Methylation of $B3GAT2$ and $ZNF793$ Are Potential Detection Biomarkers for Barrett’s Esophagus

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Abstract

Background: Barrett’s esophagus (BE) is a preneoplastic condition in which normal esophageal squamous epithelium (SQ) is replaced by specialized intestinal metaplasia. It is the presumed precursor for esophageal adenocarcinoma (EAC) as well as the strongest risk factor for this cancer. Unfortunately, many patients with BE go undiagnosed under the current BE screening guidelines. The development of noninvasive and accurate BE detection assays could potentially identify many of these undiagnosed BE patients.

Methods: DNA methylation is a common epigenetic alteration in BE. Therefore, we conducted a genome-wide methylation screen to identify potential BE biomarkers. Samples from SQ ($N = 12$), stomach ($N = 26$), and BE ($N = 29$) were analyzed and methylation levels at over 485,000 CpG sites were compared. Pyrosequencing assays were used to validate the results and MethyLight assays were developed to detect the methylated alleles in endoscopic brushings.

Results: We discovered two genes, $B3GAT2$ and $ZNF793$, that are aberrantly methylated in BE. Clinical validation studies confirmed $B3GAT2$ and $ZNF793$ methylation levels were significantly higher in BE samples (median = 32.5% and 33.1%, respectively) than in control tissues (median = 2.29% and 2.52%, respectively; $P < 0.0001$ for both genes). Furthermore, gene-specific MethyLight assays could accurately detect BE ($P < 0.0001$ for both) in endoscopic brushing samples.

Conclusion: $B3GAT2$ and $ZNF793$ are hypermethylated in BE, and the methylation status of these genes can be used to detect BE in tissue samples.

Impact: These findings support the development of methylated $B3GAT2$ and $ZNF793$ as biomarkers for noninvasive assays for the detection of BE. Cancer Epidemiol Biomarkers Prev; 24(12): 1890–7. ©2015 AACR.

Introduction

Esophageal adenocarcinoma (EAC) is among the most rapidly increasing cancers in western populations, with a rise in incidence from 0.63/100,000 in 1980 to 3.32 cases/100,000 in 2010 (1). In 2012, there are estimated to have been approximately 17,500 people in the United States affected by esophageal cancer resulting in over 15,000 deaths (2).

EAC is thought to arise from Barrett’s esophagus (BE), a preneoplastic condition in which the normal squamous lining of the lower esophagus is replaced by specialized intestinal-type metaplasia. BE can progress to EAC through a multistep sequence involving low-grade dysplasia, high-grade dysplasia, and finally EAC. Being the strongest risk factor for EAC, in a recent large cohort study, the presence of BE was found to confer an 11-fold increased risk of EAC compared with the general population (3). Epidemiologic studies suggest BE is a prevalent condition, occurring in approximately 1.6% of the population (4) and is most common in people who are older (5), male (6), obese (7), and who have chronic gastro-esophageal reflux disease (GERD; ref. 8). Because of the increased risk for EAC, people with BE are placed in surveillance programs that entail regular gastrointestinal endoscopy exams with biopsies of the BE segment to assess for dysplasia. The value of these surveillance programs, however, has been questioned because of its relatively high resource utilization, invasive nature, and the mixed results of studies assessing their impact on EAC incidence or survival (3, 6, 9, 10). One factor that likely has affected the impact of BE surveillance programs is that the majority of patients with BE remain undiagnosed and therefore are not enrolled in surveillance programs. It is estimated that 95% of patients with a new diagnosis of EAC do not have a
previous diagnosis of BE and are not under surveillance (11–14). The consequence of undiagnosed BE patients not being under surveillance can be appreciated by studies that demonstrate improved survival in EAC patients diagnosed during surveillance compared with EAC patients diagnosed at the time of symptoms (11–13). Thus, there is a need to develop accurate, less-invasive methods for diagnosing BE that could be used in population-based screening programs. This need led us to carry out a series of studies to identify potential detection biomarkers for BE.

