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Aberrant Vimentin Methylation Is Characteristic of Upper Gastrointestinal Pathologies

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Abstract

Background—We have previously established aberrant DNA methylation of Vimentin exon-1 (VIM methylation) as a common epigenetic event in colon cancer and as a biomarker for detecting colon neoplasia. We now examine VIM methylation in neoplasia of the upper gastrointestinal tract.

Methods—Using a quantitative real-time Methylation-Specific PCR assay we tested for VIM methylation in archival specimens of esophageal and gastric neoplasia.

Results—We find that acquisition of aberrant VIM methylation is highly common in these neoplasms, but largely absent in controls. The highest frequency of VIM methylation was detected in lesions of the distal esophagus, including 91% of Barrett's esophagus (BE, n=11), 100% of high grade dysplasia (HGD, n=5), and 81% of esophageal adenocarcinoma (EAC, n=26), but absent in controls (n=9). VIM methylation similarly was detected in 87% of signet ring (n=15) and 53% of intestinal type gastric cancers (n=17). Moreover, in tests of cytology brushings VIM methylation proved detectable in 100% of BE cases (n=7), 100% of HGD cases (n=4), and 83% of EAC cases (n=18), but was absent in all controls (n=5).

Conclusions—These findings establish aberrant VIM methylation as a highly common epigenetic alteration in neoplasia of the upper gastrointestinal tract, and demonstrate that Barrett's esophagus, even without dysplasia, already contains epigenetic alterations characteristic of adenocarcinoma.

Impact—These findings suggest VIM methylation as a biomarker of upper gastrointestinal neoplasia with potential for development as molecular cytology in esophageal screening.

Keywords

Barrett's Esophagus; Esophageal Cancer; Gastric Cancer; Vimentin; Methylation

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INTRODUCTION

Acquisition of aberrant DNA methylation in CpG-rich DNA islands is a common event in a variety of human cancers, and in some instances is a mechanism of gene silencing. (1) Aberrantly methylated DNA can also serve as a tumor biomarker that can be detected in tumor tissues, as well as in blood, stool and urine of patients with certain types of cancer.(2–6) We have previously reported that methylation of a CpG island overlapping the first exon of the human Vimentin (VIM) gene is detected in 53–83% of colon tumors, but is virtually absent in normal colonic epithelium. Based on these findings, detection of VIM methylation in either fecal DNA or in blood has been adapted as a method for early detection of colon cancers.(7,8) In this study, we investigated whether VIM methylation has a role in upper gastrointestinal tract carcinogenesis, and if so, whether it also can be developed as biomarker for clinical detection of upper gastrointestinal neoplasms. To do this, we developed a quantitative real-time-based Methylation-Specific PCR for assay of VIM methylation and then interrogated a range of lesions of the upper gastrointestinal tract in an archive of formalin fixed and paraffin embedded (FFPE) tissue. We further tested whether VIM methylation could be detected in endoscopically acquired cytology brushings from upper gastrointestinal lesions.

MATERIALS AND METHODS

FFPE Tissues Specimens

Archival normal and neoplastic tissue specimens were obtained from the Department of Pathology at University Hospitals Case Medical Center using a tissue procurement protocol approved by University Hospitals Case Medical Center Institutional Review Board. Before use, diagnostic slides of all samples were reviewed by a gastrointestinal pathologist [JW] for confirmation of the recorded diagnoses, and particularly for confirmation of diagnoses of high grade dysplasia. After confirmation of diagnoses, specimens for this study were prepared via manual microdissection of unstained slides or punch biopsies of tissue blocks. The presence of intestinal metaplasia in designated esophageal biopsies was required for a diagnosis of Barrett's Esophagus according to published guidelines.(9) Demographic and clinical data for all FFPE specimens used in this study is included in Supplementary Table 1.

FFPE Tissue DNA

DNA was purified using QIAamp DNA micro kit (QIAGEN) according to the manufacturer's protocol with the following modifications: Initial incubation in buffer ATL with Proteinase K was carried out at 60°C instead of 56°C and proceeded for 4 days instead of 16 hours. An additional 1.5 µl of Proteinase K was added after 3–24 hours of incubation. The DNA was eluted from columns in 50 µl of distilled water and used immediately for bisulfite conversion, or frozen at –80°C until use.

