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Cancer Epidemiology, Biomarkers & Prevention

Research Article

Aberrant Vimentin Methylation Is Characteristic of Upper Gastrointestinal Pathologies

Helen Moinova[1], Rom S. Leidner[1], Lakshmeswari Ravi[1], James Lutterbaugh[1], Jill S. Barnholtz-Sloan[3], Yanwen Chen[3], Amitabh Chak[1,3], Sanford D. Markowitz[1,3], and Joseph E. Willis[2,3]

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 vimentin methylation in neoplasia of the upper gastrointestinal tract.

Methods: Using a quantitative real-time methylation-specific PCR assay, we tested for vimentin methylation in archival specimens of esophageal and gastric neoplasia.

Results: We find that acquisition of aberrant vimentin methylation is highly common in these neoplasms, but largely absent in controls. The highest frequency of vimentin methylation was detected in lesions of the distal esophagus, including 91% of Barrett's esophagus ($n = 11$), 100% of high-grade dysplasia (HGD, $n = 5$), and 81% of esophageal adenocarcinoma (EAC, $n = 26$) but absent in controls ($n = 9$). Vimentin 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 vimentin methylation proved detectable in 100% of Barrett's esophagus 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 vimentin methylation as a highly common epigenetic alteration in neoplasia of the upper gastrointestinal tract and show that Barrett's esophagus, even without dysplasia, already contains epigenetic alterations characteristic of adenocarcinoma.

Impact: These findings suggest vimentin methylation as a biomarker of upper gastrointestinal neoplasia with potential for development as molecular cytology in esophageal screening. *Cancer Epidemiol Biomarkers Prev; 21(4); 594–600. ©2012 AACR.*

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% to 83% of colon tumors but is virtually absent in normal colonic epithelium. On the basis of these findings, detection of vimentin 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 vimentin 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 vimentin methylation and then interrogated a range of lesions of the upper gastrointestinal tract in an archive of formalin-fixed, paraffin-embedded (FFPE) tissue. We further tested whether vimentin methylation could be detected in endoscopically acquired cytology brushings from upper gastrointestinal lesions.

Authors' Affiliations: Departments of [1]Medicine and [2]Pathology, and [3]Case Comprehensive Cancer Center, Case Western Reserve University and Case Medical Center, Cleveland, Ohio

Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

H. Moinova and R.S. Leidner contributed equally to the study.

Corresponding Authors: S.D. Markowitz, Case Western Reserve University, 10900 Euclid Avenue, Wolstein Research Building, Room 3-128, Cleveland, OH 44106. Phone: 216-368-1976; Fax: 216-368-8928; E-mail: SXM10@cwru.edu; and J.E. Willis, Department of Pathology, Case Medical Center, 11100 Euclid Avenue, Cleveland, OH 44106. Phone: 216-844-8292; Fax: 216-844-4895; E-mail: Joseph.Willis@case.edu

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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 (Cleveland, OH) using a tissue procurement protocol approved by University Hospitals Case Medical Center Institutional Review Board (IRB). Before use, diagnostic slides of all samples were reviewed by a gastrointestinal pathologist

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(J.E. Willis) for confirmation of the recorded diagnoses and particularly for confirmation of diagnoses of high-grade dysplasia (HGD). 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 are included in Supplementary Table S1.

FFPE tissue DNA

DNA was purified by 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 to 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 methylation-specific PCR assay

To create a template for methylation-specific PCR, DNA samples were subjected to bisulfite conversion and purified by an Epitect kit (QIAGEN) according to the manufacturer's protocol. Four microliters of bisulfite-converted DNA at a concentration of 0.2 to 25 ng/μL was used as a template for real-time methylation-specific 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 vimentin. For both actin and vimentin real-time PCR, a mixture of DNA from 4 colon cancer cell lines that are each fully methylated across the vimentin 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 C_t values into nanogram DNA amounts. Vimentin methylation was calculated as percentage ratio of the amount of methylated DNA measured by vimentin quantitative PCR, divided by total bisulfite-converted DNA amount in the sample, as measured by actin quantitative PCR. The real-time methylation-specific PCR reactions were carried out in triplicates in 20 μL volume using the LightCycler PCR Master Mix (Roche) with 400 nmol/L each primer and 200 nmol/L 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 minutes, followed by 50 cycles of 30 seconds at 95°C and 60 seconds at primer-specific annealing/extension temperature (see Table 1).

