DNA Methylation as an Adjunct to Histopathology to Detect Prevalent, Inconspicuous Dysplasia and Early-Stage Neoplasia in Barrett’s Esophagus

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Abstract

Purpose: Endoscopic surveillance of Barrett’s esophagus is problematic because dysplasia/early-stage neoplasia is frequently invisible and likely to be missed because of sampling bias. Molecular abnormalities may be more diffuse than dysplasia. The aim was therefore to test whether DNA methylation, especially on imprinted and X-chromosome genes, is able to detect dysplasia/early-stage neoplasia.

Experimental design: 27K methylation arrays were used to find genes best able to differentiate between 22 Barrett’s esophagus and 24 esophageal adenocarcinoma (EAC) samples. These were validated using pyrosequencing on a retrospective cohort (60 Barrett’s esophagus, 36 dysplastic, and 90 EAC) and then in a prospective multicenter study (98 Barrett’s esophagus patients, including 28 dysplastic and 9 early EAC) designed to utilize biomarkers to stratify patients according to their prevalent dysplasia/EAC status.

Results: Genes (23%) on the array, including 7% of X-linked and 69% of imprinted genes, have shown statistically significant changes in methylation in EAC versus Barrett’s esophagus (Wilcoxon $P < 0.05$). 6/7 selected candidate genes were successfully internally (Pearson’s $P < 0.01$) and externally validated (ANOVA $P < 0.001$). Four genes (SLC22A18, PIGR, GJA12, and RIN2) showed the greatest area under curve (0.988) to distinguish between Barrett’s esophagus and dysplasia/EAC in the retrospective cohort. This methylation panel was able to stratify patients from the prospective cohort into three risk groups based on the number of genes methylated (low risk: $< 2$ genes, intermediate: 2, and high: $> 2$).

Conclusion: Widespread DNA methylation changes were observed in Barrett’s carcinogenesis including $\approx 70\%$ of known imprinted genes. A four-gene methylation panel stratified patients with Barrett’s esophagus into three risk groups with potential clinical utility.

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Introduction

Patients with Barrett’s esophagus have a substantially increased risk of progression to esophageal adenocarcinoma (EAC) compared with the general population (RR: 11.3, 95% CI: 8.8–14.4; ref. 1). The incidence of EAC has increased 7-fold in the past 30 years (3.6–25.6 cases per million; ref. 2) and the prognosis is poor with a median survival of about 11 months because of late presentation (3). Because of the improved survival in those diagnosed when the disease is confined to mucosa or submucosal layers, patients with Barrett’s esophagus are recommended to undergo endoscopic surveillance for the early detection of cancer (4, 5). The cost-effectiveness and risk:benefit ratio to the patient of endoscopy has been questioned time and again as the risk of progression is relatively low (1, 6, 7); around 0.3% according to the recent estimates (8). The intermediate dysplastic stages between Barrett’s esophagus and EAC are the most reliable markers of progression; however, the histologic presence of dysplasia is subjective because of known sampling bias during endoscopy together with a high inter and intraobserver variability (9, 10). The wide variation in progression rates in patients categorized as having low grade dysplasia (LGD) has been highlighted by 2 recent studies. In a Dutch study, the incidence rate of high-grade dysplasia (HGD) or EAC in individuals with confirmed low-grade dysplasia was high at 13.4% (95% CI: 3.5–23.2) per patient per annum (11); whereas in a US study, the progression rate of individuals with LGD was similar to that of nondysplastic patients which is a 16-fold difference (12). In patients with HGD, data from a
randomized radiofrequency ablation intervention trial suggest a rate of progression of 19% per year in the nontreatment arm (13). Hence there is a pressing need for biomarkers that can accurately detect prevalent dysplasia in flat Barrett’s mucosa and predict those patients most likely to progress to cancer.

