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Methylated *Eyes Absent 4 (EYA4)* Gene Promotor in Non-neoplastic Mucosa of Ulcerative Colitis Patients with Colorectal Cancer: Evidence for a Field Effect

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Abstract

Background—Aberrant methylation of the *EYA4* gene (*mEYA4*) highly discriminates ulcerative colitis (UC) cases with colorectal neoplasia from UC controls in both tissue and stool. It is not known if *mEYA4* is also present in nonadjacent non-neoplastic mucosa (NNM) of UC patients with colorectal neoplasia.

Methods—Formalin-fixed tissues from 25 UC cases with colorectal cancer (CRC) and 25 UC controls with neither CRC nor dysplasia were matched on gender, age, disease duration, disease extent, and coexistence of primary sclerosing cholangitis. DNA was extracted from sections of CRC and NNM from cases and UC control mucosae. Bisulfite-treated DNA was amplified using real-time methylation-specific PCR. The Wilcoxon rank-sum test assessed differences in *mEYA4* levels from CRC, NNM, and control samples. Logistic regression was used to estimate sensitivity and specificity.

Results—Sufficient DNA was available for 20 cases and 20 controls. The median *mEYA4* level (with interquartile range) was 2 (0–5.7) in control mucosae but 93 (38.5–306) in CRC ($P < 0.0001$) and 27.4 (3–140) in NNM ($P = 0.0009$). At 95% specificity, *mEYA4* was present in 80% of CRC and 50% of NNM tissue samples. The odds ratio of *mEYA4* in NNM as an indicator of synchronous CRC was 19 (95% confidence interval, 2–170).

Conclusions—The authors demonstrate significantly higher *mEYA4* levels in NNM and synchronous CRC from UC cases than in colorectal mucosae of UC controls without neoplasia. The finding of this CRC-associated field change has important implications to the use of *mEYA4* as a potential UC surveillance marker in tissue or stool.

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Keywords

inflammatory bowel disease/complications; neoplasms/genetics; field cancerization defect; biological markers; DNA methylation

Patients with inflammatory bowel disease, specifically chronic ulcerative colitis (UC) and Crohn's colitis, are at increased risk of colorectal cancer (CRC) and dysplasia.^{1,2} Chronic inflammation seems to promote UC-associated CRC (UC-CRC),^{3,4} and cancer risk increases with extent and duration of disease, concomitant primary sclerosing cholangitis, and presence of pseudopolyposis.⁵ UC-CRC arises from both endoscopically visible polypoid and flat dysplastic precursors.⁶ Low-grade flat dysplasia⁷ and high-grade polypoid dysplasia⁸ can predict synchronous UC-CRC. UC-CRC is also more likely than sporadic CRC to be synchronous at presentation.⁹

Across multiple diseases, investigators have sought to understand the etiological molecular mechanisms for multicentric neoplasms to develop within regions of common carcinogen exposure, including chronic inflammation. Originated by Slaughter et al,¹⁰ the field cancerization defect (or field effect) hypothesis has important implications in the UC-CRC disease model. An early report of abnormal *p53* staining in cancerous, dysplastic, and histologically normal mucosa from colectomy specimens suggested clonal expansion of an early founder mutation that confers a growth advantage.¹¹ Local and segmental field cancerization has been confirmed by mapping *p53* and *KRAS* mutations in non-dysplastic crypts adjacent to UC-CRCs.¹² Field cancerization is also supported by analysis of telomere length in non-neoplastic distant colon segments of UC patients with cancer or high-grade dysplasia.¹³

Aberrant methylation of gene promoters represents another molecular change that may be part of this field phenomenon. At least 1 of *TGFB2*, *SLIT2*, *HS3ST2*, or *TMEFF2* was found to be methylated in 12 of 30 non-neoplastic mucosae adjacent to inflammatory bowel disease-associated neoplasms and also in inflamed but normal mucosae of 15 of 20 patients at high risk of inflammatory bowel disease-associated neoplasia.¹⁴ Methylation of some markers, such as *RUNX3* and *MINT1*, are found in both CRC and nonadjacent non-neoplastic mucosa (NNM) of patients with UC.¹⁵ We have previously shown that aberrant methylation of the *Eyes Absent 4* gene (*mEYA4*) highly discriminates UC cases with colorectal neoplasia from UC controls without neoplasia in both tissue¹⁶ and stool.¹⁷ It is not yet known if *mEYA4* is confined to neoplastic tissue or if it is also found in NNM of UC patients with CRC.

