Dysregulation of Alternative Splicing was Associated with the Pathogenesis of Ulcerative Colitis

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Research Article

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Abstract

Background: Although numerous risk loci of Ulcerative Colitis (UC) in the human genome have been identified, the pathogenesis of UC remains not fully understood. Recently, multiple transcriptomic analyses have shown that aberrant gene expression in UC patients' colon tissues were associated with the disease progressing. A pioneer study also demonstrated that altered post-transcriptional regulation may be involved in the progression of UC.

Methods: Herein, we provided a comprehensive analysis of alternative splicing (AS) signature on UC patients. We analyzed three datasets, which include 74 tissue samples from UC patients in total, and identified over 2,000 significant AS events in these datasets.

Results: Skipped Exon (SE) and Alternative first exon (AFE) were found to be the top two significantly altered AS events. Immune response related pathways were significantly enriched. Genes that showed significantly AS events were more likely to be dysregulated at the expression level.

Conclusion: Overall, these results suggested that alteration of AS may play crucial roles in the pathogenesis of UC.

Summary

We presented a landscape of AS events in UC based on a combination analysis of two cohorts. Our results indicated that the dysregulation of AS may play roles in determining the pathogenesis of UC.

Introduction

Ulcerative Colitis (UC), a subtype of inflammatory bowel disease (IBD), has become a global emergence disease [21]. The pathogenesis of UC is complicated, including interactions among microbial shifts, the host's genetic background, and the environmental cues, resulting in activating mucosal immune system in a chronic way [15, 22]. Although multiple genome-wide association studies have identified hundreds of associated genetic risk loci in patients with UC [8], the pathogenesis of UC remains not fully understood.

Recently, several transcriptomics-focused studies have been carried out to determine the alteration of gene expression in patients with different subtypes of IBD. Planell et al. performed transcriptomic analysis of colonic biopsies from patients with UC and the healthy control using microarrays. They found that several genes and pathways were permanently dysregulated despite of the histological recovery of the patients [25]. Another microarray-based study demonstrated that expression of Interferon-γ and interleukin-17 were comparably elevated in both inflamed and unaffected colon mucosa from patients, indicating that inflammation response was not limited to the endoscopic lesions in patients with IBD [33]. Smith et al. identified dysregulation of BRINP3 may play roles in the pathogenesis of UC [28]. A more recent study that focused on analyzing colonic mucosal transcriptome of patients with long-duration UC also identified that genes and pathways were dysregulated in long-duration UC patients compared to

short-duration UC patients [20]. Overall, all these studies suggested that transcriptional regulation may play crucial roles in determining the etiology of UC.

Alternative splicing (AS) of the precursor mRNA works as one of the essential mechanisms for increasing the protein diversity as well as regulating the intricate protein-RNA interaction network [32, 6]. Almost 95% of the total human genes with multi-exons, were discovered to involve AS events [4]. More and more evidence demonstrated that AS plays crucial roles in many biological events, such as oncogenic processes including cell proliferation, cell apoptosis, hypoxia, immune escape as well as metastasis [7] [24]. AS were also found to play essential roles in basic developmental process and tissue identity [2]. Recently, a pioneer study, which focused on profiling the AS events in IBDs, showed 47 splicing factors and 33 intron retention events were dysregulated in the mucosal tissue of patients with IBD patients [13]. Another array-based pioneer study also found more than 392 genes were differentially expressed in long-duration UC patients, which were associated with a dysregulated AS network [20]. However, the former study lacks a comprehensive analysis due to lacking next generation sequencing approach, the latter study drew special attention to the comparison between long-duration UC and short-duration UC.

In order to provide a landscape of how AS is involved in the pathogenesis of UC, we first obtained a public mRNA-seq data from NCBI GEO dataset (GSE137344), which included 44 mRNA expression data from UC patients and 37 mRNA expression data from the controls. Using Miso and related AS event analysis software [16], we identified 8 types and 2,385 significant AS events in UC patients compared to the control. 110 biological pathways were significantly enriched for these AS events in the UC patients, of which some were highly involved in chronic inflammation/immune response pathways. We next performed another mRNA-seq experiment on colon tissues from our own cohort of UC patients and the control (4 UC vs 4 control). 57% genes that were identified to involve significant AS events in our experiment also had significant AS events in the GSE137344 study. Immune response related pathways were found to be the most significantly enriched terms in the validation experiment dataset as well. To further demonstrate the potential role of AS in the UC progression, we also performed a validation experiment on comparing the AS of the two clusters of UC patients with different disease progressions. Overall, we provided a comprehensive analysis of AS events on UC patients combining multiple datasets. We believed our results could shed a light on understanding the mechanism of post-transcriptional regulation in the pathogenesis of UC.

