Methylated *B3GAT2* and *ZNF793* are potential detection biomarkers for Barrett's esophagus

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Abstract

Background—Barrett's esophagus (BE) is a pre-neoplastic 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.

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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=28), 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.

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 pre-neoplastic 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 multi-step 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 to the general population (3). Epidemiological studies suggest BE is a prevalent condition, occurring in approximately 1.6% of the population (4) and is most common in people that are older (5), male (6), obese (7), and who have chronic gastro-esophageal reflux disease (GERD) (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 due to 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 to 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 the current study, we conducted a genome-wide analysis of normal esophageal squamous epithelium (SQ), stomach (cardia and fundus) and BE and identified two genes, beta-1,3-glucuronyltransferase 2 (*B3GAT2*) and zinc finger 793 (*ZNF793*), as aberrantly methylated genes in non-dysplastic BE compared to 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 histological subtypes of interest that would be subjected to DNA extraction. In those sections with mixed histology (e.g. Barrett's and squamous esophagus), 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 esophagogastroduodenoscopy for GERD indications and 2 of them had Los Angeles Grade B erosive esophagitis. All BE brushing specimens were obtained from patients that had a previously confirmed diagnosis via upper endoscopy and histology. The brushings were obtained at the time of the endoscopic exam using a through-the-scope cytology brush. 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 manufacturers' instructions (Life Technologies). 250 ng 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 'Beta-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 beta-values in BE group to the mean of beta-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; Table 1). Assays were initially chosen based on the following criteria: 1) assay primer binding accuracy (>70%) as determined by the primer design software, 2) amplicon size ~100bp, and 3) 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 EpiTect 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 (Supplemental 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 (Supplemental Figure S2–5). The pyrosequencing assays were then run on a second independent set of samples ("validation cohort") (Figure 2, Supplemental Figure 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_080742.2) and methylated *ZNF793* (NM_001013659) were designed using ABI Primer Express software Version 5.0. The locations of primers and probes are indicated in Figure 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 *et al.* 2006 (21). A complete list of all MethyLight primer and probe sequences is provided in Table 2.

The MethyLight PCR reaction mixture consisted of the 2X 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 uL. 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 Bio-Rad CFX manager software version 3.1 and Cq was determined with the Single Threshold method (BioRad). In order 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. Intra-assay 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

$100 \times \left(methylation / control \right)$, 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 assay, a methylation-independent control reaction that has been previously described (20).

Statistical Analysis

Statistical analysis of the results of pyrosequencing and MethyLight assays assessing methylation levels in the different tissue types was performed with a two-tailed Wilcoxon-Mann-Whitney test. The association of methylation status between adjacent CpG sites was measured with Spearman rank correlation test. Both tests were performed using GraphPad Prism version 6.02 (GraphPad Software Inc.). The association of methylation status of CpG sites with potential confounding factors, such as age and gender, was tested using the univariate linear regression model and P value < 0.05 was considered significant. ROC curve analysis and cut-off value determination were performed by YC and JBS using SAS version 9.4 (SAS Institute Inc.).

RESULTS

B3GAT2 and *ZNF793* are aberrantly methylated in Barrett's esophagus (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 to normal SQ with established cutoff values: difference in beta-value > 0.4, and false discovery rate q < 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-glucuronyltransferase 2. Three other CpG loci (probe IDs cg24588375, cg02711801, cg23296010) that demonstrated large differences in methylation between BE and control samples were located in the promoter region of *ZNF793*, which encodes zinc finger protein 793 (Fig1; Supplemental Table S2). We have tested the association of methylation status of CpG sites with potential confounding factors, such as age and gender, for which we have complete datasets, using the univariate linear regression model (P <0.05 was considered significant). We didn't find age or gender to be significant confounding variables ($P = 0.2322$ for age, $P = 0.7741$ for gender). We didn't test race as a confounding variate because > 96% of our subjects from which our samples were obtained are Caucasians.

In order 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 as well as 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 (Supplemental 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 beta-value results (Supplemental Fig. S2).

We also found that CpG sites adjacent (within ~20bp) 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' (Figure 1, Supplemental Fig.3 and 4).

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*_cg16556906 methylation (median methylation level = 2.29%), while 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, Supplemental Fig.6). Similarly, all 49 control samples had very low-level *ZNF793* methylation (median=3.13%, 2.02% and 2.42% for *ZNF793*_cg24588375, cg02711801 and cg23296010, respectively), while higher *ZNF793* methylation levels were detected in BE cases (median=29.9%, 31.0% and 30.2% respectively) (Fig. 2B, C and 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 included the lack of an accurate pyrosequencing assay, lack of confirmation of the methylation status determined with the HM450 array using pyrosequencing, and lack of confirmation of differential methylation between BE and the control tissues in the validation cohort studies. Therefore, we focused on *B3GAT2* and *ZNF793* for the development of highly sensitive MethyLight assays.