Aberrant DNA methylation is shown to be a characteristic of cancer and these changes are known to occur early during transformation (14). It has already been shown in a number of studies that DNA methylation changes occur during progression from Barrett’s esophagus to EAC and that these alterations have the potential to be utilized as biomarkers (15–20). These studies have mostly employed a candidate approach based on known methylation targets in other cancers. However high-throughput array-based platforms are now available to identify DNA methylation changes and we have employed this approach to find candidate biomarkers in Barrett’s carcinogenesis. Imprinted genes and the X-chromosome are both epigenetically controlled by DNA methylation (21), but have never been examined specifically in the context of biomarkers for EAC. Methylation changes in these genes may be ideal biomarkers as physiologic inactivation of one allelic copy has already occurred because of imprinting and via X-inactivation in females.

Hence, in this study we conducted DNA methylation screening of Barrett’s esophagus and EAC samples using arrays to determine candidate biomarkers. We analyzed imprinted and X-chromosome genes separately and purposefully separated males from females to allow meaningful conclusions to be drawn. We conducted robust internal and external validation using pyrosequencing for both retrospective and prospective validation, we have shown that methylation changes can be used alongside histopathology to detect patients who have no visible signs of dysplasia/cancer at high risk of progression.

**Translational Relevance**

In Barrett’s esophagus, endoscopic detection of dysplasia and its confirmation by histology suffers from sampling bias and a high inter and intraobserver variability. DNA methylation is known to play a role in cancer development and hence in this study we hypothesized that it could be used as an adjunct for detecting and diagnosing dysplasia/early cancer as molecular changes are more diffuse than dysplastic lesions, which can be easily missed during endoscopy. Using Illumina Infinium arrays to select a methylation signature and pyrosequencing for both retrospective and prospective validation, we have shown that methylation changes can be used alongside histopathology to detect patients who have no visible signs of dysplasia/cancer at high risk of progression.

**DNA extraction and bisulphite conversion**

For the methylation arrays, high molecular weight DNA was isolated from fresh-frozen tissue using standard proteinase-K phenol/chloroform extraction. Samples with A260/A280 of less than 1.8 and a fragment size of less than 2 kb were discarded. A volume corresponding to 1 μg of DNA was measured using a Quant-iT PicoGreen® dsDNA kit (Invitrogen Ltd) according to the manufacturer’s instructions. Bisulphite modification was done using the EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation).

DNA extraction for pyrosequencing assays was also carried out using the above-mentioned protocol. DNA extraction from formalin-fixed paraffin-embedded (FFPE) tissues was carried out using the QIAamp DNA Micro Kit (Qiagen) using the manufacturer’s instructions. One microgram of DNA was bisulphite modified and eluted in 30 μL of elution buffer.

**Illumina Infinium assay**

The Infinium assay (Illumina) was run using the automated protocol at Cambridge Genomic Services. Samples were denatured before whole-genome amplification (WGA) using 0.1N NaOH. Multisample amplification master mix (MSM) was then added to the DNA samples and incubated at 37°C for 20 hours. The amplified DNA was fragmented by vortexing, precipitated using isopropanol and dispensed onto the BeadChips which were incubated at 48°C for 20 hours in hybridization buffer to allow for the DNA to hybridize. Unhybridized DNA was washed off and single-base extension was carried out with extended primers and labeled nucleotides using the TECAN Freedom Evo liquid handling robot. The BeadArray Reader (Illumina) was used to read the signal and output files were generated using GenomeStudio Software (Illumina).