Results

Types of Alternative Splicing in the GSE137344 public dataset

Eight types of alternative splicing (AS) events were discovered, which include alternative 3’splice site (A3SS), alternative 5’splice site (A5SS), alternative first exon (AFE), alternative last exon (ALE), mutually exclusive exons (MXE), retrained introns (RI), skipped exon (SE), and tandem 3’ UTR (**Figure 1A**). Compared to the normal samples, we identified 2,358 significant AS events in eight different AS types in total (**Table S1**). Among these types, SE type has the most significant events, but the ratio over the total

number of SE events is rather low (**Figure 1B**). In fact, 8.1% of tandem 3'UTR events and 7.2% of ALE events are recognized as significant AS events, over numbering others. The distribution and intersection of gene symbols between 8 events types for related AS events are shown in **Figure.1C**. Since most of ALE and AFE AS events were identified across multiple genes, ALE events and AFE events were mapped to over 5,000 and 3,000 genes respectively, and hence having the most intersections.

Pathway Analysis of the GSE137344 public dataset

The AS events related to UC contribute to the enrichment of 110 different biological pathways (**Table S2**). Most of pathways was associated with only one splicing type. Only antigen processing and presentation and ribosome are affected by three splicing type (**Figure 2A**). "Systemic lupus erythematosus (SLE)" was the most significantly enriched term with a p-value less than $5 * 10^{-20}$. Interestingly, a recent study showed that patients with SLE had a greater prevalence of IBD than matched controls [27]. The second most enriched term was "Alcoholism", with a p-value less than $3 * 10^{-6}$. We then combined related AS events pathway analysis results with RNA-seq expression results. And we found two inflammation related genes containing UC related AS events in these pathways. HDAC6 and LIPA were observed with an ALE event and a tandem 3'UTR event, respectively. According to the previous study, HDAC6 was found to be involved in alcoholism pathway which was associated with chronic inflammation [17]. LIPA was previously associated with steroid biosynthesis which was also considered to be involved in modulating inflammation [14]. Two genes in different sample groups showed significant differential expression on RNA level (**Figure 2B, 2C**).

Splicing types in the 8-sample mRNA-seq experiment.

To validate AS events that indeed exists comprehensively in the UC, we performed another RNA-seq experiment on four UC patients and four normal samples from Shengjing Hospital of China Medical University. Same strategies were applied in the data analysis of validation RNA-seq experiments. Eventually, A set of 2,352 significant AS events were discovered in our dataset (**Table S3**). Interestingly, SE and AFE were still found to be the top two AS types, while MXE was the least one (**Figure 3A**). But the ratio over the total number of all events type were relatively different comparing to the result from public dataset (**Figure 3B**). AFE and ALE were identified across multiple genes, which exhibited the most intersections (**Figure 3C**). Interestingly, we also noticed that 57% genes that were identified to exhibit significant AS events in our dataset also showed significant AS events in the public dataset (**Figure S1**), though they are from different tissues. This result indicated that the AS regulation could be more related to the disease progression than the tissues.

Combined analysis of expression and splicing in the 8-sample experiment.

mRNA-seq experiments on those 8 samples identified over 1,500 differentially expressed genes (p-adj < 0.05, log2Foldchange > 0.5) in UC patients compared to the control (**Figure S2**). Principal component analysis (PCA) based on the mRNA expression was performed using the top 2000 gene expression data (**Figure 4A**). We also performed a PCA analysis on the AS events in our cohort in order to characterize the

AS events between the disease and normal samples. We summarized 1,731 related AS events which occurred across all 8 samples with Percent Spliced In (PSI) values generated by MISO software. PC1-PC3 accounted for 60% of the variance (**Figure 4B**). The biological difference between UC disease patients and normal samples is captured by the first, the second and third principal component (PC). These results indicated that AS patterns and the expression profiles could both demonstrate the biological differences between the samples from the patients and the normal.

We next examined whether the expression level of the genes that showed significant AS events also had significant changes of expression. Venn diagram showed that 140 of 667 downregulated genes and 233 of 846 upregulated genes also had AS events in UC patients, indicating some of the gene expression changes may be due to the dysregulation of AS (**Figure 4C**).

Biological Process Pathway analysis

We performed biological process pathway analysis on our 8-sample experiment based on the gene list of 2,352 significant AS events. Since only less than 200 related genes had MXE, RI and tandem 3' UTR event types, we failed to identify any significantly enriched biological process among these genes. Among the other five AS type events, multiple pathways were highly enriched in terms of biological process (**Figure 5**). Among those pathways, "immune response" was enriched most significantly. Besides, LIPA, which was identified to be differentially expressed as well as had significant AS events in the public dataset, was also identified to involve a tandem 3'UTR event and showed the significant differential expression on RNA level in the validation dataset (**Table S4**) (**Figure S3**). These results suggested that the dysregulated AS, which was similar to the expression level itself, was strongly associated with altered immune response in UC patients.

