

Hypermethylation of Tachykinin-1 Is a Potential Biomarker in Human Esophageal Cancer

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Abstract Purpose: Our aim was to investigate whether and at what stage hypermethylation of the *tachykinin-1* (*TAC1*) gene is associated with human esophageal neoplastic transformation.

Experimental Design: *TAC1* promoter hypermethylation was examined by real-time methylation-specific PCR in 258 human esophageal specimens and 126 plasma samples from patients or tissues at various stages of neoplastic evolution.

Results: *TAC1* hypermethylation in tissue samples showed highly discriminative receiver-operator characteristic curve profiles, clearly distinguishing esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) from normal esophagus ($P < 0.0001$). Both frequencies and normalized methylation values of *TAC1* tissue methylation were significantly higher in Barrett's metaplasia (BE), dysplastic Barrett's esophagus, EAC, and ESCC than in normal esophagus ($P < 0.01$). The frequency of *TAC1* hypermethylation increased dramatically and early during neoplastic progression, from 7.5% in normal esophagus to 55.6% in BE from patients with Barrett's metaplasia alone, 57.5% in dysplastic Barrett's esophagus, and 61.2% in EAC. There was a significant relationship between *TAC1* hypermethylation and BE segment length, a known clinical risk factor for neoplastic progression. Twelve (50%) of 24 ESCC exhibited *TAC1* hypermethylation. Overall patient survival correlated significantly with *TAC1* methylation status in ESCC patients (mean survival, 22 versus 110 months; $P = 0.0102$, log-rank test), but not in EAC patients. Both mean normalized methylation values and frequency of *TAC1* hypermethylation in plasma samples were significantly higher in EAC patients than in control subjects. Treatment of KYSE220 ESCC and BIC EAC cells with 5-aza-2'-deoxycytidine reduced *TAC1* methylation and increased *TAC1* mRNA expression.

Conclusions: *TAC1* promoter hypermethylation is a common event in both major histologic types of human esophageal carcinoma, occurs early, correlates with other progression risk factors in esophageal adenocarcinogenesis, and is a tissue biomarker of a poor prognosis in ESCC. Circulating methylated *TAC1* promoter DNA also offers potential as a biomarker for the diagnosis of EAC.

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The tachykinin-1 (*TAC1*) gene has been mapped to chromosome 7q21-22 (1), a locus that frequently undergoes loss of heterozygosity in human cancers, including esophageal adenocarcinoma (EAC; refs. 2, 3). However, loss of heterozygosity is only one mechanism of gene inactivation; other common mechanisms include point mutation, homozygous deletion, and promoter methylation (4). It is now well established that promoter methylation correlates with transcriptional silencing in cancers (5), including esophageal squamous cell cancer (ESCC) and EAC (6, 7). Recently, data from our laboratory showed that the *TAC1* promoter was methylated in 16 (47%) of 34 human colon cancers, and that the demethylating agent 5-aza-2'-deoxycytidine (5-Aza-dC) reversed *TAC1* promoter hypermethylation and restored *TAC1* mRNA expression in colon cancer cell lines (8).

Esophageal cancer ranks sixth in incidence among cancers worldwide, with 400,000 new cases being diagnosed per year

Table 1. Clinicopathologic characteristics and methylation status of *TAC1* in human esophageal tissues

Clinical characteristics	No. samples	Age (y), mean	NMV		Methylation status (cutoff 0.12)			
			Mean	P*	Frequency (%)	UM	M	P†
Barrett's segment								
SSBE (<3 cm)	14	62.3	0.1186	<0.05	28.6	10	4	<0.05‡
LSBE (≥3 cm)	16	62.8	0.3391		87.5	2	14	
Histology								
Normal esophagus	67	64.4	0.0506		7.5	62	5	
BE	60	63.7	0.2212	<0.05§	63.3	22	38	
Ba	36	62.5	0.2145	<0.05§	55.6	16	20	>0.05
Bt	24	65.5	0.2313	<0.05§	75.0	6	18	
Dysplasia in Barrett's esophagus	40	65.3	0.1967	<0.05§	57.5	17	23	
Low-grade dysplasia	19	65.3	0.2361	<0.05§	63.2	7	12	>0.05
High-grade dysplasia	21	65.2	0.161	<0.05§	52.4	10	11	
EAC	67	65.1	0.227	<0.05§	61.2	26	41	
Well differentiation	10	66.2	0.2625	<0.05§	60.0	4	6	>0.05‡
Moderate differentiation	24	66.1	0.262	<0.05§	75.0	6	18	
Poor differentiation	22	65.5	0.211	<0.05§	50.0	11	11	
Unknown	11	61	0.1505	<0.05§	54.5	5	6	
ESCC	24	62.1	0.1896	<0.05§	50.0	12	12	
Well differentiation	3	61.7	0.0883	<0.05§	33.3	2	1	>0.05‡
Moderate differentiation	10	62.3	0.1362	<0.05§	50.0	5	5	
Poor differentiation	4	63.8	0.1746	<0.05§	50.0	2	2	
Unknown	7	61.1	0.3178	<0.05§	57.1	3	4	
Stage of EAC patients								
I	7	63	0.3038		71.4	2	5	>0.05‡
II	15	65.2	0.2231		53.3	7	8	
III	25	64.6	0.1845		56.0	11	14	
IV	7	66.3	0.2649		71.4	2	5	
Lymph node metastasis in EAC patients								
Negative	25	64.9	0.2451	>0.05	56.0	11	14	>0.05
Positive	25	64.6	0.1957		56.0	11	14	
Smoking status of EAC patients								
Never	6	58.5	0.3761		83.3	1	5	>0.05‡
Former	24	68.5	0.1961		62.5	9	15	
Current	13	60.8	0.2464		46.2	7	6	
Alcohol drinking status of EAC patients								
Never	16	65.3	0.2111		62.5	6	10	>0.05‡
Former	15	63	0.2276		53.3	7	8	
Current	10	65.7	0.2542		60.0	4	6	

