Case Report

Case Report of Molecular Signature in Poorly Differentiated Esophageal Adenocarcinoma

Andrew M. Kaz1,2,3,11, Christopher T. Rhodes4,11, ChenWei Lin2, Shu-Wei Angela Huang6, Chao-Jen Wong5, Richard S. Sandstrom6, Yufeng Wang4, Amitabh Chak7,8, Joseph E. Willis9, William M. Grady2,3, and Chin-Hsing Annie Lin4,10,*

Affiliations:
1 Gastroenterology Section, VA Puget Sound Medical Center, Seattle, WA
2 Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA
3 Division of Gastroenterology, University of Washington School of Medicine, Seattle, WA
4 Department of Biology, University of Texas at San Antonio, San Antonio, TX
5 Human Biology Division, Fred Hutchinson Cancer Research Center, Seattle, WA
6 Department of Genome Sciences, University of Washington School of Medicine, Seattle, WA
7 Division of Gastroenterology, Case Western Reserve University School of Medicine, Cleveland, OH
8 Division of Oncology, Case Comprehensive Cancer Center, Cleveland, OH
9 Division of Pathology, Case Western Reserve University School of Medicine, Cleveland, OH
10 Neuroscience Institute, University of Texas at San Antonio, San Antonio, TX
11 These authors contributed equally to this work.

*Corresponding author: Chin-Hsing Annie Lin, Department of Biology, University of Texas at San Antonio, San Antonio, TX 78249. Email: annie.lin@utsa.edu


Copyright: © 2017 Andrew M. Kaz, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Competing Interests: The authors declare no competing financial interests.

Received Feb 22, 2018; Revised Apr 17, 2018; Accepted Apr 23, 2018. Published May 21, 2018

ABSTRACT

Esophageal adenocarcinoma (EAC) arises from Barrett’s metaplasia (BE) in a process involving discrete genetic mutations and epigenetic alterations that remain poorly defined. An intriguing model underlying BE and EAC is that epithelial progenitor cells persist at the gastroesophageal junction, implicating dysregulation of differentiation in the pathogenesis of BE and EAC. Herein, we chose to elucidate the molecular signatures of primary poorly differentiated EAC cases. Using RNA-Seq to analyze aberrant gene expression, we found that expression of genes involved in morphogenesis and keratinocyte differentiation were decreased, whereas expression of genes involved in cell death and proliferation were increased in patients with poorly differentiated EAC. In addition, the levels of two histone methyltransferases were increased in these poorly differentiated EAC cases, including the “suppressor of variegation 39 homolog” (SUVM39H) and the “enhancer of zeste homolog 2” (EZH2). SUVM39H and EZH2 are responsible for trimethylation of histone 3 lysine 9 (H3K9me3) and lysine 27 (H3K27me3), respectively. Using chromatin immunoprecipitation and deep-sequencing (ChIP-Seq) to decipher abnormal epigenetic regulation, we identified putative targets aberrantly enriched with H3K9me3 or H3K27me3 in these poorly differentiated EAC cases. Moreover, comparative analysis of DNA methylation, ChIP-Seq, and RNA-Seq data suggests that dysregulation of histone 3 lysine 27 tri-methylation (H3K27me3) was the most common epigenetic alterations associated with gene expression change in a collection of poorly differentiated EAC. Importantly, our result implicates that the abundance and colocalization of H3K27me3 and primitive epithelial marker KRT-7 would present a unique signature of the poorly differentiated EAC cases. The significance of this
case report highlights that defects in epithelial differentiation paradigm underscore the epigenetic dysregulation via H3K27me3, also presents potential biomarkers for detection of the poorly differentiated EAC cases.

**Keywords**: epigenetic repression; DNA methylation; histone modification; esophageal adenocarcinoma (EAC); chromatin immunoprecipitation (ChIP); histone 3 lysine 9 trimethylation (H3K9me3); histone 3 lysine 27 trimethylation (H3K27me3)

**INTRODUCTION:**
Esophageal adenocarcinoma (EAC) is an epithelial-derived cancer that arises from Barrett’s esophagus (BE), a pre-malignant condition whereby the squamous-lined esophagus is replaced by intestinal-type epithelia (1). The incidence of EAC has been increasing rapidly in many Western nations, possibly related to the concomitant increase in obesity and associated risk factors in these countries (2, 3). The diagnosis of EAC is most often made late in the disease course, limiting the possibility of curative surgery (4). Primarily for this reason, the prognosis in individuals with EAC remains poor, with a five-year survival rate of 15% or less (5). A better understanding of the molecular alterations involved in the initiation and progression of BE and EAC might improve the rate of early diagnosis. Previous groups, including Krause et al (6) and The Cancer Genome Atlas (TCGA) (http://tcgadata.nci.nih.gov/) have published molecular data involving EAC cases, yet there have been no reports to date focusing on poorly differentiated EAC cases. Compelling evidence has shown that precancerous lesions, such as Barrett’s metaplasia, are derived from residual embryonic stem cells (ESCs) or epithelial progenitor cells lacking differentiation (7). In light of the potential relationship between stem or progenitor cells and BE/EAC, we focused our attention on poorly differentiated EAC cases in this study.