We next performed gene ontologies (GO) analysis on the 8-sample dataset based on the enrichment of 201 unique AS events that were only discovered in the normal group or in the UC group. The top 10 enriched GO biological process terms (Figure 6A, Table S5) reflect the immune system response and cell chemotaxis in the UC patients. GNLY is one of the genes that has unique AS events in term GO:0061844. The product of this gene is a member of the saposin-like protein (SAPLIP) family. It is an antimicrobial protein that present in the granules of human cytotoxic T lymphocytes, as well as in the natural killer (NK) cells, that can also activate antigen-presenting cells through TLR4 [29]. In Figure 6B, we presented different AS events of this gene between the normal and the UC patients using the read coverage track figure (Figure 6B). Two 3' intron retention events were identified only in the normal tissues. As the recent studies showed intron retention may affect the transcription efficiency [23], we speculated that this unique AS event in the control may limit the expression of this gene, while the UC patients may abandon this AS event to increase the transcription. Finally, we also compared the AS events of two clusters of UC patients with different disease progression status from the GSE130038 study. We identified 111 significant AS events between the two clusters. Pathway enrichment analysis also identified certain GO terms that are related to the progression of the UC (Figure 6C). These results suggested that AS may play vital roles in the UC pathogenesis, such as acting as an indicator of the disease progression.

Discussion

Dysregulation of AS is strongly associated with the pathogenesis of many human diseases. Emerging evidence have showed that targeting the cellular mRNA splicing may lead to development of novel therapeutics [34]. In fact, multiple studies have already demonstrated the potential roles of dysregulated AS in many human diseases, including cancer, immune and infectious diseases, and neurodegenerative disorders [31, 26, 18]. Indeed, two pioneer studies [20] [13] have already identified dysregulation of AS may be associated with the pathogenesis of IBD. However, neither these two studies did the enrichment analysis for these AS related genes, nor these studies focused on the comparison between disease samples and control samples using a non-array-based method. In fact, one of these two studies only paid attention to the intron retention events, while our study demonstrated all eight major types of AS events. In addition, we also analyzed two different datasets and found many AS related genes were overlapped between two cohorts, indicating that dysregulation of AS was strongly associated with the pathogenesis of UC comprehensively.

In our study, we found SE and AFE are the top two most significantly enriched AS events in both datasets. This indicated that the UC patients' transcriptome may be much more complex and chaotic compared to the healthy controls. Both SE and AFE will result in aberrant mRNA isoform, which will possibly activate a feedback response to the transcription, for instance, degradation [3]. In fact, many genes which showed significant AS events, also were changed significantly in terms of expression level. Although we cannot conclude whether the expression change is due to the dysregulated AS events, we still could conclude a significant association. Among the 23,519 genes detected in our mRNA-seq dataset, 1,513 of them were significantly dysregulated (padj < 0.05); In contrast, among the 4,514 genes with AS events, 373 of them were significantly dysregulated at expression level. These results suggested a significantly association that genes who showed AS events were more likely to have altered gene expression (Fisher's exact test p-value = 0.0001) as well.

Among the AS related significantly enriched pathways in our validation dataset, immune response and related pathways owned the largest network and most significantly enriched terms. We were particularly interested in two genes HDAC6 and LIPA, as both genes showed significant AS events (tandem 3'UTR and ALE) and were also involved in inflammation and immune response pathways. HDAC6 was one of the histone deacetylase family members. Studies have supported that targeting HDAC6 as an anti-inflammatory strategy for treating colon inflammation, which possibly progresses to IBD [9, 19]. LIPA encodes the lysosomal acid lipase, which functions in the lysosome for catalyzing the hydrolysis of triglycerides and cholesteryl esters. A recent study demonstrated lysosomal cholesterol hydrolysis works as a critical process for preventing metabolic inflammation. LIPA works as the key regulator, as inhibition of LIPA causes defective clearance of apoptotic lymphocytes [30]. In the analysis of two datasets, both expression level and AS pattern of LIPA were significantly dysregulated. Overall, our results suggested that targeting the dysregulated AS of HDAC6 and LIPA may be a good opportunity for developing novel therapies in the future.

Although we presented a comprehensive AS analysis on ileum and colon tissues of UC patients, our studies had certain limitations. First, we did not include a validation of AS events experimentally though some of the AS events were also identified in our validation experiments. Secondly, whether these AS events affect protein diversity will also need further experimental validation. Finally, a RIP-seq of CLIP-seq study should be performed to detecting the potential responsible splicing factors, for better illustrating the underlying mechanism that how AS events were altered in UC patients.

Conclusion

Nonetheless, our data suggested a strong association between dysregulation of AS and the pathogenesis of UC. Future drug screening study could focus on these AS related genes and AS related splicing factors.

Materials And Methods

Sample Information and Collection

A total of 4 patients with UC from Shengjing Hospital of China Medical University were collected between June 2020 and September 2020, and served as the experimental group. All eligible patients had an established diagnosis of UC according to endoscopic and histologic assessments. Colonic biopsy specimens were taken from the rectum, ulcer margin of sigmoid colon and inflamed portions.