Accordingly, we aimed to measure *mEYA4* levels in tissue sections of both primary tumors and NNM from UC patients with CRC and in mucosae from UC patients without CRC or dysplasia. We further sought to estimate the value of *mEYA4* determination in NNM of patients with UC for predicting synchronous CRC.

MATERIALS AND METHODS

Patients and Tissue Samples

Patients with UC for >10 years who developed CRC were identified (UC-CRC cases). For each case, pathology slides from the surgical resection were recalled to confirm the diagnosis of UC, and complete patient chart reviews were then performed. Patients who did not have UC confirmed by review of the pathology or whose duration of disease was <10 years as documented in the clinical chart were excluded. A total of 25 UC-CRC cases, representing a subset of the UC-CRC cases described previously,¹⁵ were examined in the current study. Complete patient chart reviews were performed on all potential UC controls. These were defined as patients with UC who did not develop CRC, who had >10 years of disease, who underwent either colectomy or colonoscopy with biopsy at the Mayo Clinic, and who did not have previous dysplasia. The final selection of UC controls was based on the frequency matching to UC-CRC cases for age, gender, extent, and duration of UC. A total of 25 UC controls, a subset of the UC control group described previously,¹⁵ were analyzed in the present study. Among case subjects, NNM was defined as nonadjacent non-dysplastic mucosa histologically involved by UC. For nonadjacent NNM UC-CRC cases and controls, DNA was extracted from all non-neoplastic paraffin tissue sections that showed evidence of chronic inflammation (1–6 blocks per patient). For DNA extraction from tumors, only the section with confirmed CRC was used.¹⁵

Assay Technique

DNA was extracted with the Gentra Puregene Tissue Kit (Qiagen, Germantown, MD) and quantified using Quant-iT Pico-Green (Invitrogen, Carlsbad, CA). From each sample, 100 ng DNA was bisulfite treated with EZ DNA Methylation Kit (Zymo Research, Irvine, CA). One microliter bisulfite-treated DNA was used as a template for methylation quantification with fluorescence-based real-time PCR (MSP). Primers for *mEYA4* were designed to target the bisulfite-modified methylated sequences of the gene promotor (IDT, Coralville, IA). A region without cytosine–phosphate–guanine sites in the β -actin gene was also quantified with real-time PCR using primers recognizing the bisulfite-converted sequence as a reference of the adequacy of bisulfite treatment and to normalize the MSP product. The MSP primers (in 5-prime to 3-prime direction) were as follows: β -actin, forward, TTT TTT TTG GTG TTT GTT TTT TTG ATT; β -actin, reverse, CAC CAA CCT CAT AAC CTT ATC ACA C; *mEYA4*, forward, CGC CAC CGA CTA CTA CGA ACT CGT; and *mEYA4*, reverse, ATA AAA ACG GAG TGG GTT TTT CGC G. PCRs for tissue DNA samples were performed with SYBR Green master mix (Roche, Mannheim, Germany) on Roche 480 LightCyclers. Bisulfite-treated CpGenome Universal Methylated DNA (Millipore, Billerica, MA) was used as a positive control and serially diluted to create standard curves for all plates. Each plate consisted of bisulfite-treated DNA samples, positive and negative controls, and water blanks. Samples that did not amplify β -actin were not included in the analysis.

Statistical Analysis

Clinical characteristics of cases and controls were compared using the Student's *t* test for continuous variables and chi-square or Fisher's exact test for categorical variables. The

primary quantitative variable was the absolute copy number of *mEYA4*, determined by MSP against standard dilutions. The Wilcoxon rank-sum test was used to compare the number of *mEYA4* copies between CRC tumors versus control tissues and NNM tissues versus control tissues. Logistic regression was performed to assess the strength of association between *mEYA4* (copies in tumors or NNM) and CRC or control status. Receiver operating characteristics curves were constructed, and the area under the curve value was calculated for each curve. From the receiver operating characteristics curves, sensitivity was estimated at 90% and 95% specificity cutoffs. The 95% confidence intervals for the sensitivity estimates were calculated using the efficient score method. Odds ratios were calculated at each cutoff and tested using a chi-square statistic. Using the univariate effect size reported in a previous study,¹⁵ we estimated that 50 patients would provide >85% power to detect an odds ratio of >10 at the 0.05 level. Analyses were performed using JMP v8.0 (SAS Institute, Cary, NC).

