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Triple-negative breast cancer cells respond to T cells severely at the alternative splicing layer

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ABSTRACT

Background: Cross talk of tumor–immune cells at the gene expression level has been an area of intense research. However, it is largely unknown at the alternative splicing level which has been found to play important roles in the tumor–immune microenvironment.
Results: Here, we re-exploited one transcriptomic dataset to gain insight into tumor–immune interactions from the point of AS level. Our results showed that the AS profiles of triple-negative breast cancer cells co-cultured with activated T cells were significantly changed but not Estrogen receptor positive cells. We further suggested that the alteration in AS profiles in triple-negative breast cancer cells was largely caused by activated T cells rather than paracrine factors from activated T cells. Biological pathway analyses showed that translation initiation and tRNA aminoacylation pathways were most disturbed with T cell treatment. We also established an approach largely based on the AS factor–AS events associations and identified LSM7, an alternative splicing factor, may be responsible for the major altered events.
Conclusions: Our study reveals the notable differences of response to T cells among breast cancer types which may facilitate the development or improvement of tumor immunotherapy.
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1. Introduction

With the development of immunotherapy such as checkpoint blockers and other therapeutic strategies, tumor–immune interactions are drawing more and more attention. The leukocyte composition of TILs (Tumor Infiltrating Lymphocytes) in breast cancer is predominantly consistent of activated $CD8^+$ cytotoxic T lymphocytes (CTLs) which play an important role in surveillance [1]. It has been reported that cancer cells escape surveillance in various ways such as loss of antigen expression and MHC components [2], shedding of NKG2D ligands [3], and development of IFN-γ insensitivity [4]. Although extensive studies have provided insights into tumor–immune cross talk at the gene expression level [5], how cancer cells respond at alternative splicing (AS) level is largely unknown.

AS means is a mechanism through which cells generate multiple messenger RNAs (mRNA) from a single genomic locus through the inclusion or exclusion of specific exons in pre-mRNA processing [6]. The comparison of mRNA with genomic sequences showed viral sequences are removed from the pre-mRNA prior to the export into the cytosol and the remaining sequences are joined together [7]. Almost all mammalian polymerase II transcripts undergo this process called pre-mRNA splicing. Due to splicing, only a small fraction of sequences from the primary transcripts are joined together to form the mature mRNA [7]. It is now clear that the vast majority of pre-mRNAs contain exons that can be

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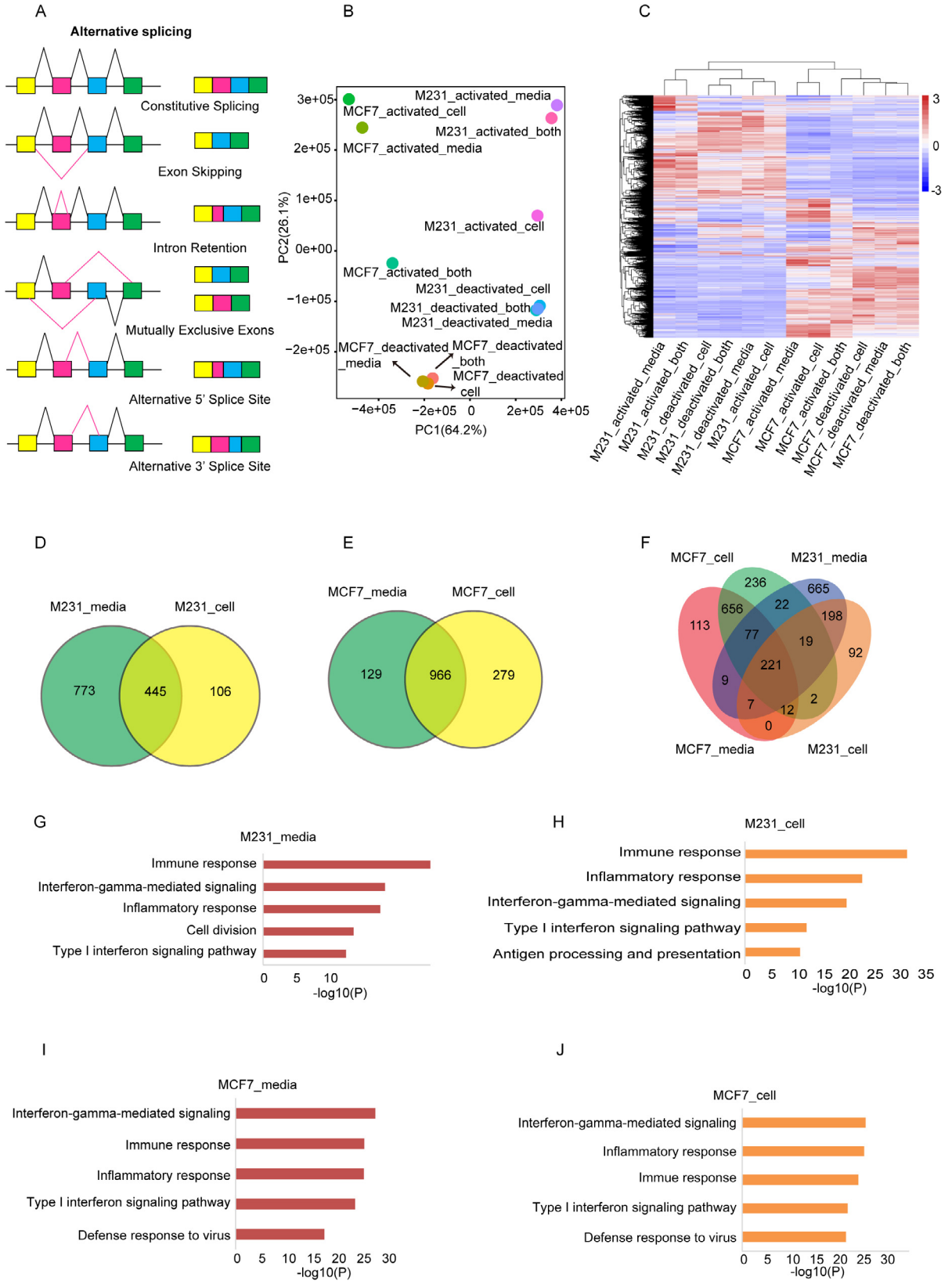