4 patients with normal distal colon confirmed by surgical pathology served as the control group.

The study was approved by the institutional review board of Shengjing Hospital of China Medical University, and informed consent was obtained from each patient.

Public Dataset Preparation

We obtained RNA-seq data from NCBI (GSE137344). This dataset included 44 mRNA expression data of of ileum tissues from Ulcerative Colitis patients and 37 mRNA expression data from the normal (Here we chose this dataset is because that though UC does not typically involve other areas of the gastrointestinal tract, further extension of the inflammatory process into the terminal ileum is common [1, 12]). Fastq files were aligned on human Hg19 genome by STAR-2.7.1a. The indexed .bam file were generated by Samtools (1.10). We analyzed the GSE130038 [10], which presented the RNA-seq data on colon tissues, using the same pipeline.

Identification of Important AS Events

For GSE137344 RNA dataset, 8 types and 60,690 AS events were identified by MISO (0.5.4) [16] for 81 samples in total, using an in-house MISO pipeline. The level of alternative splicing events was defined as Percentage Spliced In (Psi). To get differentially expressed alternative splicing events between UC disease patients and normal samples, we applied Wilcoxon test on all alternative splicing events with at least 3 patients and 1 normal sample. Significant events were defined as ones with p-values < 0.05. Among

them, 2,385 AS events have wilcox test p-values less than 0.05 between the 44 UC samples and 37 normal samples and thus were defined as related/significant AS events. We removed AS events which occurred in less than 3 patients and 1 normal sample.

Same method was applied in validation experiment part. In our validation experiment RNA-seq dataset, 8 types and 94,815 AS events were identified for 8 samples in total. 2,352 AS events are significant with wilcox test p-values less than 0.05 between the 4 UC samples and 4 normal samples. AS events which occurred in less than 2 patients and 1 normal sample were also removed.

Pathway Analysis

Pathway analysis for the GSE137344 data was performed using Enrichr with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway library. Only the pathways with p-value less than 0.05 were considered related. In validation experiments pathway analysis, WebGestalt (2019) and SUMER were applied. We selected Over-Representation Analysis (ORA) as enrichment method and geneontology biological process as functional database in WebGestalt analysis. The enriched category with the gene sizes less than 5 and false discovery rate (FDR) above 0.05 was removed in WebGestalt results.

The result of SUMER was input into Cytoscape (3.8.0) to modify color and text size of the network.

Gene Ontology Analysis

Gene ontology analysis in validation experiment was performed using Enrichr and the bar plot of top 10 enriched terms was created by Enrichr Appyter.

RNA-seq analysis pipeline

RNA-seq library was prepared using the NEBNext Ultra RNA with Poly-A selection kit and was sequenced on an Illumina Hi-Seq 4000 (Genergy, Shanghai). Kallisto [5] software was used to quantify RNA-seq counts. Differential gene expression was determined with log2foldchange >1.5 and P < 0.05 genes with >1 count per million. Any genewith a P-value greater than False Discovery Rate (FDR), after Benjamini–Hochberg correction for multi-testing, was deemed significantly differentially expressed under the test conditions as compared to the controls.

Visualization

We used Plotly to generate the Sankey diagram. UpSet plots were generated by ComplexHeatmap [11] in RStudio (1.2.5042). In order to avoid random error, we randomly selected and merged RNA-seq alignment data of three Ulcerative Colitis disease samples and three normal samples respectively from GSE137344 RNA data to generate genome Sashimi plots by IGV (version 2.8.3). Other graphs were plotted in RStudio (1.2.5042). Venn diagram was plotted using an online software from http://bioinformatics.psb.ugent.be/webtools/Venn/.

Declarations

Ethics approval and consent to participate

The study was approved by the institutional review board of Shengjing Hospital of China Medical University, and informed consent was obtained from each patient (2020Yc002). All the experiment protocol for involving human data was in accordance with the guidelines of national/international/institutional or Declaration of Helsinki in the manuscript.

Consent for publication

All authors consent

Availability of data and material

Data for 4 UC validation sequencing are available from the corresponding author on request. Please contact author for data requests.

Competing interests

The authors declare no competing financial interests.

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Authors' contributions

Y. Tan designed research and collected the patients’ samples; D. Li performed the informatic analysis, and Y. Tan wrote the paper.

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Authors' information

Not applicable.