Aberrant DNA methylation is a common epigenetic alteration in a variety of human cancers, including EAC, and associated precursors, like BE (15, 16). Thus, aberrantly methylated genes have the potential to serve as detection biomarkers for BE and EAC. In this study, we conducted a genome-wide analysis of normal esophageal squamous epithelium (SQ), stomach (cardia and fundus), and BE and identified two genes, β-1,3-galacturonyl-transferase 2 (B3GAT2) and zinc finger 793 (ZNF793), as aberrantly methylated genes in nondysplastic BE compared with control tissue. Using orthogonal assays, we confirmed the high prevalence of methylated B3GAT2 and ZNF793 in BE and the lack of methylation at these loci in control tissues. Consequently, we developed MethyLight assays and demonstrated methylated B3GAT2 and ZNF793 could be used to accurately detect BE in endoscopic brushing samples. These findings suggest that methylated B3GAT2 and ZNF793 have the potential to be used as detection biomarkers in noninvasive assays for BE.

Materials and Methods

Tissue samples

Formalin-fixed paraffin-embedded (FFPE) slides and cores were obtained from Case Western Reserve University/University Hospitals of Cleveland (Cleveland, OH), the University of Michigan Medical School (Ann Arbor, MI), and University of North Carolina School of Medicine (Chapel Hill, NC), following protocols approved by the Institutional Review Board of each institution. An expert pathologist (JEW or MW) reviewed H&E-stained sections in order to confirm the diagnosis and to clearly identify areas enriched for the different histologic subtypes of interest that would be subjected to DNA extraction. In those sections with mixed histology (e.g., BE and SQ), care was taken to separate these areas using microdissection with a sterile razor blade or to identify an area with a high proportion of the histology of interest that would be targeted for a core extraction. A total of 12 SQ, 12 cardia/carditis, 16 fundus, and 29 BE samples from 44 patients were subjected to analysis using the Illumina HumanMethylation450 BeadChip arrays (HM450 arrays), followed by pyrosequencing-based assays for technical validation. Further, an independent set of samples (21 SQ, 14 cardia, 14 fundus, and 33 BE from 66 patients) were used in pyrosequencing-based assays to validate the HM450 array results.

Endoscopic brushing specimens were collected as previously reported (17). A total of 30 SQ, 14 cardia, 10 BE samples from were used to develop the B3GAT2 and ZNF793 MethyLight screening assays. Twenty-three of the control samples had esophagealadrenocarcinoma for GERC indications and 2 of them had Los Angeles Grade B erosive esophagitis. All BE brushing specimens were obtained from patients who had a previously confirmed diagnosis via upper endoscopy and histology. The brushings were obtained prior to passage of the endoscope through the area of interest. Demographic information on the subjects from which the clinical samples were obtained can be found in Supplementary Table S1.

Sample preparation

Genomic DNA was extracted from FFPE cores and tissue sections using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions with a modification of incubation time of 7–8 days in proteinase K for FFPE cores. Genomic DNA was eluted into a 50 μl total volume and quantified with a ND-1000 spectrophotometer (NanoDrop Technologies) or using the Quant-iT PicoGreen DNA Assay Kit following the manufacturer's instructions (Life Technologies). Two hundred and fifty nanograms of DNA from each sample was bisulfite converted using the EZ DNA Methylation Kit (ZymoResearch) and eluted in either an 8 μl volume for HM450 arrays or a 40 μl volume for pyrosequencing.

Genome-wide methylation arrays

Bisulfite converted DNA was processed with Infinium HD FFPE DNA Restore Kit, according to the manufacturer's instructions (Illumina Inc.). The DNA samples were submitted to the Genomics Core at the Fred Hutchinson Cancer Research Center (FHCRC) and processed to run the HM450 arrays following the manufacturer's instructions (Illumina Inc.). Data acquisition, normalization, filtering, and analysis were conducted as previously described (15). Methylation values from the HM450 arrays are reported as “β values,” where 0.0 is equivalent to 0% methylation and 1.0 is equivalent to 100% methylation at a given CpG dinucleotide (18). Differentially methylated loci were identified by comparing mean β values in BE group to the mean of β values in SQ control tissues for a particular array CpG site. To account for multiple comparisons, false discovery rate q values were calculated to determine the significance of differentially methylated loci (19): a false discovery rate q value <0.05 was used as the threshold for statistically significant results in this set of studies.