Detection of methylated *B3GAT2* and *ZNF793* in endoscopic brushings from Barrett's esophagus

In light of our findings that *B3GAT2* and *ZNF793* were more highly methylated in BE than in control esophageal and gastric tissues, we developed quantitative MethyLight assays for methylated *B3GAT2* and *ZNF793* under the consideration that they could be used as detection biomarkers for BE in DNA extracted from endoscopic brushings, which contained a mixture of cell types. To determine the sensitivity and specificity of these assays for BE detection, we used a set of brushing samples from the stomach and esophagus, consisting of 44 control samples including 30 SQ and 14 cardia samples, and 10 BE samples. As shown in Fig. 3A, detectable *B3GAT2* methylation was found in 8 out of 30 SQ samples and 10 out of 14 cardia samples. The median *B3GAT2* methylation was 0.00% and 0.516% of input DNA in SQ and cardia samples respectively. *B3GAT2* methylation was significantly higher in the BE samples (N=10) compared to the control samples (median = 6.43%, $P < 0.0001$ for BE vs.control). The *B3GAT2* MethyLight assay was found to have a highly discriminative ROC curve profile (Fig. 3B), clearly distinguishing BE from control. The MethyLight assay for methylated *B3GAT2* was found to be 50% sensitive and 100% specific for detecting and

discriminating BE from control samples when applying a cutoff value of 8.2% methylation (Fig. 3B; Table 3).

When applying a similar approach to the assessment of *ZNF793* methylation, we found 7 out of 30 SQ samples and 11 out 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% $P < 0.0001$ for BE vs. SQ; $P = 0.0005$ for BE vs. cardia; $P < 0.0001$ 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

Esophageal adenocarcinoma (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 Barrett's esophagus (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 esophagus with biopsies 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) (22), male gender (23), frequent heartburn (5, 22, 23), chronic gastro-esophageal reflux disease (GERD) (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, non-endoscopic 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 (26) 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 to detect methylated *B3GAT2* and *ZNF793* in endoscopic brushing samples. Our results demonstrate that methylated *B3GAT2* or *ZNF793* can accurately detect BE from a mixed cell 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 *et al* 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) (27). Given the known molecular heterogeneity of BE, our results and those of Moinova *et al* raise the possibility that a panel assay with all three genes could be a highly sensitive and specific detection assay for BE.

Although the biological function of the *B3GAT2* and *ZNF793* genes in human carcinogenesis is not currently known, aberrant DNA hypermethylation 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 CRC (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 biological 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 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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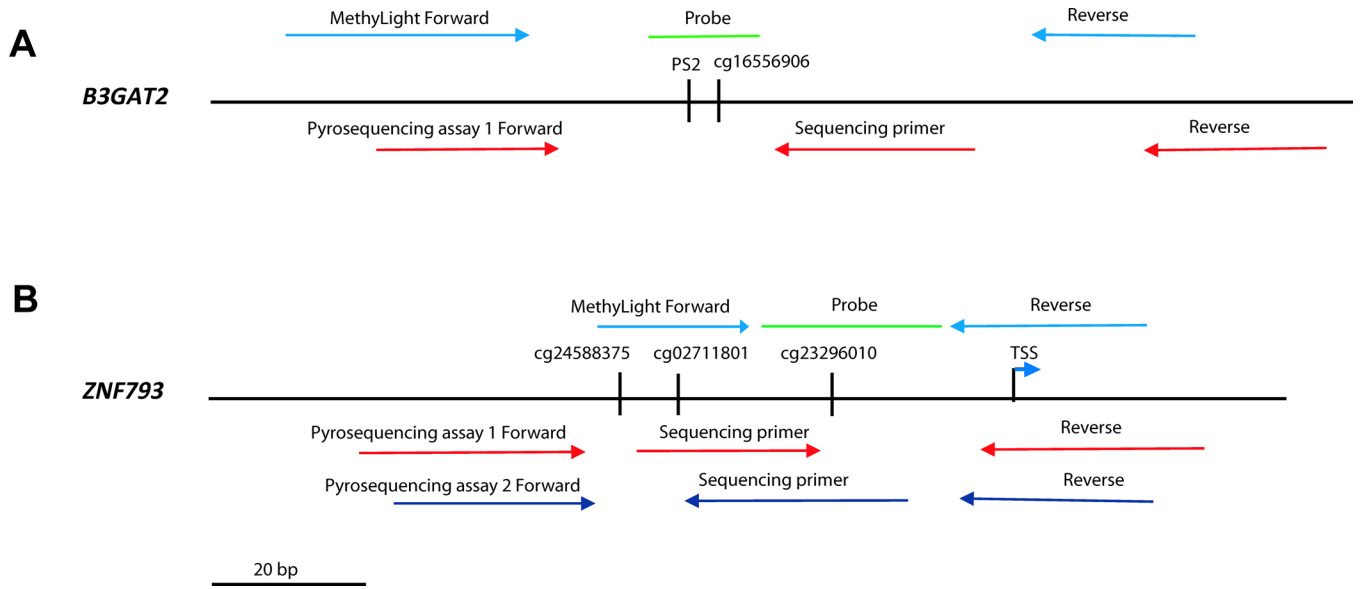
Figure 1.
Location of pyrosequencing assay primers, MethyLight primers and probes, and CpG probes on the HumanMethylation450 arrays for (A) *B3GAT2* and (B) *ZNF793*.

