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Subtypes of Barrett's Esophagus and Esophageal Adenocarcinoma Based on Genome-wide Methylation Analysis

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Abstract

Objective: To determine if DNA methylation subtypes exist in esophageal adenocarcinoma (EAC) and its precursor Barrett's esophagus (BE).

Design: We performed genome-wide DNA methylation profiling on samples of non-dysplastic BE from cancer-free patients (N=59), EAC (N=23), normal squamous esophagus (N=33) and normal fundus (n=9), and identified methylation subtypes using a recursively partitioned mixture

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model. We assessed genomic alterations for 9 BE and 22 EAC samples with massively parallel sequencing of 243 EAC-associated genes, and analyzed transcriptome data to identify epigenetically repressed genes in EAC. We used 5-AZA treatment in EAC cell lines to assess the role of DNA methylation in gene silencing. The biological consequences of gene silencing were determined by shRNA knock-down in SK-GT-4 cells. Finally, we assessed the response to anti-cancer therapies in representative EAC cell lines (JH-EsoAd1, OE33, SK-GT-4 and OE19).

Results: We identified and validated 4 methylation subtypes of EAC and BE. The high methylator subtype (HM) had more activating events in *ERBB2* ($P < .05$, Student t test) and a higher global mutation load ($P < .05$, Fisher's exact test), compared to other subtypes. We demonstrated that *PTPN13* is aberrantly methylated and transcriptionally silenced only in the HM subtype and in 57% of EACs. 5-AZA treatment restored *PTPN13* mRNA level in OE33 and ESO26 cell lines, which coincided with a significant decrease in the promoter methylation ($P < .05$, Welch's t test). Inhibition of *PTPN13* expression in EAC cells promoted several hallmark behaviors of EAC including proliferation, colony formation and migration, and further, resulted in increased phosphorylation of ERBB2/EGFR/Src kinase pathways. Finally, we found a subtype-specific response to anti-cancer therapies including topotecan, SN-38 and palbociclib, in EAC cell lines.

Conclusions: Identification of DNA methylation subtypes in EAC provides novel insight into the molecular heterogeneity of BE and EAC and the functional role of epigenetic alterations in EAC tumorigenesis. Characterization of these subtypes reveals they have the potential to be used to guide the clinical management of EAC patients.

Graphical Abstract

SUBTYPE COLOR ASSIGNMENT

	JH-EsoAd1	OE33	OACM5.1C	OE19	FLO (LM)
EC50	9.937	10.71	2.902	0.1298	0.7969

Keywords

dysplasia; gastrointestinal cancer; methylation

INTRODUCTION:

Esophageal adenocarcinoma (EAC) is a significant cause of cancer-related mortality in the US with a dismal 15% 5-year survival rate¹. Of additional concern is that the incidence-based mortality rate for EAC has increased dramatically over the last three decades². Thus, there is a substantial need to advance our understanding of the molecular pathology of EAC and to use this understanding to improve our ability to prevent and manage EAC.

It is well established that virtually all EAC arises from Barrett's esophagus (BE), which is a premalignant condition characterized by intestinal metaplasia of the distal esophagus believed to arise secondary to chronic gastroesophageal reflux³. Studies over the last half-century have refined our understanding of the BE→EAC sequence and shown it to be a multi-step process that involves the progression of non-dysplastic Barrett's esophagus (NDBE, or BE) to low-grade dysplasia (LGD), high-grade-dysplasia (HGD) and then EAC⁴. A primary mechanism driving this sequence is the accumulation of genetic and epigenetic alterations. Recently, studies of the genome of BE and EAC have revealed that mutations in multiple genes, including *TP53*, *CDKN2A*, *SMAD4*, *ERBB2*, *PIK3CA*, and *ELMO1*, are common in EAC and also present in BE, although at a lower frequency⁵⁻⁹. Of particular note, *TP53* mutations appear to play a role in BE progression presumably by affecting genomic stability¹⁰⁻¹². Furthermore, Stachler et al identified two molecular classes of EAC that differ by the presence of whole genome doubling and mutation patterns¹¹. More recently, Secrier et al. showed three distinct molecular subtypes of EAC based on mutational signatures¹³. These studies demonstrate that there is inter-lesional molecular heterogeneity among EACs and BE, as has been shown for other cancers including colorectal, breast, and gliomas¹⁴⁻¹⁶.

In addition to genetic alterations, epigenetic alterations have been found to be common in BE and EAC. CpG island DNA methylation in the promoter regions, a specific type of epigenetic modification, can lead to the silencing of a subset of tumor suppressor genes and presumably drive cancer formation.¹⁷ The majority of studies published to date on DNA methylation in BE and EAC have been limited to the assessment of small panels of candidate genes but have established that aberrantly methylated genes are present in both BE and EAC, and that they likely contribute to the progression of BE→EAC¹⁸⁻²². More recent studies have used HumanMethylation27 and HumanMethylation450 (HM450) arrays to identify global methylation alterations in BE and EAC, but these studies are limited by a lack of validation cohorts.²⁰²³²⁴ A recently published paper by TCGA found molecular features including DNA hypermethylation in esophageal carcinoma, including EAC²⁵. However, whether such DNA methylation features exist in BE and whether methylation based subtypes of EAC and BE exist requires further study at this time.