Abbreviations: UM, unmethylated; M, methylated.

*Student's *t* test.

† Excludes cases with unknown status.

‡ Fisher's exact test.

§ Comparisons made with normal esophagus.

|| χ^2 for independence test.

(9). This malignancy exists in two principal forms, each 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 (9). 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 (~14% 5-year survival; ref. 10). Therefore, to improve outcome, it is important to discover novel early detection biomarkers and new targets for chemoprevention and therapy. There is a growing body of evidence showing that abnormal methylation of DNA is an early event in carcinogenesis and can serve as an early cancer detection or progression biomarker (5). Based on these findings, we hypothesized that *TAC1* expression was silenced via promoter hypermethylation in human esophageal cancers, that this was an early event in the genesis

of EAC, and that *TAC1* hypermethylation could serve as a potential early detection biomarker for EAC.

To test these hypotheses, we studied methylation of the *TAC1* gene promoter by real-time quantitative methylation-specific PCR in 258 human endoscopic esophageal specimens and 126 plasma samples from patients or tissues at various stages of neoplastic evolution. The effect of a DNA methyltransferase inhibitor, 5-Aza-dC, on the reexpression of natively methylated and epigenetically silenced *TAC1* was also studied in esophageal cancer cell lines. Our results show that *TAC1* is silenced by promoter hypermethylation, and that this hypermethylation is a common event in both major histologic types of human esophageal carcinoma, occurs early during Barrett's-associated esophageal neoplastic progression, and is significantly associated with a poor prognosis in ESCC patients. In addition, our data show that circulating hypermethylated *TAC1* DNA is associated with the presence of EAC.

Materials and Methods

Tissue and plasma samples. In the current study, endoscopic biopsies of 67 normal esophagi, 60 Barrett's metaplasias without dysplasia [BE, including 36 obtained from patients with Barrett's only (Ba) and 24 from patients with Barrett's accompanied by EAC (Bt)], 40 dysplasias occurring in BE (including 19 low grade and 21 high grade), 67 EAC, and 24 ESCC were obtained from 195 patients. All normal esophageal specimens were located at least 7 cm away from any BE or other esophageal pathology. In addition, plasma samples were obtained from 35 age- and gender-matched noncancer/non-Barrett's control subjects, 10 additional patients with Ba, 20 with dysplastic Barrett's esophagus, and 61 with EAC. Among these specimens, matched biopsy and plasma samples were available from 17 control subjects, 3 patients with Ba, 8 with dysplastic Barrett's esophagus (five low grade and three high grade), and 32 with EAC. EAC was staged according to the sixth edition of the tumor-node-metastasis staging system (11). 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 done. Biopsies were obtained using a standardized biopsy protocol, as previously described (7). Research tissues were taken from grossly apparent Barrett's epithelium or from mass lesions in patients manifesting these changes at endoscopic examination, and histology was confirmed using parallel immediately adjacent aliquots obtained at endoscopy. Biopsy and plasma specimens were stored in liquid nitrogen or at -80°C , respectively, before DNA extraction. Clinicopathologic characteristics are summarized in Tables 1 and 2 for tissue and plasma specimens, respectively.

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% 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). Freely circulating DNA in plasma was isolated as previously described (12).

Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen). 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 quantitative methylation-specific PCR, as described previously (8). Promoter methylation levels of TAC1 were determined using quantitative methylation-specific PCR with the ABI 7900 Sequence Detection System (Applied Biosystems), using primers and probes as described previously (8). A standard curve was generated using serial dilutions of CpGenome Universal Methylated DNA (Chemicon). The normalized methylation value (NMV) was defined as follows: $\text{NMV} = (\text{TAC1-S}/\text{TAC1-FM})/(\text{ACTB-S}/\text{ACTB-FM})$, where TAC1-S and TAC1-FM represent TAC1 methylation levels in the sample and universal methylated DNAs, respectively, while ACTB-S and ACTB-FM correspond to β -actin in the sample and universal methylated DNAs, respectively.

