Hypermethylation of the *AKAP12* Promoter is a Biomarker of Barrett's-Associated Esophageal Neoplastic Progression

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Abstract

The A-kinase anchoring protein 12 (AKAP12) is a kinase scaffold protein with known tumor suppressor activity. Recently, *AKAP12* promoter hypermethylation was reported in gastric and colorectal cancers. We examined *AKAP12* promoter hypermethylation using real-time methylation-specific PCR in 259 human esophageal tissues. *AKAP12* hypermethylation showed highly discriminative receiver-operator characteristic (ROC) curve profiles, clearly distinguishing esophageal adenocarcinoma (EAC) from esophageal squamous cell carcinoma and normal esophagus ($P < 0.0001$). *AKAP12*-normalized methylation values were significantly higher in Barrett's metaplasia (BE), dysplastic Barrett's, and EAC than in normal esophagus ($P < 0.0000001$). *AKAP12* hypermethylation frequency was zero in normal esophagus but increased early during neoplastic progression, to 38.9% in BE from patients with Barrett's alone, 52.5% in dysplastic Barrett's metaplasia, and 52.2% in EAC. *AKAP12* hypermethylation levels were significantly higher in normal esophageal epithelia from patients with EAC (mean = 0.00082) than in normal esophagi from patients without Barrett's or esophageal cancer (mean = 0.00007; $P = 0.006$). There was a significant correlation between *AKAP12* hypermethylation and BE segment length, a known clinical neoplastic progression risk factor. In contrast, only 2 (7.7%) of 26 esophageal squamous cell carcinomas exhibited *AKAP12* hypermethylation. Treatment of BIC and OE33 EAC cells with 5-aza-2′-deoxycytidine reduced *AKAP12* methylation and increased *AKAP12* mRNA expression. *AKAP12* mRNA levels in EACs with unmethylated *AKAP12* (mean = 0.1663) were higher than in EACs with methylated *AKAP12* (mean = 0.0668). We conclude that promoter hypermethylation of *AKAP12* is a common, tissue-specific event in human EAC, occurs early during Barrett's-associated esophageal neoplastic progression, and is a potential biomarker for the early detection of EAC. (Cancer Epidemiol Biomarkers Prev 2008;17(1):111–7)

Introduction

The A-kinase anchoring protein 12 (AKAP12; also known as Gravin, and its rodent orthologue src-suppressed C-kinase substrate, SSeCKS), a multivalent anchoring protein and an important regulator of the β2-adrenergic receptor complex, controls cell signaling, cell adhesion, mitogenesis and differentiation, and possesses tumor suppressor activity (1-5). The *AKAP12* gene maps to chromosome 6q24-25.2, a locus that frequently contains deletions in human cancers (2, 6-8). Downregulation of AKAP12 expression has been reported in various human cancers, including those of the breast, prostate, ovary, stomach, and colon (8-12). It is now well-established that promoter hypermethylation correlates with silencing of gene transcription in cancers (13), including esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC; refs. 14, 15). Furthermore, there is a growing body of evidence showing that abnormal methylation of DNA is an early event in carcinogenesis and could serve as an early detection biomarker in cancer (13). Recently, *AKAP12* promoter CpG island hypermethylation was reported in gastric and colorectal cancers, and it was shown that the demethylating agent 5-aza-2′-deoxycytidine (5-Aza-dC) reversed *AKAP12* promoter hypermethylation and restored *AKAP12* expression in colorectal and gastric cancer–derived cell lines (11, 12). Thus, it is clear that *AKAP12* hypermethylation is involved in tumorigenesis in the human digestive tract; however, it is not known whether or at what neoplastic stage this epigenetic alteration contributes to human esophageal tumorigenesis.