**Materials and Methods**

**Patient samples**

For the retrospective studies (methylation arrays and retrospective external validation) all patient samples (H&E slides, endoscopic biopsies, and surgical resection specimens), were obtained from patients who had attended Cambridge University Hospitals NHS Trust and provided individual informed consent (ethics: 04/Q2006/28, 09/H0308/118). For the prospective study, patients with Barrett’s esophagus undergoing surveillance or tertiary referral for further evaluation of HGD or early EAC were recruited after obtaining informed consent from Cambridge University Hospitals NHS Trust, Queens University Hospital Nottingham, and Amsterdam Medical Centre (ethics: 10/H0305/52). Pathology was verified for all cases according to the Royal College of Pathologists UK guidelines by an experienced upper GI pathologist (Dr. Maria O’Donovan) and for dysplasia and EAC a minimum of 2 experienced pathologists reviewed the cases (referring hospital + Dr. Maria O’Donovan). All Barrett’s esophagus samples were confirmed to have intestinal metaplasia and all EACs for a cellularity of greater than equal to 70%. Patient demographics are available in Supplementary Tables S1–S3.

**DNA extraction for pyrosequencing assays** was also carried out using the above-mentioned protocol. DNA extraction from formalin-fixed paraffin-embedded (FFPE) tissues was carried out using the QIAamp DNA Micro Kit (Qiagen) using the manufacturer’s instructions. One microgram of DNA was bisulphite modified and eluted in 30 μL of elution buffer.
Array data analysis and selecting targets

Signal-to-noise ratio ranking. Barrett’s esophagus and EAC samples were separated into 2 groups and ranking of all genes was done using the "Signal2Noise" metric (GSEA software, Broad Institute). Signal2Noise uses the difference of means scaled by the SD.

$$\frac{(\mu_A - \mu_B)}{(\sigma_A + \sigma_B)}$$

where $\mu$ is the mean and $\sigma$ is the SD. The larger the signal-to-noise ratio, the larger the difference of means (scaled by SD); hence more distinct methylation is seen for each phenotype and more the gene acts as a "class marker." Imprinted genes and those on the X-chromosome were also analyzed separately. The final list of genes can be obtained from Supplementary Table S4.

Wilcoxon test. As a further check to test for differential methylation, a two-sided Wilcoxon test was conducted for each probe on the array. Variance of probes with low or high methylation is in general lower than variance of probes with medium methylation (22). So tests for differential methylation tend to preferentially select probes whose values are confined to the extremes of the scale. To reduce this effect, we conducted a Gaussian normalization before the Wilcoxon tests to reduce heteroscedasticity. The values’ ranks, normalized between 0 and 1, were taken to be probabilities from a Gaussian distribution and transformed to variables using the distribution’s quantile function. The $P$-values were adjusted for multiple testing using the false discovery rate method of Benjamini & Hochberg (23). We were interested in probes that had both statistically significant and large absolute differences in methylation. Therefore, for each probe, we also calculated the difference between the median of the methylation values in the 2 phenotypes. A probe’s rank in the ordered list of Wilcoxon $P$-values and its rank in the ordered list of absolute difference in medians were averaged. The probes were arranged in descending order of this average.

The purpose of using 2 different tests to look for targets was to avoid false positives and to ensure that the selected targets not only have a statistically significant but a large absolute difference in methylation that was reproducible using pyrosequencing which is generally attributed to have an error margin of ±5%. The targets appearing high up in both these analyses were then selected for validation.

Genes were selected for validation based on the following criteria: present in both of the lists, biologic importance in EAC and/or other cancers, probe’s proximity to the promoter and relatively low density of CpGs in the vicinity so that it would be possible to design robust pyrosequencing assays (Fig. 2A and B, Supplementary Table S4).

Pyrosequencing assays

Pyrosequencing assays were designed using PSQ Assay Design Software (version 1.0.6, Biotage; Supplementary Table S5). Genomic DNA sequences were obtained from NCBI map viewer (build 36). All PCR reactions were carried out in volumes of 25 μL using IMMOLASE™ DNA Polymerase (Biotine). Bisulphate-converted DNA of 0.75 μL was used as a template for each reaction. 20 μL of each PCR reaction was mixed with 60 μL of bead mix composed of 3 μL streptavidin-coated beads solution (GE Healthcare), 20 μL nuclease-free water, and 37 μL PyroMark binding buffer (Qiagen) in a 96-well plate and left on a shaking platform for 10 minutes. The pyrosequencing reaction plate was prepared by adding 1.5 μL of 10 μmol/L sequencing primer and 43.5 μL of PyroMark Annealing Buffer (Qiagen) into each of the wells. The pyrosequencing vacuum machine (Biotage) was used to wash and denature the DNA bound to streptavidin-coated beads before being released into the pyrosequencing reaction plate. The plate was heated to 80°C for 3 minutes and then cooled down to room temperature to allow the sequencing primer to anneal onto the single-stranded DNA and the sequencing reaction was carried out according to the manufacturer’s protocol.