Cumulative evidence has demonstrated that changes in chromatin status involving either DNA or histone methylation can alter gene expression patterns critical for neoplastic initiation, tumor progression, and tumor heterogeneity (8-10). Aberrant DNA methylation has been noted to occur in virtually all cancers including EAC, whereas alterations in histone modification in EAC have not been well characterized (6, 8, 11-17). Modifications of histones affect the balance between gene activation and repression in part by affecting chromatin activity. In this study, we demonstrated increased expression of two histone methyltransferases in EAC cases, including the “enhancer of zeste homolog 2” (EZH2) and the “suppressor of variegation 39 homolog” (SUVR39H). These methyltransferases are responsible for catalyzing trimethylation of histone 3 lysine 27 (H3K27me3) and lysine 9 (H3K9me3), respectively (18, 19). During development and lineage commitment, H3K9me3 is recognized as a negative regulator of transcription and helps establish and maintain cell-type specific gene expression patterns (20, 21). EZH2 is an enzymatic component of the complex of Polycomb group (PcG) proteins, which are recruited by PcG response elements (PREs) (22-24). PREs function in both gene repression and activation as the same PREs that recruit PcG proteins, resulting in H3K27me3-regulated gene repression, also encode enhancers for activation of expression (25). Consequently, several developmental disorders and cancers are complicated by dual function of PREs/EZH2/H3K27me3 involving both activation and repression (26). Yet, neither H3K9me3 nor H3K27me3 has been thoroughly examined in EAC. To investigate this further, we used ChIP-Seq analysis to determine putative targets aberrantly enriched with H3K27me3 and/or H3K9me3 in poorly differentiated EAC cases. We also combined ChIP-Seq and RNA-Seq data to evaluate the effect of histone methylation on gene expression. Next, because considerable evidence suggests that promoter DNA hypermethylation and chromatin modification through H3K9me3 and H3K27me3 work in concert to repress gene expression (8, 18, 27-30), we employed a combination of ChIP-Seq and genome-wide methylation analysis to identify which genes were associated with aberrant DNA methylation, H3K9me3, H3K27me3, or a combination of all three. Given the growing evidence that the expression of loci affected by repressive chromatin marks are not necessarily repressed, we assessed the relationship of gene expression, aberrant H3K27me3 or H3K9me3 enrichment, and aberrant DNA methylation in the EAC. Our findings suggest a novel mechanism, in which altered expression of a set of differentiation-associated genes is correlated with aberrant H3K27me3 enrichment in a collection of poorly...
differentiated EAC. Strikingly, cytokeratin 7 (KRT-7) representing primitive epithelia, is dramatically altered in morphology and highly co-localized with H3K27me3 in poorly differentiated EAC. This finding implicates that KRT-7 and H3K27me3 can be utilized as an additional biomarker for the characterization of poorly differentiated EAC.

**MATERIALS and METHODS:**

**Tissue Samples**

Human esophageal specimens clinically classified as poorly differentiated EAC and normal SQ were obtained from the Cooperative Human Tissue Network (CHTN), an NCI-supported resource. The histology of SQ and EAC specimens was analyzed using H&E staining with Harris Hematoxylin, bluing in 0.2% ammonia hydroxide, and Eosin B. All methods were carried out in accordance with relevant guidelines and regulations approved by the IRB prior to initiating these studies. All experimental protocols were approved by EH&S and biosafety committees of the University of Texas at San Antonio, Fred Hutchinson Cancer Research Center, and Case Western Reserve University.

**Immunohistochemistry**

Frozen EAC and SQ specimens were sectioned at 12 μm, blocked with 10% goat serum for 30 minutes, and incubated 4 °C overnight with antibody specific for KRT-7 (Lifespan Biosciences, Cat#LS-B7164, mouse anti-human KRT-7, 1:200 dilution), H3K9me3 (Active Motif #39162, 1:500 dilution), H3K27me3 (Active Motif #39155, 1:500 dilution). On the subsequent day, sections were washed with 0.5% TritonX-100/1X PBS. Following wash, sections were incubated with AlexaFlour-488 conjugated mouse secondary antibody and AlexaFlour-594 conjugated rabbit secondary antibody (Life Technologies, Cat#A21200 and Life Technologies, Cat#A11091; 1:1000 dilution) for 1 hour. All sections were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Cat# H-1200). Images were acquired on a Nikon eclipse80i fluorescent microscope (10X). Higher magnification images were acquired on a Zeiss 710 confocal microscope with 20X and 40X objectives, and were processed using the associated analysis software, Zen. Scale bars and cell size measurement were processed by ImageJ (https://imagej.nih.gov/ij/). All steps of imaging quantification were performed in ImageJ following manufacturer's instruction (https://imagej.nih.gov/ij/docs/pdfs/examples.pdf). Mean fluorescence intensity (MFI) was determined by splitting RGB Color images into 8-bit greyscale images using the Split Channels tool.