Pyrosequencing

The differentially methylated CpGs between SQ and BE cases identified by the HM450 DNA methylation array analysis were validated by pyrosequencing-based studies. For B3GAT2 and ZNF793 pyrosequencing assays, primers and probes targeting sequences surrounding the specific CpG dinucleotide identified on the arrays were designed with the PyroMark Primer Assay Design 2.0 software (Qiagen; Fig. 1 and Table 1). Assays were initially chosen based on the following criteria: (i) assay primer binding accuracy (>70%) as determined by the primer design software, (ii) amplicon size ~100 bp, and (iii) no potential for primer-dimer binding. The PCR amplification step was performed using the Qiagen PyroMark PCR kit (Qiagen) following the manufacturers' instructions. The optimal annealing temperatures were determined by temperature gradient PCR with Epitext 100% methylated and unmethylated control DNA (Qiagen). For each pyrosequencing assay, a series of standard DNA samples with known percentages of methylated DNA (0%, 20%, 40%, 60%, 80%, and 100%) were used to confirm the accuracy of the assays before assessing the clinical samples (Supplementary Fig. S1). Assays that exceeded r 2 0.85 were considered to be accurate for use in the validation studies. The pyrosequencing assays were first run using the same samples contained on the HM450 arrays in
order to assess the accuracy of the HM450 array results at the CpG sites of interest (Supplementary Fig. S2–S5). The pyrosequencing assays were then run on a second independent set of samples (“validation cohort”; Fig. 2 and Supplementary Fig. S6). Potential biomarkers were eliminated from further analysis if the findings from the HM450 arrays were not confirmed using the pyrosequencing assays on the same samples used for the HM450 arrays (“technical validation experiments”) or if the pyrosequencing assay results did not significantly differentiate BE from control tissues in the validation cohort samples.

MethyLight PCR
We developed MethyLight PCR assays for detecting methylated B3GAT2 and ZNF793 in DNA from tissue biopsies and brushings. The primer and probe sequences for methylated B3GAT2 (NM_001013659) and methylated ZNF793 (NM_080742.2) were designed using ABI Primer Express software Version 5.0. The locations of primers and probes are indicated in Fig. 1. We also used a methylation-independent ALUC4 control reaction to normalize input DNA amounts, following a previously described protocol (20). ALUC4 has been shown to be an accurate locus for normalization of DNA input for MethyLight assays by Weisenberger and colleagues (21). A complete list of all MethyLight primer and probe sequences is provided in Table 2.

The MethyLight PCR reaction mixture consisted of the 2× ITAQ Universal Probes Supermix (BioRad), and locus specific primers and probes. The primer and probes were used at final concentrations of 900 and 250 nmol/L, respectively. Bisulfite-converted DNA was used as a template for MethyLight PCR assay in a final reaction volume of 20 μL. Each MethyLight PCR reaction was performed using the CFX96 Touch Real-Time PCR Detection System (BioRad). The thermocycler conditions were: 95°C for 15 minutes followed by 49 cycles of 95°C for 15 seconds and 60°C for 1 minute. 100% methylated EpiTect Methyl DNA and 100% unmethylated EpiTect Unmethyl DNA (Qiagen) were used as the positive and negative control samples. All samples were run in duplicate in at least two independent reactions. Data were analyzed using the BioRad CFX Manager software version 3.1 and Cq was determined with the Single Threshold method (BioRad). To establish high stringency for the MethyLight PCR to detect methylated alleles only, an initial experiment was conducted using the positive and negative control samples, and a signal detection threshold was adjusted so that only methylated alleles were detected. Intraassay variation in concentration measured by %CV was less than 15%.

The methylation status of B3GAT2 and ZNF793 in the brushing samples was reported as a relative methylation percentage (RM%). RM% was calculated as a percentage ratio of 100 × (methylation/control), in which ‘methylation’ refers to the amount of methylated B3GAT2 or ZNF793 as determined by MethyLight assays, and ‘control’ refers to the amount of total bisulfite-converted DNA, as measured by ALUC4 MethyLight.