Esophageal cancer ranks sixth among cancers worldwide, with 400,000 new cases diagnosed each year (16). This malignancy exists in two principal forms, each

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possessing distinct pathologic characteristics: ESCC, which occurs at high frequencies in many developing countries, especially in Asia; and EAC, which is more prevalent in Western countries, with a rapid rate of increase in recent years (16). Although significant advances have been made in the treatment of esophageal cancers, these aggressive malignancies commonly present as locally advanced disease with a very poor prognosis (i.e., with ~14% 5-year survival; ref. 17). In order to improve outcome, it will be valuable to discover novel early detection biomarkers and targets for chemoprevention and therapy. We hypothesized that *AKAP12* might be inactivated via promoter hypermethylation in human esophageal cancers, and that this event occurred early in the genesis of EAC, making it a potentially useful early detection biomarker.

In the current study, we investigated whether and when promoter hypermethylation of the *AKAP12* gene was involved in esophageal tumorigenesis by performing real-time methylation-specific PCR in 259 endoscopic esophageal biopsy specimens of differing histologies. In addition, the effect of a DNA methyltransferase inhibitor, 5-Aza-dC, on reactivation of epigenetically silenced *AKAP12* was also studied in esophageal cancer cell lines. Our results indicate that promoter hypermethylation of *AKAP12* is a common event in EAC but not in ESCC, and occurs early during Barrett's-associated esophageal neoplastic progression.

Materials and Methods

Tissue Samples. The 259 specimens examined in the current study comprised 66 normal esophageal specimens [including 19 obtained from non-Barrett's/non–esophageal cancer patients (NE), 20 from ESCC patients (NEcS), and 27 from EAC patients (NEcA)], 60 non-dysplastic Barrett's metaplasias [BE, including 36 obtained from patients with Barrett's alone (Ba) and 24 from patients with Barrett's accompanied by EAC (Bt)],

Table 1. Clinicopathologic characteristics and methylation status of *AKAP12* in human esophageal tissues

Clinical characteristics*	No. of samples	Age (y) mean	NMV †		Methylation status (cutoff 0.05) ‡			
			Mean	*P*	Frequency (%)	UM	M	*P*
Histology								
Normal esophagus	66	64.3	0.00042		0	66	0	
NE	19	64.1	0.00007		0	19	0	
NEcA	27	66.7	0.00082	<0.05§,‖	0	27	0	
NEcS	20	61.3	0.00021		0	20	0	
Barrett's metaplasia	60	63.7	0.116	<0.01§,¶,**	48.3	31	29	
Barrett's from non-EAC patients	36	62.5	0.1122	<0.01§,¶,**	38.9	22	14	>0.05 ††
Barrett's from EAC patients	24	65.5	0.1216	<0.01§,¶,**	62.5	9	15	
Dysplasia in Barrett's esophagus	40	65.3	0.1344	<0.01§,¶,**	52.5	19	21	
Low-grade dysplasia	19	65.3	0.1505	<0.01§,¶,**	52.6	9	10	>0.05 ††
High-grade dysplasia	21	65.2	0.1199	<0.01§,¶,**	52.4	10	11	
EAC	67	65.1	0.1157	<0.01§,¶,**	52.2	32	35	<0.0001 ‡‡
ESCC	26	62.5	0.01	<0.05§,¶	7.7	24	2	
Barrett's segment								
Short-segment (<3 cm)	14	62.3	0.0543	<0.05§	28.6	10	4	>0.05 ‡‡
Long-segment (≥3 cm)	16	62.8	0.1879		56.3	7	9	
Stage of EAC patients								
I	7	63	0.081	>0.05§§	42.9	4	3	>0.05 ‡‡
II	15	65.2	0.145		53.3	7	8	
III	25	64.6	0.09		48	13	12	
IV	7	66.3	0.161		85.7	1	6	
Lymph node metastasis in EAC patients								
Negative	25	64.9	0.129	>0.05§	56	11	14	>0.05 ††
Positive	25	64.6	0.094		48	13	12	
Smoking status of EAC patients								
Never	6	58.5	0.083	>0.05§§	50	3	3	>0.05 ‡‡
Former	24	68.5	0.095		54.2	11	13	
Current	13	60.8	0.146		61.5	5	8	
Alcohol drinking status of EAC patients								
Never	16	65.3	0.0652	>0.05§§	50	8	8	>0.05 ‡‡
Former	15	63	0.1581		73.3	4	11	
Current	10	65.7	0.1156		40	6	4	

*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.
§ Student's *t* test.
‖ Comparisons made with NE or NEcS.
¶ Comparisons made with normal esophagus.
** Comparisons made with ESCC.
†† χ^2 for independence test.
‡‡ Fisher's exact test.
§§ Kruskal-Wallis test.