Esophageal brushings for molecular cytology

Lesional brushings were obtained during research endoscopy from subjects with Barrett's esophagus, HGD, and esophageal adenocarcinoma (EAC) under an IRB-approved protocol at the Case Medical Center. Under the same protocol, brushings were obtained from patients without Barrett's esophagus; from both the distal esophagus and gastric cardia. Diagnosis in all cases and controls was established by histopathologic biopsy review. Demographic and clinical data for all patients in this study are included in Supplementary Table S2. Endoscopically collected cytobrushes were clipped into nuclease free 0.5 mL Cryo-Safe tubes and immediately snap frozen on dry ice for transport to gas phase liquid nitrogen storage until use. The QIAamp 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

Table 1. Primers and probes used in real-time PCR

Gene	Primer/probe	Primer/probe sequence	Annealing/ extension	Size, bp	Notes
VIM	Forward primer	TCGTTTCGAGGTTTTCGCGTTAGAGAC	68°C	217	Primers from ref. (2)
	Reverse primer	CGACTAAAACTCGACCGACTCGCGA			
	Probe	[6FAM]-CGGGAGTTAGT[+T]CGCGTTATCGTCGTCGTTT-[BHQ1]			
Actin	Forward primer	GGATAGGATAGTTTTATTTTTAG	57°C	217	
	Reverse primer	ATACAAAACTATACTCAACCAA			
	Probe	5′-6FAM-ACCACCACCCAACACACAATAACAAACACA-BHQ1-3′			Probe from ref. (8)

NOTE: [+N] denotes locked nucleic acid (LNA) bases.

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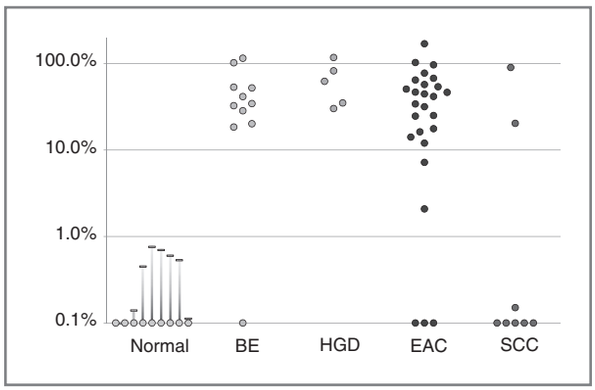

Figure 1. Vimentin methylation in Barrett's esophagus (BE) and esophageal neoplasias. Shown is percentage of vimentin methylation relative to total actin DNA detected in each sample. Circles denote individual samples. Samples in which no vimentin methylation was detected are depicted as having 0.1% methylated 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 vimentin methylation. Normal-normal squamous mucosa; SCC-squamous cell cancer of the esophagus.

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 vimentin 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 vimentin methylation, ranging from 10% to 100% of total tumor DNA (Fig. 1). As tumor tissues contained both cancer and normal stromal elements, we interpret these levels as suggesting vimentin methylation was likely present in all of the cancer cells. Two additional EAC cases showed lower levels of vimentin methylation between 1% and 10%, for an overall detection rate of 88%. High-level vimentin methylation was detected in 2 of 9 squamous carcinomas of the esophagus, ranging from 10% to 100% of total tumor DNA. The difference between squamous cancer and adenocarcinoma subtypes was statistically significant, $P = 0.003$ (the Fisher exact test, 2 sided). No vimentin 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 vimentin methylation in the development of EAC, we analyzed FFPE tissue DNA from cases of Barrett's Esophagus. Barrett's esophagus 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 Fig. 1, high-level vimentin methylation ranging from 10% to 100% was detected in FFPE tissue DNA from 10 of 11 (91%) cases of Barrett's esophagus without dysplasia and 5 of 5 Barrett's esophagus cases with HGD. These findings suggested that vimentin methylation is an early and frequent epigenetic alteration in the Barrett's esophagus sequence from metaplasia to dysplasia to EAC in the distal esophagus.