Table 1. Pyrosequencing primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cpg site</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Sequeceing primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>B3GAT2</td>
<td>cg03556906</td>
<td>AGTTAAGGATGAGGATGAGGT</td>
<td>AAAACTCTGCCCTCTCCATT</td>
<td>AATACAAAAAATGATTTATCC</td>
</tr>
<tr>
<td>ZNF793</td>
<td>cg24588375</td>
<td>ATTTAATTGAGATTTTGAGGT</td>
<td>AAAACTCTGCCCTCTCCATT</td>
<td>ATACAAAAAATGATTTTATCC</td>
</tr>
<tr>
<td>ZNF793</td>
<td>cg23926010</td>
<td>GAAATTTAGGATTTTGAGGT</td>
<td>AAAACTCTGCCCTCTCCATT</td>
<td>ATACAAAAAATGATTTTATCC</td>
</tr>
</tbody>
</table>

*All sequences are listed in the 5’-3’ orientation.
Barrett’s Esophagus Detection Biomarkers

Figure 2.
The majority of Barrett’s esophagus (BE) samples have higher levels of methylated B3GAT2 (cg16556906) and ZNF793 (cg24588375, cg0271801, and cg23296010) compared with control epithelial tissue, including normal esophagus (SQ) and stomach (cardia and fundus). Results of pyrosequencing assays for methylated B3GAT2 and ZNF793 run on an independent validation set of tissue samples (“Tissue Type”). Pyrosequencing assay results for CpG sites that are differentially methylated in the majority of BE compared with normal SQ and normal gastric epithelium (cardia and fundus) are shown for (A) cg16556906, located in B3GAT2; (B) cg24588375, located in ZNF793; (C) cg0271801, located in ZNF793; and (D) cg23296010, located in ZNF793. Horizontal bars indicate the median of each sample group (**, BE vs. SQ, cardia, fundus, or all control tissues, \(P < 0.0001\), determined by a two-tailed Wilcoxon–Mann–Whitney test).

Results

B3GAT2 and ZNF793 are aberrantly methylated in BE

We conducted genome-wide DNA methylation profiling using the Illumina HumanMethylation450 (HM450) Beadchip arrays on DNA samples from normal esophageal squamous epithelium (SQ; \(N = 12\)), cardia (\(N = 12\)), fundus (\(N = 16\)), and BE (\(N = 29\)). After data normalization, filtering, and analyses as previously described (15), we identified 17 methylated CpGs that were significantly aberrantly methylated in BE compared with normal SQ with established cutoff values: difference in \(\beta\)-value > 0.4 and false discovery rate \(q\)-value < 0.05. The CpG site (probe ID cg16556906) with one of the greatest differences in methylation values between BE and control samples (SQ, cardia, and fundus) was located in the promoter region of B3GAT2, a gene that encodes \(\beta\)-1,3-glucuronotransferase 2. Three other CpG loci (probe IDs cg24588375, cg02711801, cg23296010) that demonstrated large differences in methylation values between BE and control samples were obtained are Caucasians.

To validate the methylation status of these four CpGs using an orthogonal method, we developed pyrosequencing assays that assessed the same CpGs on the HM450 arrays and adjacent CpGs...
that were located within approximately 100 bp of the array CpG sites (Fig. 1). After determining the accuracy of the pyrosequencing assay design (Supplementary Fig. S1), we used these assays to assess the methylation status of B3GAT2 and ZNF793 in a subset of the cases that were used on the HM450 array studies. The pyrosequencing results correlated well with the HM450 array β-value results (Supplementary Fig. S2).

We also found that CpG sites adjacent (within ~20 bp) to the CpG identified in the HM450 array studies had similar methylation levels within the same sample (N = 55 pairs of samples, Spearman rank correlation test, P value < 0.0001). Of interest, a second CpG for B3GAT2 was identified using the pyrosequencing assays that exhibited a slightly higher specificity than the CpG site identified from the HM450 array. We name this CpG "PS2" (Fig. 1 and Supplementary Figs. S3 and S4).