Real-time quantitative reverse-transcription PCR. To determine TAC1 mRNA levels, one-step real-time quantitative reverse transcription-PCR was done using a Qiagen QuantiTect Probe reverse transcription-PCR kit (Qiagen) and the ABI 7900 Sequence Detection System (Applied Biosystems). Primers and probes were the same as previously reported (8). β -Actin was used to normalize data. A standard curve was generated using serial dilutions of qPCR Reference Total RNA (Clontech). The normalized mRNA value was calculated according to the following formula for relative expression of target mRNA: $(\text{TarS}/\text{TarC}) / (\text{ACTB-S}/\text{ACTB-C})$, where TarS and TarC represent levels of mRNA expression for the target gene in the sample and control mRNAs, respectively, while ACTB-S and ACTB-C correspond to amplified β -Actin levels in the sample and control mRNAs, respectively.

5-Aza-dC treatment of esophageal cancer cell lines. To determine whether TAC1 inactivation was due to promoter hypermethylation in esophageal cancer, two esophageal cancer cell lines (KYSE220 and BIC) were subjected to 5-Aza-dC (Sigma) treatment as previously described (13, 14). 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 milliliter of cells was added every 24 h for 6 days. DNAs and RNAs were harvested on day 6.

Data analysis and statistics. Receiver-operator characteristic (ROC) curve analysis (15) was done using NMVs for the 67 EAC, 24 ESCC, and 67 normal esophagi by Analyse-it software (version 1.71, Analyse-it

Table 2. TAC1 hypermethylation in 126 plasma samples from patients with contrasting clinical status

Clinical status of patient	No. samples	Age (y) mean	NMV		Methylation status (cutoff 0.12)			
			Mean	P	Frequency (%)	UM	M	P
Control subject	35	65	0.0336		8.6	32	3	<0.05*†
BE	10	61	0.0046	<0.05 ††	0.0	10	0	
Dysplastic Barrett's esophagus	20	64.5	0.0018	<0.01 ††	0.0	20	0	<0.01*†
Low-grade dysplasia	10	62.4	0.0016	<0.01 ††	0.0	10	0	
High-grade dysplasia	10	66.6	0.002	<0.01 ††	0.0	10	0	
EAC	61	64.2	0.2237	<0.05§	29.5	43	18	
Stage of EAC patients								
I	3	65.3	0.6469	>0.05	33.3	2	1	
II	7	60.7	0.5139		57.1	3	4	
III	15	64.4	0.1595		26.7	11	4	
IV	7	67.1	0.3584		42.9	4	3	
Lymph node metastasis in EAC patients								
Negative	11	63.7	0.4449	>0.05 †	56.0	6	5	>0.05*
Positive	15	65.1	0.1596		56.0	11	4	

*Fisher's exact test.

† Comparisons made with EAC patients.

‡ Mann-Whitney U test.

§ Student's t test (comparisons made with normal esophagus).

|| Kruskal-Wallis test.

Software). Using this approach, the area under the ROC curve identified optimal sensitivity and specificity levels at which to distinguish normal from malignant esophageal tissues, and corresponding NMV thresholds were calculated for *TAC1*. The cutoff value determined from this ROC curve was applied to determine the frequency of *TAC1* methylation in each tissue type included in the present study. For all other tests, Statistica (version 6.1; StatSoft) was used. Differences with $P < 0.05$ were deemed significant.

Results

***TAC1* promoter hypermethylation in different esophageal tissues.** *TAC1* promoter hypermethylation was analyzed in 24 ESCC and 67 EAC, 40 dysplastic Barrett's esophagi (19 low grade and 21 high grade), 60 BE (36 Ba and 24 Bt), and 67 normal esophagi. *TAC1* promoter hypermethylation showed highly discriminative ROC curve profiles, which clearly distinguished both ESCC ($P < 0.0001$) and EAC ($P < 0.0001$) from normal esophagus. ROC curves with corresponding area under the ROC curves for *TAC1* of ESCC versus normal esophagus, EAC versus normal esophagus, and both types of esophageal tumors versus normal esophagus are shown in Fig. 1.

The tissue cutoff NMV for *TAC1* (0.12) was chosen from the ROC curve to achieve a high sensitivity while still keeping specificity above 90%. Mean NMV and frequency of *TAC1* hypermethylation for each tissue type are shown in Table 1. The NMV of *TAC1* was significantly higher in ESCC, EAC, dysplastic Barrett's esophagus (high grade and low grade), Ba, Bt, and BE than in normal esophagus ($P < 0.0000001$, Student's *t* test). Similarly, increased frequencies of *TAC1* hypermethylation were observed in Ba (55.6%), dysplastic Barrett's esophagus (57.5%), and EAC (61.2%) relative to normal esophagus (7.5%), yielding a sensitivity of 61.2% (41 of 67) and a specificity of 92.5% (62 of 67) for the diagnosis of EAC. Both *TAC1* hypermethylation frequency and mean NMV were higher in Bt than in Ba (75% versus 55.6% and 0.2313 versus 0.2145, respectively). Among 15 cases with corresponding normal esophagus, BE, and EAC, one (no. 2) was unmethylated, three (nos. 1, 3 and 13) were methylated only in EAC, three (nos. 5, 16, and 17) were methylated only in BE, and the remaining eight were methylated in both BE and EAC (Fig. 2A). Among 41 cases with corresponding normal esophagus and esophageal tumors, four (100%) of four cases (nos. 22, 23, 33, and 36) showing methylation in normal esophagus were also methylated in corresponding tumor (Fig. 2B), and the *TAC1* NMVs of esophageal tumors (mean 0.209) were significantly higher than those of corresponding normal esophagus (mean 0.056; $P < 0.0000001$, Student's paired *t* test; Fig. 2C). Twelve (50%) of 24 ESCC exhibited *TAC1* hypermethylation.