**Flow Cytometry**

The EAC specimens and SQ tissues were dissociated into single cells by Accutase, and passed through a 40 μm cell strainer. Cells were subsequently fixed by 4% paraformaldehyde, permeabilized, incubated with FC block for 10 minutes, then 4 °C overnight incubation with antibody against KRT-7 and H3K27me3. Subsequently, cells were washed with Perm/Wash buffer (BD Biosciences, Cat#554723), then incubated with anti-mouse AlexaFluor-488 and anti-rabbit PE for one hour, wash three times with Perm/Wash buffer, and re-suspended in PBS prior to flow analysis. For analysis, unstained cells for gate placement and compensation beads for spectral compensation were included. Briefly, a drop of OneComp eBeads (eBioscience 01-1111-42) was incubated with 1 ul of fluorochrome-conjugated anti-mouse antibody for 15 – 30 min on ice and protected for light. Beads were washed with 1 ml of cell staining buffer and re-suspended in 300 ul of same buffer prior running the samples on an LSR-II cytometer. Flow cytometry data was acquired on a LSR-II (BD Biosciences) configured with an argon 488 laser with a 505 LP dichroic and 525/50 filter to detect Alexa fluor 488 and a green 510 laser with a 735 LP dichroic and a 575/26 filter to detect PE. Compensation and data analysis was performed using FlowJo software (Tree Star, Inc, Ashland, OR). Because the amount of specimens available for analysis is limited, the analysis was acquired under the same cell number for each case (10,000 cells) to overcome this sample size discrepancy.

**Western Blot Analyses**

Human esophageal cell lines and cross sections of snap-frozen esophageal specimens were homogenized using a glass Tenbroeck tissue grinder in 1 ml Buffer A (250mM sucrose; 10mM Tris-HCl, pH 8.0; 10mM MgCl₂; 1mM EGTA) containing 1X protease inhibitor to extract cytoplasmic proteins. The resulting pellets were further homogenized in 1 ml RIPA buffer.
containing 1X protease inhibitor to isolate nuclear fractions. The protein concentrations in cytoplasmic and nuclear fractions were quantified using the Bradford protein assay (Bio-Rad #500-0001). For western blots, equal amounts of protein from the nuclear fraction were denatured in final 1X SDS stop buffer and subjected to SDS-PAGE with antibody against H3K27me3 (Upstate #07-449, 1:1000), EZH2 (Active Motif #39639, 1:500), SUZ12 (Active Motif #07-379, 1:1000), SUV39H1 (Cell Signaling #2991), H3K9me3 (Active Motif #39162), and γ-tubulin (Sigma #T5326; 1:1000). Subsequently, HRP-conjugated secondary IgG (Cell Signaling #70745; 1:5000) and an enhanced chemiluminescence kit (ECL plus; GE#RPN2232) were used for detection.

Chromatin Immunoprecipitation (ChIP), Sequencing (ChIP-Seq), and qChIP-PCR
Esophageal adenocarcinoma specimens from two patients (both poorly differentiated) and normal human esophageal tissues were crosslinked in 1.1% formaldehyde before chromatin shearing using the Diagenode Bioruptor. The resulting sheared 200-500 bp chromatin fragments were incubated with H3K9me3 (Millipore #07-473) or H3K27me3 (Millipore #07-449) antibody-conjugated Protein A Dynabeads (Life Technology #10002D) overnight. For normalization, an aliquot of sheared chromatin fragments was incubated with antibody against unmodified H3 antibody-conjugated Protein A Dynabeads. Subsequently, enriched chromatin fragments were eluted, de-crosslinked, and purified for library preparation (Illumina Library Kit, Cat#15026486) for the Illumina HiSeq2500 sequencer. DNA obtained from each ChIP pull-down was sequenced to high depth of 150 million tags with 50 bases pair-end sequencing. We performed additional runs of ChIP-Seq using two different commercial antibodies against H3K9me3 (Active Motif #39162) and H3K27me3 (Active Motif #39155). For qChIP-PCR validation, 2 ng ChIP DNA from H3-ChIP (normalization) and H3K27me3-ChIP for Real-Time PCR was carried out using SYBR green PCR mix (Applied Biosystems ABI) on ABI7900HT detection system to obtain cycle threshold (Ct, log2 scale). After normalization by subtraction of Ct counts between H3-ChIP and H3K27me3-ChIP for each SQ and EAC case, the enrichment of H3K27me3 in each case was calculated by ΔCt (log2). We evaluated enrichment using 2 SQ and 3 EAC cases. Primers were designed using ABI Primer Express Software version 3.0 for sequences within peak start and end by MACS peak call.

Read Alignment and Peak Calling
The resulting reads were compiled, processed, and aligned to human genome hg38 for peak detection and motif enrichment by using MACS2. The UCSC human genome reference (hg38) was derived from Genome Reference Consortium Human Reference 38 (GRCh38) assembly. Unique paired-end reads were aligned to hg38 using Bowtie version 1.0.1 (31), allowing for 2 mismatches per read, and selecting best stratum per hit. Normalized peaks were generated using MACS2 version 2.1.0.20140616 with both unmodified H3 and SQ ChIP-DNA as input, using bam paired-end as format, and FDR of 0.01, which incorporates multiple test correction. Peaks were associated to the nearest transcription start site using BEDOPS closest-features with closest option and Gencode v21 human annotation. The complete sets of ChIP-Seq enrichment data are provided with accompanying GSE files.

Gene Ontology, Network, and Pathway Analysis
Gene ontology analysis was performed using the DAVID functional analysis tool (32). Molecular networks and pathways analyses were constructed using Kyoto Encyclopedia of Genes and Genomes (KEGG) and Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, Redwood City, CA, USA). These loci, known as focus genes, were grouped into ontology classes. Ingenuity Knowledge Base, a repository of biological and chemical interactions, was used as a reference set for network inference. Direct or indirect interactions between genes and gene products were inferred based on experimentally observed relationships supported by at least one reference from the literature.