Thus, to determine if there are molecular subtypes of BE and EAC based on DNA methylation patterns, we performed genome-wide DNA methylation profiling using HM450 arrays in 59 BE, 23 EAC, 33 normal SQ and 9 fundus samples. We identified 4 distinct methylation subtypes. We assessed genomic alterations and integrated methylome and transcriptome data to characterize the methylation subtypes. Using the primary EAC tissue samples and EAC cell lines, we assessed whether methylation-based subtyping in EAC

could provide novel insights into the functional role of epigenetic alterations and could potentially be helpful to guide treatment decisions for EAC.

MATERIALS AND METHODS

(Supplemental Methods contain details on experiment design, methods, reagents and analysis)

Primary tissue sample collection

All primary EAC samples were obtained from University Hospitals Cleveland Medical Center (Cleveland, OH) following Institutional Review Board approval. Fresh frozen normal esophagus tissues (N=7) were obtained from GICaRes Biorepository (University of Washington Department of Medicine; Division of Gastroenterology) following Institutional Review Board approval. Formalin fixed paraffin-embedded (FFPE) slides and cores from a total of 33 normal squamous (SQ) from BE, 9 fundus from EAC patients, 59 nondysplastic Barrett's (NDBE) samples from cancer-free patients, and 23 EAC samples, as well as an independent set of 16 fresh frozen EAC biopsy samples were obtained via the Barrett's Esophagus Translational Research Network (BETRNet). All the FFPE BE/EAC samples were reviewed by an expert GI pathologist and were determined to be >70% BE or EAC. See Supplemental Table 1 for the demographic information of BETRNet samples.

EAC Cell lines

The established EAC cell lines ESO26, OE33, SK-GT-4, FLO-1, OACM5.1C, and OE19 were purchased from the European Collection of Authenticated Cell Cultures (ECACC) through Sigma Aldrich. Cells were cultured in standard conditions according to the Repository's instructions. The JH-EsoAd1 was a kind gift from Hector Alvarez²⁶. The cell lines were authenticated by the suppliers prior to being sent to our laboratory.

DNA preparation and HM450 array data acquisition and preprocessing

DNA extraction and bisulfite conversion were performed as described previously²⁷. DNA samples were processed and run on HumanMethylation450 (HM450) arrays following the manufacturer's instructions (Illumina Inc.). Raw intensity data was read, preprocessed and batch corrected in R (v3.2.2). After subsequent filtering, 426,718 probes were left for analysis. Additional HM450 array datasets include: Level 1 HM450 array data from 87 EAC and 11 adjacent normal tissue samples obtained from TCGA on 11/13/2015 (<https://tcga-data.nci.nih.gov/tcga/>), and 125 EAC samples from the Gene Expression Omnibus (study ID: GSE72872)²³.

Subtype Identification

The most variable probes (MVPs, N = 1515) were identified among BETRNet EACs. Recursive-partitioning mixture model (RPMM) clustering (RPMM R package, v.1.2.0) was performed on MVP methylation to identify methylation subtypes.²⁸

Targeted Sequencing of BETRNet samples

Preprocessing was performed on DNA libraries using Picard tools and Burrows-Wheeler Alignment tool as described prior²⁹³⁰. Mutation analysis for single nucleotide variants (SNV) was performed using MuTect v1.1.4³¹³². We used the SomaticIndelDetector tool that is part of the GATK for indel calling. Selection of likely somatic non-synonymous mutations was performed as described in Supplemental Methods. Copy number variants were called then tangent normalized, with prior filtering of high-variance capture intervals, using the tool ReCapSeg v1.4.4.³³

Exome sequencing analysis of TCGA samples

Whole exome sequencing files were downloaded from TCGA and used as input for MutSig (<http://www.broadinstitute.org/cancer/cga/mutsig>) and GISTIC (www.broadinstitute.org/cancer/cga/gistic) analysis as previously described.³⁴³⁵

Integrative Analysis of Repressed Genes and Pathways

Level 3 gene expression data from TCGA EAC samples was obtained from Firehose (<https://confluence.broadinstitute.org/display/GDAC/Home>). We then applied stringent workflows to identify significant, substantial, and biologically meaningful evidence of epigenetic and epitranscriptomic repression (Supplemental Methods and Supplemental Figure 11). For differentially regulated gene sets, we performed network analyses with Panther (<http://www.pantherdb.org>) and DAVID (<https://david.ncifcrf.gov/>)³⁶³⁷.

Quantitative methylation-specific PCR (qMS-PCR)

PTPN13 qMS-PCR assays were performed to validate HM450 array results in clinical samples and EAC cell lines using C-LESS as control. Detailed methods including primer sequences for qMS-PCR assays, and the calculation of the relative methylation of *PTPN13* are provided in Supplemental Methods and Supplemental Table 17.

RNA extraction

RNA extraction from primary tissue samples and EAC cell lines was performed as previous described¹⁹.

Quantitative real-time PCR

The mRNA level of *PTPN13* was determined by a SYBR assay. Taqman gene expression assay for *GUSB* expression (*Applied Biosystems*) was used for normalization of cDNA loading. Detailed methods and primer sequences are provided in Supplemental Methods and Supplemental Table 17.

5-Aza-2'-deoxycytidine (5-Aza) treatment

OE33 and ESO26 cell lines were treated with 5-Aza as previous described¹⁹ and described in Supplemental Methods.