BE was defined as long segment (LSBE) if it was ≥ 3 cm in length, or short segment (SSBE) if < 3 cm, according to generally accepted criteria (16). The mean NMV of *TAC1* was significantly higher in LSBE (mean 0.339) than in SSBE (mean 0.119; $P = 0.00544$, Student's *t* test, Fig. 3A). Similarly, the segment lengths of BEs with hypermethylated *TAC1* promoters were significantly longer than the segment lengths of BEs with unmethylated promoters (mean 5.56 cm versus 2.25 cm; $P = 0.00256$, Student's *t* test; Fig. 3B), and the frequency of *TAC1* hypermethylation was significantly higher in LSBE than in SSBE (87.5% versus 28.6%; $P = 0.0022$, Fisher's exact test; Table 1).

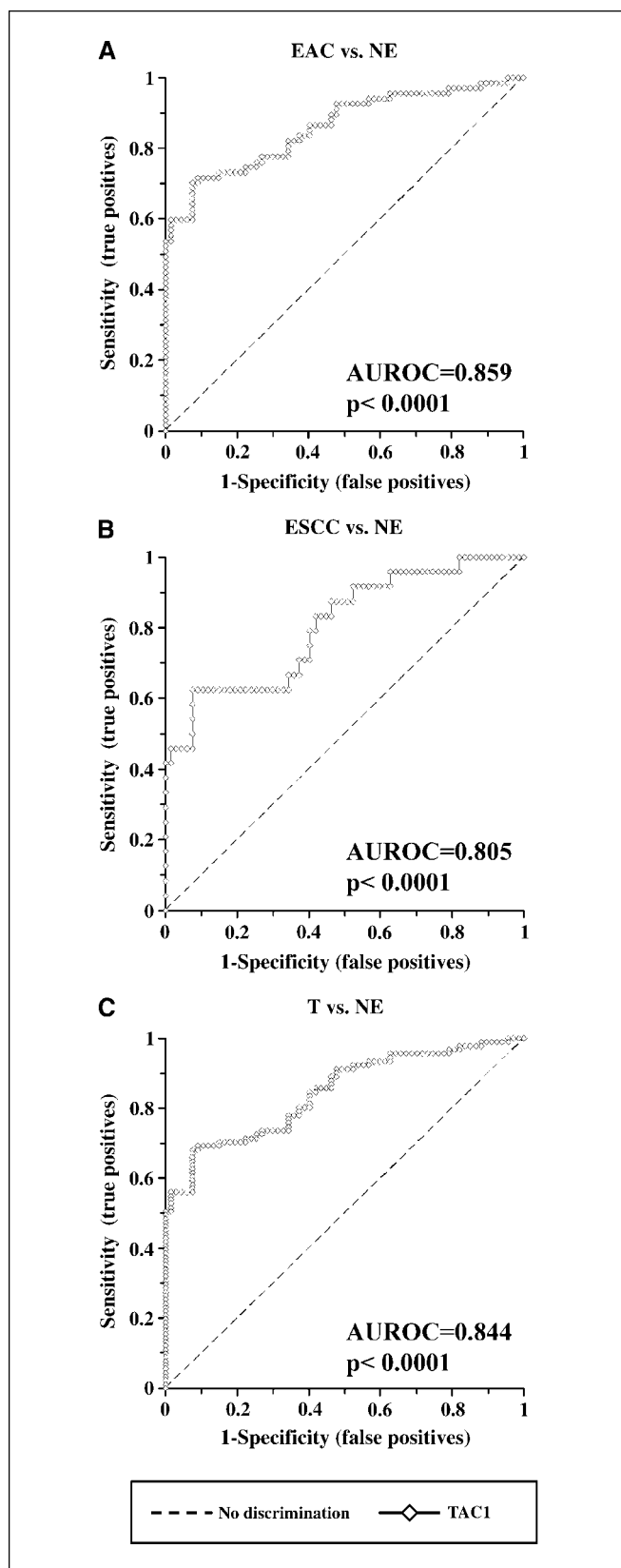
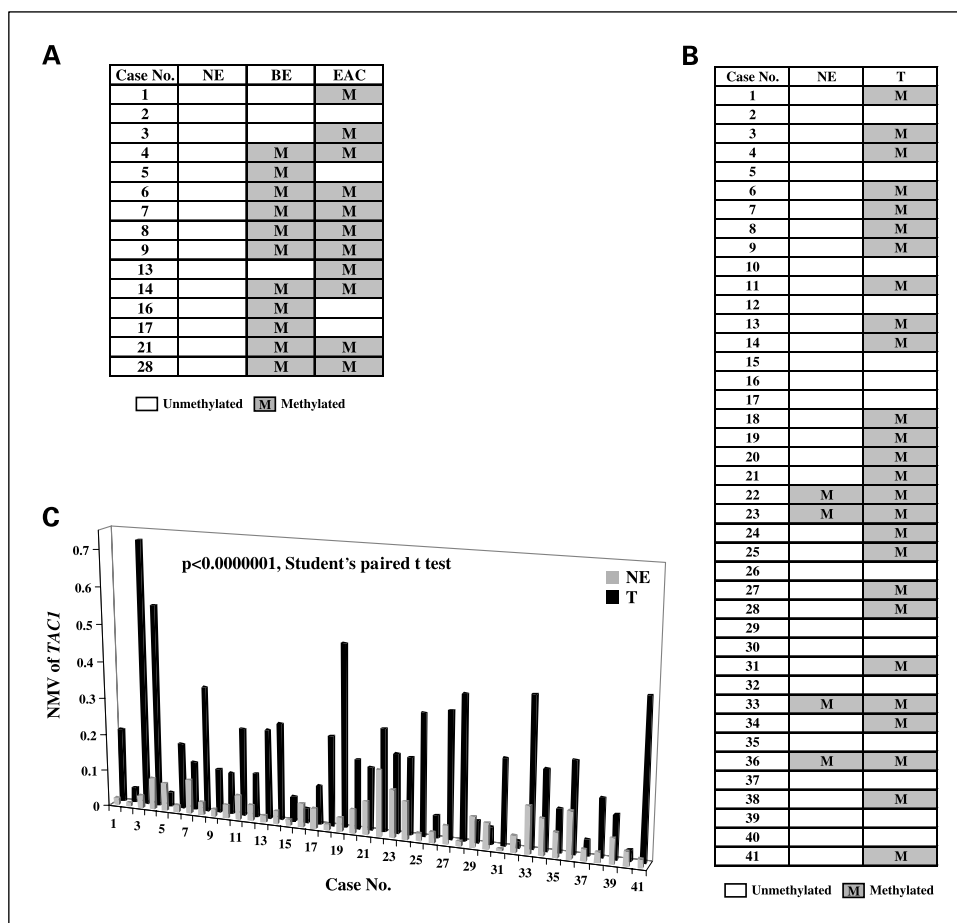


