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Endoglin Promoter Hypermethylation Identifies a Field Defect in Human Primary Esophageal Cancer

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Abstract

Endoglin (ENG) is a 180-kDa transmembrane glycoprotein that functions as a component of the transforming growth factor-β receptor complex. Recently, *ENG* promoter hypermethylation was reported in several human cancers. We examined *ENG* promoter hypermethylation using real-time quantitative methylation-specific PCR in 260 human esophageal tissues. *ENG* hypermethylation showed highly discriminative receiver-operator characteristic curve profiles, clearly distinguishing esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) from normal esophagus (N) ($p<0.01$). Interestingly, *ENG* normalized methylation values were significantly higher in ESCC than in N ($p<0.01$) or EAC ($p<0.01$). *ENG* hypermethylation frequency was 46.2% in ESCC and 11.9% in N, but increased early and sequentially during EAC-associated neoplastic progression, to 13.3% in Barrett's metaplasia (BE), 25% in dysplastic BE (D), and 26.9% in frank EAC. *ENG* hypermethylation was significantly higher in N from ESCC patients (mean = 0.0186) than in N from EAC patients (mean = 0.0117; $p < 0.05$). Treatment of KYSE220 ESCC cells with the demethylating agent, 5-aza-2′-deoxycytidine, reversed *ENG* methylation and reactivated *ENG* mRNA expression. We conclude that promoter hypermethylation of *ENG* is a frequent, tissue-specific event in human ESCC and exhibits a field defect with promising biomarker potential for the early detection of ESCC. In addition, *ENG* hypermethylation occurs in a subset of human EAC, and early during BE-associated esophageal neoplastic progression.

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Informed consent was obtained from the subject(s) and/or guardian(s)

Using real-time quantitative methylation-specific PCR, we examed promoter hypermethylation of Endoglin (ENG) in 260 endoscopic esophageal biopsy specimens of differing histologies. Results demonstrate that hypermethylation of ENG is a common, tissue-specific event in human esophageal squamous cell carcinomas (ESCC) and exhibits a field defect in normal mucosa with potential biomarker implications for the early detection of ESCC.

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Introduction

Esophageal cancer ranks as the eighth most common cancer worldwide, with 482,000 new cases in 2008, and is the sixth most common cause of cancer death with 407,000 deaths[1]. This malignancy exists in two principal forms, each possessing distinct pathological characteristics: esophageal squamous cell carcinoma (ESCC), which occurs at high frequencies in many developing countries, especially Asia, and including China[2]; and esophageal adenocarcinoma (EAC), which is more prevalent in Western countries. These aggressive malignancies commonly present as locally advanced disease with a very poor prognosis (*i.e.,* with approximately 17% 5-year survival)[3], although significant advances have occurred in treatment. In order to improve outcome, it will be vitally important to discover early events that can serve as detection biomarkers or targets for chemoprevention and therapy.

Endoglin/CD105 (ENG) is a 180-kDa transmembrane glycoprotein that functions as a component of the transforming growth factor-β receptor complex [4, 5]. ENG is expressed predominantly in proliferating vascular endothelial cells, where it plays a critical role in vascular remodeling and angiogenesis [6–10]. Germline mutations in the *ENG* gene can lead to an autosomal dominant vascular dysplasia, hereditary hemorrhagic telangiectasia type 1 syndrome [7, 11]. Its critical role in angiogenesis has prompted investigators to evaluate the role of ENG in cancer progression and metastasis. Intratumour microvessel density assessed by ENG staining strongly correlates with prognosis in different cancer patients [9, 10, 12, 13]. Although *ENG* hypermethylation has been reported in human lung, colonrectal and breast cancers [14–16], there has been only one study that evaluated *ENG* hypermethylation in 2 ESCC patients and 16 ESCC cell lines [17]. Therefore, to further investigate *ENG* hypermethylation in human esophageal carcinogenesis, we investigated whether and at which neoplastic stage promoter hypermethylation of *ENG* is involved in human esophageal carcinogenesis, using real-time quantitative methylation-specific PCR (qMSP) analyses of 260 endoscopic esophageal biopsy specimens of differing histologies. We also evaluated the effect of the demethylating agent, 5-aza-2′-deoxycytidine (5-Aza-dC), on reactivation of epigenetically silenced *ENG* in esophageal cancer cells. Our results establish that promoter hypermethylation of *ENG* is a common event in ESCC but not in EAC and occurs early during Barrett's-associated esophageal neoplastic progression.