Gene knock-down by small hairpin RNA transfection

pGIPz-based short hairpin RNA (shRNA) constructs specifically targeting human *PTPN13* mRNA were provided by the Genomics Shared Resource Core at the Fred Hutchinson Research Center (FHCRC, Seattle, WA). The lentiviral transfection was performed as described before³⁸. The target sequences of each *PTPN13* shRNA construct were confirmed by direct sequencing and listed in Supplemental Table 17.

Cell Proliferation Assays

Cells were plated at a density of 1000 cells per well onto 96-well plates at day 0. Every day until day 4, CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, U.S.A) was added and incubated at 37°C for 1 hour. Optical density was measured at 490 nm using a plate reader (Molecular Devices, Sunnyvale, CA).

Colony Formation Assays

Cells were trypsinized into a single-cell suspension. A total of 50 and 100 cells were plated in each well of a 6-well plate and maintained for 14 days to allow colony formation. Colonies were fixed with methanol and stained with 0.01(w/v) crystal violet. Two independent experiments were performed.

Cell Migration/Invasion Assay

Cell motility was measured on FlouroBlok Inserts with 8-mm pores (24 well format; Corning Incorporated, Corning, NY) and cell invasiveness was measured on the same Inserts containing 100μL of 300μg/mL of matrigel diluted in serum free media, according to the manufacturer's instructions. Briefly, cells (5×10^4) were seeded onto insert membranes without and with matrigel. After incubation at 37°C for 24 hours, cells that did not migrate or invade through the pores of the FlouroBlok Inserts were manually removed. Membranes were washed, fixed and stained. Cells present at the bottom of the membrane were counted using a computer program after fluorescent images of the membranes were taken. Three independent experiments were performed.

Western blotting

Western blotting experiments were conducted as described previously³⁹. Details on the antibodies are provided in Supplemental Methods.

In vitro drug sensitivity assays

The in vitro drug sensitivity assays were conducted as described previously⁴⁰. The detailed methods are provided in Supplemental Methods. The following compounds were used for drug treatment: SN-38 and topotecan (Sigma Aldrich), palbociclib (Sellekchem). The compounds were diluted to working concentration in DMSO (Sigma Aldrich).

Statistical analysis and data visualization

Analyses applied R/Bioconductor, MutSig, GISTIC, PANTHER, and DAVID statistical software. R/Bioconductor libraries included minfi, sva, qvalue, RPMM, ggplot2, and ComplexHeatmap. Essential scripts available on GitHub (<https://github.com/GradyLab/>)

GE_methylation_subtypes). Binomial tests were used to assess enrichment. Correlations between multiple molecular datatypes (eg. promoter methylation vs. gene expression) used Spearman tests, unless indicated otherwise. We evaluated genomic event count data using Fisher's exact test. Benjamini-Hochberg and FDR-adjustment were used to adjust p-values. Student t test was used (1) to compare the average (mean) difference in *PTPN13* methylation or gene expression (2) to compare proliferation and migration data between two groups. When the two groups had unequal variances and unequal sample sizes, Welch's t test was used instead. Reported *P* values are two-sided; $P < 0.05$ was considered as significant. GraphPad Prism version 7.0 (GraphPad Software Inc., La Jolla, CA) was used to calculate *P* values and EC50 values in drug sensitivity experiments.

Results

Genome wide DNA methylation analysis of BE and EAC reveals distinct methylation subtypes

Molecular subclasses based on DNA methylation alterations have been recently identified in several cancer types, including colorectal cancer and gliomas¹⁶⁴¹. Herein, we conducted studies to determine if DNA methylation subtypes are present in EAC and its precursor BE.

First, we applied a model-based clustering method (RPMM) to HM450 datasets from $N = 23$ EAC samples collected through BETRNet. RPMM is suitable for β -distributed datasets including the HM450 platform²⁸, and it has been used to identify molecular subtypes in cancers of colon and breast⁴¹⁴². We performed clustering using the 1515 most variable CpG probes (MVPs, β -value SD > 0.25 , Supplemental Table 2). Interestingly, MVPs were significantly enriched for intragenic CpGs in both promoter (55%, $P < 10^{-15}$) and body regions (37%, $P < 10^{-6}$), and they were further enriched for probes in CpG islands (70%, $P < 10^{-15}$).

Clustering using RPMM revealed four methylation subtypes among EAC samples (Figure 1A), denoted as high (HM), intermediate (IM), low (LM) and minimal (MM) methylator based on the underlying MVP methylation patterns. Considering a CpG β -value > 0.6 as hypermethylated, HM harbors the most hypermethylated CpG probes ($N = 981$), followed by IM ($N = 181$), LM ($N = 58$), and MM ($N = 39$). To validate the subtype assignments, we also conducted multidimensional scaling (MDS) on the 1515 MVPs and found the subtype designations from RPMM were largely concordant with two-dimensional MDS clustering patterns (Supplemental Figure 1).

Notably, the methylation pattern in MM is similar to what is observed in the normal SQ and fundus (Figure 1A and Supplemental Figure 2A). We found uniformly lower methylation levels of MVPs in normal matched tissues compared to their adjacent EACs ($N=7$ pairs, Supplemental Figure 2B).

To validate our findings of methylation subtypes in EAC samples from the BETRNet cohort, we used an independent set of 87 EACs and 11 normal matched samples from the Cancer Genome Atlas (TCGA) (See Supplemental Table 3 for TCGA EAC cohort information). Clustering analysis with the MVPs available after preprocessing ($N = 1479 / 1515$) again

revealed four distinct clusters corresponding to the HM, IM, LM, and MM subtypes (Figure 1B). Similar to the BETRNet EAC cohort, these subtypes largely reflect global hypermethylation in the HM and IM, compared to the LM and MM subtypes (Supplemental Figures 3).

