens were stored in liquid nitrogen prior to DNA extraction. Clinicopathologic characteristics are summarized in Table I.

Cell lines

OE33 EAC and KYSE220 ESCC cells were cultured in 47.5% RPMI 1640, 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, CA). Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA). DNAs and RNAs were stored at −80°C prior to analysis.

Bisulfite treatment and real-time methylation-specific PCR

One microgram DNA was treated with bisulfite to convert unmethylated cytosines to uracils prior to MSP using an EpiTect Bisulfite Kit (Qiagen, Valencia, CA). Promoter methylation levels of *CDH13* were determined by real-time quantitative MSP with an ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA), using primers and probes as follows: *CDH13*-forward: 5′-TTTGGGAAGTTGGTTGGTTGGC-3′; *CDH13*-reverse: 5′-ACTAAAAACGCCCGACGACG-3′ and probe: 5′-TATGTT TAGTGTAGTCGCGTGTATGAATGAA-3′. β-actin was used for normalization of data. Primers and probe for β-actin were the same as previously reported.[17] A standard curve was generated using serial dilutions of CpGenome Universal Methylated DNA (CHEMICON, Temecula, CA). Normalized methylation value (NMV) was defined as follows: NMV = (*CDH13-S*/*CDH13-FM*)/(*ACTB-S*/*ACTB-FM*), where *CDH13-S* and *CDH13-FM* represent *CDH13* methylation levels (derived from the standard curve) in sample and fully methylated DNAs, respectively, while *ACTB-S* and *ACTB-FM* correspond to β-actin in sample and fully methylated DNAs, respectively.

Real-time quantitiative RT-PCR

To determine *CDH13* mRNA levels, one-step real-time quantitative RT-PCR was performed using a Qiagen QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany) and an ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers and probe for *CDH13* were as follows: *CDH13*-forward: 5′-ATGTTGGCAAGGTAGTCGATAGTG-3′; *CDH13*-reverse: 5′-ACGCTCCCTGTGTTCTCATTG-3′ and probe: 5′-CC AGAAAGGTCCAAGTTCCGGCTCACT-3′. β-actin was used for normalization of data. Primers and probe for β-actin were the same as previously reported.[17] A standard curve was generated using serial dilutions of qPCR Reference Total RNA (Clontech, Mountainview, CA). 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 target gene mRNA expression (derived from the standard curve) in sample and control mRNAs, respectively, while *ACTB-S* and *ACTB-C* correspond to amplified *ACTB* levels in sample and control mRNAs, respectively.

5-Aza-dC treatment of esophageal cancer cell lines

To determine whether *CDH13* inactivation was due to promoter hypermethylation in esophageal cancer, 2 esophageal cancer cell lines (KYSE220 and OE33) were subjected to 5-Aza-dC (Sigma, St. Louis, MO) treatment as previously described.[31,32] Briefly, 1 × 10^5 cells/ml were seeded onto a 100-mm dish and grown for 24 hr. Then, 1 μl of 5 mM 5-Aza-dC per ml of cells was added every 24 hr for 4 days. DNAs and RNAs were harvested on day 4.

Data analysis and statistics

Receiver-operator characteristic (ROC) curve analysis[33] was performed using NMVs for the 67 EAC, 26 ESCC and 66 NE specimens by Analyse-it© software (Version 1.71, Analyse-it Software, Leeds, UK). Using this approach, the area under the ROC curve (AUROC) identified optimal sensitivity and specificity levels at which to distinguish normal from malignant esophageal tissues (NE *vs*. EAC), yielding a corresponding NMV threshold with which to dichotomize the methylation status of *CDH13*. The threshold NMV value determined from this ROC curve was applied to determine the status of *CDH13* methylation in all tissue types included in the study. For all other statistical tests, Statistica (version 6.1; StatSoft, Tulsa, OK) was employed. Differences with $p < 0.05$ were considered significant.