Methylated controls of 0%, 50%, and 100% were prepared for all the assays and used with every run. DNA synthesized by PCR was used for this. Primers were designed using the NCBI Primer Designing Tool (24) to amplify a region greater than but containing the sequence to be analyzed by pyrosequencing (Supplementary Table S6). Genomic DNA isolated from normal squamous esophagus was used as a template. All PCRs were conducted in 50 μL duplicates. One reaction was used for in vitro methylation. Briefly 40 μL of the PCR reaction was mixed with 5 μL of 10 × NEBuffer 2, 2.5 μL of 3.2 mmol/L L-adenosylmethionine (SAM), 4 U (1 μL) of CpG Methyltransferase (M.SssI; NEB) and incubated for 2 hours at 37°C. After 2 hours, another 0.5 μL of 3.2 mmol/L SAM, 2 U (0.5 μL) of M.SssI, and 0.5 μL of water were added and incubated overnight at 37°C. Both reactions (in vitro methylated and unmethylated) were then purified using the QiAquick PCR purification Kit (Qiagen). These were then bisulphite converted as mentioned before and mixed to generate a 50% methylated control along with 0% and 100% methylated controls.

Statistical analysis

To compare the difference between groups (Barrett’s esophagus, Barrett’s esophagus with dysplasia and EAC) a t test or one-way ANOVA was used for continuous variables (age, segment length, and methylation) and χ² test for categorical variables (gender). A $P < 0.05$ was considered statistically significant. Receiver operating characteristic (ROC) curves were used to evaluate the distinguishing ability of each gene and the 4 gene signature and area under the curve (AUC) was reported. Methylation cut-off points for each gene were selected on the basis of individual ROC curves to have the best accuracy. All analyses were done using GraphPad Prism and SPSS19.0.

Results

Array data analysis

Illumina HumanMethylation27 BeadChips were used to assess and compare methylation levels of 27,578 individual CpG loci spanning 14,475 genes and 110 miRNA promoters in 22 Barrett’s esophagus and 24 EAC samples (GEO accession no: GSE32925). Signal-to-noise ratio and
two-sided Wilcoxon tests were used to rank genes showing the greatest difference in methylation (both hypermethylation and hypomethylation) between the Barrett’s esophagus and EAC, and from this a “class marker” gene set was identified that was able to clearly distinguish between the 2 phenotypes (Fig. 1). Twenty-three percent of all the genes present on the array showed a statistically significant difference in methylation (Wilcoxon $P < 0.05$). On the whole, hypermethylation was observed to be slightly more prevalent (1,764/14,475 to 12.18%) as compared with hypomethylation (1,590/14,475 to 10.98%) in EAC versus Barrett’s esophagus (Wilcoxon $P < 0.05$). Out of the 51 imprinted genes present on the array (25) 17 (33.33%) showed hypermethylation and 18 (35.29%) hypomethylation in EAC versus Barrett’s esophagus (Wilcoxon $P < 0.05$; which comes to a total of 68.62% of all the imprinted genes present on the array). Separate analyses were done for males and females for genes on the X-chromosome to cater for the effects of X-inactivation in females. Genes on the X-chromosome showed similar levels of hyper and hypomethylation in EAC compared with Barrett’s esophagus (22 genes each hyper and hypomethylated out of a total 600, Wilcoxon $P < 0.05$). Most methylation changes were confined to within known CpG islands. Detailed results can be seen in Table 1.