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Figures

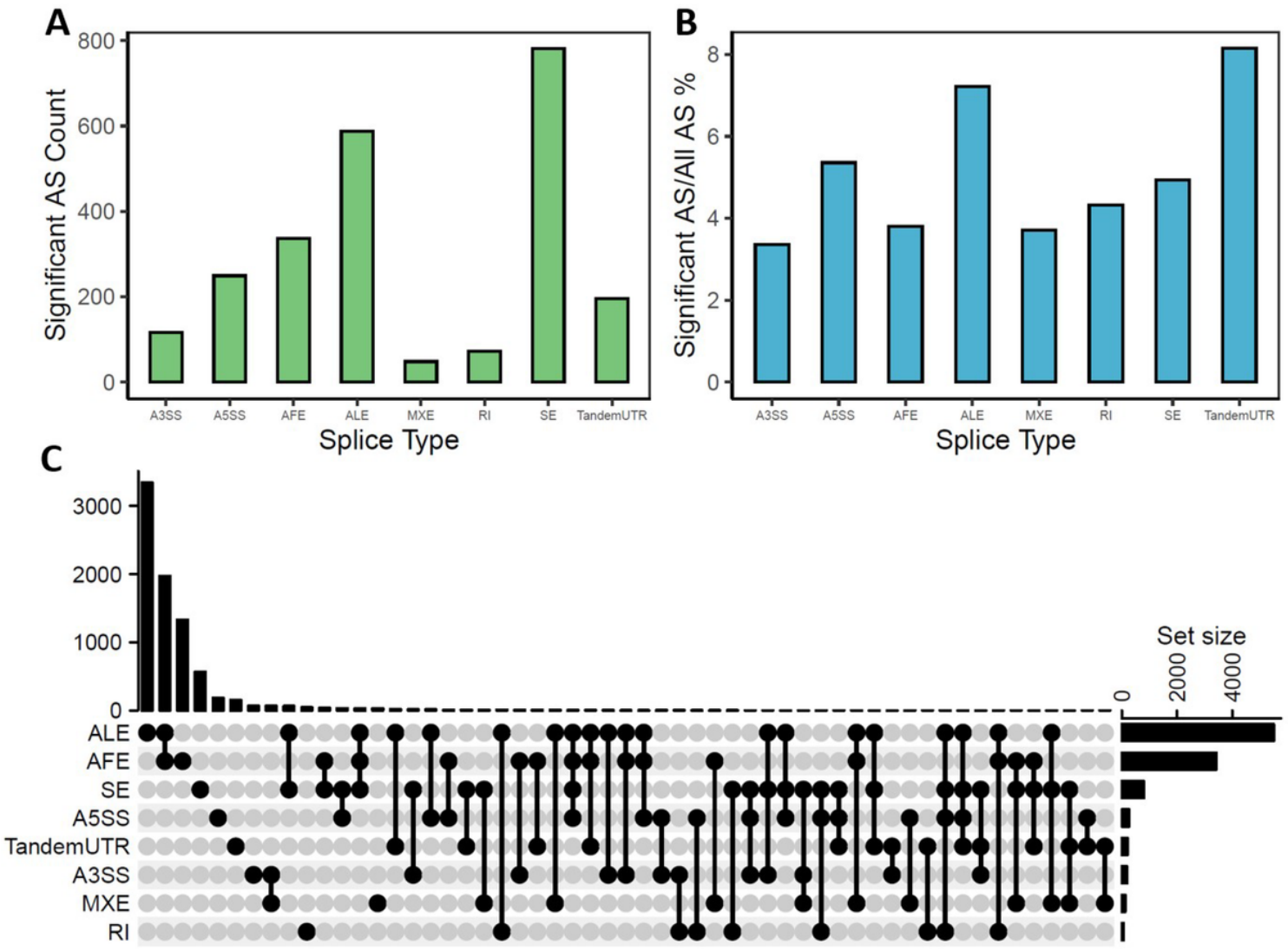

Figure 1

Alternative splicing identification in Ulcerative Colitis disease samples and normal samples. A. Significant AS event counts in different AS types. B. The ratio of related AS events over all AS events in different AS types. C. Distribution of AS types and intersection between gene symbols of 2,385 related AS events.