Genome-wide DNA Methylation Arrays
HumanMethylation450 (HM450) BeadChips (Illumina #WG-314-1003) were used to compare patterns of DNA methylation in 18 squamous esophagus and 24 EAC samples. Due to limited sample size, only 2 EAC specimens were used for streamline analysis of DNA methylation, RNA-Seq, and qChIP-PCR. Data from the
BeadChips was exported into the GenomeStudio Methylation Module software (Illumina), which contains information on probe location, CpG island location, and methylation values. Methylation values from the HM450 arrays are reported as ‘beta values’ \( \beta = \frac{M+U}{M+2U} \), where 0.0 is equivalent to 0% methylation and 1.0 is equivalent to 100% methylation at a given CpG dinucleotide. We also converted beta values to ‘M values’ \( M = \log_2(\frac{M}{U}) \), which are logarithmic scores similar to those used in gene expression microarrays, for statistical calculations. Quality control and filtering of probes was performed as previously described (33). For differential methylation analysis (DMA), probes with \( \beta \geq 0.20 \) were considered differentially methylated between EAC and SQ.

**Gene Expression Analysis**

Total RNA was extracted using TRIzol reagent (Invitrogen/Life Technologies #15596-026) and sequencing libraries were generated with Illumina RNA-Seq library preparation kit with Ribozero. RNA deep-sequencing for paired-end 36 base pair reads was run at Illumina HiSeq2500; subsequently, 325 million pass filtered reads of each specimen were aligned to both hg19 and hg38. DESeq was used to normalize raw read counts. Cuffdiff was applied to analyze the differential gene expression between EAC and control (SQ) using a log2(fold change) of 1.0 as a cut-off for gene expression. The criteria for increased gene expression was a log2(fold change) > 1; and log2(fold change) < -1 for all EAC specimens. Heatmaps were generated using the function ‘heatmap.3’ within the R program, and results used unsupervised hierarchical clustering to identify similarities between genes and between esophageal samples. All heatmaps were generated by unsupervised hierarchical clustering with Euclidian distance metric to construct dendograms. For qRT-PCR validation, total RNA was extracted by TRIzol (Invitrogen) reagent and cDNA was synthesized by SuperScript II kit (Invitrogen) according to the manufacturer’s instructions. The transcript expression level was measured by Real-Time PCR with SYBR Green detection on ABI7900HT system (Applied Biosystems). S16 RNA was used as internal control for normalization (\( \Delta Ct \)). The differential expression was measured by subtraction of \( \Delta Ct \) between SQ and EAC specimens to obtain \( \Delta \Delta Ct \) (log2). Primers were designed using ABI Primer Express Software version 3.0

**Comparative Analysis**

Comparative analysis of DNA methylation, ChIP-Seq, and RNA-Seq data across data sets was performed with R (https://cran.r-project.org/). R codes are provided in supplementary information. Heatmaps were rendered using heatmap.3 function and unsupervised clustering with Euclidian distance metric (https://github.com/obigriffith/biostar-tutorials/blob/master/Heatmaps/heatmap.3.R).

**RESULTS:**

RNA-Seq demonstrates changes in gene expression in poorly differentiated esophageal adenocarcinoma cases. Previous work demonstrating that BE cells may be derived from embryonic stem cells led us to focus our study on poorly differentiated EAC cases without neoadjuvant chemotherapy (Figure 1) (7). We utilized RNA-Seq (> 300 million tag reads/each case) to compare gene expression patterns between poorly differentiated EAC and normal human SQ specimens. Overall, we found 861 and 1019 genes with increased vs. decreased expression in EAC compared to SQ, respectively (Supplemental Table 1). Gene Ontology analysis revealed that genes involved in epithelial differentiation, tissue development/morphology, and epithelial-mesenchymal transition (EMT) were decreased in EAC (Figure 2A). In contrast, numerous genes showed increased expression in the EAC cases, including genes involved in apoptosis/cell death, cellular growth/proliferation, inflammatory/immune responses, metabolic processes (i.e. ATP production; AMP-associated kinase; phospholipase), Rho-associated protein kinase, ubiquitin enzyme, and solute family of aspartate/glutamate carrier (Figure 2B). We utilized RT-qPCR to validate randomly selected genes, for which the expression levels were mostly consistent with RNA-Seq analysis (EAC1 and EAC2), whereas an additional EAC case (EAC3) containing a mix of undifferentiated cells, metaplasia,
and SQ showed somewhat inconsistent expression levels compared to the other cases (Figure 2C). This is not a surprising result considering the heterogeneity within the tumor specimen. When we specifically looked at genes involved in metabolism and chromatin activity, distinct sets of genes were either up- or down-regulated in EAC compared to SQ (Figure 3 and 4; Supplemental Table 1), suggesting dynamic alterations in chromatin status and metabolic processes. Importantly, the transcripts of EZH2 and SUV39H were increased in poorly differentiated EAC as compared to SQ (Figure 4A).