Bisulfite conversion of the genomic DNA and real-time MS-PCR assay

To create a template for methylation-specific PCR, DNA samples were subjected to bisulfite conversion and purified using an Epiect kit (QIAGEN) according to the manufacturer's protocol. 4 µl of bisulfite-converted DNA at a concentration of 0.2–25ng/µl was used as a template for real-time MS-PCR assay. To normalize input DNA amounts, a companion real-time PCR assay was designed against bisulfite converted Actin gene sequences that lack CpG dinucleotides and so are not modified by methylation. The assay for Actin was designed to generate an amplification product of the same size as the assay for methylated VIM. For both Actin and VIM real-time PCR a mixture of DNA from 4 colon cancer cell lines that are each fully methylated across the VIM CpG island, was used to generate a dilution standard curve that was run with all real-time assays and used as part of data

analysis in BioRad CFX manager software to convert the Ct values into ng DNA amounts. VIM methylation was calculated as percentage ratio of the amount of methylated DNA measured by VIM qPCR, divided by total bisulfite-converted DNA amount in the sample, as measured by Actin qPCR. The real-time MS-PCR reactions were performed in triplicates in 20 µl volume using Light Cycler PCR master mix (Roche) with 400nM each primer and 200nM probe (sequences in Table 1). Amplifications were done in 96-well plates in CFX96 Real-Time System (BioRad) under the following conditions: 95°C for 10 min, followed by 50 cycles of 30sec at 95°C and 60 sec at primer-specific annealing/extension temperature (see Table 1).

Esophageal brushings for molecular cytology

Lesional brushings were obtained during research endoscopy from subjects with BE, HGD and EAC under an Institutional Review Board approved protocol at the Case Medical Center. Under the same protocol, brushings were obtained from patients without BE; from both the distal esophagus and gastric cardia. Diagnosis in all cases and controls was established by histopathological biopsy review. Demographic and clinical data for all patients in this study is included in Supplementary Table 2. Endoscopically collected cytobrushes were clipped into nuclease free 0.5ml cryo-safe tubes and immediately snap frozen on dry ice for transport to gas phase liquid nitrogen storage until use. The QIA amp Micro kit protocol with carrier RNA (QIAGEN) was used according to manufacturer's protocol with overnight 56°C lysis of cells trapped within the cytobrush. DNA yields from clinical samples were quantitated using the Qubit fluorometer (Invitrogen). The esophageal brushings DNA was used for vimentin methylation assay by identical methods as described for FFPE DNA.

RESULTS

Vimentin methylation in Esophageal Cancer

We assessed VIM methylation in FFPE tissue DNA from esophageal cancers, including adenocarcinoma (EAC), the primary subtype in the distal esophagus, and squamous carcinoma, the primary subtype in the proximal esophagus. Out of 26 cases of EAC examined, 21 cases (81%) showed high level VIM methylation, ranging from 10%–100% of total tumor DNA (Fig. 1). As tumor tissues contained both cancer and normal stromal elements we interpret these levels as suggesting VIM methylation was likely present in all of the cancer cells. Two additional EAC cases showed lower levels of VIM methylation between 1%–10%, for an overall detection rate of 88%. High level VIM methylation was detected in 2 of 9 squamous carcinomas of the esophagus, ranging from 10%–100% of total tumor DNA. The difference between squamous cancer and adenocarcinoma subtypes was statistically significant, $p=0.003$ (Fisher's exact test, two-sided). No VIM methylation was detected in 9 normal esophageal samples of squamous mucosa (Fig. 1).