Next, we assessed the methylation status of the four CpGs identified by the HM450 array and the additional B3GAT2 site using pyrosequencing on an independent set of samples that included control cases (Validation cohort; N = 49; 21 SQ, 14 cardia, 14 fundus) and BE (N = 33). As shown in Fig. 2A, all control cases except one cardia sample showed very low-level B3GAT2_c16556906 methylation (median methylation level = 2.29%), whereas DNA from BE without dysplasia samples had significantly higher methylation levels (median methylation level = 32.5%, P < 0.0001 for BE vs. SQ, cardia, or fundus). The results from the validation cohort confirmed that CpG site PS2 in B3GAT2 had higher methylation levels in BE cases (median methylation level = 45.4%, P < 0.0001 for BE vs. SQ, cardia, or fundus; Supplementary Fig. S6). Similarly, all 49 control samples had very low-level ZNF793 methylation (median = 3.13%, 2.02%, and 2.42% for SQ vs. cardia vs. fundus; Fig. 2B-D). The difference in ZNF793 methylation was statistically significant (P < 0.0001 for comparing BE vs. SQ, cardia, or fundus for each CpG).

Of note, assessment of other candidate CpG sites from the HM450 array studies was performed; however, they are not discussed because they did not meet criteria during the validation process to be used in the studies of endoscopic brushing samples described below. Reasons for their failure in the validation process include the lack of an accurate pyrosequencing assay design, low-level methylation, we found 7 out of 30 SQ samples and 11 out of 14 cardia samples had detectable levels of methylated ZNF793. The median ZNF793 methylation was 0.00% and 0.282% of input DNA in SQ and cardia samples, respectively. As with B3GAT2, ZNF793 methylation was found to be significantly higher in BE samples compared to control samples (median = 28.2% vs. SQ; median = 0.005 for BE vs. cardia; median = 0.0001 for BE vs. all controls; Fig. 3C). Methylated ZNF793 exhibited a highly discriminative ROC curve (Fig. 3D). The MethyLight assay for methylated ZNF793 was found to be 70% sensitive and 100% specific for BE detection when applying a cutoff value of 2.2% methylation (Table 3).

To better understand how this information can be used clinically, we also calculated the cutoff values and specificity for both markers when the sensitivity is increased (Table 3). For B3GAT2, when the sensitivity is set at 100%, the cutoff methylation value is 0.534% and the specificity is 70.5%. Similarly, when the sensitivity of ZNF793 is set at 100%, the methylation value cut-off is 0.194% and the specificity is 72.7%.

**Discussion**

EAC has emerged as one of the most rapidly increasing cancers in Western countries and often has a poor prognosis. Early detection of EAC and its precursor BE holds promise to limit the incidence of EAC and to decrease EAC-related deaths. Currently, the diagnosis of BE depends on endoscopic examination of the esophageal mucosa. BE screening is currently recommended only for those individuals at high risk for BE. Clinical and demographical features associated with an increased risk of BE include age (>40 years; ref. 22), male gender (23), frequent heartburn (5, 22, 23), chronic gastro-esophageal reflux disease (GERD; ref. 5), and obesity (7). For this reason, medical societies such as the American Gastroenterological Association (AGA) and American College of Gastroenterology (ACG) suggest screening for BE in individuals with multiple risk factors for BE (4, 24). However, population-based studies suggest that the majority of people with BE do not have these risk factors and therefore would not be recognized based on a screening strategy using known risk factors (25). Patients with unrecognized BE appear to be at increased risk for EAC and their identification and subsequent enrollment in surveillance programs may lead to improved EAC related mortality. Unfortunately, the current method for diagnosing BE, upper endoscopy with esophageal biopsies, is suboptimal for screening the general population due to its cost and invasiveness.
To address the limitations of endoscopy for screening, recent studies have described a minimally invasive, nonendoscopic approach to collect cells. This device, called "an esophageal balloon," consists of a tethered capsule that is swallowed and then releases a sponge to be pulled up through the esophagus, collecting cells in transit. BE cells collected with this device can be identified using biomarker assays such as immunostaining for the protein trefoil factor 3 (17) or assays for mutant TP53 (18, 19). In our study, two genes, B3GAT2 and ZNF793, were discovered to be aberrantly methylated in BE through a genome-wide methylation screen and were validated by pyrosequencing-based assays. MethyLight assays were then developed and used to assess DNA methylation in the BE samples compared to the SQ and gastric samples for methylated B3GAT2 (A) and ZNF793 (C). The ROC results are shown in panels B and D.