**Identifying targets for validation**

To ensure that the selected targets for validation would have a statistically significant and large absolute difference in methylation and hence be suitable as biomarkers, the results of signal-to-noise ratio ranking were compared with the results of the Wilcoxon tests. The top 7 genes present in both the lists fulfilling the aforementioned selection criteria (see methods) were selected for validation (Fig. 2A). For RGN, which is an X-inactivated gene (p11.3-Xp11.23), it was observed that methylation levels were different in males compared with females in normal tissues (normal squamous esophageal epithelium). Therefore, separate analyses were done for both the genders for RGN in the pathologic external validation samples. TCEAL7 on the other hand, also on the X-chromosome, did not seem to be affected by DNA methylation-associated X-inactivation and therefore the analysis for males and females were combined in all subsequent experiments (Fig. 2B and C).

**Internal validation**

The 7 genes selected were first internally validated using pyrosequencing assays on the same samples that were run on the methylation arrays. The assays were designed to analyze the same DNA sequence which was probed by the arrays. Pearson’s correlation was used to assess whether the
and dysplastic Barrett's esophagus compared with nondysplastic Barrett's esophagus and EAC.

Generating a methylation cut-off

RIN2 and EAC. The biggest change in methylation occurred at the onset of dysplasia and for the biggest change in methylation was observed for all the selected biomarker genes, it was a gradual increase, whereas for this occurred between dysplasia/EAC and nondysplastic Barrett's esophagus. In this occurred between dysplasia and EAC.

Table 1. DNA methylation changes observed from the Illumina Infinium 27K array analysis comparing 24 EAC and 22 Barrett’s esophagus cases (total number of probes on the array = 27,578, total number of genes = 14,475)

<table>
<thead>
<tr>
<th>Trends (EAC vs. Barrett’s esophagus)</th>
<th>Number of probes</th>
<th>Percentage of probes</th>
<th>Number of genes</th>
<th>Percentage of genes</th>
<th>Number of probes within CpG islands</th>
<th>Number of probes outside of CpG Islands</th>
</tr>
</thead>
<tbody>
<tr>
<td>All genes</td>
<td>Hypermethylation</td>
<td>1,952</td>
<td>7.1</td>
<td>1,764</td>
<td>12.18</td>
<td>1,389</td>
</tr>
<tr>
<td></td>
<td>Hypomethylation</td>
<td>1,740</td>
<td>6.3</td>
<td>1,590</td>
<td>10.98</td>
<td>1,114</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>3,692</td>
<td>13.4</td>
<td>3,354</td>
<td>23.16</td>
<td>2,503</td>
</tr>
<tr>
<td>Imprinted genes</td>
<td>Hypermethylation</td>
<td>33</td>
<td>8.5</td>
<td>17</td>
<td>33.33</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Hypomethylation</td>
<td>27</td>
<td>6.9</td>
<td>18</td>
<td>35.29</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>60</td>
<td>15.4</td>
<td>35</td>
<td>68.62</td>
<td>53</td>
</tr>
<tr>
<td>X-chromosome genes (males only)</td>
<td>Hypermethylation</td>
<td>24</td>
<td>2.2</td>
<td>22</td>
<td>3.66</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Hypomethylation</td>
<td>24</td>
<td>2.2</td>
<td>22</td>
<td>3.66</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>48</td>
<td>4.4</td>
<td>44</td>
<td>7.33</td>
<td>32</td>
</tr>
</tbody>
</table>

Results from pyrosequencing matched with the results from the arrays (Fig. 3). Six of 7 genes successfully validated which were SLC22A18 (tumor suppressing subtransferable candidate 5, a paternally imprinted gene; P < 0.0001, coefficient = 0.9), PI G R (polymeric immunoglobulin receptor; P < 0.0001, coefficient = 0.9), GJA12 (gap junction protein, gamma 2; P < 0.0001, coefficient = 0.9), RIN2 (Ras and Rab interactor 2; P < 0.01, coefficient = 0.7), RGN (senescence marker protein-30, X-linked gene; P < 0.0001, coefficient = 0.9), and TCEAL7 (transcription elongation factor A-like 7, X-linked gene; P < 0.0001, coefficient = 0.9). ATP2B4, however, failed to validate (P = 0.6, coefficient = 0.1) as shown in Supplementary Fig. S1.