Materials and Methods

Tissue Samples

The 260 specimens examined in the current study comprised 67 normal esophageal specimens {N, including 19 obtained from non-Barrett's/non-esophageal cancer patients (NE), 20 from ESCC patients (NEcS), and 28 from EAC patients (NEcA)}, 60 non-dysplastic Barrett's metaplasias (BE), 40 dysplastic Barrett's specimens (D), 67 EACs, and 26 ESCCs. 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 [18]. Research tissues were obtained from grossly apparent Barrett's epithelium or from mass lesions in patients manifesting these changes at endoscopic examination, and histology was confirmed using parallel aliquots taken from identical locations at endoscopy. All biopsy specimens were stored in liquid nitrogen prior to DNA extraction.

Cell Lines

The KYSE220 ESCC cell line was obtained from collaborators at Toyama University (Prof. Yutaka Shimada) and cultured in 47.5% RPMI 1640, 47.5% F-12 supplemented with 5% fetal bovine serum.

DNA and RNA Extraction

Genomic DNA and total RNA were extracted from biopsies and cultured cells using DNeasy Tissue Kits (Qiagen, Valencia, CA) and TRIzol reagent (Invitrogen, Carlsbad, CA), respectively. DNAs and RNAs were stored at −80°C before analysis.

Bisulfite Treatment and Real-Time Methylation-Specific PCR

DNA was treated with bisulfite to convert unmethylated cytosines to uracils prior to qMSP, as described previously[15]. Promoter methylation levels of *ENG* were determined by real-time qMSP with the ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), using primers and probes as described previously [15]. Normalized methylation value (NMV) was defined as follows: NMV = (*ENG-S*/*ENG-FM*)/(*ACTB-S/ACTB-FM*), where *ENG-S* and *ENG-FM* represent *ENG* methylation levels 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 *ENG* mRNA levels, one-step real-time quantitative RT-PCR was performed using a Qiagen QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany) and the ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). *β-actin* was used for normalization of data. Primers and probe for *ENG* and *β-actin* were the same as previously reported (2006a). 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 *= (ENG-S/ENG-C)/(ACTB-S/ACTB-C,* where *ENG-S* and *ENG-C* represent levels of mRNA expression for *ENG* 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 *ENG* inactivation was due to promoter hypermethylation in esophageal cancer, KYSE220 cells were subjected to 5-Aza-dC (Sigma, St. Louis, MO) treatment as previously described[19, 20]. Briefly, 1×10^5 cells/ml were seeded onto a 100 mm dish and grown for 24 h. Then, 1 μl of 5mM 5-Aza-dC per ml of cells was added every 24 hours for 4 days. DNAs and RNAs were harvested on day 4.

Data Analysis and Statistics

Receiver-operator characteristic (ROC) curve analysis (1982z) was performed using NMVs for the 67 EAC, 26 ESCC and 67 N specimens by Analyse-it© software (Version 1.71, Analyse-it Software, Leeds, UK). With this approach, the area under the ROC curve (AUROC) identified optimal sensitivity and specificity levels at which to distinguish normal from malignant esophageal tissues, yielding corresponding NMV thresholds defining methylation status of *ENG*. The threshold NMV value determined from this ROC curve was applied to determine the status of *ENG* methylation in all tissue types included in the present study. For all other statistical tests, Statistica (version 6.1; StatSoft, Inc., Tulsa, OK) was used. Differences with $p<0.05$ were considered significant.

Results

ENG Promoter Hypermethylation in Esophageal Tissues

Promoter hypermethylation of *ENG* was analyzed in 67 N (including 19 NE, 20 NEcS and 28 NEcA), 60 BE, 40 D, 67 EAC, and 26 ESCC samples. *ENG* promoter hypermethylation showed highly discriminative ROC curve profiles and AUROCs, clearly distinguishing ESCC from both N and EAC ($p<0.01$ and $p<0.01$, respectively; Figure 1A and 1B), as well as NEcS from NEcA ($p<0.01$; Figure 1C), but not EAC from N (data not shown).