For the MethyLight assays, the primer locations are indicated by the light blue arrows and the probe location is indicated by the green line. For the pyrosequencing assay, assay 1 is designated by the red arrows and assay 2 is designated by the blue arrows. The internal arrow is the sequencing primer. The CpG probe identities (beginning with 'cg') from the HM450 array are shown beneath the small vertical lines. The second *B3GAT2* CpG was discovered using a pyrosequencing assay and does not have a corresponding HM450 array identifier. We have named this CpG "PS2" and shown its location and proximity to theHM450 CpGprobe found in the initial data analysis. The DNA strand shown is in the 5'—3' orientation and is the top strand.
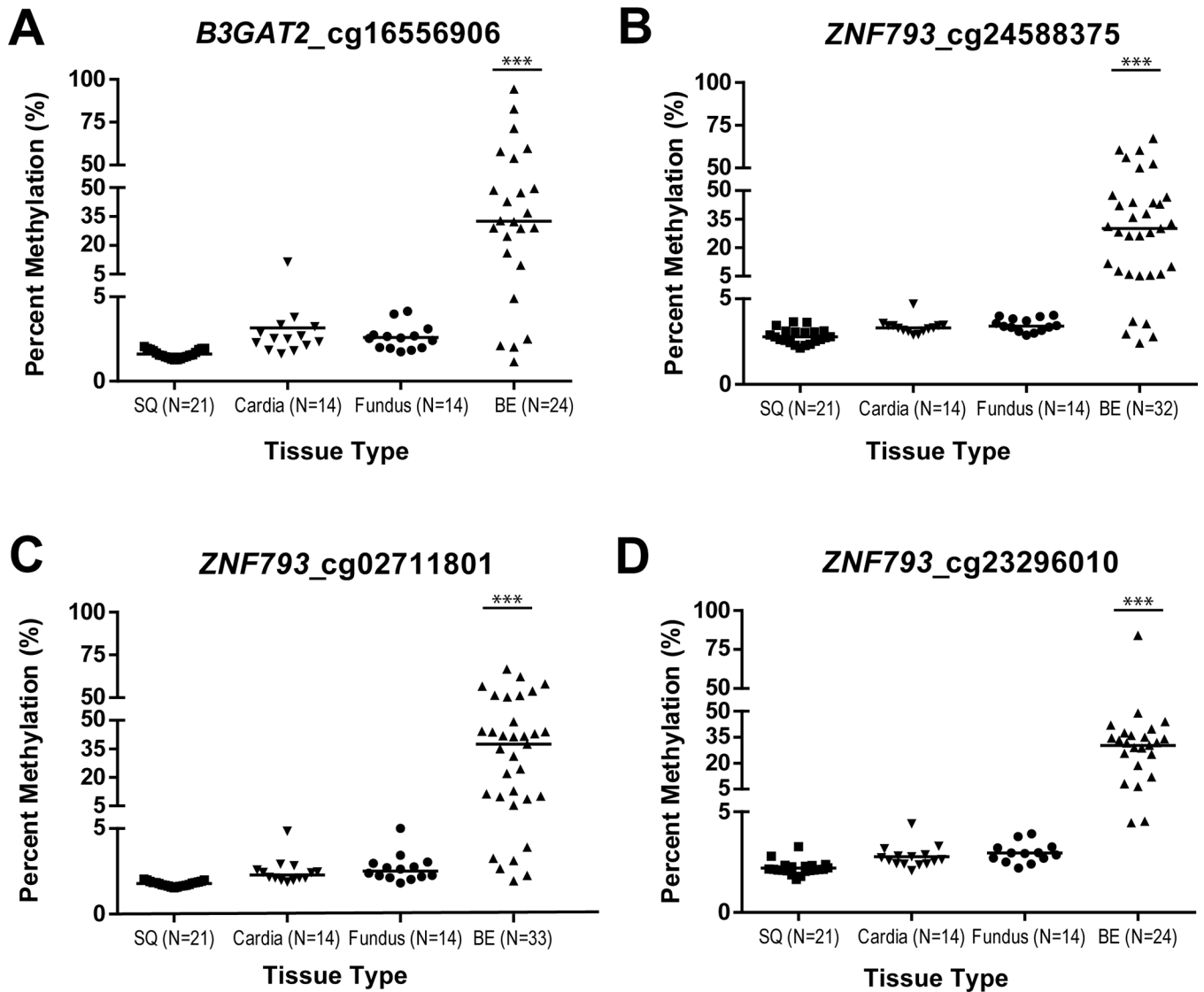
Figure 2.
The majority of Barrett's esophagus (BE) samples have higher levels of methylated *B3GAT2* (cg16556906) and *ZNF793* (cg24588375, cg02711801, and cg23296010) compared to control epithelial tissue, including normal esophagus (SQ) and stomach (cardia and fundus). Results of pyrosequencing assays 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 