Retrospective external validation

External validation by pyrosequencing was carried out on an independent set of retrospectively collected 60 Barrett’s esophagus, 36 Barrett’s esophagus with dysplasia, and 90 EAC samples (Fig. 4). All of these cases had the histopathologic diagnosis confirmed on the actual biopsy used for analysis. This validation set also enabled an assessment to be made of when in the disease pathogenesis the methylation changes occurred. A statistically significant increase in methylation was observed for all the selected biomarker genes in EAC and/or nondysplastic Barrett’s esophagus compared with nondysplastic Barrett’s esophagus (ANOVA P < 0.001). An ANOVA test was also done to confirm this trend separately for EAC samples with and without neoadjuvant chemotherapy (P < 0.001). For SLC22A18, PI G R, TC E A L 7, and RIN2 genes, it was a gradual increase, whereas for RGN the biggest change in methylation occurred at the onset of dysplasia and for GJA12 this occurred between dysplasia and EAC.

Generating a methylation cut-off

As an increase in DNA methylation was observed in EAC and dysplastic Barrett’s esophagus compared with nondysplastic Barrett’s esophagus, ROC curves were used to detect the power of the 6 genes individually and then in combination to differentiate between dysplastic Barrett’s esophagus/EAC and nondysplastic Barrett’s esophagus followed by PI G R (AUC = 0.963), SLC22A18 (AUC = 0.954), RIN2 (0.922), RGN (AUC = 0.865) but only in males and lastly TC E A L 7 (AUC = 0.788). The greatest AUC of 0.988 (P < 0.01) was obtained using the 4 gene combination (SLC22A18 + PI G R + GJA12 + RIN2) which had a sensitivity of 94% and a specificity of 97% (Fig. 6A).

Prospective validation

The methylation cut-offs selected for the 4 genes using ROC curves (SLC22A18, PI G R, GJA12, and RIN2) were then tested on a prospective cohort of 98 patients (including 17 LGD and 20 HGD/EAC) undergoing Barrett’s esophagus surveillance endoscopy in 3 tertiary referral centers to enrich for dysplasia and EAC. Random quadrantal biopsies every 2 cm were taken according to international guideline (26) along with 3 extra biopsies for DNA methylation taken randomly from within the Barrett’s esophagus segment. For the analysis, the biopsy with the highest methylation value per gene was selected taking advantage of the likely molecular field effect. A patient was categorized according to their highest histopathologic diagnosis (LGD<HGD<EAC) on any surveillance biopsy taken at that endoscopy. The data have shown that the risk of both dysplasia and EAC increased with the number of genes methylated (Fig. 6B). 17.6% of the cases in the more than 2 gene-methylated group were dysplastic (low-grade dysplasia only). In the group with 2 genes methylated, the proportion of dysplastic cases increased to 42.3% including 11.5% high-grade dysplasia/EAC. In the group with less than 2 genes methylated,
14.5% of cases had LGD and 32.8% had HGD/EAC (combined cases of dysplasia and EAC: 47.3%). It should be noted that these data were derived from minimal sampling (3 biopsies for methylation study regardless of segment length) compared with the quadratic biopsies taken every 2 cm to determine the histopathologic diagnosis. Clinical variables such as age (t test $P = 0.5$) and sex (chi-square $P = 0.5$) did not alter the risk for prevalent dysplasia and EAC. The mean segment length in nondysplastic Barrett’s esophagus was observed to be 7.3 cm (range 2–14 cm) and 7.2 cm (range 2–16 cm) in cases with dysplasia/EAC ($t$ test $P = 0.9$).

