

Frequent Methylation of *Eyes Absent 4* Gene in Barrett's Esophagus and Esophageal Adenocarcinoma

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Abstract

Most esophageal adenocarcinomas arise within Barrett's esophagus but the cause of this increasingly prevalent condition remains unknown. Early detection improves survival and discriminant screening markers for Barrett's esophagus and cancer are needed. This study was designed to explore the natural history of *eyes absent 4* (*EYA4*) gene methylation in the neoplastic progression of Barrett's esophagus and to evaluate methylated *EYA4* as a candidate marker. Aberrant promoter methylation of *EYA4* was studied by methylation-specific PCR using bisulfite-treated DNA from esophageal adenocarcinomas, Barrett's esophagus, and normal epithelia, and then confirmed by sequencing. Eight cancer cell lines were treated with the demethylation agent 5-aza-2'-deoxycytidine, and *EYA4* mRNA expression with and without treatment was quantified by real-time reverse-transcription PCR. *EYA4*

hypermethylation was detected in 83% (33 of 40) of esophageal adenocarcinomas and 77% (27 of 35) of Barrett's tissues, but only in 3% (2 of 58) of normal esophageal and gastric mucosa samples ($P < 0.001$). The unmethylated cancer cell lines had much higher *EYA4* mRNA expression than the methylated cancer cell lines. Demethylation caused by 5-aza-2'-deoxycytidine increased the mRNA expression level by a median of 3.2-fold in methylated cells, but its effect on unmethylated cells was negligible. Results indicate that aberrant promoter methylation of *EYA4* is very common during tumorigenesis in Barrett's esophagus, occurs in early metaplasia, seems to be an important mechanism of down-regulating *EYA4* expression, and represents an intriguing candidate marker for Barrett's metaplasia and esophageal cancer. (Cancer Epidemiol Biomarkers Prev 2005;14(4):830-4)

Introduction

The incidence of esophageal adenocarcinoma is rapidly increasing in the Western world (1-3). Because most esophageal adenocarcinomas are detected at advanced stages, mortality rates have remained high with a median 5-year survival of only ~10% (4). Nearly all such cancers arise within Barrett's esophagus, an intestinal-type metaplasia that has replaced the normal esophageal squamous epithelium following injury by gastroesophageal reflux (5-7). In some, Barrett's esophagus undergoes transformation to dysplasia and then to cancer (5-7). However, fewer than 5% of patients with esophageal adenocarcinoma have had an antecedent diagnosis of Barrett's metaplasia (8, 9). As such, refinements in endoscopic surveillance programs for patients with known Barrett's metaplasia will not result in substantial benefit at the population level unless detection of the Barrett's precursor is meaningfully increased. A hope for early detection of Barrett's esophagus and early-stage esophageal adenocarcinoma may lie in the identification of discriminant and stable markers for these lesions (10).

Inactivation of tumor suppressor genes by aberrant promoter methylation has been considered as a potential mechanism of tumorigenesis in esophageal and other cancers, but highly informative methylation markers for esophageal cancer have not been identified (11-14). *Eyes absent 4* (*EYA4*) is one of four members of the *EYA* gene family that is homologous to the *eyes absent* gene in *Drosophila* (15-17). *Eyes absent* works as a key regulator of ocular differentiation and may modulate apoptosis

(15, 16). In humans, inherited mutations in *EYA* genes have been associated with syndromic developmental abnormalities (18-22). A potential role of *EYA4* gene in human cancer was recently suggested by a microarray-based methylation analysis that showed *EYA4* is frequently methylated in colorectal cancer but not methylated in normal mucosa (23). There are no prior reports of *EYA4* methylation in esophageal cancer.

The present study was undertaken to evaluate methylated *EYA4* as a candidate marker for Barrett's esophagus and esophageal adenocarcinoma and to explore the relationship between methylation status and expression of this gene.

Materials and Methods

Approval of this study was obtained from the Institutional Review Board of Mayo Foundation.