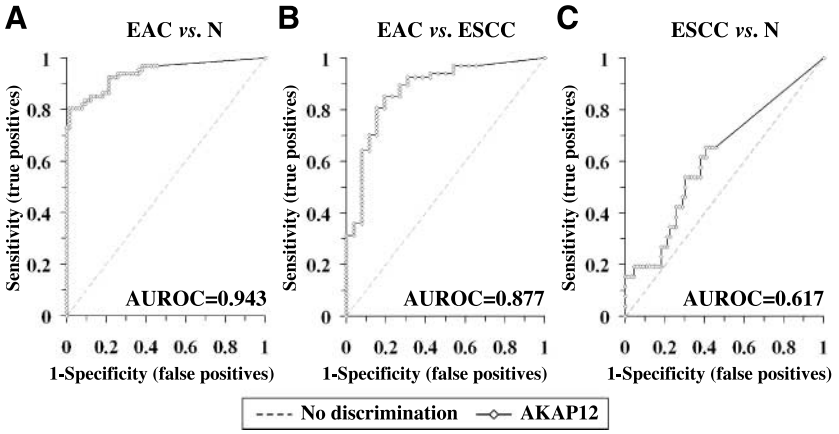

Figure 1. ROC curve analysis of NMV. ROC curve analysis of *AKAP12* NMVs of normal esophagus (N) versus EAC (**A**), ESCC versus EAC (**B**), and N versus ESCC (**C**). The area under the ROC curve (AUROC) conveys this biomarker's accuracy in distinguishing EAC from normal specimens and from ESCC in terms of its sensitivity and specificity.

40 dysplastic Barrett's specimens (including 19 low-grade and 21 high-grade dysplasias), 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 (15). 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 before DNA extraction. Clinicopathologic characteristics are summarized in Table 1.

Cell Lines. Three EAC (BIC, OE33, and SEG) and nine 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, 47.5% F12 supplemented with 5% fetal bovine serum.

DNA and RNA Extraction. Genomic DNA and total RNA were extracted from biopsies and cultured cells using a DNeasy Tissue Kit (Qiagen) and TRIzol reagent (Invitrogen), respectively. DNA and RNA 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 methylation-specific PCR, as described previously (12). The promoter methylation levels of *AKAP12* were determined by real-time quantitative methylation-specific PCR with the ABI 7700 Sequence Detection System (Applied Biosystems), using primers and probes as described previously (12). Normalized methylation value (NMV) was defined as follows: NMV = (*AKAP12-S / AKAP12-FM*) / (*ACTB-S /*