Barrett's esophagus (BE)compared to normal squamous epithelium (SQ) and normal gastric epithelium (cardia and fundus) are shown for A) cg16556906, located in *B3GAT2*; B) cg24588375, located in *ZNF793*; C) cg02711801, 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 Wicoxon-Mann-Whitney test).
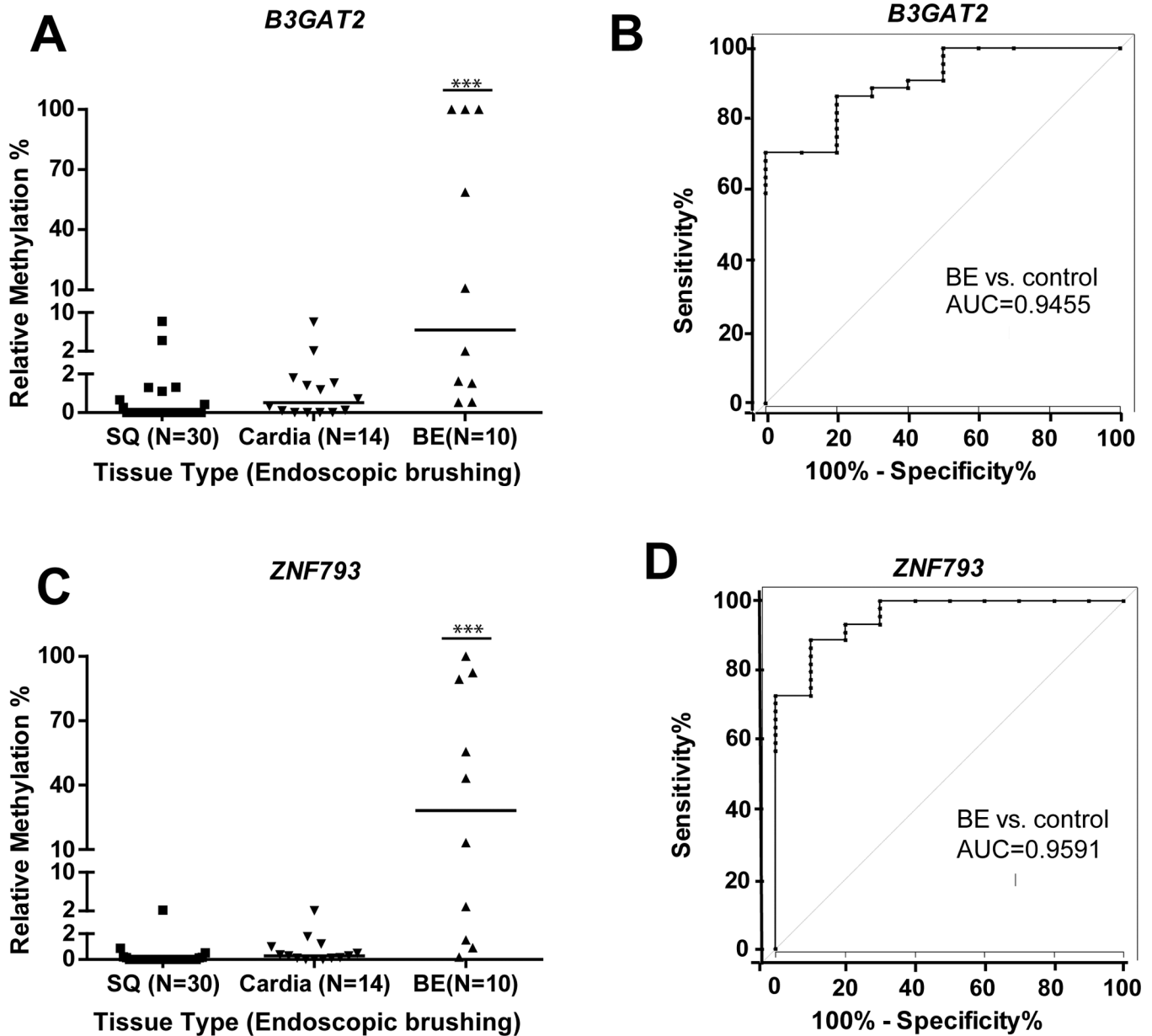
Figure 3.
MethyLight assay results for methylated *B3GAT2* and methylated *ZNF793* run on a set of endoscopic brushings. MethyLight assays for methylated *B3GAT2* and*ZNF793* were developed and used to assess DNA extracted from endoscopic brushing samples from Barrett's esophagus (BE), as well as control epithelial tissue, including normal esophagus (SQ) and stomach (cardia). The results for the assays reveal higher levels of methylation in the Barrett's esophagus 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. The MethyLight assay for methylated *B3GAT2* was found to be 50% sensitive with a 100% specificity for BE vs. normal esophagus or cardia (designated "control"),and the MethyLight assay for methylated *ZNF793* was found to be 70% sensitive with a 100% specificity for BE