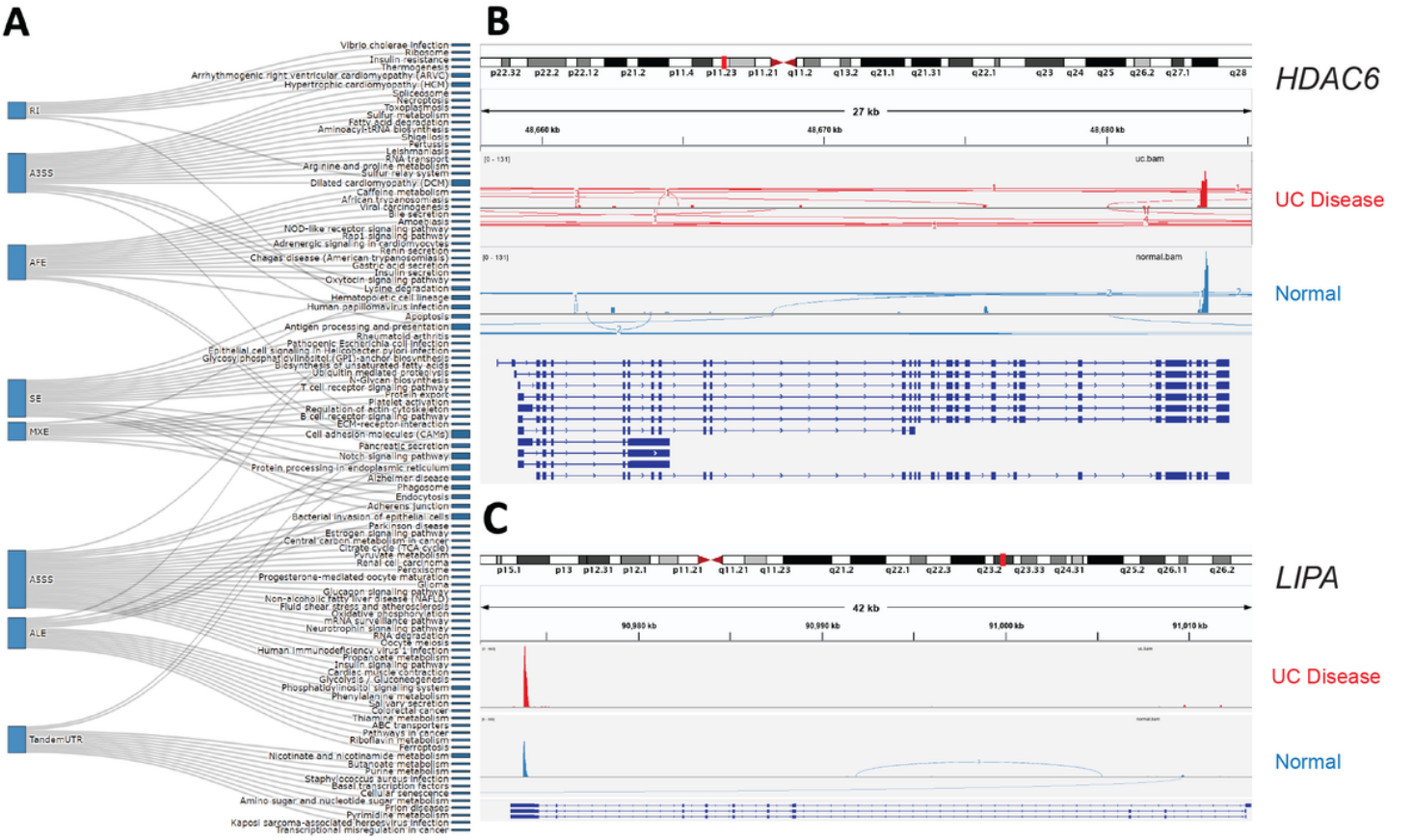

Figure 2

Pathway Analysis. (A) The biological pathways associated with the related AS events in different splice type. (B) HDAC6 mRNA expression in UC samples and normal samples. (C) LIPA mRNA expression in UC samples and normal samples.