Finally, we observed no correlation between available demographic features (age, gender, or tobacco use in TCGA EACs; age or gender in BETRNet EACs) and methylation subtype membership ($P > 0.1$, Spearman test). We then conducted survival analyses in independent data sets from TCGA (87 EACs) and the Gene Expression Omnibus (GSE72872, 123 EACs), but found no significant correlation either with or without adjustment for pathologic stage of the tumor ($P > 0.1$, Cox Proportional Hazard testing) (Supplemental Figure 4).

Methylation-based subtypes in BE

To investigate the presence of the methylation subtypes in BE, the known precursor of EAC, we applied RPMM-based clustering in 59 non-dysplastic Barrett's esophagus (NDBE) samples from cancer-free patients. Remarkably, with the same MVPs identified in EAC, we again identified four subtypes in NDBE (Figure 1A, Supplemental Figure 5). The number of hypermethylated MVPs (mean β -value > 0.6) in the HM, IM, LM, and MM subtypes was 751, 88, 37, and 32, respectively, and MVP methylation patterns across MM BEs was similar to matched SQ and fundus (Figure 1A).

Somatic genomic alterations in EAC and BE methylation subtypes

In order to further characterize the EAC methylation subtypes, we assessed genomic alterations in the BETRNet EAC cohort. We utilized massively parallel sequencing with a targeted sequencing panel containing all exons of 243 of the most commonly altered genes in gastroesophageal cancer (Supplemental Tables 4 and 5, Figure 2B)¹⁰¹¹. We identified a total of 62 likely pathogenic somatic mutations in 29 candidate genes, 74 gene level copy number gains (28 of which were considered high level amplifications, $\log_2$ ratio > 2), 335 gene level single copy deletions, and 64 gene level multi-copy deletions (Supplemental Table 4B). We identified frequent *TP53* mutations in all of the methylation subtypes. Although the number of samples in each subtype was small, an elevated frequency of *ERBB2* events (COSMIC mutations or amplifications) was found in the HM group (6/7, 86%), but only one case was found in the IM and MM subtypes (Supplemental Table 5). To assess the clinical relevance of the high frequency of *ERBB2* alterations in the HM subtype, we assessed *ERBB2* expression in a subset of the BETRNet EACs (N=18) using a standard *ERBB2*/HER2 IHC assay (Supplemental Methods). Although the significance of the findings is limited by the number of samples available for the *ERBB2* IHC assay, we observed the greatest frequency of increased HER2 cases in the HM subtype (HM: N=2/5 or 40%, IM: 1/3 or 33%, LM: 0/6, MM: 0/2).

We next assessed genomic alterations in a subset of NDBE samples (N = 9) with the sequencing panel described above (Supplemental Figure 6, Supplemental Table 4C). Recognizing the number of samples is limited and may impact on the interpretation of our results, we did not identify significant differences in gene alteration frequency between the methylation subtypes of the BE samples.

To confirm our finding of an increased rate of *ERBB2* events in the HM subtype, we utilized the exome sequencing data from the TCGA EAC samples. Overall likely *ERBB2* activating genomic alterations (recurrent COSMIC mutations or amplifications) occurred in 7/25 (28%) of HM subtype, 8/26 (31%) of IM subtype, 1/20 (5%) of LM subtype, and 2/16 (12.5%) of MM subtype (Supplemental Table 6). In order to determine if a larger number of samples with expanded sequencing coverage would allow discovery of other alterations unique to a given methylation subtype, we next assessed whether there were subtype-specific events both mutations and copy number alterations. We observed a trend for increased loss of function *ARID1A* mutations in the HM subtype (6/25, 24%) compared to the IM (2/26, 8%), LM (1/20, 5%), and MM (1/16, 6%) subtypes ($P = 0.1 - 0.2$, Fisher's exact test). Amplifications in *CDK6* were found in 5/26 (19%) of IM samples, while only in 1 sample for all other subtypes. High-level *MDM2* amplifications were rare but found exclusively within the MM subtype (3/16, 19%).

Notably, we also observed significant differences in the overall mutation loads between the EAC methylation subtypes (Figure 2A). The highest overall mean mutation load was seen in the HM subtype (10.9 mut/MB) followed by the IM subtype (6.87 mut/MB). The LM (5.19 mut/MB) and MM (5.54 mut/MB) subtypes had the lowest loads ($P = 0.09, 0.02, 0.03$ for HM vs IM, LM, and MM respectively, Student's t-test). Two HM samples were outliers with very high mutation loads (Supplemental Figure 7). After removal of these samples, the HM subtype had a considerably lower mutation load of 7.78 mut/MB but was still significantly higher than the LM and MM subtype mutation loads ($P = 0.02$ and 0.03 , respectively, Student's t-test). The HM subtype also had a higher proportion of mutations that were small insertions or deletions compared to the other subtypes (15.4% vs 6.0%, 7.5%, and 6.9% for IM, LM, and MM, respectively; $P < 0.0001$ HM vs any other subtype).