vs. normal esophagus or cardia (designated "control"). Of note, combining methylated *B3GAT2* and *ZNF793* resulted in a sensitivity of 70% with a 100% specificity for BE. 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 Wicoxon-Mann-Whitney test).

Table 1

Pyrosequencing primers[*]

Gene	CpGSite	Forward Primer	Reverse Primer	Sequencing Primer
B3GAT2	cg16556906	AGTAAGGGATTGAGAGTAGGT	AAAAACTTTCCTTCCTCACATT	AATAAAAACCCTAAAAATTATATCC
ZNF793	cg02711801	ATTTAATTGAGATTTTGAGGTTGTTAGT	AAAAACCCAAAATCCTATTCACTAAAA	CCAAAATCCTATTCACTAAAAAATT
ZNF793	cg24588375	ATTTAATTGAGATTTTGAGGTTGTTAGT	AAAAACCCAAAATCCTATTCACTAAAA	CCAAAATCCTATTCACTAAAAAATT
ZNF793	cg23296010	GAAATTTAGAATTTTGTTTATTGGAAGG	ATATAAAATCCTCCCATCCTAAATAACC	GTGGAGGTTGATAGTTTTAAAG

[*]
*
All sequences are listed in the 5'—3' orientation.

Table 2

MethyLight primers and probes[a]

Gene	Primer/probe	Primer/probe sequence
B3GAT2	**Forward**	TGAGAAGATAAGAGTAAGGGATTGAGAGT
	Reverse	CACATTCCCGCCGAAATAAC
	Probe	FAM-CGCGCGAATATAGTC-MGBNFQ[b]
ZNF793	**Forward**	GTCGGGTGGCGGTTGATAG
	Reverse	TCGTCCCATCCTAAATAACCGATA
	Probe	FAM-TTTAAAGTCGTAATTGAGTTTGC-MGBNFQ[b]
ALUC4	**Forward**	GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA
	Reverse	ATTAACTAAACTAATCTTAAACTCCTAACCTCA
	Probe	FAM-CCTACCTTAACCTCCC-MGBNFQ[b]

[a] All sequences are listed in the 5'-3' orientation.

[b] MGBNFQ refers to a Minor Groove Binder non-fluorescent quencher in the 3' terminus of the probe.

Table 3

Sensitivity and specificity calculations for Barrett's esophagus vs. normal esophagus or normal stomach using data from MethyLight assays for methylated *B3GAT2* and *ZNF793* run on endoscopic brushings.

Gene	Cutoff Value(RM%)[*]	Sensitivity	Specificity
B3GAT2	0.534	100.0%	70.5%
	1.51	80.0%	86.4%
	8.21	50.0%	100.0%
ZNF793	0.194	100.0%	72.7%
	0.90	90.0%	88.6%
	1.15	80.0%	93.2%
	2.20	70.0%	100.0%

[*] RM%=relative methylation percentage