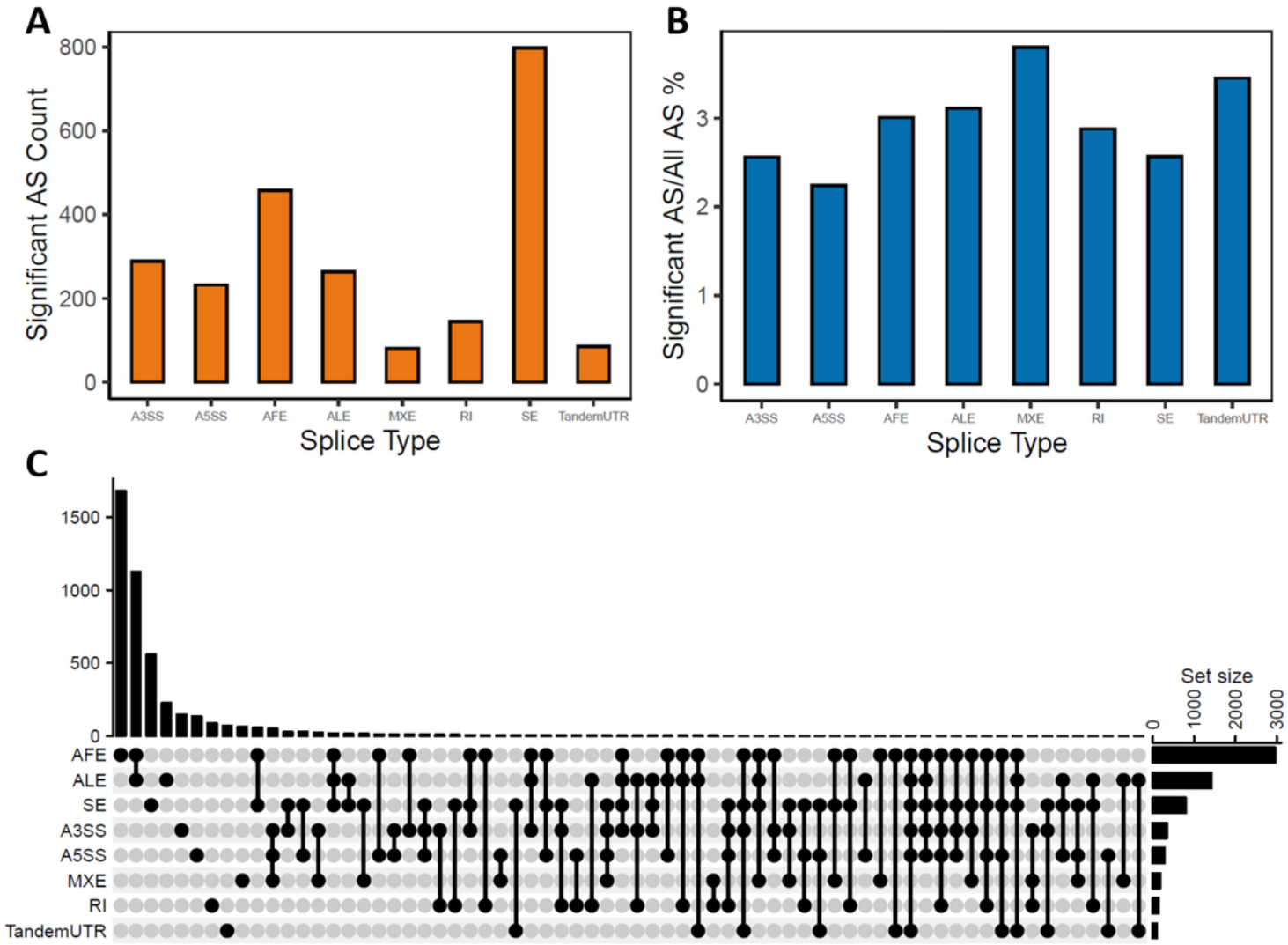

Figure 3

Alternative splicing identification in Ulcerative Colitis disease samples and normal samples in validation experiments. A. Significant AS event counts in different AS types. B. The ratio of related AS events over all AS events in different AS types. C. Distribution of AS types and intersection between gene symbols of 2,352 related AS events.