Fig. 1. ROC curve analysis of NMV. A, normal esophagus (NE) versus EAC. B, normal esophagus versus ESCC. C, normal esophagus versus both types of esophageal tumors (T). The area under the ROC curve (AUROC) for *TAC1* hypermethylation conveys its accuracy in distinguishing normal esophagus from EAC, ESCC, and esophageal tumor in terms of sensitivity and specificity.

Fig. 2. Methylation status of *TAC1* in matched esophageal tissue samples. **A**, among 15 cases with matched normal esophagus, BE, and EAC, one case (2) was unmethylated in all tissues, three (1, 3 and 13) were methylated (M) only in EAC, three (5, 16, and 17) were methylated only in BE, and the remaining eight were methylated in both BE and EAC. **B**, among 41 cases with matched normal esophagus and EAC, four of four cases (22, 23, 33, and 36) showing methylation in normal esophagus were also methylated in tumor. **C**, *TAC1* NMVs of EAC (mean 0.209) were significantly higher than those of matched normal esophagi (mean 0.056; $P < 0.0000001$, Student's paired *t* test).



Overall patient survival correlated with *TAC1* methylation status in ESCC patients, but not in EAC patients (Fig. 3C and D). ESCC patients with hypermethylation of *TAC1* (defined by a *TAC1* NMV higher than the cutoff value of 0.12) had significantly shorter survivals than did patients without *TAC1* hypermethylation (mean survival, 22 months versus 110 months; $P = 0.0102$, log-rank test; Fig. 3C).

No significant associations were observed between *TAC1* promoter hypermethylation in tissues and patient age (data not shown), smoking or alcohol consumption status, tumor stage or lymph node metastasis, tumor differentiation, or histologic type of esophageal carcinoma (EAC versus ESCC; Table 1).

***TAC1* hypermethylation in freely circulating plasma DNA from patients of contrasting clinical statuses.** To further elucidate the potential value of *TAC1* hypermethylation as a biomarker, we measured *TAC1* methylation levels in 126 plasma samples from 35 control subjects, 10 additional patients with Ba, 20 with dysplastic Barrett's esophagus, and 61 with EAC. The cutoff NMV (0.12) for dichotomization of plasma *TAC1* data was chosen from the ROC curve using the following criteria: $\leq 10\%$ of plasma samples from control subjects hypermethylated and $>20\%$ of plasma samples from EAC patients hypermethylated. We chose achieving 90% specificity as our top priority, based on the following reasoning: Additional true positives diagnosed by plasma methylated *TAC1* levels represent new, potentially life-saving, usually asymptomatic diagnoses that would not have been made without the plasma assay.