Mapping vimentin methylation during Barrett's esophagus progression

To further investigate the timing of VIM methylation in the development of esophageal adenocarcinoma, we analyzed FFPE tissue DNA from cases of Barrett's Esophagus (BE). BE is a specialized intestinal-type metaplasia of the distal esophagus, which can progress through dysplasia to ultimately give rise to EAC.(10,11) As shown in Figure 1, high level VIM methylation ranging from 10%–100% was detected in FFPE tissue DNA from 10 of 11 (91%) cases of BE without dysplasia and 5 of 5 BE cases with high grade dysplasia (HGD). These findings suggested that VIM methylation is an early and frequent epigenetic alteration in the BE sequence from metaplasia to dysplasia to EAC in the distal esophagus.

In 4 of the cases studied, the specimen of BE and/or BE with HGD was derived from a biopsy obtained concurrently and adjacent to an EAC specimen that we had also studied (Supplementary Fig 1). In all 4 cases, the high level of VIM methylation detected in the EAC was also detected in the synchronous BE and/or BE with high grade dysplasia tissue (Supplementary Fig. 1). We did note one instance of an individual with two concurrent biopsies of BE, in which one of the two biopsies tested negative for VIM methylation (data not shown).

In five additional cases we were able to identify a previous FFPE tissue sample obtained during routine clinical surveillance for BE, spanning 1 to 7 years of follow-up (Supplementary Fig. 2). In two individuals in which BE progressed over time, in one case to HGD and in the other to EAC, both the initial BE specimen and the progressed specimen showed VIM methylation. In two additional individuals in which BE did not progress, both the early and later specimens showed VIM methylation. Only in one individual, VIM methylation was detected in 2 concurrent biopsies of BE, but was absent in a BE specimen obtained from the same individual 4 years earlier (patient 14: supplementary Fig. 2 and data not shown). This is consistent with a model of VIM methylation as an early epigenetic event in carcinogenesis and of EAC arising from an initiated field of BE.

Detection of BE associated lesions by “Molecular Cytology”

The findings of high level VIM methylation in archival specimens of BE, HGD, and EAC lesions of the esophagus suggested that VIM methylation might be useful as a biomarker to assist in detection of these diseases. To explore this concept, we developed a “molecular cytology” assay in which DNA was extracted from cytology brushings of the esophagus, and tested for VIM methylation. Using this approach, detectable VIM methylation was found in 7 of 7 BE patients, 4 of 4 HGD patients, and 15 of 18 EAC patients. Reflecting cellular dilution of brushing samples in some cases, levels of VIM methylation detected ranged from 3% to 75% of input DNA (Fig 2). In contrast, no VIM methylation (above a 0.1% detection limit) was detected in esophageal brushings from any of 5 control individuals with normal upper endoscopies (Fig 2). As a further control, brushings from these individuals’ gastric cardia were also tested, and again, these specimens were all negative for VIM methylation (Fig 2). The finding of VIM methylation in cases but not in controls was statistically significant for each of the groups tested using generalized linear models with contrasts ($p=0.00049$ for all comparisons; $p=0.006$ for BE versus controls, $p=0.011$ for HGD versus controls, $p=0.0067$ for EAC versus controls). The finding of methylation in cases but not in controls remained statistically significant for each of the groups tested even if a stringent cut-off of 10% methylation is used to classify cases as “VIM-methylated” (which would be 100-fold above the detection limit that separates cases from controls in this study) using generalized linear models with contrasts ($p=0.0125$ for all comparisons, $p=0.001$ for BE versus controls, $p=0.0083$ for HGD versus controls, $p=0.0028$ for EAC versus controls). In 4 additional and separate cases (two HGD and two EAC) we obtained both a directed brushing of the esophageal lesion of interest and an undirected “blind” brushing along the entire length of the esophagus. In all 4 of these cases, VIM methylation was easily and well detected in the undirected sample, as well as the targeted brushing (data not shown). This preliminary observation suggests that testing VIM methylation in undirected brushings of the esophagus could potentially be used as a less invasive alternative to conventional endoscopic screening for BE.