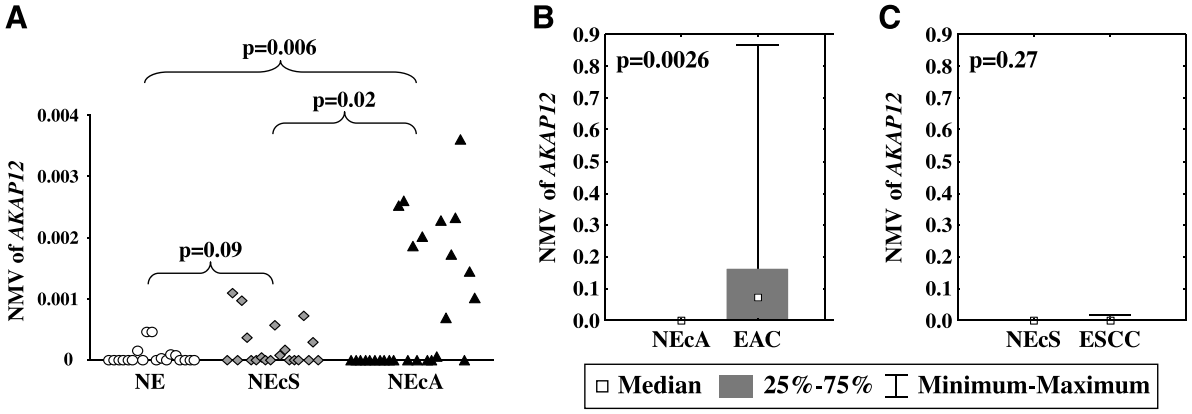

Figure 2. Methylation levels of *AKAP12* in histologically normal esophageal epithelium from patients with contrasting clinical status and in matched esophageal samples. **A.** *AKAP12* hypermethylation was apparent at relatively low levels, but it was significantly higher in normal esophageal epithelium from EAC patients (NEcA; mean, 0.00082) than in normal esophagus from non-Barrett's/non–cancer patients (NE; mean, 0.00007; $P = 0.006$), or normal esophagus from ESCC patients (NEcS; mean, 0.00021 and $P = 0.02$), although not in NEcS versus NE ($P = 0.09$; Student's t test). **B.** *AKAP12* NMVs in EAC (mean, 0.1241) were significantly higher than those in matching NEcA (mean, 0.0008; $P = 0.0026$, Student's paired t test). **C.** *AKAP12* NMVs in ESCC (mean, 0.0018) did not differ significantly from those in matching NEcS (mean, 0.0002; $P = 0.27$, Student's paired t test).

ACTB-FM), where *AKAP12-S* and *AKAP12-FM* represent *AKAP12* methylation levels in sample and fully methylated DNAs, respectively, whereas *ACTB-S* and *ACTB-FM* correspond to *β-actin* in sample and fully methylated DNAs, respectively.

Real-time Quantitative Reverse Transcription-PCR. To determine *AKAP12* mRNA levels, one-step real-time quantitative reverse transcription-PCR was performed using a Qiagen QuantiTect Probe RT-PCR Kit (Qiagen) and the ABI 7700 Sequence Detection System (Applied Biosystems). Primers and probe for *AKAP12* were as follows: *AKAP12*-forward, 5′-CGCCACCAAGCTCCTACA-3′; *AKAP12*-reverse, 5′-GCCATTTAGGTCACCCTCCTG-3′; and probe, 5′-AAGAATGGTCAGCTGTCCACCATCA-3′. β-Actin was used for normalization of data. Primers and probe for β-actin were the same as previously reported (12). A standard curve was generated using serial dilutions of qPCR Reference Total RNA (Clontech). 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 *AKAP12* inactivation was due to promoter hypermethylation in esophageal cancer, two esophageal cancer cell lines (BIC and OE33) were subjected to 5-Aza-dC (Sigma) treatment as previously described (18, 19). Briefly, 1×10^5 cells/mL were seeded onto a 100 mm dish and grown for 24 h. Then, 1 μL of 5 mmol/L 5-Aza-dC per mL of cells was added every 24 h for 4 days. DNAs and RNAs were harvested on day 4.

Data Analysis and Statistics. ROC curve analysis (20) was done using NMVs for the 67 EAC, 26 ESCC, and 66 normal specimens by Analyse-it software (version 1.71). Using this approach, the area under the ROC curve identified optimal sensitivity and specificity levels at which to distinguish normal from malignant esophageal tissues, yielding corresponding NMV thresholds defining methylation status of *AKAP12*. The threshold NMV value determined from this ROC curve was applied to determine the status of *AKAP12* methylation in all tissue types included in the present study. For all other statistical tests, Statistica (version 6.1; StatSoft, Inc.) was used. Differences with $P < 0.05$ were considered significant.

Results

***AKAP12* Promoter Hypermethylation in Esophageal Tissues.** Promoter hypermethylation of *AKAP12* was analyzed in 66 normal esophageal specimens (including 19 NE, 20 NEcS, and 27 NEcA), 60 BE (including 36 Ba and 24 Bt), 40 dysplastic Barrett's (including 19 low-grade and 21 high-grade dysplasias), 67 EAC, and 26 ESCC. *AKAP12* promoter hypermethylation showed highly discriminative ROC curve profiles and area under the ROC curves, clearly distinguishing EAC from both normal specimens and ESCC (Fig. 1A and B), but not ESCC from normal specimens (Fig. 1C).