Thus, any true positive cases diagnosed in this manner constitute an improvement over current management. In contrast, false-positive diagnoses from plasma methylated *TAC1* levels represent a potential detriment because they would result in unnecessary endoscopic procedures, needlessly increase costs, and lead to complications that would not have occurred without the plasma assay. Therefore, we considered it our highest priority to minimize false positives (i.e., to maximize specificity), even at the cost of diminishing true positives (i.e., sensitivity). Mean *TAC1* hypermethylation NMVs and frequencies using the cutoff NMV of 0.12 in plasma samples are shown in Table 2. The *TAC1* NMV in plasma was significantly higher in EAC patients (mean 0.2237) than in control subjects (mean 0.0336; $P < 0.05$, Student's *t* test), patients with Ba (mean 0.0046; $P < 0.05$, Mann-Whitney *U* test), or patients with dysplastic Barrett's esophagus (mean 0.0018; $P < 0.01$, Mann-Whitney *U* test). Similarly, *TAC1* hypermethylation frequencies in plasma samples were significantly higher in EAC patients (29.5%) than in control subjects (8.6%; $P < 0.05$, Fisher's exact test) or patients with dysplastic Barrett's esophagus (0%; $P < 0.01$, Fisher's exact test), but not in patients with Ba (0%; $P = 0.056$, Fisher's exact test), yielding a sensitivity of 29.5% (18 of 61) and a specificity of 91.4% (32 of 35) for the diagnosis of EAC.

The methylation status of *TAC1* in matched tissue and plasma samples are summarized in Supplementary Table S1. Among 17 control subjects with matched tissue and plasma

samples, 14 were unmethylated in both tissue and plasma, whereas three were methylated in plasma but unmethylated in tissue. Among 32 EAC patients with matched tissue and plasma samples, 10 were methylated in both tissue and plasma, 4 were unmethylated in both tissue and plasma, 14 were methylated in tissue but unmethylated in plasma, and 4 were unmethylated in tissue but methylated in plasma.

No significant associations were observed between *TAC1* promoter hypermethylation in plasma samples and patient age (data not shown), survival (data not shown), tumor stage, or lymph node metastasis (Table 2).

***TAC1* methylation and mRNA levels in esophageal cancer cell lines after 5-Aza-dC treatment.** All 12 (three EAC and nine ESCC) esophageal cancer cell lines showed high *TAC1* NMV levels, above the cutoff level of 0.12 (Fig. 4A). KYSE 220 and BIC, which exhibited some of 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 *TAC1* was diminished, whereas the mRNA level of *TAC1* was increased, in both KYSE220 and BIC cells (Fig. 4B).

Discussion

By alternatively spliced transcription, *TAC1* encodes the neuropeptides substance P, neurokinin A, and neuropeptide K and γ , which act through two types of transmembrane

G-protein-coupled receptors, denoted neurokinin-1 and neurokinin-2 (1). The precise involvement of *TAC1* in carcinogenesis remains to be fully elucidated. The biological activity of *TAC1* gene products may vary among different histologic tumor types (17). Substance P has been shown to have proliferative and antiapoptotic effects via the mitogen-activated protein kinase cascade and nuclear factor- κ B (18, 19). Conversely, substance P inhibits melanoma formation in a murine model by a mechanism involving antitumor immunity (20). In the gastrointestinal tract, tachykinins regulate smooth muscle contractility, epithelial ion transport, vascular permeability, and immune function (21). The *TAC1* gene locus shows a high frequency of loss of heterozygosity in EAC (2, 3). In the current study, we systematically investigated hypermethylation of the *TAC1* gene promoter in primary human esophageal lesions of differing histologic types and grades. Our results show that *TAC1* promoter hypermethylation occurs frequently in both human EAC and ESCC. The frequency of *TAC1* hypermethylation was extremely low in normal esophagus but increased at the very early preneoplastic stage of Ba, while being maintained in dysplastic Barrett's esophagus and EAC. There was no significant association between *TAC1* promoter hypermethylation and histologic subtype of esophageal carcinoma (EAC versus ESCC). These results suggest that hypermethylation of *TAC1* occurs early in many subjects, that the frequency of this epigenetic event increases during esophageal carcinogenesis,