**Discussion**

This study has identified widespread changes in DNA methylation, which distinguish between Barrett’s esophagus and EAC. Use of an array-based strategy has enabled us to identify novel genes previously unknown to play a role in this disease. We hypothesized that methylation of imprinted and X-chromosome genes might provide candidate biomarkers as one copy is already inactivated. The analysis showed that almost 70% imprinted genes had altered methylation status in EAC and one of these, SLC22A18, was in the final stratification panel. Robust internal and external validation using pyrosequencing allowed us to select a 4-gene panel with an excellent ROC to distinguish between nondysplastic Barrett’s esophagus and dysplastic Barrett’s esophagus/EAC samples (AUC = 0.988). This panel enabled us to stratify patients into 3 (low, intermediate, and high) risk groups based on the number of methylated genes identified from analysis of a limited number of biopsies by virtue of the field effect.

A number of previous studies have looked at DNA methylation changes in Barrett’s carcinogenesis. However, none of the genes such as $p16$, $APC$ (27), and $MGMT$ (19) and a previously identified 8-gene panel (28) were shown in this current study to be differentially methylated in EAC versus Barrett’s esophagus. One reason for this might be that most biomarker studies have used a candidate, rather than an array-based approach, and compared the Barrett’s esophagus-associated disease states (dysplasia and EAC) to the normal squamous epithelium of the esophagus, whereas we have compared dysplasia/EAC to Barrett’s esophagus in our study (17, 29, 30). Also these studies have focused on predicting the future risk of cancer, whereas in our study we have focused on detecting dysplasia and early cancer in patients where it is not picked by histopathology. Metaplastic Barrett’s esophagus resembles intestinal rather than the squamous esophageal epithelium; and there is the possibility that the differences in DNA methylation observed between the normal squamous esophageal epithelium and Barrett’s esophagus/dysplasia/EAC might purely reflect differences in tissue morphology rather than playing any role in carcinogenesis. For this reason, we included 2 duodenum samples as control in our array-based methylation scan. If the methylation level of a gene was similar in both Barrett’s esophagus and duodenum; it was deemed that gene was involved in the maintenance of the columnar intestinal type epithelium rather than in the...
development of cancer. There were also methodologic differences in the assays used; previous studies have employed methylation-specific PCR (MSP) whereas here we used pyrosequencing which is a more quantitative method that has gained widespread acceptance (31). Lack of external validation has also been a problem in a number of recent studies (32, 33).

In this study, more hypermethylation was seen in cancer compared with hypomethylation (Table 1), in keeping with the fact that promoter hypermethylation is a well-established phenomenon in cancer. We also observed greater methylation changes to occur within known CpG islands. However, in a recent publication comparing the normal squamous mucosa with Barrett’s mucosa in 3 patients, methylation changes were reported to occur more frequently outside of CpG islands (34). It should, however, be noted that the majority of probes on the Illumina Infinium platform are positioned around promoter sites and 60% of human genes are associated with promoters spanning CpG islands. The recent availability of comprehensive genome-wide coverage of methylation changes will enable further light to be shed on this.

Figure 3. Internal validation. Beta values from the Illumina Infinium array (y-axis) are plotted against the % methylation from pyrosequencing (x-axis; N = 12).
documented in other cancers such as breast (36) and hepatocarcinomas (37) but we have shown for the first time that this can have a biomarker potential.