In summary, looking across the BETRNet and TCGA EAC cohorts, we identified 32 HM, 31 IM, 26 LM, and 20 MM samples. A combined analysis across the BETRNet and TCGA EAC cohorts demonstrated results largely consistent with what was observed in the individual datasets (Table 1 and Supplemental Table 7). We observed that recurrent COSMIC *ERBB2* mutations were enriched in the HM subtype, with a higher number of mutated samples (6/32, 19%) compared to the IM (3/31, 10%), the MM subtype (0/20) or LM subtype (0/26) (Fisher's exact test, $P = 0.03$ for both HM vs. LM and HM vs. MM). The number of *ERBB2* amplified samples was significantly greater than observed in the LM subtype (1/26, 4%) (Fisher's exact test, $P = 0.02$). *ARID1A* alterations also showed a suggestive strong correlation between methylation subtype, being highest in the HM subtype (7/32, 22%), followed by the IM (3/31, 10%), LM (3/26, 11%), and MM (1/20, 5%) subtypes, respectively.

Integrative analysis of EAC subtype-specific gene and pathway repression

We analyzed HM450 methylation and RNA seq datasets performed on TCGA EAC to identify genes differentially regulated by promoter methylation and miRNA expression across the EAC methylation subtypes (Supplemental Figure 8; Supplemental Methods).

The HM EAC cases had the greatest total ($N = 69$ genes, Spearman $\rho < 0$, p-adj. < 0.05) as well subtype-specific ($N = 50$ genes, 72%) epigenetically repressed genes (Figure 2C,

Supplemental Table 8). Among these were putative tumor suppressors implicated in various cancer types, including *PTPN13*, *TUSC1*, *RGS6*, *TIAM1*, and *HUNK*^{43–47} (Supplemental Figure 9). Gene enrichment tests of HM-specific repressed genes showed enrichment of pathways important in cancer (Supplemental Table 9). By contrast, using the same search criteria, we found no epigenetically repressed genes in MM cases, and only 3 in LM cases.

We also compared differentially expressed miRNAs and their putative target genes across methylation subtypes (Figures 2D–E, Supplemental Methods, Supplemental Figures 10–13, Supplemental Table 10). Gene enrichment analysis of DETGs repressed by miRNAs in HM cases compared to MM cases (Binomial test, $p\text{-adj} < 0.05$, Supplemental Table 11–14) revealed subtype-specific repression of cancer pathways in the HM subtype, including the WNT pathway and cell adhesion and signaling (see details in Supplemental Results and Methods).

Aberrant methylation of *PTPN13* in a subset of EAC led to its transcriptional repression

We decided to focus on one particular gene *PTPN13*, to study functional consequences of gene silencing in EAC. *PTPN13* was chosen because its promoter hypermethylation was negatively correlated with repressed gene expression in the HM and IM subtypes. *PTPN13* encodes a nonreceptor-type protein tyrosine phosphatase. It is a putative tumor suppressor gene in breast cancer⁴⁸ and non-small cell lung cancer⁴⁹. We used quantitative methylation-specific PCR to assess *PTPN13* methylation in the gene promoter region in an independent set of clinical samples, including normal esophagus (N=7) and EAC (N=16). We detected low level of *PTPN13* methylation (median 0.07 ± 0.012) in normal samples and significantly elevated *PTPN13* methylation in EAC samples (Welch's t test $P=0.03$). Using the relative methylation level >0.1 as a cutoff threshold for methylated *PTPN13*, it was methylated in 56% of EAC samples (Figure 3A). We further analyzed the *PTPN13* mRNA expression levels in 14 EAC samples with available mRNA, and correlated them with the samples' *PTPN13* methylation status (Figure 3B). The *PTPN13* gene expression was heterogeneous in EAC samples, likely reflecting a mixture of stromal and tumor tissue and the small sample size. (Note: Assessment of normal squamous epithelium contamination with *KRT14* expression revealed the two outliers had high levels of contamination. See Supplemental Figure ***) We observed 75% of *PTPN13* methylated EAC samples showed low mRNA level (relative expression <0.5), while 67% of unmethylated EAC samples showed high mRNA level (relative expression >8). To determine the role of DNA methylation in regulation *PTPN13* gene expression, we treated ESO26 and OE33 cell lines, which have methylated *PTPN13* and no detectable mRNA level, with 5-azacytidine (5-Aza). As shown in Figure 3C–D, 5-Aza treatment restored *PTPN13* gene expression in both cell lines, and this restoration of gene expression was associated with a significant decrease in the promoter DNA methylation (Student t test, $P<0.5$).

Inhibition of *PTPN13* in EAC cells led to increased cell growth, colony formation and migration.

To directly investigate the functional roles of *PTPN13* in EAC, we measured the promoter methylation and gene expression of *PTPN13* in 7 EAC cell lines. We found *PTPN13* was methylated with no detectable mRNA level in 4 EAC cell lines (ESO26, FLO-1, OE33 and

ESO51) and that SK-GT-4 had higher PTPN13 mRNA level than any other cell line (Figure 4A). Therefore, we chose SK-GT-4 for shRNA knockdown studies to investigate the effect of *PTPN13* loss on EAC cells. We used 4 PTPN13-specific shRNAs with confirmed target sequences and chose to use one that achieved > 85% reduction of *PTPN13* mRNA levels in transfected SK-GT-4 cells vs non-specific scrambled (NSC) control (Supplemental Figure 13). To determine the functional consequences of repressed *PTPN13* in EAC cells, we performed several in vitro assays. In comparison with parental SK-GT-4 cells, or SK-GT-4-NSC, stable transfection of SK-GT-4 cells with shRNA-PTPN13 had significantly increased growth at day 4 (Student t test, $P < 0.01$; Figure 4B). SK-GT-4 shRNA-PTPN13 clones also had increased capacity to form colonies compared to SK-GT-4-NSC control cells (Figure 4C). Further, suppression of *PTPN13* in SK-GT-4 cells significantly increased the migration of SK-GT-4 compared to the mock transfected cells or NSC control cells (Student t test $P < 0.05$, Figure 4D). However, we did not observe significant differences in cell invasion capability. Taken together, these results suggest that inhibition of *PTPN13* in EAC cells promoted cell growth, colony formation and migration.