Vimentin methylation in adenocarcinoma of pancreas, duodenum and stomach

The finding of highly frequent VIM methylation in esophageal lesions raised the possibility that VIM methylation might typify epithelial neoplasia throughout the upper gastrointestinal tract, as well as in the colon. To test this, we examined a set of FFPE tissue samples of

cancers from the stomach, pancreas, duodenum, and small intestine. Similar to our findings in the esophagus, VIM methylation also proved commonly present in gastric cancers. High levels of VIM methylation (10%–100%) were identified in thirteen of fifteen (87%) signet ring gastric cancers and in nine of 17 (53%) intestinal type gastric cancers (Fig. 3A). The difference between these two gastric cancer types was not statistically significant ($p=0.061$, Fisher's exact test, two-sided). Low level VIM methylation (1%–10%) was further detected in FFPE tissue specimens from an additional 3 intestinal type gastric cancers. VIM methylation was not detected in any of 5 FFPE normal gastric mucosa samples from cancer free individuals (Fig 5A, normal 1), and was also not detected in 5 of 7 FFPE accompanying normal gastric mucosa samples from individuals with gastric cancer (Fig 3A, normal 2). In the remaining 2 gastric cancer cases, trace (<1%) VIM methylation was detected in the accompanying FFPE normal gastric mucosa specimens. This suggests that either trace cancer cells were admixed with the normal mucosa, or that the cancers may have developed from a field of initiated cells, marked by early acquisition of VIM methylation. Supporting this latter possibility is the finding that in one individual who harbored both a gastric cancer and a gastric dysplasia, both lesions were positive for VIM methylation (data not shown). VIM methylation was not limited to stomach, but was also detected in FFPE tissue samples from cancer of the duodenum, pancreas and distal small intestine, (Fig 3B). Similar to our observations in individuals with stomach cancer, trace levels of less than 1% of VIM methylation were detected in accompanying normal duodenal FFPE tissue specimens collected from duodenal cancer cases.

DISCUSSION

These results demonstrate that aberrant VIM methylation is a common epigenetic event in neoplasias of the upper gastrointestinal tract. We particularly observed the highest frequency of VIM methylation in neoplasia of the lower esophagus, with high level VIM-methylation detected in 91% of BE, 100% of HGD, and 81% of EAC. These findings establish VIM methylation as a highly frequent DNA alteration in BE and BE derived neoplasias. The high frequency of VIM methylation across BE, HGD, and EAC, the co-synchronous detection of VIM methylation in BE and adjacent EAC specimens, and the presence of high level VIM methylation in BE lesions several years prior to VIM methylated HGD and EAC, provide further molecular evidence of BE as the precursor lesion for EAC cancers. Moreover, detection of VIM-methylation in cytology brushings from BE, HGD, and EAC, and the absence of readout in brushings from normal controls, suggests that VIM methylation may provide a very clean marker for a “molecular cytology” approach to screening for these diseases.

EAC has steadily increased in incidence over recent decades. With an 85% mortality rate, this cancer is the most rapidly increasing cause of cancer mortality from solid tumors in the American population.(12–15) There has thus been substantial interest in development of screening approaches for early detection of EAC and its premalignant precursor lesion, Barrett's esophagus. However, the majority of EACs develop in patients without prior symptoms. Thus current clinical practices that initiate endoscopic screening based on herald symptoms have not significantly impacted EAC mortality.(11) This has prompted efforts to develop minimally invasive screening procedures for EAC and BE.(16) One such approach has, for example, used an ingested sponge for sampling of esophageal cells, coupled with immunocytochemistry for trefoil factor 3, a biomarker for detection of BE changes.(17,18) Our finding of VIM methylation as a highly frequent, highly specific, early biomarker of BE that is readily detectable in esophageal brushings by a highly sensitive methylation specific PCR, provides the initial evidence to support a molecular cytology approach to minimally invasive clinical screening and early detection of asymptomatic premalignant stage disease based on this biomarker. Moreover, our detection of VIM methylation in 87% of signet ring

and 53% of intestinal type gastric cancers makes VIM methylation among the most common DNA alterations associated with gastric cancer. Although gastric cancer accounts for fewer annual deaths in the US than esophageal cancer, gastric cancer remains a significant cause of cancer mortality, especially worldwide.(13,19) VIM methylation may accordingly add to the panel of other methylated markers that have been described for detection of this disease.(20)