Tissues and Cell Lines. Forty adenocarcinomas, 35 Barrett's lesions, and 58 normal esophageal or gastric epithelia were collected. The clinical characteristics of these tissues are shown in Table 1. All samples had been formalin-fixed and paraffin-embedded and were obtained at Mayo Clinic (Rochester, MN).

Three esophageal adenocarcinoma cells (SEG-1, BIC-1, and OE33) and five other adenocarcinoma cell lines from the upper gastrointestinal tract (one liver cancer cell HUH7 and four pancreatic cancer cells, BXP3, ASPC-1, CAPAN2, and PANC-1) were studied. SEG-1, BIC-1, HUH7, ASPC-1, and PANC-1 were grown in DMEM supplemented with 10% fetal bovine serum. OE33, CAPAN2, and BXP3 were grown in RPMI 1640 supplemented with 10% fetal bovine serum.

Microdissection and DNA Extraction. Tissue sections were reexamined by a pathologist who circled out histologically distinct lesions to direct careful microdissection. Genomic DNA from both tissues and cell lines was extracted using Qiagen DNA Mini Kit (Qiagen, Valencia, CA) with some minor modification.

Received 7/13/04; revised 9/20/04; accepted 1/3/05.

Grant support: A generous grant from Charles Oswald.

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Table 1. Clinical characteristics of subjects studied

	Cancer	Barrett's	Normal	
Number	40	35	58	
Median age (range)	64 y (37-82 y)	65 y (37-82 y)	62 y (20-78 y)	
Sex (M/F)	36/4	30/5	35/23	
Pathology and other information	Grade			
	2	Nondysplasia	11	Esophagus 27
	3			Stomach 31
	4	Dysplasia	24	
	Stage			
	I	Low grade	10	Adjacent to Barrett's 28
	II	High grade	14	From normal individuals 30
	III, IV			

Bisulfite Treatment. Sodium bisulfite converts unmethylated cytosine residues, but not methylated one, to uracil. One microgram of DNA was denatured with 0.3 mol/L NaOH in a total volume of 55 μ L for 20 minutes at 42°C. Six-hundred-microliter mix of 10 mmol/L hydroquinone (Sigma, St. Louis, MO) and 4 mol/L sodium bisulfite (Sigma) were added to each denaturing reaction and the mixture was incubated at 55°C for 4 hours in the dark. Five microliters of Glassmilk (Qbiogene, Carlsbad, CA) were added to bind the DNA for 10 minutes at room temperature. Glassmilk pellets were washed with 70% ethanol thrice. Then, 20 μ mol/L NaOH/90% ethanol were added to desulfate the bisulfite-treated DNA for 10 minutes at 37°C. After pelleting the Glassmilk, 90% ethanol was added to wash the DNA twice. After the last washing ethanol has been completely removed, the bisulfite-treated DNA was resuspended in 30 μ L TE buffer (pH 7.5).

Methylation-Specific PCR. Methylation-specific PCR was done using a previously described method (24). The methylation-specific primers were 5'-CGC CAC CGA CTA CTA CGA ACT CGT A-3' (sense) and 5'-ATA AAA ACG GAG TGG GTT TTT CGC G-3' (antisense). The unmethylation-specific primers were 5'-TCA CCA CCA ACT ACT ACA AAC TCA TA-3' (sense) and 5'-GTT AAA TAA AAA TGG AGT GGG TTT TTT GTG-3' (antisense). Each primer was designed to contain three to four CpG dinucleotides so that methylation-specific primers only amplified fully methylated DNA and unmethylation-specific primers only amplified unmethylated DNA. The specificity of this set of primers was checked by *in vitro* methylated DNA and unmethylated DNA. The product of *EYA4* methylation primers, which covers 16 CpGs, was located in a typical CpG island just before its exon 1 (position 81,361-81,462, Genbank accession no. AL121959). The schematic graph of the 5' region of *EYA4* gene is shown in Fig. 1.