The cutoff NMV for *ENG* (0.02) was identified from ROC curves (ESCC *vs.* N) as maximizing both sensitivity and specificity. Mean NMV and frequency of *ENG* hypermethylation for each tissue type are shown in Table 1. NMVs of *ENG* were significantly higher in ESCC than in N or EAC ($p<0.01$, Mann-Whitney U test; Table 1). Moreover, NMV of *ENG* was significantly higher in NEcS (mean = 0.0186) than in NEcA (mean = 0.0115 and $p < 0.05$, Mann-Whitney U test; Table 1). Similarly, the frequency of *ENG* hypermethylation was significantly higher in ESCC than in N (46.2% *vs.* 11.9%, $p < 0.001$), and was sequentially increased in BE (13.3%), D (25%), and EAC (26.9%) *vs.* N (11.9%; $p > 0.05$, $p > 0.05$ and $p < 0.05$, respectively, Chi-square for independence test). *ENG* hypermethylation frequency was higher in ESCC than in EAC, although these differences did not achieve statistical significance (46.2% *vs.* 26.9%, $p = 0.074$). There was no significant different for *ENG* hypermethylation by either NEcS vs. NE, or NEcA vs. NE. In the current study, the mean NMV in NEcS was not signigicantly higher than in NE. This finding could have resulted from differences in sample sizes between these two groups.

No significant associations were observed between *ENG* promoter hypermethylation and patient age, survival, Barrett's segment length, EAC tumor stage, lymph node metastasis, or smoking or alcohol consumption (data not shown).

ENG Methylation and mRNA Levels in KYSE220 Cells after 5-Aza-dC Treatment

KYSE220 cells were subjected to demethylation by 5-Aza-dC treatment. After 5-Aza-dC treatment, the NMV of *ENG* was diminished and *ENG* mRNA levels were increased (Figure 2).

Discussion

In the current study, we systematically investigated hypermethylation of the *ENG* gene promoter in primary human esophageal lesions of contrasting histological types. Our results demonstrate that *ENG* promoter hypermethylation occurs frequently in human ESCC (46.2%), but only in a smaller subset of EAC (26.9%). However, *ENG* hypermethylation occurs early and increases sequentially during esophageal adenocarcinogenesis, from 11.9% in N (and 10.7% in NEcA) to 13.3% in BE, 25% in D, and 26.9% in EAC. Interestingly, methylation levels of *ENG* were significantly higher in NEcS than in NEcA, suggesting that *ENG* exhibits a field defect with potential biomarker value for ESCC lurking nearby, even when analyzing non-neoplastic esophageal mucosa. In addition, *ENG* was hypermethylated more frequently in ESCC than in EAC. Taken together, these findings imply that hypermethylation of *ENG* is a common event in ESCC, occurs early in some subjects during the development of EAC, increases in frequency during Barrett's-associated esophageal adenocarcinogenesis, and is a cell type-specific event (*i.e.,* more common in ESCC than in EAC). Further evidence supporting this tissue specificity was provided by ROC curves, which clearly distinguished ESCC from EAC but not EAC from N. Further support for tissue specificity was evident from our finding that mean *ENG* NMVs were significantly higher in ESCC than in EAC. Thus, *ENG* hypermethylation appears to constitute a critical field-defect event in human ESCC.

Despite extensive knowledge on intratumour microvessel density assessed by ENG staining as a prognostic factor in different cancer patients [9, 10, 12, 13], limited data are available on *ENG* hypermethylation in tumor cells[14–17]. *ENG* was significantly downregulated in non-small cell lung cancer based on Affymetrix GeneChip assay, and its promoter was aberrantly methylated in 5 (71%) of 7 lung cancer cell lines, in 11 (69%) of 16 primary lung tumors, and in 4 (80%) of 5 normal lung tissues based on combined bisulfite restriction analysis [14]. Based on qMSP assays, *ENG* was methylated in 3 of 34 colon cancers, but not in normal colonic mucosae[15]. In a large cohort of invasive breast cancers, lack of *ENG* expression in the tumor cell compartment correlated with *ENG* gene methylation and poor clinical outcome, and its expression in breast tumor cells suppressed invasion and metastasis [16]. *ENG* was previously shown to be methylated in 2 ESCC patients and 16 ESCC cell lines by non-quantitative methylation-specific PCR, and its expression suppressed invasion in ESCC cells [17]. To our knowledge, the current study is the first to quantitatively measure methylation of *ENG* in a large cohort of human esophageal cancers.