Results

CDH13 promoter hypermethylation in esophageal tissues

Promoter hypermethylation of *CDH13* was analyzed in 66 NE, 60 BE (including 36 Ba and 24 Bt), 40 D (including 19 LGD and 21 HGD), 67 EAC, and 26 ESCC. *CDH13* promoter hypermethylation showed highly discriminative ROC curve profiles and AUROCs, clearly distinguishing both EAC and ESCC from NE (Figs. 1*a* and 1*b*), as well as EAC from ESCC (Fig. 1*c*).

The cutoff NMV for *CDH13* (0.06) was identified from the ROC curve (EAC *vs*. NE) to achieve the highest possible sensitivity while maintaining 100% specificity. Mean NMV and frequency of *CDH13* hypermethylation for each tissue type are shown in Table I. NMVs of *CDH13* were significantly higher in ESCC, EAC, D, HGD, LGD, BE, Ba and Bt than in NE ($p < 0.001$, Mann–Whitney U test). The frequency of *CDH13* hypermethylation was significantly higher in BE (70%), D (77.5%) and EAC (76.1%) than in N (0%; $p < 0.0001$, $p < 0.0001$ and $p < 0.0001$, respectively; Fisher's exact test). Interestingly, both *CDH13* hypermethylation frequency and mean NMV were significantly higher in Bt than in Ba (87.5% *vs*. 58.3%, $p = 0.021$ and 0.3871 *vs*. 0.2623, $p = 0.045$, respectively). The mean *CDH13* NMV in EAC (0.2722) was significantly higher than that in matching NE (0.0034) for 27 cases in which matching NE and EAC were available ($p < 0.00001$, Wilcoxon matched pairs test). In contrast to EAC, only five (19.2%) of 26 ESCCs manifested hypermethylation of *CDH13*. There was no significant difference in mean *CDH13* NMV between tumor and normal tissue in 13 cases for which matching ESCC (0.0337) and NE (0.0131; $p = 0.6$, Wilcoxon matched pairs test) were available. Both *CDH13* hypermethylation frequency and mean NMV were significantly higher in EAC than in ESCC (76.1% *vs*. 19.2%, $p < 0.0001$ and 0.2392 *vs*. 0.0458, $p < 0.0001$, respectively), as well as in D *vs*. ESCC (77.5% *vs*. 19.2%, $p < 0.0001$ and 0.3383 *vs*. 0.0458, $p < 0.0001$, respectively) and in BE *vs*. ESCC (70% *vs*. 19.2%, $p < 0.0001$ and 0.3122 *vs*. 0.0458, $p < 0.0001$; Table I).

According to generally accepted criteria,[34] BE was defined as long-segment (LSBE) if it was equal to or greater than 3 cm in length, or short-segment (SSBE) if less than 3 cm. The mean NMV of *CDH13* was significantly higher in LSBE than in SSBE (0.4071 *vs*. 0.131; $p < 0.01$, Student's *t*-test, Table I and Fig. 2*a*). Similarly, segment lengths of BEs with methylated *CDH13* promoters (mean = 5.83 cm) were significantly longer than segment lengths of BEs with unmethylated *CDH13* promoters (mean = 1.83 cm; $p < 0.001$, Student's *t*-test; Fig. 2*b*), and the frequency of *CDH13* hypermethylation was significantly higher in LSBE than in SSBE (87.5% *vs*. 28.6%; $p < 0.01$, Fisher's exact test; Table I).

No significant associations were observed between *CDH13* promoter hypermethylation and patient age (data not shown), survival (log-rank test, data not shown), tumor stage, lymph node metastasis, smoking or alcohol consumption (Table I).