Horizontal bars indicate the median methylation level of each sample group. In panels A and B (**, BE vs. SQ, cardia, fundus, or all control tissues, P < 0.0001, determined by a two-tailed Wilcoxon–Mann–Whitney test).

Table 3. Sensitivity and specificity calculations for BE versus normal esophagus or normal stomach using data from MethyLight assays for methylated B3GAT2 and ZNF793 run on endoscopic brushings.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cutoff value (RM%)</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>B3GAT2</td>
<td>0.534</td>
<td>100.0%</td>
<td>70.5%</td>
</tr>
<tr>
<td></td>
<td>1.51</td>
<td>80.0%</td>
<td>86.4%</td>
</tr>
<tr>
<td></td>
<td>8.21</td>
<td>50.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>ZNF793</td>
<td>0.194</td>
<td>100.0%</td>
<td>72.7%</td>
</tr>
<tr>
<td></td>
<td>0.90</td>
<td>90.0%</td>
<td>88.6%</td>
</tr>
<tr>
<td></td>
<td>1.15</td>
<td>80.0%</td>
<td>93.2%</td>
</tr>
<tr>
<td></td>
<td>2.20</td>
<td>70.0%</td>
<td>100.0%</td>
</tr>
</tbody>
</table>

*RM%, relative methylation percentage.

Although the biologic function of the B3GAT2 and ZNF793 genes in human carcinogenesis is not currently known, aberrant DNA hypermethylatation in the B3GAT2 promoter region was recently reported in colon cancer, especially in the CpG island methylator phenotype (CIMP) subtype (28, 29). In fact, B3GAT2 has been included in a panel of markers to define CIMP status in colorectal cancer (29). We now demonstrate the aberrant DNA methylation in the promoter region of B3GAT2 and ZNF793 likely occurs in BE, the earliest recognized step in EAC carcinogenesis. Although we have found that methylated B3GAT2 and ZNF793 are common in BE, we do not know if this methylation affects the expression of these genes. Our findings also warrant further investigation into the biologic functions of these two genes in the pathogenesis of EAC.

It is also worth noting that we observed a wide distribution of B3GAT2 and ZNF293 relative methylation levels in the population including normal squamous epithelial cells and cardia cells, and therefore have the potential to be used as biomarkers for BE. Consistent with our findings, Moinova and colleagues recently showed methylated vimentin (VIM) is a potential biomarker of upper gastrointestinal neoplasia and BE (27). In a feasibility study using esophageal brushing samples, methylated VIM detected all BE cases (N = 7) and was not found in any control samples (N = 5; ref. 27). Given the known molecular heterogeneity of BE, our results and those of Moinova and colleagues raise the possibility that a panel assay with all three genes could be a highly sensitive and specific detection assay for BE.

Table 3. Sensitivity and specificity calculations for BE versus normal esophagus or normal stomach using data from MethyLight assays for methylated B3GAT2 and ZNF793 run on endoscopic brushings.
pyrosequencing studies in the validation cohort and in the BE endoscopic brushing samples. At this time because of inherent limitations in the assays we used and because of the way in which we collected our samples, we can not determine whether the range of methylation in BE samples we observed is secondary to differences in the proportion of BE cells in the different samples or is secondary to different molecular classes of BE (e.g., low methylation BE vs. high methylation BE). Although speculative based on our results, if there are BE samples that differ in the B3GAT2 or ZNF793 methylation status, it is possible that methylated B3GAT2 or methylated ZNF793 could be used as predictive markers for progression to EAC. Further studies are needed to investigate this possibility.

In summary, using the HM450 arrays, we have demonstrated that four CpGs associated with the two genes B3GAT2 and ZNF793 are commonly and specifically aberrantly methylated in BE, the early precursor of EAC. These methylated CpGs were validated using orthogonal assays run on independent sample sets. These findings provided motivation for investigation into the potential application of these methylated genes as diagnostic biomarkers for BE using a noninvasive assay. Consequently, we developed Methylight assays to detect methylated B3GAT2 and ZNF793 and showed them to accurately detect BE in esophageal brushing samples. Collectively, our findings support further investigation of methylated B3GAT2 and ZNF793 as cytology-related biomarkers for BE detection.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


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