Chromatin repressive marks H3K9me3 and H3K27me3 are enriched in poorly differentiated esophageal adenocarcinoma. The histone methyltransferases EZH2 and SUZ12 (a component of the PRC2 complex within the PcG) have previously been associated with leukemia, colon cancer, and breast cancer (18, 27-29). Our RNA-Seq analysis demonstrated increased mRNA expression of both SUV39H1 and EZH2 in the EAC cases compared to SQ cases (Figure 4A). We confirmed elevated protein expression of EZH2 and SUV39H1, but not SUZ12, in nuclear cell fractions from all three EAC samples compared to SQ (Figure 4B). Next, we compared the levels of H3K9me3 and H3K27me3 in the chromatin fractions from a normal squamous (SQ) esophageal tissue sample to esophageal adenocarcinoma (EAC) specimens from three patients. Levels of H3K9me3 and H3K27me3 were consistently increased in all EAC samples compared to the SQ sample (Figure 4B). Our results indicate that the abundance of EZH2 and SUV39H1 correlates with the aberrant accumulation of H3K27me3 and H3K9me3, respectively, in EAC.

H3K27me3 enrichment is co-localized with primitive epithelial marker KRT-7. KRT-7 is present in specific glandular-type of primitive epithelia (7), and localizes to the junctional epithelium in the normal squamous (SQ) esophagus (Figure 5A). Consistent with RNA-Seq results demonstrating increased expression of KRT-7

Figure 1: Representative H&E-stained sections of a normal squamous esophagus and two poorly differentiated EAC tissues. (A-C) magnification 4X. (D-F) magnification 20X. The normal squamous esophagus is shown in Panels A and D. B and E: EAC case 1. C and F: EAC case 2.
in poorly differentiated EAC (Supplemental Table 1), KRT-7 becomes more abundant and diffuse in EAC compared to SQ as shown by immunostaining (Figure 5A, B). Using confocal imaging, the morphology of KRT-7 positive cells changed significantly between SQ and the poorly differentiated EAC specimens (Figure 5C-H). Intriguingly, KRT-7 positive cells were highly co-localized with H3K27me3 that appeared enlarged size in the poorly differentiated EAC (Figure 6A-I). Using flow cytometry for quantification, we confirmed increased KRT-7 and approximately 86% of co-localization of KRT-7 and H3K27me3 in poorly differentiated EAC cases (data not shown). Yet, the abundant co-localization between H3K9me3 and KRT-7 was not as profound as H3K27me3 and KRT-7 (Figure 7A-D). Our finding suggests co-immunostaining of KRT-7 and H3K27me3 as an additional characterization for poorly differentiated EAC cases.

Genome-wide ChIP-Seq for poorly differentiated esophageal adenocarcinoma cases demonstrates that putative target genes are enriched with H3K9me3 and H3K27me3. To assess the genomic loci subjected to aberrant H3K27me3 and/or H3K9me3 enrichment in poorly differentiated EAC cases, we performed genome-wide ChIP-Seq (chromatin immunoprecipitation and deep-sequencing) for three independent EAC specimens and SQ tissues with antibodies specific to H3K27me3 or H3K9me3. After normalization to SQ controls, independent EAC cases displayed common and distinct putative targets, which are aberrantly enriched with H3K9me3 or H3K27me3. As H3K27me3 is known to be a broadly distributed chromatin mark, we identified the enrichment of H3K27me3 in approximately 57% in the intergenic regions while 43% were enriched with H3K27me3 at promoter regions, 5’-UTR, 3’-UTR, or coding regions. We identified 3449 H3K27me3-enriched genes that were common targets between the two poorly differentiated EAC specimens (EAC1 and EAC2) compared to the normal SQ specimen (Supplemental Table 2). For the 3449 genes aberrantly enriched with H3K27me3, significant GO terms include cellular differentiation and morphology, signaling pathway, and tissue development (Figure 8A). We then acquired additional poorly differentiated EAC case (EAC3) for H3K27me3 ChIP-Seq to uncover a total of 1039 common targets of H3K27me3 across 3 independent specimens. Among 1039 genes commonly enriched with H3K27me3, Gene Ontology (GO) and Ingenuity Pathway Analysis (IPA) revealed their functions in cellular morphogenesis/organization, molecular transport, protein synthesis, and signaling (Figure 8B). We validated ChIP-Seq results by ChIP-qPCR for selected H3K27me3 putative targets to confirm that enrichment of H3K27me3 is dominant in EAC cases compared to SQ specimens (Figure 8C, D). Significant gene sets enriched with H3K27me3 in these independent EAC cases led us to determine the correlation between aberrant gene repression (RNA-Seq) and enrichment of H3K27me3 (ChIP-Seq) in these poorly differentiated EAC cases compared to SQ. We identified differential gene expression correlated with H3K27me3 enrichment (Figure 9) that highlight the contrasting roles of PREs and support the notion that EZH2/H3K27me3 does not absolutely suppress gene expression.

We also sought to determine whether H3K9me3 was aberrantly enriched in EAC compared to SQ specimens. We found only 96 common genes enriched by H3K9me3 across three EAC specimens, suggesting distinct epigenetic landscape of H3K9me3 in independent EAC cases. Although there were no significantly overrepresented GO terms for the 96 H3K9me3-enriched genes, IPA predicted that putative targets of H3K9me3 have known functions in cell
For each different EAC case, GO and IPA also identified that distinct gene sets enriched with H3K9me3 were predominantly involved in either gap junction/GPCR signaling, post-

**Figure 3**: RNA-Seq analysis for two poorly differentiated EAC cases compared to normal squamous esophagus for genes involved in metabolism. A: Heatmap depicts genes involved in various metabolic processes are either up- or down-regulated in EAC compared to SQ cases. B: Scatter plot illustrates genes involved in metabolism with more than 8-fold increase or decrease at expression level in two EAC cases compared to SQ. Size of points indicates the sum of the absolute values of EAC gene expression values. x and y axes represent expression values in units of log2(fold change) for EAC1 and EAC2, respectively.
transcriptional modification, or regulation of gut-brain axis and gastrointestinal hormones, such as cholecystokinin and peptide YY (Figure 10A, B).