Ethical Considerations

The Mayo Clinic Institutional Review Board approved this research protocol.

RESULTS

Sufficient DNA for *EYA4* and β -actin amplifications was available for 20 cases and 20 controls (Table 1). Respectively, in cases and controls, 71% and 75% were male; mean (SD) age was 53 (14.1) and 51 (13.1). Mean (SD) UC duration was 20 years (9.8 years) and 21 years (8.5 years), disease was extensive in 17 (85%) and 15 (75%), and primary sclerosing cholangitis was present in 4 (20%) and 2 (10%). None of these characteristics were significantly different between cases and controls. Control subjects were significantly more likely to have active inflammation at the time of colectomy ($P = 0.007$).

For cases, data were abstracted regarding the location of both NNM and tumor samples. The majority (75%) of NNM samples were obtained in an anatomical segment distinct from the tumor, e.g., for tumors in the cecum, samples were obtained from a colorectal segment other than the cecum. Median (interquartile range) tissue levels of *mEYA4* were not significantly affected by distance from the tumor: 5 (1–192) methylated copies in the same segment versus 62 (7–141) copies in distinct segments, $P = 0.3$.

Tissue *mEYA4* levels were substantially and significantly higher in primary tumors and NNM from UC cases with CRC than in UC control mucosae. The median number of *mEYA4* copies (with interquartile range) was 2 (0–5.7) in UC control mucosae, 93 (39–306) in case with CRC tumors ($P < 0.0001$ versus control), and 27 (3–140) in case with NNM ($P = 0.0009$ versus control) (Fig. 1). Receiver operating characteristics curves plotted from the logistic regression models are shown in Figure 2. Among DNA samples taken from case with CRC tumors, *mEYA4* copy number was strongly associated with case versus control status with an area under the curve of 0.94. When UC-NNM case levels were modeled, *mEYA4* was also strongly associated with case versus control status with an area under the curve of 0.80. No difference was observed when models were corrected by dividing the *mEYA4* copy number by the copy number of β -actin, indicating that the DNA input into the MSP reaction was uniform and that quantification of the MSP products was accurate.

At 95% specificity, *mEYA4* was elevated in primary tumor tissue from 16 of 20 patients with CRC (sensitivity for CRC 80% [56%–93%]). In 10 of 20 patients with CRC, levels of *mEYA4* were elevated in NNM samples (sensitivity for CRC 50% [28%–72%]). The tissue level of *mEYA4* in NNM was a strong predictor of synchronous CRC, odds ratio 19 (2–170).

DISCUSSION

This study demonstrates that a CRC-associated field change of aberrantly methylated *EYA4* occurs in patients with UC. The finding of *mEYA4* in random NNM samples was predictive of synchronous CRC.

Based on our earlier observations,¹⁶ *mEYA4* is typically present in colorectal neoplasia tissue from patients with UC but absent in mucosa from UC controls without neoplasia. More recently, our preliminary data suggested feasibility of *mEYA4* assay in stool for the detection of both CRC and precancers in patients with UC or Crohn's disease.¹⁷ The findings in the present study have additional important implications for the use of *mEYA4* as a potential stool or tissue marker in UC surveillance. Aberrant methylation of *EYA4* may augment the predictive value of conventional white light colonoscopy with random biopsies. However, because we have detected *mEYA4* in NNM tissue from UC cases with CRC, an individual with persistent *EYA4* methylation may be at increased risk of UC-CRC, despite normal pathology findings on surveillance biopsies. If the strong predictive association between *mEYA4* field changes and CRC risk holds in corroborative observations, then assay of *mEYA4* has potential to serve as a useful adjunct to routine histology.

The mechanisms underlying field cancerization effects in UC require elucidation. A key question is whether or not the diffuse methylation changes reflect clonal expansion, as is suggested by studies that mapped DNA mutations. Investigators examining mutations in 5 exons of the *p53* gene have demonstrated clonal expansion across multiple colonic crypts.^{11,12} We have previously sequenced the UC-CRC tissues of the case patients for mutations in *p53*, *KRAS*, *BRAF*, *APC*, and *PIK3CA*; however, only 14 of 25 tumors contained a mutation on any of the genes sequenced.¹⁷ Therefore, we chose not to repeat mutation sequencing in the UC-NNM tissues in the present study. A recent study that followed the expansion of mutant non-neoplastic colonic epithelial stem cells did not show a consistent methylation pattern.¹⁸ These findings suggest that methylation markers may not reflect clonality or that the methylation markers studied may be subject to epigenetic drift. Alternatively, the studied markers may undergo dynamic methylation changes that control tissue-specific gene regulation. Clonal expansion of UC-CRC may also be limited to the same or adjacent anatomical segment as the primary tumor^{11,12}; in contrast, distant synchronous UC-associated¹² and sporadic¹⁹ neoplasms do not necessarily share clonal ancestry by mutation or DNA mismatch repair analysis.