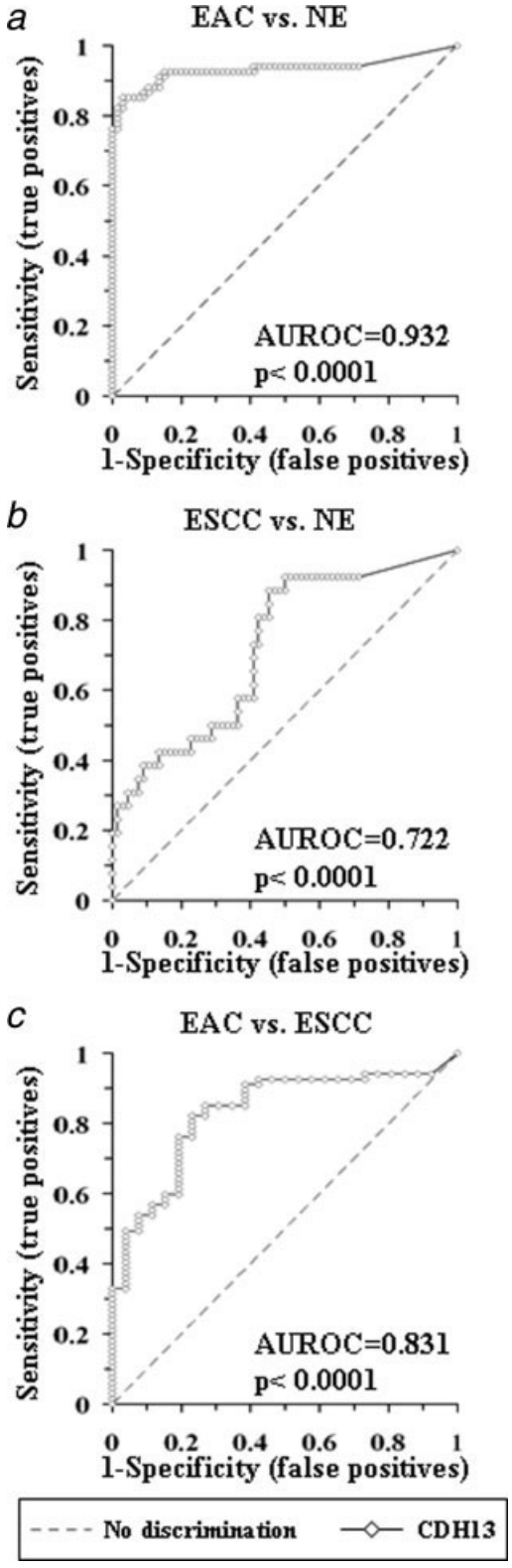

Figure 1 – Receiver-operator characteristic (ROC) curve analysis of normalized methylation value (NMV). ROC curve analysis of *CDH13* NMVs in esophageal adenocarcinoma (EAC) *vs*. normal esophagus (NE) (*a*), esophageal squamous cell carcinoma (ESCC) *vs*. NE (*b*) and EAC *vs*. ESCC (*c*). The high area under the ROC curve (AUROC) conveys the accuracy of this biomarker in distinguishing EAC from NE and from ESCC in terms of its sensitivity and specificity.