In 4 of the cases studied, the specimen of Barrett's esophagus and/or Barrett's esophagus with HGD was derived from a biopsy obtained concurrently and adjacent to an EAC specimen that we had also studied (Supplementary Fig. S1). In all 4 cases, the high level of vimentin methylation detected in the EAC was also detected in the synchronous Barrett's esophagus and/or Barrett's esophagus with HGD tissue (Supplementary Fig. S1). We did note one instance of an individual with 2 concurrent biopsies of Barrett's esophagus, in which one of the 2 biopsies tested negative for vimentin methylation (data not shown).

In five additional cases, we were able to identify a previous FFPE tissue sample obtained during routine clinical surveillance for Barrett's esophagus, spanning 1 to 7 years of follow-up (Supplementary Fig. S2). In 2 individuals in which Barrett's esophagus progressed over time, in one case to HGD and in the other to EAC, both the initial Barrett's esophagus specimen and the progressed specimen showed vimentin methylation. In 2 additional individuals in which Barrett's esophagus did not progress, both the early and later specimens showed vimentin methylation. Only in one individual, was vimentin methylation detected in 2 concurrent biopsies of Barrett's esophagus, but was absent in a Barrett's esophagus specimen obtained from the same individual 4 years earlier (patient 14, Supplementary Fig. S2 and data not shown). This is consistent with a model of vimentin methylation as an early epigenetic event in carcinogenesis and of EAC arising from an initiated field of Barrett's esophagus.

Detection of Barrett's esophagus associated lesions by "molecular cytology"

The findings of high-level vimentin methylation in archival specimens of Barrett's esophagus, HGD, and EAC lesions of the esophagus suggested that vimentin 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 vimentin methylation. Using this approach, detectable vimentin methylation was found in 7 of 7 patients with Barrett's esophagus, 4 of 4 patients with HGD, and 15 of 18 patients with EAC. Reflecting cellular dilution of brushing samples in some cases, levels of vimentin methylation detected ranged from 3% to 75% of input DNA (Fig. 2). In contrast, no vimentin methylation (above a 0.1% detection limit) was detected in esophageal brushings from any of 5 control individuals with

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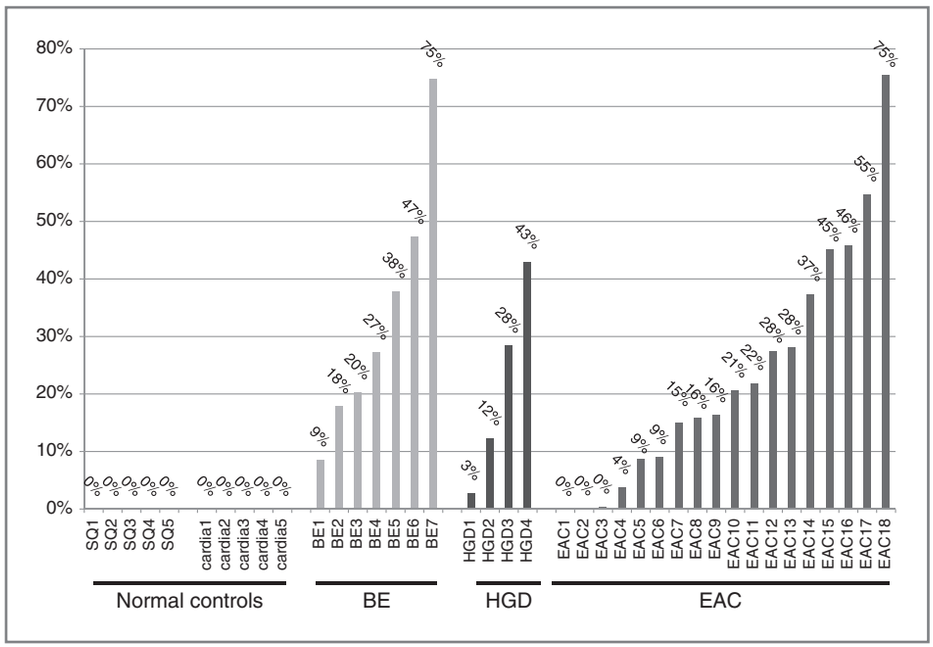

Figure 2. Percentage of vimentin methylation in targeted esophageal brushings from individuals with BE, EAC, as well as from normal controls who were sampled in both squamous esophagus (SQ) and gastric cardia (cardia).