Methylated *EYA4* may act as a record of mitotic age²⁰ and better reflect cumulative inflammatory or other environmental exposures. Chronic inflammation seems to alter microbial composition, possibly promoting tumorigenesis. In an interleukin 10-deficient murine colitis model, expansion of genotoxic colibactin-producing *Escherichia coli* strains was related to the presence of inflammation rather than introduction of a carcinogen,

azoxymethane.²¹ Importantly, colibactin-producing *E. coli* have been shown to induce aneuploidy and double-stranded DNA breaks in mammalian cells.²² Although the direct connections between inflammation, microbial factors, and DNA methylation are not yet fully understood, gene silencing due to aberrant methylation was observed following a defined double-stranded DNA break in the tumor suppressor gene *E-cadherin*.²³ The tyrosine phosphatase domain of EYA proteins dephosphorylates histone protein H2AX, implicating EYA in DNA repair. Although this has been directly studied in embryonic development,²⁴ this observation also implicates EYA proteins in carcinogenesis.²⁵ Because we have previously shown reversible methylation-dependent downregulation of *EYA4* expression,¹⁶ it may be that methylation of the *EYA4* promotor silences a tumor suppressor function.

Although methylation of many gene promoters may correlate with active inflammation,²⁶ we have demonstrated that *mEYA4* levels were not influenced by acute inflammatory activity at the time point of colectomy; these results are similar to those reported for UC-CRC-associated methylation markers.¹⁵

This study has both strengths and limitations. DNA did not amplify in 5 case and 5 control samples; as a result, the total number of samples analyzed was below the intended target. Despite the change in estimated statistical power, the results remained statistically significant. Samples were randomly selected from a large well-characterized cohort, and controls were tightly matched on known risk factors for colitis-associated cancers. However, patients were enrolled at a tertiary referral center and may not be representative of the general at-risk population. Additionally, the use of available archived tissue blocks did not permit detailed mapping of the field size. It is possible that the samples contained stromal DNA; however, this would be expected to dilute the difference in *mEYA4* copy numbers between NNM and control samples, which was still quite significant. Use of paraffin-embedded tissue may result in excessive DNA fragmentation,²⁷ and DNA recovery rates and yield of *mEYA4* may be higher with frozen tissues. Although aberrantly methylated DNA is a very robust and chemically stable biomarker, it was beyond the scope of our aims to make direct comparisons to other classes of biomarkers for which there are promising preliminary data.^{28–33}

Methylated *EYA4* in NNM biopsies may serve as a useful predictive marker of synchronous CRC in patients with UC; these novel findings warrant further investigation. Our results also have potential clinical relevance to the use and interpretation of stool tests that incorporate *mEYA4* and other methylation markers for surveillance CRC in UC.

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FIGURE 1.

Copy number of *mEYA4* in tissues from patients with UC. A, Tumor tissue in UC cases with CRC versus colorectal mucosae in UC controls without CRC or dysplasia. B, Nonadjacent NNMs in cases with CRC versus mucosae in UC controls.

FIGURE 2.
Detection of CRC based on tissue *mEYA4* levels in (A) primary tumors and (B) NNM:
receiver operating characteristics curves plotted.

TABLE 1

Patient Characteristics

	Cases (n = 20)	Controls (n = 20) ^a
Men	14	15
Mean age (SD), yr	53 (14.1)	51 (13.1)
Mean UC duration (SD), yr	20 (9.8)	21 (8.5)
Extensive disease (%) ^b	17 (85)	15 (75)
Primary sclerosing cholangitis (%)	4 (20)	2 (10)
Inflammation score ¹⁵	0.19	0.78 ^c

^aβ-Actin did not amplify in samples from 5 cases and 5 controls.

^bProximal to the splenic flexure.

^c*P* = 0.0007, all other comparisons nonsignificant.