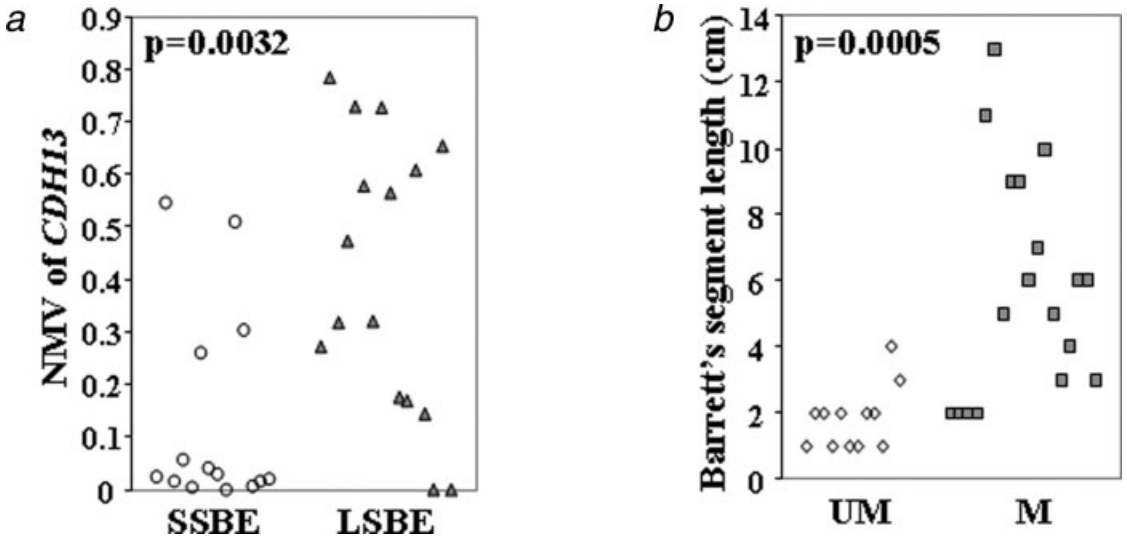

Figure 2 – Correlation between Barrett's segment length and *CDH13* hypermethylation. (*a*), Normalized methylation value (NMV) of *CDH13* was significantly higher in long-segment BE (LSBE, mean = 0.4071) than in short-segment BE (SSBE, mean = 0.131; $p = 0.0032$, Student's *t*-test). (*b*), Positive *CDH13* hypermethylation status was significantly correlated with BE segment length ($p = 0.0005$, Student's *t*-test).

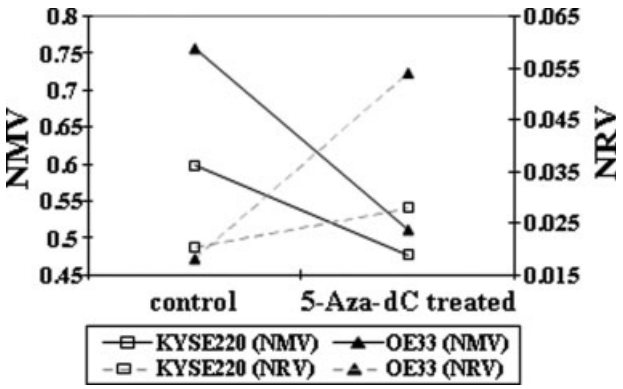

Figure 3 – CDH13 methylation level and mRNA expression in esophageal cancer cell lines after treatment with the demethylating agent 5-aza-2′-deoxycytidine (5-Aza-dC). KYSE220 and OE33 EAC cells were subjected to 5-Aza-dC treatment. In both cell lines, after 5-Aza-dC treatment, the NMV of *CDH13* was diminished, while the normalized mRNA value (NRV) of *CDH13* was increased.

CDH13 methylation and mRNA levels in esophageal cancer cell lines pre- and post-5-Aza-dC treatment

KYSE220 ESCC and OE33 EAC cells were subjected to 5-Aza-dC treatment. After 5-Aza-dC treatment, the NMV of *CDH13* was diminished and the mRNA level of *CDH13* was increased in both KYSE220 and OE33 cells (Fig. 3).

Discussion

In the current study, we systematically investigated hypermethylation of the *CDH13* gene promoter in cell lines and primary human esophageal lesions of contrasting histological types and grades by qMSP. Our results demonstrate that *CDH13* promoter hypermethylation occurs frequently in human EAC, but not in ESCC. In addition, our data show that *CDH13* hypermethylation increases early during esophageal adenocarcinogenesis, from 0% in NE to 58.3% in BE, 77.5% in D and 76.1% in EAC. These results imply that hypermethylation of *CDH13* occurs early in most subjects, that its frequency increases during adenocarcinogenesis, and that it is tissue-specific (*i.e.*, common in EAC but rare in ESCC). Further evidence supporting this tissue specificity is provided by ROC curves, which clearly distinguished EAC from ESCC. Similarly, support for tissue specificity is evident from the finding that both *CDH13* hypermethylation frequency and mean *CDH13* NMV were significantly higher in EAC than in ESCC. In addition, the low frequency (19.2%) of *CDH13* hypermethylation in ESCC, as determined in the current study, is consistent with previous findings by other groups.[24,29] Thus, *CDH13* hypermethylation appears to constitute a critical event in only one of the two esophageal cancer subtypes.