One microliter bisulfite-modified DNA was amplified in a total volume of 25 μ L containing 1 \times PCR buffer (Perkin-Elmer, Boston, MA), 1.5 mmol/L MgCl₂, 100 μ mol/L of each deoxynucleotide triphosphate, 200 nmol/L of each primer, and 1 unit of AmpliTaq Gold polymerase (Perkin-Elmer). Amplification included hot-start at 95°C for 12 minutes, denaturing at 95°C for 30 seconds, annealing at certain temperatures for 30 seconds, extension at 72°C for 30

seconds for 35 cycles, and a final 10-minute extension step. The annealing temperatures for methylation and unmethylation reactions were 65°C and 55°C. The PCR product sizes were 102 and 108 bp for methylation and unmethylation reactions, respectively. Bisulfite-treated human genomic DNA and *in vitro* methylated DNA were used as positive controls for unmethylation and methylation, respectively. PCR products were confirmed by automated sequencing. All PCR reactions have been done twice.

Demethylation of Cancer Cells. Cells were grown in low density for 12 to 24 hours in six-well plates and then treated with 5 μ mol/L 5-aza-2'-deoxycytidine (DAC; Sigma) or mock treated with PBS for 96 hours. The dose and timing of DAC were based on prior tests and published studies (25, 26).

Quantitative Real-time Reverse Transcription-PCR Analysis. The mRNA expression of *EYA4* in the esophageal adenocarcinoma cell lines with or without DAC treatment was carefully quantified. Briefly, RNA from these cells was extracted with RNeasy Mini kit (Qiagen) according to the manufacturer's protocol and was quantified by spectrophotometer. Reverse transcription was done on 2 μ g total RNA using Omniscript RT Kit (Qiagen) with some minor modifications. cDNA was amplified by real-time PCR in an iCycler (Bio-Rad, Hercules, CA). The reverse transcription-PCR primers of *EYA4* were 5'-AAC TGA GGC AGC CAC TCT GT-3' (sense) and 5'-TCC CCA CAG CTG TAT CCT TC-3' (antisense). Because *EYA4* has four transcript variants and exon 5, 16, and 20 are often absent or replaced by splicing (15), we designed the antisense primer on exon 7 and the sense primer on exon 9 so that our PCR product could avoid the influence of splicing and differ cDNA from DNA. *GAPDH* was used as an internal reference gene for normalizing the cDNA input (27). The primers for *GAPDH* were 5'-CAT CAC CAT CTT CCA GGA GCG-3' and 5'-TGA CCT TGC CCA CAG CCT TG-3' (28). The product length of *EYA* and *GAPDH* was 250 and 442 bp, respectively. The mRNA expression ratio of *EYA4* was defined as the ratio of the fluorescence emission intensity value of *EYA4* reverse transcription-PCR products to that of *GAPDH* PCR products multiplied by 100. Fluorogenic quantitative real-time PCR assays were done in a reaction volume of 25 μ L containing 1 \times iQ SYBR Green Supermix (Bio-Rad), 40 nmol/L each primer, and 1 μ L cDNA under the following conditions: 95°C for 3 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 45 seconds.

Amplification was done in 96-well plates. Each plate consisted of cDNA samples and multiple water blanks as well as positive and negative controls. Separate amplification assays were done for *EYA4* and *GAPDH*; each assay was done in duplicate. Serial dilutions of positive controls were used to make standard curves for each plate. Melt curve was conducted for each reaction to guarantee that only one identical product was amplified and the PCR products were further confirmed by agarose gel electrophoresis.



Figure 1. The schematic graph of the 5' regions of *EYA4* gene. Vertical bars indicate CpG sites. The shaded bar above CpG sites indicates the first exon. The black bar below the CpG sites indicates the region analyzed by methylation-specific PCR.

Statistical Analysis. Fisher's exact test and χ^2 test were used to analyze the data obtained by methylation-specific PCR and were done using SAS statistical software (SAS Institute, Inc., Cary, NC).