Inhibition of *PTPN13* in EAC cells led to enhanced ERBB2/EGFR tyrosine kinase signaling

PTPN13 encodes a nonreceptor-type protein tyrosine phosphatase, although its biological functions in EAC have not been elucidated. Thus, we determined if inhibition of *PTPN13* in EAC cells would affect the tyrosine phosphorylation status of a panel of key kinases implicated in EAC. As shown in Figure 4E, the phosphorylation of EGFR-Tyr1068, ERBB2-Tyr1248 and Src-Tyr416 was increased in *PTPN13* knock-down SK-GT-4 cells in basal condition and was further enhanced upon EGF stimulation, compared to the NSC control. These data demonstrate that inhibition of *PTPN13* enhanced EGFR/ERBB2 and Src kinase signaling. We also assessed PTPN13 protein expression in primary EAC tissues. With a limited sample size (N=14), we observed activation of EGFR at Tyr1068 in 2 EACs with methylated *PTPN13* and reduced gene/protein expression (Supplemental Figure 14). Further, we examined the expression of PTPN13 and phospho-ERBB2 (Tyr1248) in EAC tissue microarrays. The immunohistochemical analyses revealed that 7.7% cases (3/39 EAC cores) had strong pERBB2 expression (Tyr1248) (score 2+) with weak or absent PTPN13 (score 60). In contrast, 41% cases (16/39) with weak or absent pERBB2 (Tyr1248) (score 2) had strong PTPN13 expression (IS>60) (Supplemental Table 16).

Efficacy of cytotoxic chemotherapy differs between EAC methylation subtypes

Given the different genomic features observed between the methylation subtypes, we hypothesized that EACs from different methylation subtypes might respond differently to anti-cancer chemotherapies. Consequently, as noted previously, we performed DNA methylation profiling on available EAC cell lines and determined the methylation subtype of each cell line (Figure 5A, Supplemental Table 15). We then tested the growth inhibitory efficacy of two commonly used chemotherapeutic drugs SN-38 (the active metabolite of irinotecan) and topotecan, both are topoisomerase I inhibitors, in cell lines representative of the methylation subtypes: JH-EsoAd1 and OE33 (HM), SK-GT-4 (LM) and OE19 (MM). Two HM cell lines responded similarly to SN-38 and topotecan, and exhibited a marked sensitivity to both chemo drugs, compared to SK-GT-4 and OE19 (Figure 5B–C).

We then evaluated whether these cell lines had differential sensitivities to targeted therapies. Agents targeting cyclin-dependent kinases (CDKs) are currently being evaluated in clinical trials and have potential for the treatment of EAC. Given the genomic alteration profiles of methylation subtypes, we chose to assess palbociclib, a highly specific CDK4/6 inhibitor. We found JH-EsoAd1 and OE33 (HM cell lines) responded similarly to palbociclib, while OACM5.1C (LM cell line) was substantially more sensitive to palbociclib (Supplemental Figure 15). The ability of palbociclib to effectively block CDK4/6 activity was determined by inhibition of Rb phosphorylation (Supplemental Figure 16).

Discussion

The development of high-throughput methods for characterizing the cancer genome and epigenome has revealed molecular heterogeneity among cancers originating from the same tissue, providing greater insight into their biology, and clinical heterogeneity in natural history and treatment response^{50–52}. We now provide further insight into that molecular heterogeneity by identifying and characterizing DNA methylation subtypes in BE and EAC (Figure 1). Our joint analysis of both methylation and genomic alterations allowed for several primary observations. First, the HM subtype showed a significantly higher overall mutation load than the other subtypes (Figure 2A). Second, integrative analyses of DNA methylation and gene expression, as well as differential microRNA and target gene expression, revealed HM-specific differences in patterns of gene and network deregulation (Figure 2). Notably, methylation subtypes in NDBE reflect similar MVP methylation patterns to those in EAC (Figure 1A). Furthermore, our integrative analysis identified a list of candidate genes that are epigenetically silenced specifically in the HM subtype, including the candidate tumor suppressor gene, *PTPN13*, a non-receptor type tyrosine phosphatase. Using combined orthogonal assays (qMSP and qRT-PCR) in an independent cohort of EAC primary tissue samples, we validated our findings that *PTPN13* methylation is uniformly low in normal esophagus tissues and that EACs show promoter hypermethylation and loss of *PTPN13* mRNA expression in a subset of EAC primary tissues as well as EAC cell lines. We provided evidence that promoter methylation was driving loss of *PTPN13* expression through studies using 5-azacytidine, a DNMT1 inhibitor (Figure 3).