The cutoff NMV for *AKAP12* (0.05) was identified from the ROC curve (EAC versus normal specimens) as maximizing both sensitivity and specificity. Mean NMV and frequency of *AKAP12* hypermethylation for each tissue type are shown in Table 1. NMVs of *AKAP12* were significantly higher in ESCC, EAC, dysplastic Barrett's, high-grade dysplasia, low-grade dysplasia, BE, Ba, and Bt than in normal specimens ($P < 0.05$, Student's *t* test). Moreover, the mean NMVs of *AKAP12* were significantly higher in NEcA (mean = 0.00082) than in NE (mean = 0.00007 and $P = 0.006$) or NEcS (mean = 0.00021 and $P = 0.02$), but not significantly higher in NEcS than in NE ($P = 0.09$, Student's *t* test; Table 1; Fig. 2A). The frequency of *AKAP12* hypermethylation was increased in

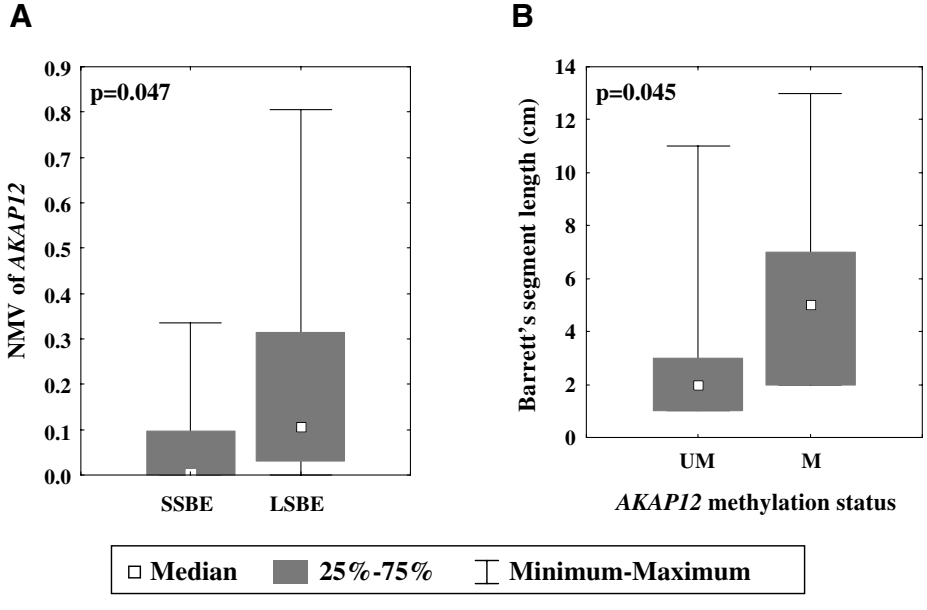

Figure 3. Correlation between Barrett's segment length and *AKAP12* hypermethylation. **A.** NMV of *AKAP12* was significantly higher in LSBE (mean, 0.1879) than in SSBE (mean, 0.0543; $P = 0.047$, Student's *t* test). **B.** Positive *AKAP12* hypermethylation status was significantly correlated with BE segment length ($P = 0.045$, Student's *t* test).