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 vimentin methylation (Fig. 2). The finding of vimentin 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 Barrett's esophagus vs. controls, $P = 0.011$ for HGD vs. controls, $P = 0.0067$ for EAC vs. controls). The finding of methylation in cases but not in controls remained statistically significant for each of the groups tested even if a stringent cutoff value of 10% methylation is used to classify cases as "vimentin 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 Barrett's esophagus vs. controls, $P = 0.0083$ for HGD vs. controls, $P = 0.0028$ for EAC vs. controls). In 4 additional and separate cases (2 HGD and 2 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, vimentin 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 vimentin methylation in undirected brushings of the esophagus could potentially be used as a less invasive alternative to conventional endoscopic screening for Barrett's esophagus.

Vimentin methylation in adenocarcinoma of pancreas, duodenum, and stomach

The finding of highly frequent vimentin methylation in esophageal lesions raised the possibility that vimentin 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, vimentin methylation also proved commonly present in gastric cancers. High levels of vimentin methylation (10%–100%) were identified in 13 of 15 (87%) signet ring gastric cancers and in 9 of 17 (53%) intestinal type gastric cancers (Fig. 3A). The difference between these 2 gastric cancer types was not statistically significant ($P = 0.061$, the Fisher exact test, 2 sided). Low level vimentin methylation (1%–10%) was further detected in FFPE tissue specimens from an additional 3 intestinal type gastric cancers. Vimentin methylation was not detected in any of 5 FFPE normal gastric mucosa samples from cancer-free individuals (Fig. 3A, 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%) vimentin 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 vimentin 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 vimentin methylation (data not shown). Vimentin 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 vimentin

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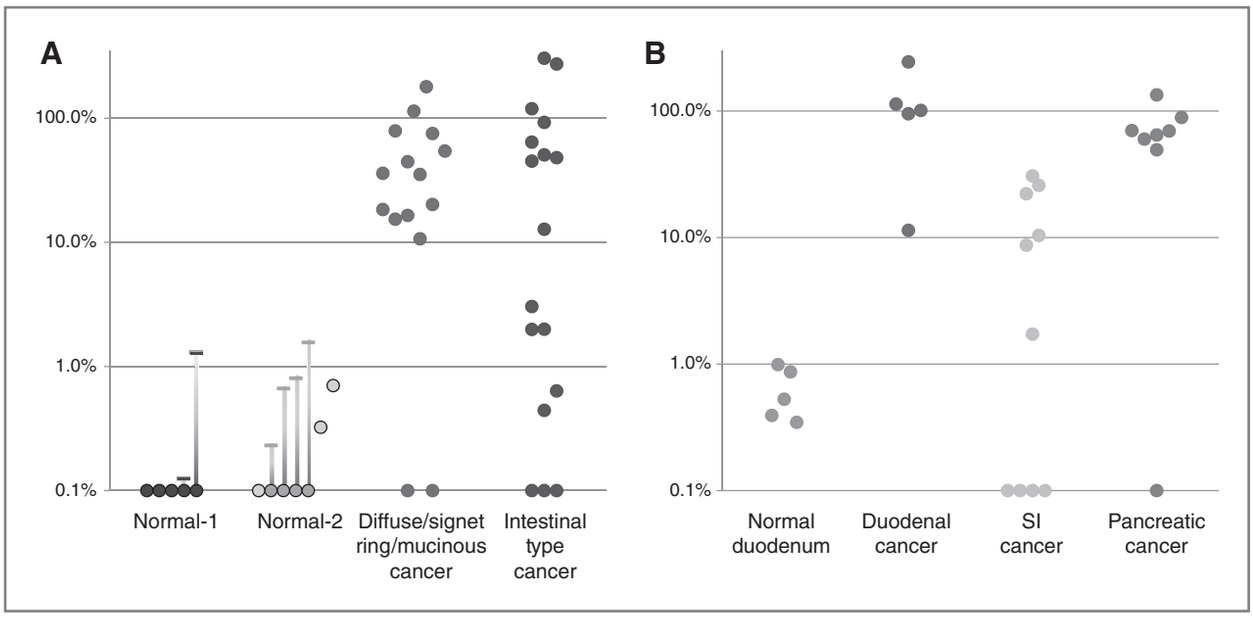