It has been reported that hypermethylation of gene promoters in histologically normal tissue can be related to the initiation of carcinomas [21–26]. For example, methylation of the *MLH1* promoter was observed in small foci of normal colonic epithelial cells from patients with colon cancer and was associated with silencing of this gene, but was not observed in sections of normal colon from healthy volunteers, suggesting that tumors with gene silencing due to epigenetic alteration may evolve from rare clones of methylated cells in normal epithelia[24]. Non-neoplastic epithelia from ESCC patients was significantly more methylated than in control esophageal epithelia from healthy volunteers in a panel of 14 promoter loci[25]. Data from our group also previously showed that *AKAP12* hypermethylation was significantly higher in NEcA than in NE or NEcS[26]. Similarly, in the current study, *ENG* hypermethylation was significantly higher in NEcS than in NEcA. Thus, our highly sensitive real-time qMSP approach allowed us to show that non-neoplastic esophageal epithelia from ESCC patients already exhibit low but abnormal levels of *ENG* promoter methylation. It can therefore be hypothesized that increased *ENG* methylation in normal esophageal cells extends their lifespan enough to put them at higher risk for future malignant evolution. Furthermore, mean *ENG* NMVs were significantly higher in ESCC than in EAC. These results also further imply that hypermethylation of *ENG* is an early and unique event, constituting a potentially powerful biomarker for early ESCC detection.

5-Aza-dC and its derivatives have demonstrated effectiveness as therapeutic anti-cancer drugs [27, 28]. In agreement with previous findings [16, 17], the current study found that methylation of *ENG* in ESCC cancer cell lines was associated with silenced or reduced expression of *ENG* mRNA. Treatment with 5-Aza-dC reactivated mRNA expression and reversed *ENG* hypermethylation in these cells. Restoration of *ENG* mRNA expression by demethylating agent treatment implies that DNA hypermethylation was responsible for silencing of *ENG*. These findings also suggest the possibility that epigenetic therapies may be useful in at least a subset of these patients. In addition, the known involvement of ENG in angiogenesis[6–10, 12] suggests the possibility that anti-angiogenesis therapy may be directed toward a subset of these patients, such as those whose tumors lack *ENG* methylation. Further studies are needed to address this possibility.

The current findings establish that hypermethylation of the *ENG* promoter, leading to gene silencing, is a common event in human ESCC. In addition, these results show that *ENG* hypermethylation occurs early during a subset of Barrett's-associated esophageal adenocarcinogenesis. Further large-scale prospective longitudinal validation studies of this biomarker as a potential predictive biomarker of ESCC are stimulated by these data.

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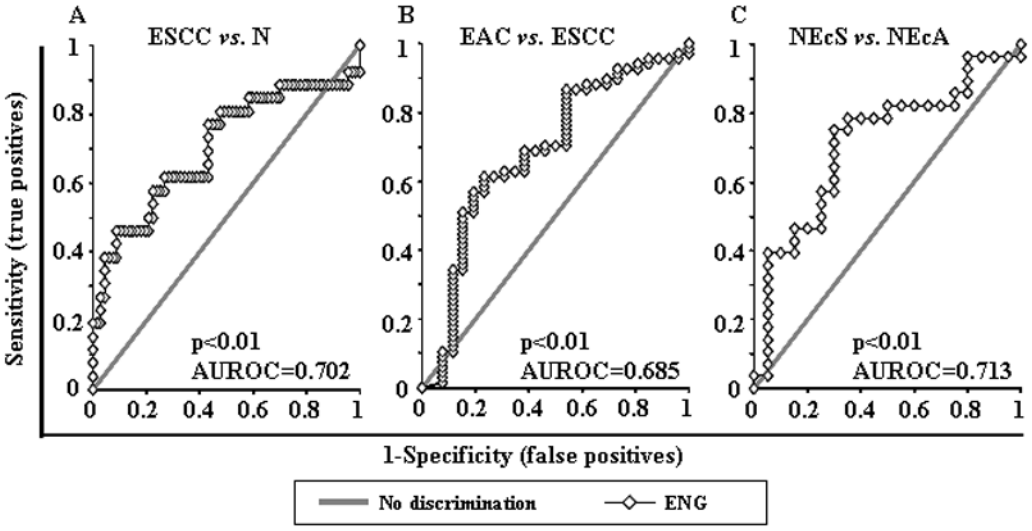