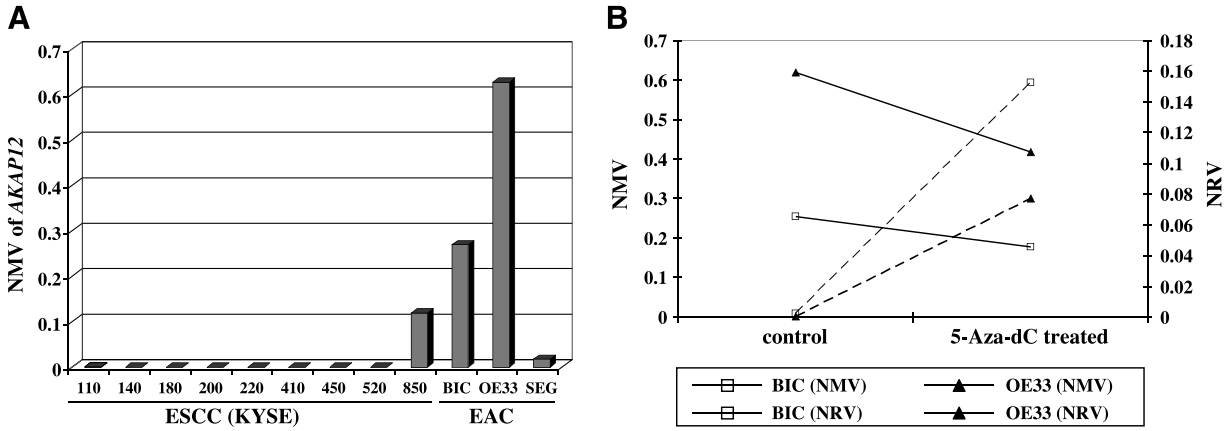

Figure 4. *AKAP12* methylation status and mRNA expression in esophageal cancer cell lines after treatment with 5-Aza-dC. **A.** One of nine ESCC and two of three EAC esophageal cancer cell lines showed high *AKAP12* methylation levels, above the threshold level of 0.05. **B.** BIC and OE33 EAC cells were subjected to 5-Aza-dC treatment. After 5-Aza-dC treatment, the NMV of *AKAP12* was diminished, whereas the normalized mRNA value (NRV) of *AKAP12* was increased in both cell lines.

Ba (38.9%), dysplastic Barrett's (52.5%), and EAC (52.2%) versus normal esophageal specimens (0%; $P < 0.0001$, $P < 0.0001$, and $P < 0.0001$, respectively, Fisher's exact test). Both *AKAP12* hypermethylation frequency and mean NMV were higher in Bt than in Ba, although these differences did not achieve statistical significance (62.5% versus 38.9%, $P = 0.073$; and 0.1216 versus 0.1122, $P = 0.82$, respectively). The mean *AKAP12* NMV of EAC (0.1241) was significantly higher than that of matching NEcA (0.0008) in 27 cases with corresponding NEcA and EAC ($P = 0.0026$, Student's paired t test; Fig. 2B). In contrast to EAC, only 2 (7.7%) of 26 ESCCs showed hypermethylation of *AKAP12*. There was no significant difference between tumor and normal tissue mean *AKAP12* NMV in 13 cases with matching ESCC (0.0018) and NEcS (0.0002; $P = 0.27$, Student's paired t test; Fig. 2C).

Both *AKAP12* hypermethylation frequency and mean NMV were significantly higher in EAC than in ESCC (52.2% versus 7.7%, $P < 0.0001$; and 0.1157 versus 0.01, $P = 0.0013$, respectively), as well as in dysplastic Barrett's versus ESCC (52.5% versus 7.7%, $P = 0.0002$; and 0.1344 versus 0.01, $P = 0.0013$, respectively) and in BE versus ESCC (48.3% versus 7.7%, $P = 0.0002$; and 0.116 versus 0.01, $P = 0.0008$; Table 1).

According to generally accepted criteria (21), BE was defined as long-segment (LSBE) if it was $\geq$3 cm in length, or short-segment (SSBE) if <3 cm. The mean NMV of *AKAP12* was significantly higher in LSBE (0.1879) than in SSBE (0.0543; $P = 0.047$, Student's t test; Table 1; Fig. 3A). Similarly, the segment lengths of BEs with methylated *AKAP12* promoters (mean = 5.62 cm) were significantly longer than the segment lengths of BEs with unmethylated *AKAP12* promoters (mean = 3.18 cm; $P = 0.045$, Student's t test; Fig. 3B), and the frequency of *AKAP12* hypermethylation was higher in LSBE than in SSBE (56.3% versus 28.6%; $P = 0.16$, Fisher's exact test; Table 1).

No significant associations were observed between *AKAP12* promoter hypermethylation and patient age (data not shown), survival (data not shown), tumor stage or lymph node metastasis (Table 1), and smoking or alcohol consumption (Table 1).