Several studies have suggested that methylation of certain genes may occur as a field change and may be associated with an increased risk of malignant progression.[17,19,20,30,35] *CDKN2A*, *ESR1* and *MYOD1* were methylated only in BE from patients who possessed dysplasia or cancer in other regions of their esophagus, but not in patients with no evidence of progression beyond BE, while *CALCA*, *MGMT* and *TIMP3* were methylated more frequently in normal stomach, normal esophageal mucosa and intestinal metaplasia from patients with distant dysplasia or esophageal cancer than from patients without dysplasia or cancer.[35] Previously, we demonstrated that hypermethylation of *p16*, *RUNX3* and *HPP1* in BE or LGD may represent independent risk factors for the progression of BE to HGD or EAC.[30] Recently, we also found that both hypermethylation frequency and NMV of the *nel-like 1*, *tachykinin-1, somatostatin* and *AKAP12* genes were higher in BE with accompanying EAC than in BE without accompanying EAC.[17–20] Interestingly, both *CDH13* hypermethylation frequency and level were significantly higher in BE with than without accompanying EAC in the current study, suggesting that *CDH13* is a biomarker of more ominous disease lurking nearby.

In this study, we also correlated *CDH13* methylation with clinicopathologic features. Despite some degree of controversy regarding the length of the BE segment as a predictive factor in BE progression, it is likely that this clinical parameter is an important predictor of neoplastic progression. In the Seattle Barrett's Esophagus Project, BE segment length was not related to cancer risk in a prospective cohort study of 309 Barrett's patients ($p > 0.2$); however, when patients with HGD at entrance were excluded, a strong trend was observed, with a 5 cm difference in length associated

with a 1.7-fold increase in cancer risk (95% CI, 0.8- 3.8-fold).[34] Significant differences in the frequency of both dysplasia and EAC were observed between SSBE and LSBE, at 8.1% *vs*. 24.4% for dysplasia ($p < 0.0001$) and 0% *vs*. 15.4% for EAC ($p < 0.0005$).[36] In a comprehensive prospective study of 889 consecutive patients, the prevalence of dysplasia and cancer differed significantly in patients with SSBE *vs*. LSBE.[37] More recently, a significantly increased risk of progression to HGD or EAC with LSBE after a mean follow-up of 12.7 years was reported.[38] In our previous studies, the *nel-like 1*, *tachykinin-1, somatostatin* and *AKAP12* genes were significantly more hypermethylated in LSBE than in SSBE.[17–20] Notably, in the current study, *CDH13* methylation also showed a strong relationship to BE segment length. The mean NMV of *CDH13* was significantly higher in LSBE than in SSBE. Similarly, the length of the BE segment was significantly greater in specimens with methylated than with unmethylated *CDH13* promoters. Thus, *CDH13* hypermethylation may constitute a molecular correlate of BE segment length, as well as a harbinger of nearby neoplastic disease. These results also suggest that epigenetic alterations, which may account for some of the biologic behavior of BE, clearly differ between LSBE and SSBE, suggesting a need for further large-scale studies.

In the current study, the mean NMV in dysplasia was higher than in EAC. This finding could have resulted from either differences in sample sizes between these two groups, or from differential contamination by nonneoplastic cells.

In accordance with previous findings in other primary cancer cell types,[28] we observed that methylation of *CDH13* in EAC and ESCC cancer cell lines was associated with silenced or reduced expression of *CDH13* mRNA. Treatment with 5-Aza-dC restored mRNA expression and reversed *CDH13* methylation in these cells. Restoration of *CDH13* mRNA expression by demethylating agent treatment implies that DNA hypermethylation was responsible for silencing of *CDH13*.

In summary, findings of the current study suggest that hypermethylation of the *CDH13* promoter is a common event in human esophageal adenocarcinogenesis, occurs early during Barrett's-associated esophageal carcinogenesis, and is associated with clinical risk factors of progression. In addition, *CDH13* hypermethylation is uncommon in human ESCC, thus making it a potential cell type-specific biomarker for EAC. Further large-scale prospective longitudinal validation studies of this alteration as a predictive biomarker for EAC development are warranted by these data.

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