Global Phospho-Proteomic Profiling of Pathway-Level Molecular Alterations in Barrett's Esophagus

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Introduction

Barrett's esophagus (BE) is a major risk factor predisposing individuals to esophageal adenocarcinoma (EAC), a highly morbid disease with poor survival despite invasive intervention[1-3]. BE refers to metaplastic change in the stratified squamous epithelium lining the distal esophagus, primarily driven by chronic gastric and bile reflux, resulting in replacement with an intestinal-like columnar epithelium.

Mapping the maladaptive cellular responses perturbed in early stage, non-dysplastic BE relative to normal squamous tissue is crucial to intercepting the pathobiological processes preceding and driving dysplasia. Liquid chromatography-tandem mass spectrometry (LC/MS) is a powerful analytical platform for exploring changes in the signaling pathways of transformed cells, and identifying actionable targets for disease management. In conjunction with modern systems biology approaches, one can quantify changes in relative protein expression and post-translational modification, making it well suited for comparative analyses of clinical specimens aimed in determining underlying biochemical alterations associated with metaplastic progression and pathogenesis[9]. In this pilot study, we leverage deep quantitative proteomic and phosphoproteomic profiling of clinical samples, in conjunction with rigorous statistical analysis, to define a robust set of signature proteins and pathways consistently differentially-expressed between non-dysplastic BE and adjacent squamous tissue across multiple affected patient cohorts. From our initial cohort, we identified markers and pathways distinguishing BE from normal tissue at a molecular level, whose association suggests potential roles in pre-malignant disease progression. These findings were supported by unbiased machine learning methods and comparative pathway and survival analysis of cross-mapped transcriptome data from independent patient cohorts. This systematic survey comparing BE versus normal epithelium reveals potential causal mechanisms governing transformation and pathogenesis that precede dysplastic transformation and EAC.

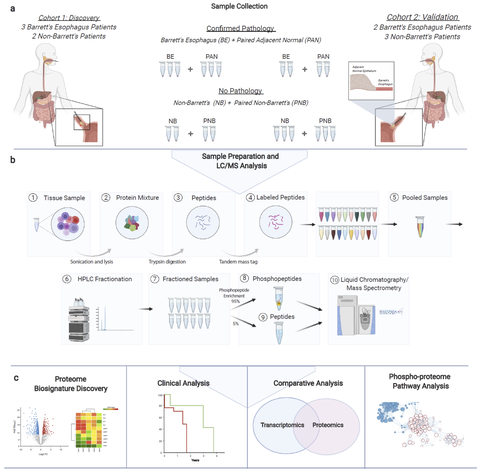


Figure 1. Depictions of the process of: (a) patient biopsy sample collection and categorization (BE=Barrett's Esophagus, PAN=paired adjacent normal, PNB=paired non-Barrett's, NB=non-Barrett's) from two cohorts; (b) sample preparation pipeline for LC/MS analysis; and (c) data analysis

Results

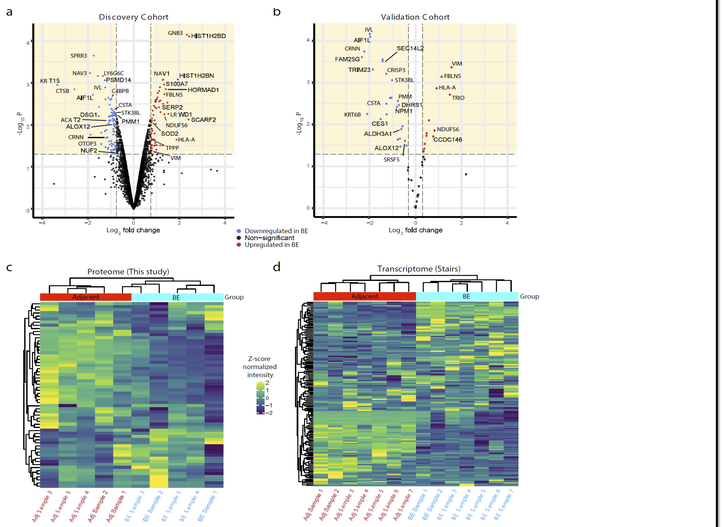


Figure 2. Volcano plots of the Barrett's esophagus signature (a) projected onto the Validation Cohort (b). Heatmap displays showing hierarchically clustered signature protein levels among BE and Adjacent samples across (c) all samples from both the discovery and validation cohorts, and (d) cognate mRNAs reported in independent transcriptomes of BE and Adjacent tissues (Stairs et al).