***AKAP12* Methylation and mRNA Levels in Esophageal Cancer Cell Lines after 5-Aza-dC Treatment.** One of nine ESCC and two of three EAC esophageal cancer cell lines showed high *AKAP12* methylation levels, above the threshold level of 0.05 (Fig. 4A). BIC and OE33 cells were subjected to 5-Aza-dC treatment. After 5-Aza-dC treatment, the NMV of *AKAP12* was diminished and the mRNA level of *AKAP12* was increased in both of these cell lines (Fig. 4B).

Correlation between Hypermethylation and mRNA Expression of *AKAP12* in EAC. To further elucidate the relationship between DNA hypermethylation and mRNA expression of *AKAP12*, we determined *AKAP12* mRNA levels in 25 EAC samples using real-time reverse transcription-PCR. *AKAP12* mRNA levels of EACs with unmethylated *AKAP12* promoters (mean = 0.1663) were higher than those of EACs with methylated *AKAP12* promoters (mean = 0.0668), with this difference barely failing to achieve statistical significance ($P = 0.057$, Student's t test; Fig. 5).

Discussion

In the current study, we systematically investigated hypermethylation of the *AKAP12* gene promoter in primary human esophageal lesions of contrasting histologic types and grades. Our results show that *AKAP12* promoter hypermethylation occurs frequently in human EAC, but not in ESCC. In addition, our data show that *AKAP12* hypermethylation increases early during esophageal adenocarcinogenesis, from 0% in normal specimens to 38.9% in Ba, 52.5% in dysplastic Barrett's, and 52.2% in EAC. Interestingly, even in nonneoplastic Barrett's mucosa, *AKAP12* seemed to serve as a biomarker of more ominous disease lurking nearby: *AKAP12* was

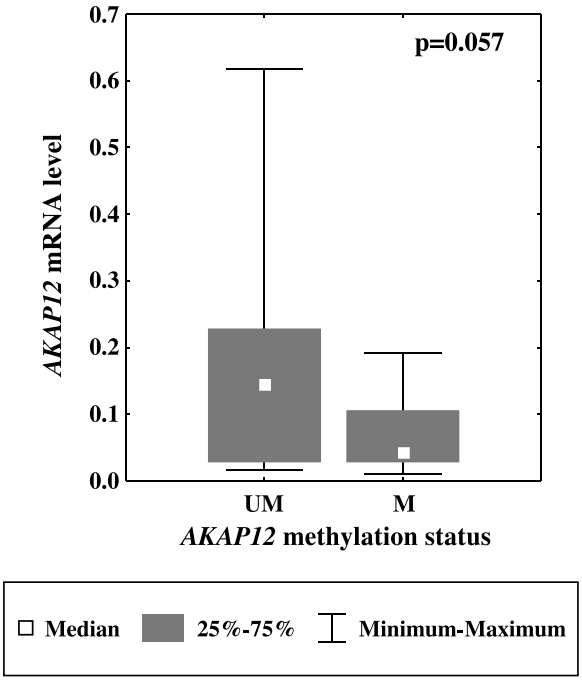

Figure 5. Correlation between *AKAP12* methylation status and mRNA expression levels in primary EAC. *AKAP12* mRNA levels in EACs with unmethylated *AKAP12* promoters (mean, 0.1663) were higher than those in EACs with methylated *AKAP12* (mean, 0.0668), with this difference barely failing to achieve statistical significance ($P = 0.057$, Student's t test).

hypermethylated more frequently in Barrett's mucosa with than without accompanying tumor, and methylation levels of *AKAP12* were higher in Barrett's with than without accompanying tumor. Furthermore, *AKAP12* methylation levels in EAC were significantly higher than in matching NEcA from the same patient. Taken together, these results imply that hypermethylation of *AKAP12* occurs early in some 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 was provided by ROC curves, which clearly distinguished EAC from ESCC but not ESCC from normal specimens. Similarly, support for tissue specificity was evident from the finding that both *AKAP12* hypermethylation frequency and mean *AKAP12* NMV were significantly higher in EAC than in ESCC. Thus, *AKAP12* hypermethylation seems to constitute a critical event unique to human EAC.