To further elucidate the biological relevance of this HM subtype specific feature, we investigated the consequences of *PTPN13* loss on several hallmark behaviors of EAC cells and showed *PTPN13* suppressed cell proliferation, colony formation and migration. Furthermore, inhibition of *PTPN13* led to increased phosphorylation in critical tyrosine sites of EGFR/ErbB2 and Src kinases at basal level, with further increases upon EGF stimulation (Figure 4). Additionally, western blotting of EAC biopsy samples reveals increased EGFR-Tyr1068 phosphorylation in a subset that harbor aberrant *PTPN13* hypermethylation and silenced expression at both mRNA and protein levels (Supplemental Figure 14). Immunohistochemistry analysis of *PTPN13* and pERBB2-Tyr1248 in EAC tissue microarrays indicated that strong phosphorylation of ERBB2 (Tyr1248) staining is only present in EACs with weak or absent *PTPN13* staining (Supplemental Table 16). In aggregate, these findings support the conclusion that *PTPN13* is a HM subtype specific tumor suppressor gene that is silenced epigenetically in HM EACs. Interestingly, our molecular characterization of DNA methylation subtypes found an increased frequency of

genomic alterations in EGFR/ERBB2 genes in HM/IM subtypes (Table 1). Collectively, our data on methylated *PTPN13* and EGFR/ERBB2 alterations reveals a novel molecular mechanism of activating oncogenic ERBB2/EGFR signaling in a subset of EAC, which highlights the importance of ERBB2/EGFR signaling pathways in the pathophysiology of a subset of EAC.

Our experiments assessing EAC cell culture susceptibility to two topoisomerase I inhibitors as well as palbociclib (Figure 5, Supplemental Figure 15) suggest that the methylation subtypes may have distinct susceptibilities to conventional and targeted therapies. While these *in vitro* results will need to be validated in additional cell lines and clinical studies, our preliminary findings suggest the DNA methylation-based subtypes are of potential clinical relevance and could eventually be used to stratify cancers by risk of progression and to optimize treatment selection for a specific individual's EAC.

An important consideration for any analysis of HM450 data is the extent to which heterogeneity in tissue sample or cell type can bias findings. We carefully considered whether variable normal tissue content could explain the methylation differences across subtypes in BE and EAC. All samples, including BE, EAC, SQ and fundus, in both the BETRNet and TCGA cohorts, contained > 70% tumor tissue as determined by expert pathologic analyses. From computational analysis of EACs, no significant differences in tumor purity between the methylation subtypes was observed except for the HM-LM comparison in the BETRNet cohort (pairwise *t*-test, $P > 0.05$, Supplemental Figure 17). We further observed no significant correlations between methylation subtype membership and important histologic (tumor nuclei percent, tumor necrosis percent, and tumor weight) or clinical features (age, gender, tobacco use and survival) ($P > 0.1$). Based on these deconvolution analyses, it is highly unlikely tumor enrichment confounds subtype membership.

In summary, we have identified four distinct DNA methylation subtypes of BE and EAC using comprehensive molecular characterization. Our integrative analysis combined with functional *in vitro* studies uncovers a novel molecular mechanism by which EAC cells activates the oncogenic kinase pathways via epigenetically silencing the tyrosine phosphatase, specifically in the HM subtype. The drug treatment experiments provided evidence that these subtypes may respond differently to the chemo- and targeted therapies. Therefore, identification of these subtypes provides novel insights into the biological mechanisms of EAC carcinogenesis and could be helpful in promoting precision medicine in EAC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

BE	Barrett's Esophagus
NDBE	non-dysplastic Barrett's Esophagus
EAC	Esophageal adenocarcinoma
LGD	low grade dysplasia
HGD	high grade dysplasia
SQ	squamous
BETRNet	Barrett's Esophagus Translational Research Network
FFPE	Formalin fixed paraffin-embedded
HM450	HumanMethylation450
HM	high methylator
IM	intermediate methylator
LM	low methylator
MM	minimal methylator
MVP	most variable probes
RPM	recursively partitioned mixture model
CIMP	CpG Island Methylator Phenotype
TCGA	the cancer genome atlas
FDR	false discovery rate
SD	standard deviation
FC	fold change
SNV	somatic nucleotide variants
CNV	copy number variants

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Figure 1.

Heatmaps of methylated CpGs in EAC and BE cases show distinct clusters based on methylation patterns. The clusters were identified with recursive partition modeling using β -value methylation at the 1515 most variable CpG probes (MVPs). (A). MVP heatmap of BETRNet samples, including EACs used for MVP discovery (N = 23), NDBE from cancer-free patients (N = 59), a representative subset of normal SQ tissues from BE patients (N = 11), and normal fundus (N = 9) from EAC patients. A comprehensive heatmap including all normal SQ samples (N = 33) is shown in Supplemental Figure 3. (B). MVP heatmap of independent validation samples from TCGA, including EACs (N = 87) and matched normal tissues (N = 11). Methylation is measured as β -values from unmethylated or 0 (dark blue) to methylated or 1 (yellow). Methylation subtype annotation: high methylator (HM, yellow); intermediate methylator (IM, coral); low methylator (LM, grey) and minimal methylator (MM, blue)

Figure 2.