Figure 3. Vimentin methylation in gastric, small intestinal (SI), pancreatic, and duodenal cancer. Shown is percentage of vimentin methylation relative to total actin DNA in each sample. Circles denote individual samples. Samples in which no vimentin methylation was detected are depicted as having 0.1% 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 vimentin methylation. A: Normal-1: normal gastric mucosa from cancer-free cases; Normal-2: normal gastric mucosa from cases with concurrent gastric cancers; B: normal: morphologically normal duodenal tissue from patients with duodenal adenoma or cancer. SI cancer, small intestinal cancer samples.

methylation were detected in accompanying normal duodenal FFPE tissue specimens collected from duodenal cancer cases.

Discussion

These results show that aberrant vimentin methylation is a common epigenetic event in neoplasias of the upper gastrointestinal tract. We particularly observed the highest frequency of vimentin methylation in neoplasia of the lower esophagus, with high-level vimentin methylation detected in 91% of Barrett's esophagus, 100% of HGD, and 81% of EAC. These findings establish vimentin methylation as a highly frequent DNA alteration in Barrett's esophagus and Barrett's esophagus–derived neoplasias. The high frequency of vimentin methylation across Barrett's esophagus, HGD, and EAC, the cosynchronous detection of vimentin methylation in Barrett's esophagus and adjacent EAC specimens, and the presence of high-level vimentin methylation in Barrett's esophagus lesions several years before vimentin-methylated HGD and EAC, provide further molecular evidence of Barrett's esophagus as the precursor lesion for EAC cancers. Moreover, detection of vimentin methylation in cytology brushings from Barrett's esophagus, HGD, and EAC, and the absence of readout in brushings from normal controls, suggests that vimentin 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 Barrett's esophagus (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 Barrett's esophagus changes (17, 18). Our finding of vimentin methylation as a highly frequent, highly specific, early biomarker of Barrett's esophagus 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 vimentin methylation in 87% of signet ring and 53% of intestinal type gastric cancers makes vimentin methylation among the

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most common DNA alterations associated with gastric cancer. Although gastric cancer accounts for fewer annual deaths in the United States than esophageal cancer, gastric cancer remains a significant cause of cancer mortality, especially worldwide (13, 19). Vimentin 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 vimentin methylation in up to 83% of colon cancers (2), vimentin methylation emerges as a high-frequency epigenetic finding associated with neoplasia in both the upper and the lower gastrointestinal tract. Vimentin methylation can be detected in DNA isolated from feces in 77% of patients with colon cancer (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 about this approach has been the finding that some individuals with vimentin methylation detected in stool DNA have normal colonoscopies (3). Our observations that vimentin methylation is also common in neoplasias of the upper gastrointestinal tract raise the intriguing possibility that in some individuals the detection of vimentin methylation in stool DNA may reflect the presence of neoplasia in the upper gastrointestinal 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 vimentin and other DNA methylation biomarkers in stool and other body fluids for early detection of neoplastic disease.

Disclosure of Potential Conflicts of Interest

A. Chak, minor ownership interest, Case Western Reserve University; J.E. Willis, minor ownership interest, patent application; R.S. Leidner, minor ownership interest, Case Western Reserve University; and S.D. Markowitz, minor ownership interest, exact sciences and patent application.

Authors' Contributions

Conception and design: H.R. Moinova, R.S. Leidner, A. Chak, S.D. Markowitz, J.E. Willis
Development of methodology: H.R. Moinova, R.S. Leidner, A. Chak, S.D. Markowitz, J.E. Willis
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.R. Moinova, R.S. Leidner, L. Ravi, A. Chak, S. D. Markowitz, J.E. Willis
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H.R. Moinova, R.S. Leidner, J.S. Barnholtz-Sloan, Y. Chen, A. Chak, S.D. Markowitz, J.E. Willis
Writing, review, and/or revision of the manuscript: H.R. Moinova, R.S. Leidner, J.S. Barnholtz-Sloan, Y. Chen, A. Chak, S.D. Markowitz, J.E. Willis
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases) Study supervision: H.R. Moinova, R.S. Leidner, A. Chak, S.D. Markowitz, J.E. Willis
Study supervision: S.D. Markowitz, J.E. Willis

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