Results

EYA4 was methylated in 83% (33 of 40) of esophageal adenocarcinomas, 77% (27 of 35) of Barrett's tissues, and 3% (2 of 58) of normal epithelial samples (Figs. 2 and 3; Table 2). The differences of EYA4 methylation across these tissue groups were statistically significant ($P < 0.001$). Furthermore, EYA4 methylation significantly differed between cancer and normal epithelia ($P < 0.001$) and between Barrett's esophagus and normal epithelia ($P < 0.001$). Differences in EYA4 methylation across cancer grades or stages were not apparent, nor were they across degrees of dysplasia in Barrett's (Table 2). No significant difference was found between methylation rates when high-grade dysplasia combined with cancer was compared with Barrett's with low-grade dysplasia and without dysplasia ($P > 0.05$), or when Barrett's without dysplasia was compared with the combination of cancer and Barrett's with dysplasia ($P > 0.05$).

EYA4 was methylated in ASPC-1, CAPAN2, PANC-1, OE33, and BIC-1 cancer cell lines, but not in SEG-1, BXP3, and HUH7 (Fig. 4). At baseline (mock treatment), expression levels of EYA4 mRNA were much lower in methylated cells than in unmethylated cells (Fig. 5A). Absent or very low levels of EYA4 expression were observed in mock-treated ASPC-1, CAPAN2, PANC-1, OE33, and BIC-1 (Fig. 5A). However, after treatment with the demethylation reagent DAC, EYA4 mRNA expression in these five methylated cells could be up-regulated by a median of 3.2-fold (Fig. 5B). Even CAPAN2, which showed no EYA4 expression at baseline, could be induced to express EYA4 after demethylation. In unmethylated cells, the effect of DAC on EYA4 expression was minimal or negative (Fig. 5B).

Discussion

Whereas various tumor suppressor genes have been reported to be methylated in Barrett's esophagus and esophageal cancer (11-14), this study shows that EYA4 is remarkable for its high frequency of methylation in esophageal adenocarcinoma (83%) and Barrett's esophagus (77%) but very low frequency in normal esophagogastric mucosa (3%). We are unaware of any genetic or epigenetic marker with comparably high expression in both Barrett's esophagus and esophageal adenocarcinoma. Variably high rates of p16 methylation have been reported in esophageal adenocarcinoma but with relatively lower rates of

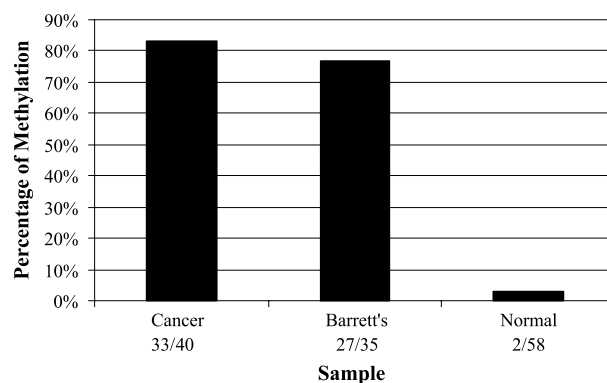


Figure 3. EYA4 methylation status in esophageal adenocarcinoma, Barrett's esophagus, and normal esophageal or gastric mucosa. The frequencies of EYA4 methylation in both esophageal adenocarcinoma and Barrett's esophagus were high, but very low in normal epithelia ($P < 0.001$).

such methylation in Barrett's esophagus (12-14). Because EYA4 methylation occurs commonly and early in Barrett's metaplasia, it represents a candidate marker for Barrett's esophagus and esophageal cancer.