Combined with our previous findings of VIM methylation in up to 83% of colon cancers (2), VIM methylation emerges as a high frequency epigenetic finding associated with neoplasia in both the upper and the lower gastrointestinal tract. VIM methylation can be detected in DNA isolated from feces in 77% of colon cancer patients,(3) and the American Cancer Society has added fecal DNA screening to their guidelines of methods endorsed for colon cancer screening.(21) However, one important question regarding this approach has been the finding that some individuals with VIM methylation detected in stool DNA have normal colonoscopies.(3) Our observations that VIM methylation is also common in neoplasias of the upper gastrointestinal tract raise the intriguing possibility that in some individuals the detection of VIM methylation in stool DNA may reflect the presence of neoplasia in the upper GI tract. This hypothesis is also consistent with observations of other investigators who have reported detection in stool DNA of other methylated or mutated markers arising from gastric and esophageal cancers.(20,22) It will be of clear interest to incorporate these hypotheses into the design of future clinical trials that examine testing for VIM and other DNA methylation biomarkers in stool and other body fluids for early detection of neoplastic disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Vimentin methylation in Barrett's Esophagus and esophageal neoplasias. Shown is percent of Vimentin methylation relative to total actin DNA detected in each sample. Circles denote individual samples. Samples in which no VIM methylation was detected are depicted as having 0.1% methylated DNA, which in most cases represented the lower limit of detection of the assay. In a few samples in which a lesser amount of input DNA was available, bars raised above the 0.1% level designate the slightly higher threshold that applied as the lower limit for detection of positive VIM methylation. Normal: Normal Squamous Mucosa; BE: Barrett's Esophagus; HGD: High-Grade Dysplasia; EAC: Esophageal Adenocarcinoma; SCC: Squamous Cell Cancer of the Esophagus.

Figure 2.

Percent Vimentin methylation in targeted esophageal brushings from individuals with: BE: Barrett's Esophagus; HGD: High Grade Dysplasia; EAC: Adenocarcinoma of the Esophagus, as well as from normal controls who were sampled in both squamous esophagus (SQ), and gastric cardia (cardia).

Figure 3.

Vimentin methylation in gastric, small intestinal, pancreatic and duodenal cancer. Shown is percent of Vimentin methylation relative to total actin DNA in each sample. Circles denote individual samples. Samples in which no VIM methylation was detected are depicted as having 0.1% methylated DNA, which in most cases represented the lower limit of detection of the assay. In a few samples in which a lesser amount of input DNA was available, bars raised above the 0.1% level designate the slightly higher threshold that applied as the lower limit for detection of positive VIM methylation.. Panel A: Normal-1: Normal gastric mucosa from cancer free cases; Normal-2: Normal gastric mucosa from cases with concurrent gastric cancers; Signet Ring Cancer: Signet Ring Gastric Cancer samples; Intestinal Type Cancer: Intestinal Type Gastric Cancer samples. Panel B: Normal, morphologically normal duodenal tissue from patients with duodenal adenoma or cancer; Duodenal Cancer: Duodenal Cancer Samples; SI Cancer: Small Intestinal Cancer Samples; Pancreatic Cancer: Pancreatic Cancer Samples.

Table 1

Primers and probes used in real-time PCR. [+N] denotes Locked Nucleic Acid (LNA) bases.

Gene	Primer/probe	Primer/probe sequence	Annealing/Extension	Size (bp)	Notes
VIM	Forward Primer	TCGTTTCGAGGTTTTCGCGTTAGAGAC	68°C	217	Primers from Ref. (2)
	Reverse Primer	CGACTAAAACTCGACCGACTCGCGGA			
	Probe	[6FAM]-CGGGAGTTAGT[+T]CGCGTTATCGTCGTCGTTT-[BHQ1]			
Actin	Forward Primer	GGATAGGATAGTTTATTTTATTTAG	57°C	217	
	Reverse Primer	ATACAAAACCTATCTCAACCAA			
	Probe	5'-6FAM-ACCAACCACCAACACACAATAACAACACA-BHQ1-3'			Probe from Ref. (8)