Characterization of methylation subtypes in EAC (**A**) Mutation loads (Mut/Mb) calculated using MutSig, and results of comparing HM to other subtypes (two-tailed Student's *t* test, $P < 0.05$ is considered as statistically significant). (**B**) BETRNet EAC sample gene alterations detected using sequencing of a panel of genes functionally important in EAC. (**C**) Venn diagram of comparing epigenetically repressed genes across subtypes. (**D**) Venn diagram comparing differentially expressed miRNAs (DEmiRs) in pairwise subtype comparisons. (**E**) Volcano plots of miR and DEmiR expression and differential expression test result (t-test, p-unadj) in three subtype comparisons (HM vs. IM, LM, and MM, respectively). X-axis is difference in mean expression between subtypes (HM minus IM, LM, or MM mean, respectively), y-axis is the $-\log_{10}(p\text{-unadj})$ from t-tests.

Figure 3.

Aberrant methylation of *PTPN13* in a subset of EAC led to its transcriptional repression. **(A)** The relative methylation of *PTPN13* (normalized to C-LESS control) in normal esophagus tissues (N=7) and EAC biopsy samples (N=16) was determined using qMS-PCR. Average relative methylation, depicted as a line, is significantly elevated in EAC than normal esophagus (Welch's t test, $P < 0.05$). The red dots indicate EAC samples with methylated *PTPN13* (See Supplemental Methods). **(B)** The relative DNA methylation level (normalized to C-LESS control) (Y-axis) and mRNA expression (normalized to GUSB) of *PTPN13* (X-axis) for 14 EAC biopsy samples with available RNA. The mRNA expression was determined using qRT-PCR with GUSB as control gene (See Supplemental Methods). The red dots indicate EAC samples with methylated *PTPN13*. **(C-D)** A significant transcriptional re-expression of the methylated *PTPN13* following 5-Aza treatment in OE33 and ESO26 cell lines. Cells were treated with 5 and 10 μ M 5-Aza for 72 hrs. *, Student t test $P < 0.05$. **(E-F)** A significant decrease in the relative methylation level of *PTPN13* following 5-Aza treatment in OE33 and ESO26 cell lines. *, Student t test $P < 0.05$.

Figure 4.

Inhibition of *PTPN13* promotes proliferation, colony formation, migration and key kinase phosphorylation in EAC cells. **(A)** The relative DNA methylation level (normalized to C-LESS control) (Y-axis) and mRNA expression (normalized to GUSB) of *PTPN13* (X-axis) in 7 EAC cell lines. The *PTPN13* mRNA level in SK-GT-4 cells is higher than other lines. **(B)** Daily proliferation assays in SKGT4-PTPN13 shRNA cells vs. non-specific scrambled control (NSC) or parental cells. **, Student t test $P < 0.01$. **(C)** Colony formation assays in NSC control cells (2 wells at left) versus PTPN13-shRNA (2 wells at right) at 50 cells or 100 cells seeded per well (original magnification 10 $\times$). **(D)** Compared to mock or NSC control cells, transfection with PTPN13-shRNA promoted migration. *, Student t test $P < .05$ in SK-GT-4 cells. **(E)** Western blotting revealed enhanced tyrosine phosphorylation in a panel of key kinases including ERBB2-Tyr1268, EGFR-Tyr1068 and SRC-Tyr416 at basal level, and further upon EGF stimulation, in PTPN13-shRNA SK-GT-4 cells compared to NSC control cells.

* Will change labeling color for subtypes

Figure 5. Treatment response in EAC cell lines representative of different methylation groups. (A) DNA methylation subtypes in EAC cell lines were determined using the HM450 array data. *In vitro* drug sensitivity curves for JH-EsoAd1 and OE33 (HM cell lines) are compared to SK-GT-4 (LM) and OE19 (MM) after 72-h exposure to a topoisomerase inhibitor, topotecan (B) and SN-38, the active metabolite of irinotecan (C). Cell survival rate (%) is expressed as the percentage of growth relative to DMSO-treated control cells ($\pm$ s.d. for three experiments).

Table 1.

Frequency of genomic alterations in selected genes across methylation subtypes in combined BETRNet and TCGA EAC cohorts. For oncogenes, genomic alterations including COSMIC hot spot mutations and gene amplifications were counted. For tumor suppressors, genomic alterations including likely loss of function mutations (nonsense, frameshift, splice site, COSMIC hot spot) and 2 copy deletions were counted. Color intensity indicates alteration frequency: white indicates 0%; light blue indicates 0 to 20%; blue indicates 20 to 50%, dark blue indicates 50 to 100%.

Gene	Pathway	Total % Altered			
		HM	IM	LM	MM
TP53	p53	78	84	74	75
ATM	p53	9	3	4	5
MDM2	p53	0	0	0	15
ARID1A	Chromatin regulation	22	16	11	5
ARID1B	Chromatin regulation	0	0	7	5
ERBB2	Receptor tyrosine kinase	41	32	4	15
EGFR	Receptor tyrosine kinase	6	13	0	0
MET	Receptor tyrosine kinase	3	0	4	0
KRAS	Receptor tyrosine kinase	12	16	15	0
NRAS	Receptor tyrosine kinase	3	3	0	0
MYB	Transcription factor	6	0	0	5
MYC	Transcription factor	13	19	8	5
CDKN2A	Cell Cycle	22	26	41	15
CDK6	Cell Cycle	3	16	7	5
RB1	Cell Cycle	6	3	4	5
SMAD2	TGF-beta	13	13	12	10
SMAD4	TGF-beta	25	32	23	25
APC	beta-Catenin	9	3	12	10