The participation of EYA4 methylation in Barrett's esophagus and esophageal cancer is a new observation. Because inactivation by aberrant promoter methylation is an important characteristic of tumor suppressor genes (29, 30), it is possible that EYA4 functions as a tumor suppressor during the evolution and neoplastic progression of Barrett's esophagus. Evidence from the present study to support this speculation includes the observations that methylation of EYA4 is associated with markedly reduced or absent mRNA expression in Barrett's esophagus and esophageal cancer and that reexpression can be induced by *in vitro* demethylation in cancer cell lines. The inactivation of tumor suppressor gene requires both alleles to be inactive (31). Because the mRNA expression level of EYA4 gene is very low in methylated cancer cell lines, both alleles of EYA4 are likely nonfunctioning, which would be consistent with a possible tumor suppressor role. Whereas all methylated cancer cell lines seemed to have an unmethylated EYA gene allele, this finding may potentially be explained by gene silencing due to one methylated allele and one genetically altered allele, or to one densely methylated allele and one sparsely methylated allele. A gene can be silenced even if only 18% CpGs in its promoter are methylated (32).

Table 2. Summary of the methylation status in tissue subjects of esophageal adenocarcinoma and Barrett's esophagus

Tissue	Methylation
Esophageal adenocarcinoma	33/40 (83%)
Grade	
2	5/7
3	17/20
4	11/13
Stage	
I	8/11
II	9/11
III, IV	16/18
Barrett's esophagus	27/35 (77%)
Nondysplasia	8/11
Low-grade dysplasia	8/10
High-grade dysplasia	11/14

NOTE: P values are not significant.

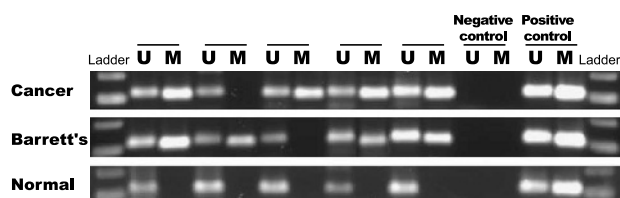


Figure 2. EYA4 methylation in esophageal adenocarcinoma (top), Barrett's esophagus (middle), and normal esophageal or gastric mucosa (bottom). PCR products in lanes U or M indicate the presence of unmethylated or methylated genes, respectively. Human genomic DNA and *in vitro* methylated DNA were used as positive controls for unmethylation and methylation. Water was used as negative control.

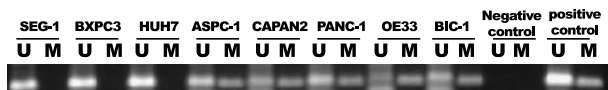


Figure 4. The methylation status of *EYA4* in cancer cell lines.

To date, few published data are available regarding the properties of the *EYA4* gene, especially its role in carcinogenesis. *EYA* genes may influence apoptosis in neoplastic cells and during development. The *EYA* gene family has been associated with the development and formation of eye, kidney, and inner ear in humans (15-22). In a recent study (33), transfection of immortalized murine myeloid cells with an *EYA2* plasmid triggered rapid apoptosis, and mitochondria were a major target of this *EYA2*-induced apoptosis. *EYA* may function with a network of other genes to induce apoptosis (33). Further investigation by plasmid transfection or other approaches will be required to elucidate the cellular function of the *EYA4* gene and its mechanisms of action.

Although most esophageal adenocarcinomas arise within Barrett's esophagus, only a small percentage of patients presenting with esophageal adenocarcinoma have had an antecedent diagnosis of Barrett's esophagus (10). Thus, in addition to early detection of esophageal cancer or dysplasia, there is a need to identify those harboring Barrett's esophagus before dysplasia and cancer have evolved so that appropriate preventative interventions can be considered. Because *EYA4* is frequently methylated in Barrett's esophagus and esophageal cancer, it may have application for screening or surveillance used alone or as part of a panel of markers. The general appeal of methylated gene markers includes the predictable site of aberrant methylation on the gene promoter region (29, 30), the chemical and biological stability of gene methylation (34), the fact that methylation is a positive signal that can be readily targeted for detection, and the availability of highly sensitive assay techniques (35, 36). Methylated genes have been studied as tumor markers in serum (35-38), sputum (39-43), urine (44-46), and stool (47-49). A recent report shows that aberrant gene methylation in normal-appearing colorectal mucosa can be detected in stool and suggests that detection of such epigenetic nonmalignant mucosal alterations might be useful in the future to identify those at increased risk for colorectal cancer (50). Likewise, given that early studies in selected patient groups suggest that genetic and epigenetic markers recovered in stool have potential to detect cancers in the upper digestive tract (49, 51-53), one could speculate that assay of methylated *EYA4* in stool could be explored as a noninvasive approach to screen for Barrett's esophagus or early-stage cancers. Some have suggested nonendoscopic brushings or balloon swabbing of the esophagus as an approach to Barrett's screening (54), and assay of methylated *EYA4* or other tumor-specific molecular markers from recovered cells may have an application with such an approach. Assay of methylated *EYA4* in serum could be of potential value in staging and postoperative surveillance of esophageal cancer.