Figure 1. Receiver-operator characteristic (ROC) curve analysis of normalized methylation value (NMV)

ROC curve analysis of *ENG* NMVs of normal esophagus (N) *vs.* esophageal squamous cell carcinoma (ESCC) (**A**), ESCC *vs.* esophageal adenocarcinoma (EAC) (**B**) and normal esophageal specimens from ESCC patients (NEcS) *vs.* normal esophageal specimens from EAC patients (NEcA) (**C**). The area under the ROC curve (AUROC) conveys this biomarker's accuracy in distinguishing EAC from N and from ESCC in terms of its sensitivity and specificity.

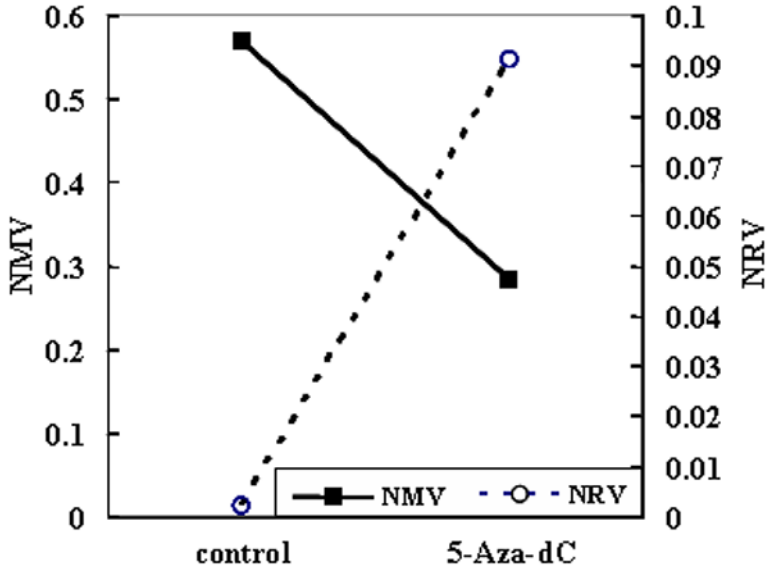

Figure 2. ENG methylation and mRNA expression in KYSE220 cells after treatment with 5-aza-2′-deoxycytidine (5-Aza-dC)

After 5-Aza-dC treatment, the NMV of *ENG* was diminished, while the normalized mRNA value (NRV) of *ENG* was increased.

Table 1

Methylation status of *ENG* in human esophageal tissues

			NMV		Methylation Status (cutoff 0.02)			
Histology	**Number of samples**	**Age (year) mean**	**mean**	**p[$]**	**Frequency**	**UM**	**M**	**p**
Normal esophagus	67	64.4	0.0137		11.9%	59	8	
NE	19	64.1	0.0117		5.3%	18	1	
NEcA	28	66.9	0.0115	> 0.05[¶]/< 0.05[*]	10.7%	25	3	> 0.05[¶]/[§]/> 0.05[*]/[§]
NEcS	20	61.3	0.0186	> 0.05[¶]	20.0%	16	4	> 0.05[¶]/[§]
Barrett's metaplasia	60	63.7	0.0123	> 0.05[**]	13.3%	52	8	> 0.05[**]/[†]
Dysplasia in Barrett's esophagus	40	65.3	0.0141	> 0.05[**]	25.0%	30	10	> 0.05[**]/[†]
EAC	67	65.1	0.0238	> 0.05[**]/< 0.01[#]	26.9%	32	18	< 0.05[**]/[†]/> 0.05[#]/[†]
ESCC	26	62.5	0.0450	< 0.01[**]	46.2%	14	12	< 0.001[**]/[†]

NE, normal esophagus from non-Barrett's/cancer patients; NEcA, NE from EAC patients; NEcS, NE from ESCC patients; EAC, esophageal adenocarcinoma; ESCC, esophageal squamous cell carcinoma; NMV: normalized methylation value; UM, unmethylated; M, methylated;

[$] Mann-Whitney U test;

[§] Fisher's exact test;

[†] Chi-square for independence test;

[¶] comparisons made to NE;

[*] comparisons made to NEcS;

[**] comparisons made to normal esophagus;

[#] comparisons made to ESCC.