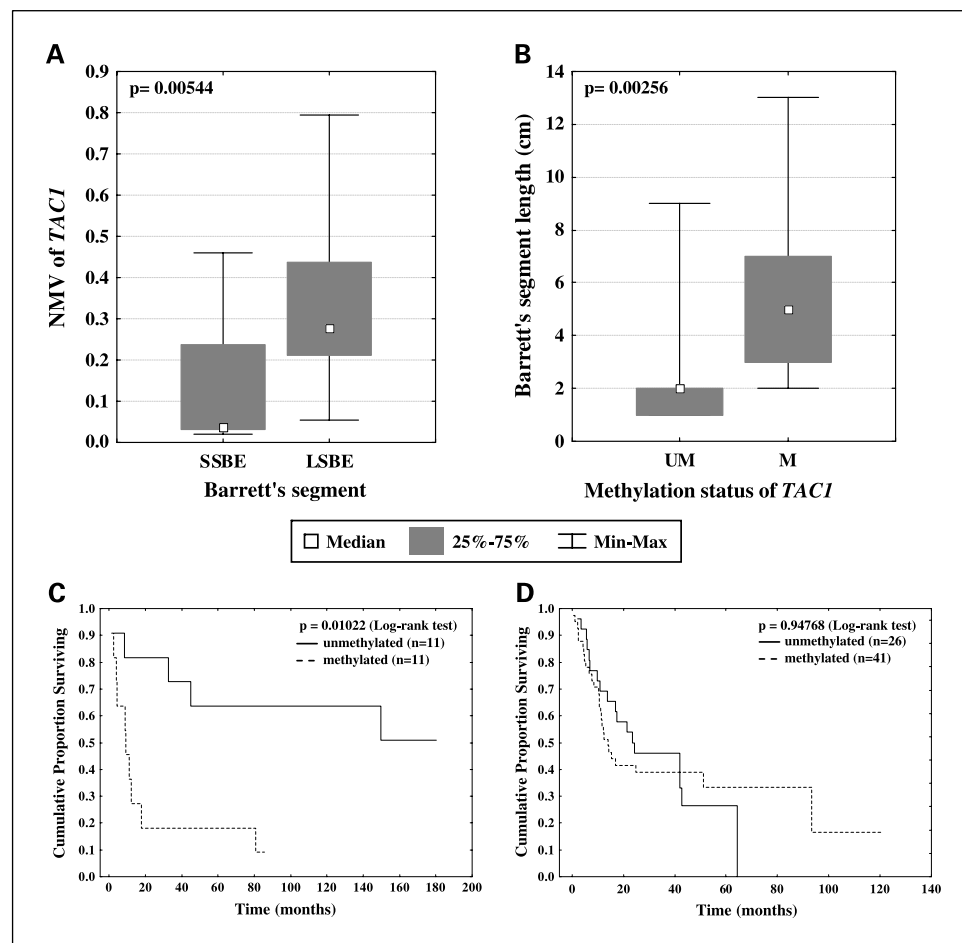


Fig. 3. Relationship between Barrett's segment length, survival, and *TAC1* hypermethylation. **A**, mean NMV of *TAC1* was significantly higher in LSBE than in SSBE ($P = 0.00544$, Student's *t* test). **B**, *TAC1* hypermethylation was associated with BE segment length ($P = 0.00256$, Student's *t* test). **C**, ESCC patients manifesting *TAC1* hypermethylation had significantly shorter survivals than did patients without *TAC1* methylation (mean survival, 22 mo versus 110 mo; $P = 0.0102$, log-rank test). **D**, no significant association was observed between *TAC1* hypermethylation and overall patient survival in EAC patients ($P = 0.94768$, log-rank test).