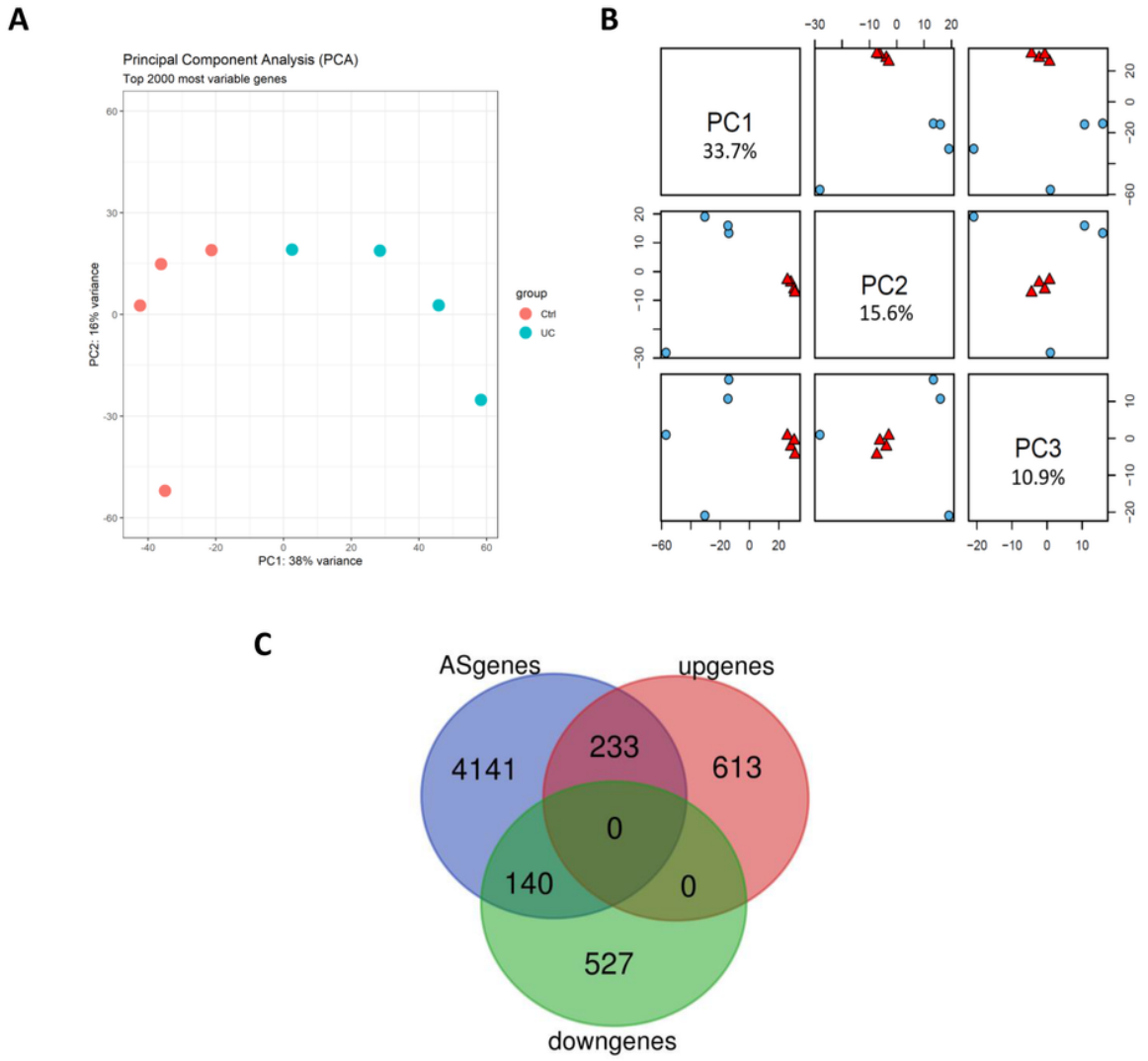

Figure 4

Combined analysis of AS events and expression profile in UC patients. (A) PCA plot of 4 UC versus 4 control expression profile using the top 2000 regulated genes as variables. (B) Scatterplot pairs of first three principal components for Percent Spliced In (PSI) values across 8 samples. The red triangles represent UC disease patients and the blue circle represent normal samples. (C). Venn diagram showed overlapped genes involved in expression regulation and AS regulation.

Figure 5

Biological process network enrichment result from Gene Ontology analysis.

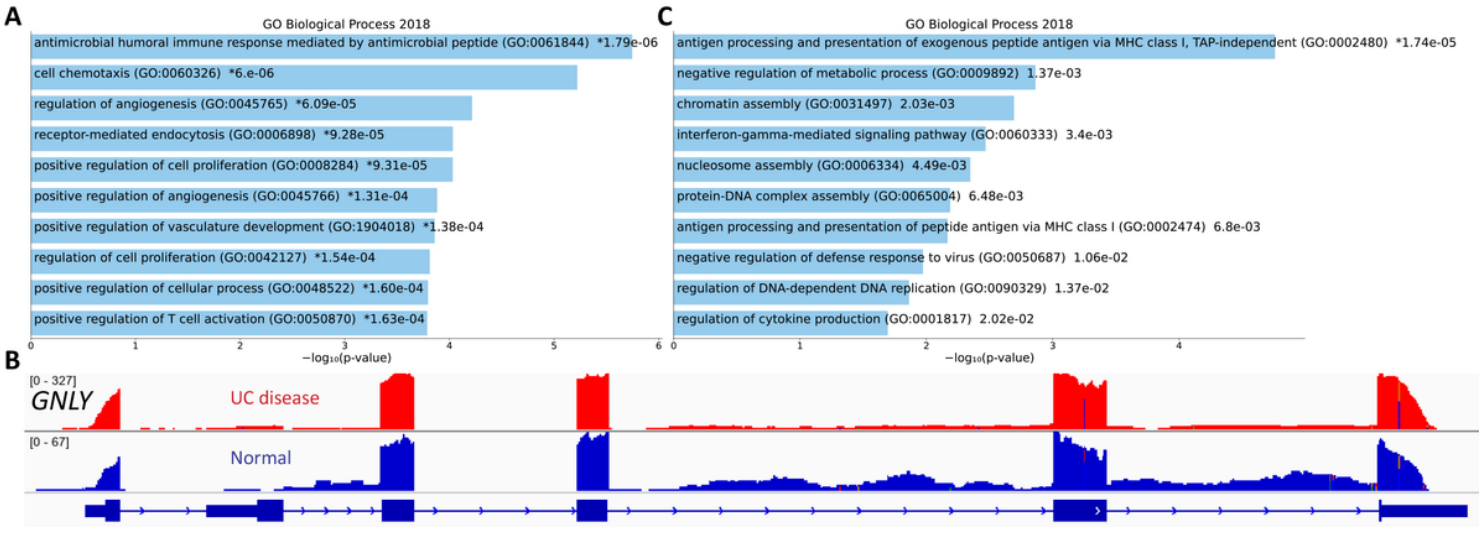

Figure 6

Analysis of GO enrichment in multiple experiments. (A) Top 10 enriched GO Biological Process terms of related genes of 201 uniquely AS events in 4 samples of UC patients. (B) Visualization of AS events on

GNLY gene. (C) Top enriched GO terms of 111 significantly expressed AS events between two clusters of UC patients with different disease severity.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure13.pdf
- TableS1.xlsx
- TableS2.xlsx
- TableS3.xlsx
- TableS4.docx
- TableS5.xlsx
- TableS6.xlsx