When we restricted our analysis to only the two poorly differentiated EAC specimens (EAC1 and EAC2), there were 128 common targets of H3K9me3-enriched genes compared to the normal SQ specimen (Supplemental Table 2). Although there were no significantly overrepresented GO terms for the 128 H3K9me3-enriched genes, IPA predicted alterations in cell survival, metabolism, and morphology pathways associated with these genes. We also found 26 genes

Figure 4: RNA-Seq analysis for two poorly differentiated EAC cases compared to normal squamous esophagus for genes involved in chromatin activity. A: Genes involved in chromatin activity or epigenetic regulation are either up- or down-regulated in EAC. Gene symbols highlight EZH2 and SUV39H1 expression levels in EAC samples. B: Western blots of nuclear fractions obtained from tissue specimens using antibodies against H3K27me3 and H3K9me3 demonstrated elevated levels of SUV39H1 and EZH2 (but not SUZ12) proteins in three EAC samples compared to a normal esophageal sample. Western blots of nuclear fractions obtained from tissue specimens using antibodies against H3K27me3 and H3K9me3 demonstrated elevated levels of the H3K27me3 and H3K9me3 marks in three EAC samples compared to a normal esophageal sample. The loading controls included both γ-tubulin and unmodified H3. The numbers obtained from normalization were relative to γ-tubulin levels, and unmodified H3 showed no significant change of total histone 3.
marked with both H3K27me3 and H3K9me3 repressive marks in the EAC1 and EAC2 cases. IPA analysis of these 26 genes and additional genes enriched with either H3K27me3 or H3K9me3 revealed molecular networks involved in cellular signaling and differentiation (Supplemental Table 2).

**Figure 5:** Primitive epithelial marker KRT-7 is abundant in poorly differentiated EAC cases. KRT-7 distribution and morphology were validated by IHC. KRT-7-positive embryonic epithelium is localized to the junctional epithelium in the normal squamous esophagus (A and C-E), but becomes more abundant and diffuse in an EAC specimen (B and F-H). Fluorescent labeling was obtained using secondary antibody AlexaFluor 488 and DAPI counter stain. A and B: 4X magnification. C - H: confocal images were acquired by 20X magnification. Scale bars = 20 µm.
A subset of genes harboring repressive histone marks are also subject to aberrant promoter DNA methylation. Epigenetic alterations, including DNA methylation and histone modifications, are highly dependent upon one another (29). Genes that are epigenetically silenced typically demonstrate: 1) deacetylated histones, 2) specific methylated histones, and 3) hypermethylated DNA promoters (29, 34, 35). In order to further explore the relationship between repressive histone methylation and DNA methylation in EAC, we used genome-wide DNA methylation arrays (Illumina HumanMethylation450 BeadChips) to

Figure 6: Confocal imaging illustrated abundant colocalization of KRT-7 and H3K27me3 in the poorly differentiated EAC. (A-C) Immunostaining for KRT-7 and H3K27me3 in sections obtained from the SQ control, 40X. (D-I) Immunostaining for KRT-7 and H3K27me3 in sections obtained from two poorly differentiated EAC, 40X. Scale bars = 20 μm.
determine the promoter methylation status of genes associated with H3K27me3 and/or H3K9me3 as determined by our ChIP-Seq studies. We performed differential DNA methylation analysis using mean methylation values of 24 EAC and 11 normal squamous esophageal specimens. Although DNMT expression levels were not significantly altered based on RNA-Seq analysis, the heatmaps clearly demonstrated that some loci associated with increased levels of DNA methylation while others with lower levels of DNA

Figure 7: Confocal imaging showed colocalization of KRT-7 and H3K9me3 in the poorly differentiated EAC. A and B: 20X magnification. C and D: 40X magnification. (A and C) Immunostaining for KRT-7 and H3K9me3 in sections obtained from the SQ control, 40X. (B and D) Immunostaining for KRT-7 and H3K9me3 in sections obtained from the poorly differentiated EAC, 40X. Scale bars = 20 μm.
Case Report
methyltransferases were associated with increased methylation, respectively. Elevated levels of these methyltransferases were associated with increased levels of their resulting chromatin-repressive marks H3K9me3 and H3K27me3 in poorly differentiated EAC compared to normal SQ tissues. Both EZH2/H3K27me3 and SUV39H/H3K9me3 are canonically associated with gene repression. Prior studies demonstrated that H3K27me3 is important for the maintenance of ESCs pluripotency via repression of genes involved in differentiation (20-24, 36-41). Although BMI and Suz12 within PcG were not derived from epithelial progenitors lacking lineage commitment (7). This evidence led us to examine poorly differentiated EAC cases with a focus on differential gene expression and epigenetic alterations. Using RNA-Seq, we found genes involved in cellular morphogenesis, keratinocyte differentiation, and epithelial junction signaling were down-regulated in patients with poorly differentiated EAC. Importantly, genes with functions in apoptosis, cell death, proliferation, and response to hypoxia were increased in these poorly differentiated EAC cases. A relatively small set of genes involved in the inflammatory response were found to be marginally increased in EAC.