Despite conflicting reports regarding the length of Barrett's esophagus as a predictive factor in BE progression, it is likely that the length of the Barrett's segment is an important predictor of neoplastic progression. Rudolph et al. showed that segment length was not related to cancer risk in a prospective cohort study of 309 patients with Barrett's esophagus followed in the Seattle Barrett's Esophagus Project ($P > 0.2$); however, when patients with high-grade dysplasia 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- to 3.8-fold; ref. 21). Weston et al. reported significant differences in the frequency of both dysplasia and EAC between SSBE and LSBE, at 8.1% versus 24.4% for dysplasia ($P < 0.0001$) and 0% versus 15.4% for EAC ($P < 0.0005$; ref. 22). Hirota et al. 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 (23). More recently, Hage et al. reported a significantly increased risk of progression to high-grade dysplasia or EAC with LSBE after a mean follow-up of 12.7 years (24). Interestingly, in the current study, *AKAP12* methylation showed a strong correlation with BE segment length. The mean NMV of *AKAP12* was significantly higher in LSBE than in SSBE. Similarly, the length of the BE segment in specimens with methylated *AKAP12* promoters was significantly greater than in samples with unmethylated *AKAP12* promoters. Thus, *AKAP12* hypermethylation may constitute a molecular correlate of BE segment length, as well as a harbinger of nearby neoplastic disease.

Previous studies have shown the importance of hypermethylation of gene promoters in histologically normal tissue as this event relates to the initiation of carcinoma (25-30). By methylation-specific *in situ* PCR and *in situ* RNA and protein analysis, methylation of the *MLH1* promoter was observed in small foci of normal colonic epithelial cells from patients with colon cancer and associated silencing of this gene, but not 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 epithelium (29).

Based on a panel of 14 promoter loci, it has recently been reported that nonneoplastic epithelium from patients with ESCC is significantly more methylated than control esophageal epithelium from healthy volunteers, and that this may contribute to progression in the dysplasia-carcinoma sequence in ESCC (30). Similarly, in the current study, *AKAP12* hypermethylation was significantly higher in NEcA than in NE or NEcS, whereas no such trend was observed for NEcS versus NE. Thus, our highly sensitive real-time quantitative methylation-specific PCR approach allowed this study to show that nonneoplastic esophageal epithelia from patients with EAC already exhibit a low but abnormal level of *AKAP12* promoter methylation. It can be hypothesized that increased *AKAP12* methylation in normal esophageal cells extends their lifespan and puts them at higher risk for future malignant transformation. These results further imply that hypermethylation of *AKAP12* is an early and unique event, constituting a potentially powerful biomarker for early EAC detection.

In accordance with previous findings (11, 12), we observed that methylation of *AKAP12* in EAC cell lines was associated with silenced or reduced expression of *AKAP12* mRNA. Treatment with 5-Aza-dC led to increased mRNA expression and concomitant reduced *AKAP12* methylation in these cells. Restoration of *AKAP12* mRNA expression by demethylating agent treatment implies that DNA hypermethylation was responsible for silencing *AKAP12*. The involvement

of CpG island hypermethylation in the silencing of *AKAP12* is also supported by our observation that *AKAP12* mRNA levels in EACs with unmethylated *AKAP12* promoters were markedly higher than those in EACs with methylated *AKAP12*.

5-Aza-dC and its derivatives have shown effectiveness as therapeutic anticancer drugs (31, 32), thus, *AKAP12* represents a novel potential target for molecularly based therapies involving demethylation in human EACs.

The current study indicates that hypermethylation of the *AKAP12* promoter, leading to gene silencing, is a common event in human EACs, occurring early during Barrett's-associated esophageal adenocarcinogenesis. In addition, *AKAP12* hypermethylation is uncommon in human ESCC, and thus represents a cell type–specific biomarker for EAC. Further large-scale prospective longitudinal validation studies of this epigenetic event as a potential predictive biomarker of EAC are stimulated by these data. These results also provide motivation for further research into potential applications of selected DNA methyltransferase or other indirect inhibitors of methylation (such as histone deacetylase inhibitors) for the prevention and treatment of esophageal cancer.

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