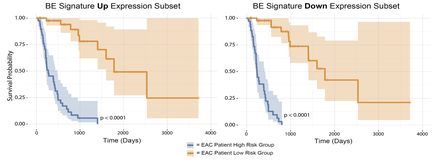


Figure 3. Kaplan-Meier plots depicting survivability of esophageal cancer patients classified according to the BE protein signature reported in this study for (left) upregulated proteins, and (right) downregulated proteins, based on classifying the corresponding transcriptomic profiles into high-risk (blue) and low-risk (orange) groups.

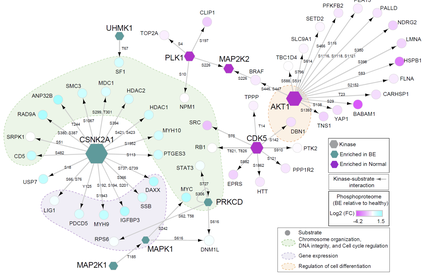


Figure 4. Protein kinase enrichment map showing differential kinase-substrate interactions inferred from motif (substrate) enrichment analysis of the tissue phosphoproteomic profiles.

Methods

Mass Spectrometry— Isotope-labeled peptides were analyzed on a ThermoScientific Q-Exactive HFX mass spectrometer connected to an EASY-nLC-1200 ultra-high pressure nanoflow chromatography system. After loading onto a reverse-phase trap, peptides were gradient separated on an EASY-Spray C18 nanocolumn over 120 min, and electro-sprayed at ~250 nL/min into the HFX instrument operated in positive ion mode. Data-dependent spectra were acquired automatically via high resolution (60,000) precursor ion scan (350-1500 m/z range) to select the 12-most intense peptides for MS/MS fragmentation by high energy dissociation (normalized collision energy of 33 @ 45,000 resolution).

Statistical Analysis and Pathway Enrichment — Bioinformatic analysis was performed using R (language and environment for Statistical Computing; http://www.R-project.org). Peptide feature intensities were log transformed and quantile normalized. LIMMA[11] R package was used for differential analysis (moderated Student t-tests), and to generate ranked lists for subsequent enrichment analysis using the Benjamini-Hochberg FDR correction. Statistical enrichment analysis was performed using fgsea R package[12] with 10,000 permutations to define empirical FDR cutoffs. Published transcriptomic datasets (raw expression values, corresponding gene names) were obtained from GEO using the GEOquery R package[13]. Volcano plots were created using the EnhancedVolcano R package[14]. Survival analysis was completed using gene expression and clinical data obtained from the cBioPortal Cancer Genome Atlas (TCGA) PanCancer Atlas.

Conclusions

In this pilot study, we determined robust proteomic and phospho-signaling patterns distinguishing BE from normal epithelium, informing upon mechanistic understanding between these distinct epithelial states. Using a multi-step discovery/validation strategy, we identified BE protein signature distinguishing clinical specimens with high correspondence to disease status. Subsequent survival analysis established the prognostic relevance of this signature to EAC by demonstrating marker association with clinical outcomes, highlighting pathophysiological importance throughout the disease course. Despite patient heterogeneity, these markers hold promise for diagnosis and monitoring disease progression.

The establishment of a BE signature based on our pilot study, supplemented by mechanistic insights from our signaling pathway-level analyses, highlights notable molecular differences between non-dysplastic BE and normal tissue that is largely recapitulated across a larger combined population of 74 BE patients. The detailed phospho-signaling mechanisms and kinase-substrate associations reported here provide unique insights, such as components of activated DNA repair mechanisms, that precede tumorigenesis and genomic instability found in overt cancer. While a large-scale thorough investigation is required to determine the functional impact of these alterations, our analyses reveal a discrete molecular state that precedes dysplasia and overt cancer.

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