Our gene expression results showed increased levels of the methyltransferases SUV39H1 and EZH2, which are responsible for H3K9me3 and H3K27me3 methylation, respectively. Elevated levels of these methyltransferases were associated with increased levels of their resulting chromatin-repressive marks H3K9me3 and H3K27me3 in poorly differentiated EAC compared to normal SQ tissues. Both EZH2/H3K27me3 and SUV39H/H3K9me3 are canonically associated with gene repression. Prior studies demonstrated that H3K27me3 is important for the maintenance of ESCs pluripotency via repression of genes involved in differentiation (20-24, 36-41). Although BMI and Suz12 within PcG were not derived from epithelial progenitors lacking lineage commitment (7). This evidence led us to examine poorly differentiated EAC cases with a focus on differential gene expression and epigenetic alterations. Using RNA-Seq, we found genes involved in cellular morphogenesis, keratinocyte differentiation, and epithelial junction signaling were down-regulated in patients with poorly differentiated EAC. Importantly, genes with functions in apoptosis, cell death, proliferation, and response to hypoxia were increased in these poorly differentiated EAC cases. A relatively small set of genes involved in the inflammatory response were found to be marginally increased in EAC.

Our gene expression results showed increased levels of the methyltransferases SUV39H1 and EZH2, which are responsible for H3K9me3 and H3K27me3 methylation, respectively. Elevated levels of these methyltransferases were associated with increased levels of their resulting chromatin-repressive marks H3K9me3 and H3K27me3 in poorly differentiated EAC compared to normal SQ tissues. Both EZH2/H3K27me3 and SUV39H/H3K9me3 are canonically associated with gene repression. Prior studies demonstrated that H3K27me3 is important for the maintenance of ESCs pluripotency via repression of genes involved in differentiation (20-24, 36-41). Although BMI and Suz12 within PcG were not derived from epithelial progenitors lacking lineage commitment (7). This evidence led us to examine poorly differentiated EAC cases with a focus on differential gene expression and epigenetic alterations. Using RNA-Seq, we found genes involved in cellular morphogenesis, keratinocyte differentiation, and epithelial junction signaling were down-regulated in patients with poorly differentiated EAC. Importantly, genes with functions in apoptosis, cell death, proliferation, and response to hypoxia were increased in these poorly differentiated EAC cases. A relatively small set of genes involved in the inflammatory response were found to be marginally increased in EAC.

Our gene expression results showed increased levels of the methyltransferases SUV39H1 and EZH2, which are responsible for H3K9me3 and H3K27me3 methylation, respectively. Elevated levels of these methyltransferases were associated with increased levels of their resulting chromatin-repressive marks H3K9me3 and H3K27me3 in poorly differentiated EAC compared to normal SQ tissues. Both EZH2/H3K27me3 and SUV39H/H3K9me3 are canonically associated with gene repression. Prior studies demonstrated that H3K27me3 is important for the maintenance of ESCs pluripotency via repression of genes involved in differentiation (20-24, 36-41). Although BMI and Suz12 within PcG were not
significantly changed in the poorly differentiated EAC cases we analyzed, previous study demonstrated aberrant expression of EZH2 and BMI in squamous cell carcinoma of esophagus (ESSC) (42). In addition, aberrant EZH2 expression is associated with P53 alteration and the progression of ESSC. Despite differential molecular signatures between the poorly differentiated EAC and ESSC, EZH2/H3K27me3 is
Figure 10: Representative functional category predicted by IPA for genes aberrantly enriched with H3K9me3 in poorly differentiated EAC cases. A: Representative genes involved in signaling pathway of gastrointestinal diseases. B: Genes have function in morphogenesis. Key at right panel: The shaded focus genes (red highlight) were enriched with H3K9me3 identified by ChIP-seq analysis. The genes without red highlight were not enriched with H3K9me3, but were involved in the network. Node shape reflects the role of each gene in the network while the direction and arrowhead shapes of each edge represent different types of interactions.
Figure 11: Overlap comparison between DNA methylation, H3K9me3, and H3K27me3. All heatmaps utilized unsupervised hierarchical clustering with Euclidian distance metric (y-axis: Gene ID; x-axis: probe ID of DNA methylation). A: Heatmap depicting loci enriched by H3K9me3 and aberrant promoter DNA methylation in EAC. B: Heatmap depicting loci enriched by H3K27me3 and aberrant promoter DNA methylation in EAC. C: Heatmap depicting loci enriched by all three repressive epigenetic marks in EAC, including H3K27me3, H3K9me3, and aberrant promoter DNA methylation. The list to the right shows the genes enriched by all three repressive epigenetic marks in EAC.
commonly involved in esophageal malignancy of ESSC and the poorly differentiated EAC and ESSC.