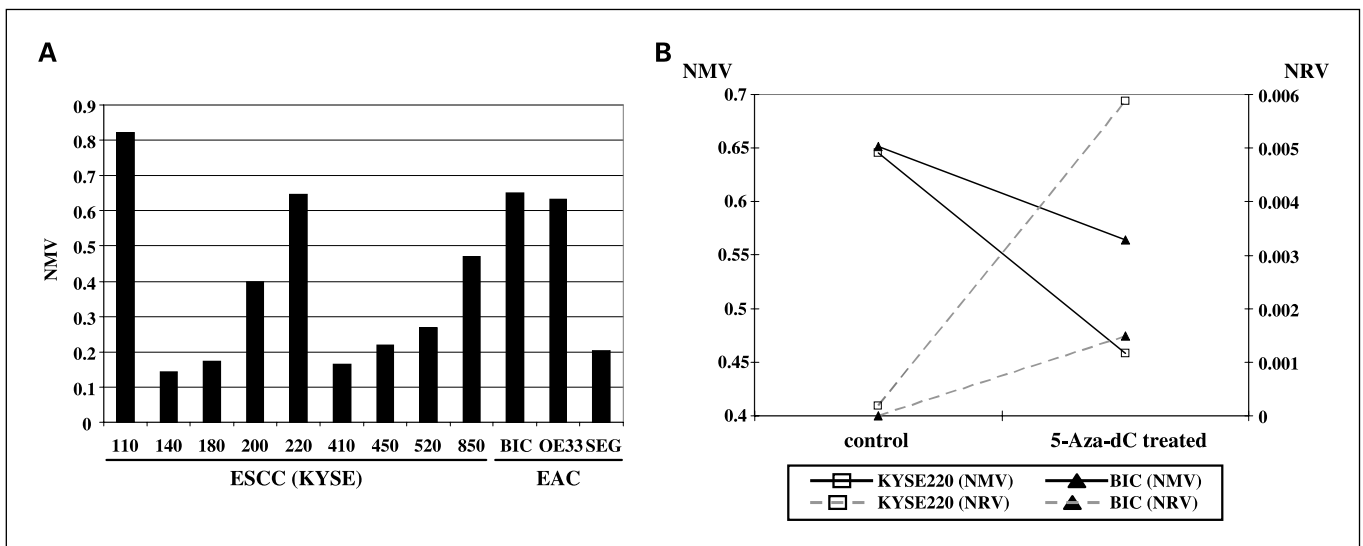


Fig. 4. *TAC1* methylation level and mRNA expression in esophageal cancer cell lines after treatment with 5-Aza-dC. **A**, 12 of 12 (three EAC, nine ESCC) esophageal cancer cell lines showed high *TAC1* NMV, exceeding the cutoff level of 0.12. **B**, KYSE 220 and BIC cells, which had some of the highest NMVs among ESCC and EAC cell lines, respectively, were subjected to 5-Aza-dC treatment. After 5-Aza-dC treatment, in both KYSE220 and BIC cells, the NMV of *TAC1* was diminished, whereas the normalized mRNA value (NRV) of *TAC1* was increased.

particularly in EAC, and that this event is highly prevalent in human esophageal cancers.

Conflicting results regarding the length of BE as a predictive factor in neoplastic progression have been reported. Although some previous studies stated that patients with SSBE can develop dysplasia (22) and EAC (16), several prospective studies showed an increased EAC risk only with LSBE (23–25). In a prospective cohort study of 309 BE patients followed in the Seattle Barrett's Esophagus Project, segment length was not related to cancer risk ($P > 0.2$); however, when patients with high-grade dysplasia at entrance were excluded, a trend was indeed observed, with a 5 cm difference in length associated with a 1.7-fold increase in cancer risk (95% confidence interval, 0.8-fold to 3.8-fold; ref. 16). Weston et al. (23) reported significant differences in the frequencies 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 ($P < 0.0005$). Hirota et al. (24) reported that the prevalence of dysplasia and cancer differed significantly between patients with SSBE and patients with LSBE in a comprehensive prospective study of 889 consecutive subjects. More recently, Hage et al. (25) reported a significantly increased risk of progression to high-grade dysplasia or EAC with LSBE after a mean follow-up period of 12.7 years. Thus, it seems likely that length of Barrett's epithelium is a contributory risk factor for both the prevalence (presence) and incidence (future development) of dysplasia and EAC. In the current study, *TAC1* methylation manifested a strong relationship to BE segment length. There were significant associations between *TAC1* promoter hypermethylation as a binary variable and BE segment length as a continuous variable ($P = 0.00256$), as well as between *TAC1* promoter hypermethylation as a continuous variable and BE segment length as a binary variable ($P = 0.00544$). The frequency of *TAC1* hypermethylation was significantly higher in LSBE (87.5%) than in SSBE (28.6%; $P = 0.0022$). Thus, *TAC1* methylation may constitute a molecular

correlate of BE segment length, in addition to its potential value as a biomarker for the prediction of BE progression.

There is increasing evidence that promoter hypermethylation has prognostic value in cancer patients, including those with esophageal cancer (12, 26, 27). Brock et al. (26) showed that hypermethylation of multiple genes was a powerful indicator of poor prognosis in EAC patients. Lee et al. (27) reported that the *Fragile Histidine Triad* gene was hypermethylated in 85 (33%) of 257 ESCC and associated with a poor prognosis in stage I to II cases. Furthermore, our previous work showed that hypermethylation of *adenomatous polyposis coli* gene DNA could be detected in circulating plasma and was associated with a poor prognosis in EAC patients (12). In the current study, *TAC1* hypermethylation in tissue was significantly associated with shortened survival in ESCC patients, and both mean NMV and frequency of *TAC1* hypermethylation in plasma were significantly higher in EAC patients than in control subjects. Thus, hypermethylation of *TAC1* seems to constitute a potentially useful biomarker of biologically aggressive disease in ESCC and EAC patients. Although *TAC1* hypermethylation in plasma DNA was observed predominantly in EAC patients, 8.6% (3 of 35) of control subjects in the current study manifested plasma *TAC1* hypermethylation. In addition, four EAC patients with plasma *TAC1* hypermethylation did not show the same alteration in their matched tumor tissues. We considered the following possible explanations for these results: (a) methylated plasma DNA could have been derived from undetected precancerous lesions in these cases; (b) apparent plasma false-positive control subjects could actually possess an increased predilection to develop malignant disease in the future; or (c) malignancies already present in other unscreened organs could have gone undetected at the time point analyzed.

In the current study, reversal of methylation and restoration of *TAC1* expression occurred in both KYSE220 and BIC esophageal cancer cell lines after 5-Aza-dC treatment.

Restoration of *TAC1* mRNA expression by 5-Aza-dC treatment is consistent with the interpretation that DNA hypermethylation was responsible for silencing of *TAC1*.

The current study suggests that hypermethylation of the *TAC1* promoter, leading to gene silencing, is a common event in human esophageal carcinomas, occurs early in Barrett's-

associated esophageal adenocarcinogenesis, and is associated with a poor prognosis in ESCC patients.

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