We looked at X-chromosome genes not only because DNA methylation plays a major role in X-inactivation in females but also because Barrett’s esophagus is more common in males who only have one copy of the X-chromosome and thus would theoretically only require one hit for the loss of the only functional allele. We were able to identify RGN, a putative tumor-suppressor gene (38, 39) that shows a successive increase in DNA methylation in the Barrett-associated metaplasia–dysplasia–adenocarcinoma sequence in males but not in females (Supplementary Fig. S2). TCEAL7 is also a candidate tumor suppressor gene shown to be epigenetically regulated in ovarian cancer and it functions by negatively regulating the NF-κB pathway (40, 41). Of the other 3 genes identified by our study; RIN2 encodes a guanine nucleotide exchange factor; PIGR encodes a poly-Ig receptor downregulation of which has been shown to be associated with more frequent lymph node metastasis in gastro-esophageal junctional tumors (42); and GJA12 encodes a gap junction protein mutations in which have recently been shown to increase the risk for secondary lymphedema after treatment for breast cancer (43). It is interesting that GJA12 seems to be methylated late in carcinogenesis, at the high-grade dysplasia/cancer transition compared with the other candidates described here.

The findings of our cross-sectional cohort study to identify putative biomarkers have potential clinical

![Diagram](image-url)
applications. For the detection of dysplasia a 4-quadrant biopsy sampling technique is employed as dysplastic lesions can be focally distributed within the Barrett’s segment without any endoscopically visible lesion. Furthermore, there is substantial intraobserver disagreement among pathologists in differentiating between low- and high-grade dysplasia (9, 10, 44). In the prospective study, we observed using our 4-gene methylation panel that DNA methylation is able to detect dysplasia/early-stage neoplasia in endoscopic biopsies even when the biopsy itself does not contain any visible dysplasia/early-stage neoplasia. This suggests that there is a field effect of methylation alterations in keeping with other research in the area of colon cancer (45, 46). The clonality of Barrett’s esophagus and evolving dysplastic lesions is still not clearly understood (47, 48) but there do seem to be widespread molecular changes before the emergence of phenotypical alterations visible by histopathology criteria (49). Our methylation panel is not intended to replace histopathology but to help reduce the chances for sampling bias and misclassification by taking advantage of the field effect and using a method that can be quantified in an objective fashion. It should be noted that there are differences between the cohorts which may explain the apparent false positives in the prospective cohort. The nondysplastic samples from the retrospective cohort were ascertained from the index biopsy, whereas the nondysplastic patients with Barrett’s esophagus in the prospective study were patients with Barrett’s esophagus under surveillance in tertiary referral centre with longer segments of Barrett’s esophagus (≥2 cm). Other studies have focused on the ability of biomarkers to predict future cancer development. Although this was not an endpoint of our multicenter trial, it will be interesting to analyze

Figure 5. ROC curves for all 6 targets. N(BE) = 32 vs. N(BED) + N(EAC) = 73. For RGN (Males only) N (BE) = 25 vs. N(BED) + N(EAC) = 51 (BE, Barrett’s esophagus; BED, Barrett’s esophagus with dysplasia; EAC, esophageal adenocarcinoma).
the predictive power of this panel as follow-up data becomes available.

Overall, we feel that this panel has the potential as an adjunct to histopathology to flag patients who harbor prevalent high-grade dysplasia and early adenocarcinoma. This needs validation in larger cohorts not skewed by referral bias in tertiary referral centers and is a promising area for further study.

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

Authors’ Contributions
Conception and design: M.A. Alvi, X. Liu, K. Ragunath, R.C. Fitzgerald
Development of methodology: M.A. Alvi, X. Liu, R. Newton, M.D. Pietro, R.C. Fitzgerald
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.A. Alvi, M. O’Donovan, K. Shariff, M.D. Pietro, J. Bergman, K. Ragunath, R.C. Fitzgerald
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.A. Alvi, X. Liu, R. Newton, L. Wernisch, N.B. Shannon, R.C. Fitzgerald
Writing, review, and/or revision of the manuscript: M.A. Alvi, X. Liu, R. Newton, J. Bergman, K. Ragunath, R.C. Fitzgerald
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.A. Alvi, X. Liu, K. Shariff, R.C. Fitzgerald
Study supervision: M.A. Alvi, R.C. Fitzgerald
Additional contributions: M. O’Donovan confirmed all histologic diagnoses

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