Chromatin regions that are enriched with H3K27me3 and/or H3K9me3 can also be affected by other histone modifications associated with gene activation (i.e. H3K4me3, H3K36me3). This raises the possibility that genes exist in a ‘bivalent chromatin state’, marked by both active and repressive chromatin marks (43). Importantly, recent publications have reported that the same DNA elements can act as either transcriptional enhancers for gene activation or as PREs for recruitment of PcG, resulting enrichment with EZH2/H3K27me3 (25, 26). Therefore, elevated level of H3K27me3 is associated with suppression of certain genes, but not with universal suppression of gene expression. The mechanism which leads to increased expression of these methyltransferases in EAC is currently unclear. It is possible that dietary or environmental factors may alter levels of the metabolites which influence these methyltransferases. For instance, S-adenosyl-L-methionine (SAM) is the common methyl donor for these methyltransferases even though the catalytic domains of these enzymes are distinct (19). Further studies analyzing the proteome and metabolome could help delineate the relation of metabolites and methyltransferases in the context of EAC.

Previous studies have noted that cancer-related genes exhibiting DNA promoter hypermethylation are enriched for the histone methyltransferases SUV39H/H3K9me3 and EZH2/H3K27me3 (29). When we compared ChIP-Seq and DNA methylation data across EAC and SQ specimens, we found a relatively small set of genes associated with both aberrant H3K9me3/H3K27me3 and DNA methylation, suggesting that triple repressive marks are uncommon in these poorly differentiated EAC cases. Comparative analyses of H3K9/H3K27 trimethylation, gene expression, and DNA methylation showed that the expression of a subset of genes was decreased in the poorly differentiated EAC cases while a set of genes was increased. This differential gene expression pattern was primarily associated with aberrant modification of H3K27me3, as opposed to H3K9me3 or DNA methylation. Importantly, dysregulation of EZH2/H3K27me3 was found in subtypes of glioblastoma containing stem cell signature (44), suggesting that EZH2/H3K27me3 is commonly upregulated in stem cell-associated and poorly differentiated cancer cases.

It is worth noting that the morphology of KRT-7 is strikingly changed and its distribution is highly colocalized with H3K27me3 in poorly differentiated EAC. This finding implicates that KRT-7 and H3K27me3 can be utilized as additional signatures for the characterization of poorly differentiated EAC tissues despite the tumor heterogeneity. In conclusion, our results suggest that epigenetic dysregulation, particularly H3K27me3, is involved in poorly differentiated EAC cases. These findings could be further explored in future studies defining epigenetic abnormalities in additional epithelial progenitor-associated EAC cases.

All ChIP-Seq and RNA-Seq data generated during the course of this study have been deposited to NIH/GEO under the accession ID: GSE94544 and GSE94545.

Author contributions
Andrew M. Kaz: DNA methylation studies, manuscript preparation; Christopher T. Rhodes: Peak calls for ChIP-Seq by MACS and SICER, integrated analysis of genome-wide data, flow cytometry, GO and IPA; Shu-Wei Angela Huang: Immunohistochemistry/confocal imaging and western blot; Richard Sandstrom: ChIP-Seq analysis and ENCODE consultant; ChenWei Lin: RNA-Seq data analysis, heatmaps and unsupervised clustering, GO and KEGG network/pathway analysis, comparative analysis of genome-wide data; Chao-Jen Wong: DNA methylation array analysis; Yufeng Wang: statistical analysis; Amitabh Chak: methylation studies; Joseph E. Willis: methylation studies, pathological assessment of tissues for microarrays; William Grady: methylation studies, manuscript preparation; Chin-Hsing Annie Lin: experimental design, materials and reagent support, ChIP and DNA purification, RNA extraction, project supervision, data collection, manuscript writing/preparation.

Acknowledgments
We thank Dr. Jianhua Ruan, Daniel Bates, and Bethany Zablotsky for technical assistance. Sequencing was performed at the High Throughput Genomics Center in the Department of Genome Sciences, University of Washington, Seattle, WA. CTR is supported in part by the SCORE grant SC3GM112543 to CAL.
**Funding**
This project was primarily supported by the SCORE grant SC3GM112543 from the National Institutes of Health (NIH/NIGMS) and TRAC award (Tenure-track Research Award Competition) to CAL; and was partially supported by US4CA163060/Barrett’s Esophagus Translational Research Network (BETRNet; NIH) to WG/AC.

**Abbreviations**
BE, Barrett’s esophagus; ChIP, chromatin immunoprecipitation; EZH2, enhancer of zeste homolog 2; EAC, esophageal adenocarcinoma; H3K9me3, histone 3 lysine 9 trimethylation; H3K27me3, histone 3 lysine 27 trimethylation; SUV39H, suppressor of variegation 39 homolog.

**Supplemental Table 1:**
List of genes up- or down-regulated in the poorly differentiated EAC cases by RNA-Seq analysis.

**Supplemental Table 2:**
List of common targets between the two EAC specimens obtained from H3K9me3 ChIP-Seq and H3K27me3 ChIP-Seq analysis. Spreadsheets display separately for putative targets of (i) H3K9me3; (ii) H3K27me3; (iii) common putative targets of H3K9me3 and H3K27me3 in both EAC1 and EAC2.

**Supplemental Table 3:**
List of common targets between ChIP-Seq and DNA methylation analysis. Spreadsheets display separately for genes with (i) H3K9me3 and DNA methylation; (ii) H3K27me3 and DNA methylation; (iii) common genes with DNA methylation, H3K9me3, and H3K27me3.

**REFERENCES**