EYA4 methylation is not unique to Barrett's esophagus and esophageal cancer, but may be specific to transformed premalignant or malignant epithelium. Our group has recently found a high frequency of tumor-specific *EYA4* methylation in sporadic colorectal neoplasms, both adenomas and cancers, and in dysplasia associated with chronic ulcerative colitis, but not in normal and inflamed colon mucosa.⁴

⁴ N.K. Osborn, et al. Hypermethylation of the *EYA4* gene in chronic ulcerative colitis-associated dysplasia and cancer: a candidate marker for surveillance?, submitted for publication.

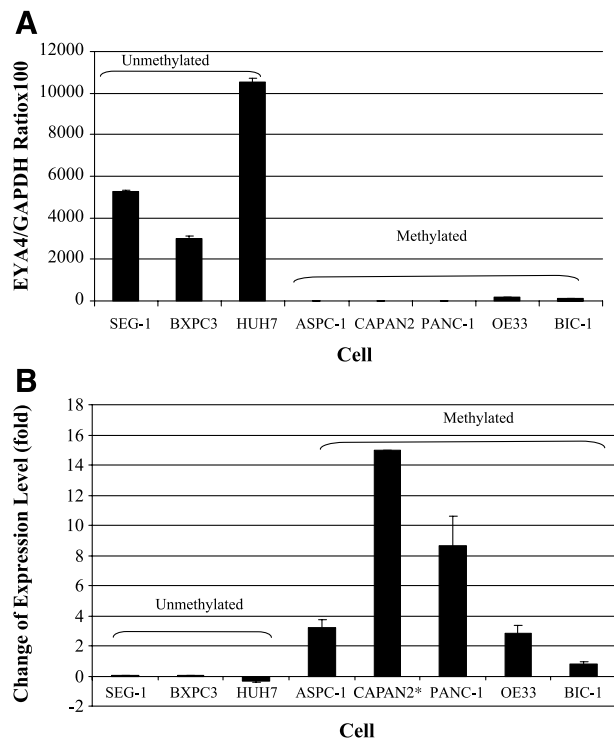


Figure 5. The *EYA4* mRNA expression quantified by real-time reverse transcription-PCR in eight cancer cells with DAC or mock treatment. **A.** At baseline (mock treatment), unmethylated cells had much higher *EYA4* mRNA expression than methylated cells. **B.** This graph shows the relative change of *EYA4* mRNA expression level induced by DAC treatment. In methylated cells, *EYA4* expression level was elevated by a median of 3.2-fold. In comparison, that *EYA4* expression change was negligible in unmethylated cells. Because baseline *EYA4* mRNA expression was not detectable in CAPAN2, its fold change could not be accurately plotted on this scale.

Based on the observations from this study, application studies on stool, serum, or other biological samples are indicated to explore the value of methylated *EYA4* as a marker for Barrett's esophagus and esophageal adenocarcinoma.

Acknowledgments

We thank Dr. David G. Beer (University of Michigan Medical School, Ann Arbor, MI) for providing SEG-1 and BIC-1 and Dr. Deborah A. Leberman (Virginia Commonwealth University, Richmond, VA) for providing OE33.

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