Fig. 1. Gene level expression analyses of TNBC and ER$^+$ cells under T cell co-culturing and conditional-media form T cell treatments. Graphical presentation of AS in Cancer (B) Principal component analysis (PCA) was performed using 18,984 genes across 6 MDA-MB-231 samples and 6 MCF7 samples under different treatments. MDA-MB-231_activated_cell: MDA-MB-231 co-cultured with activated CD8$^+$ cell; MDA-MB-231_activated_media: MDA-MB-231 co-cultured with activated CD8$^+$ media; MDA-MB-231_activated_both: MDA-MB-231 co-cultured with activated CD8$^+$ cells and media. (C) Unsupervised clustering of MDA-MB-231 samples and MCF7 samples with different treatments using expression profiles in (B). (D) Overlapping genes of MDA-MB-231_cell genes and MDA-MB-231_media genes. MDA-MB-231_cell genes: significantly differentially expressed genes between MDA-MB-231 co-cultured with activated CD8$^+$ cells and MDA-MB-231 co-cultured with deactivated CD8$^+$ cells. MDA-MB-231_media genes: significantly differentially expressed genes between MDA-MB-231 co-cultured with activated CD8$^+$ media and MDA-MB-231 co-cultured with deactivated CD8$^+$ media. (E) Overlapping of MCF7_cell genes and MCF7_media genes. MCF7_cell genes: significantly different expressed genes between MCF7 co-cultured with activated CD8$^+$ cells and MCF7 co-cultured with deactivated CD8$^+$ cells. MCF7_media genes: significantly different expressed genes between MCF7 co-cultured with activated CD8$^+$ media and MCF7 co-cultured with deactivated CD8$^+$ media. (F) Venn plot showing the overlapping genes in (D and E). GO biological processes enriched in MDA-MB-231_media genes (G), MDA-MB-231_cell genes (H), MCF7_media genes (I), MCF7_cell genes (J).

alternatively included into the mature mRNA or removed from it, which is called alternative splicing. This process is implemented and regulated largely by the expression of the core splicing factors and alternative splicing factors [8]. These factors splice a pre-mRNA by multiple ways to finally produce several mRNAs which can be translated into different protein variants with different functions (Fig. 1A). Recently, it is evidenced that AS regulated almost all the cancer hallmarks, including escaping surveillance [9]. It is well-known that the aberrant expression of HLA (human leukocyte antigen) molecules on the surface of tumor cells produced at the AS level is one of the escaping mechanism [10]. For example, a splice isoform of MHC-I with exon 7 missing, which stimulates the response of cytotoxic T lymphocytes, is more effective in hampering melanoma growth in mice than the canonical variant [11]. Thus, systematically studying the tumor–immune interactions at the AS level would increase our understanding of the molecular mechanisms of T cell surveillance and cancer cell escape.

Aminoacylation process transfer RNAs (tRNAs) with amino acid establishes the rules of the genetic code which are catalyzed by an ancient group of 20 enzymes known as aminoacyl tRNA synthetases (AARSs) [12]. It has been proved that the etiology of cancer is connected to specific aminoacyl tRNA synthetases [12,13]. For example, tryptophanyl-tRNA synthetase (WARS) directly regulates angiogenesis as pro- and anti-angiogenic cytokines [14]. EPRS (glutamyl-prolyl tRNA synthetase) negatively regulates this process via translational suppression of vascular endothelial growth factor-A [15]. Tyrosyl-tRNA synthetase is secreted and cleaved into two distinct cytokines that work in angiogenesis as well as in the immune response [16,17]. The specific roles of aminoacylation processes in the immune response of tumor cells are largely unknown.

Here, we characterized the AS profile response to T cell treatment in ER^+ (Estrogen receptor positive) and triple-negative breast cancer cells (TNBC) by re-exploiting a previous study [5]. We found remarkable differences in AS profile alteration in responding to T cells in ER^+ and TNBC. We also found that translation initiation and tRNA aminoacylation pathways were significantly altered during T cell treatment in TNBC. Using our established approach, we found that the expression of LSM7 was involved in the major proportion of altered AS events in TNBCs.

2. Materials and methods

2.1. Data used

To gain insight into tumor–immune cell cross talk, we reanalyzed one previously published dataset (GSE73527). The overall design of this study was to co-culture MDA-MB-231 or MCF7 cells with PBMCs (peripheral blood mononuclear cells) alone or the conditioned media or a combination of both for 24 h and then harvest for further analysis. PBMCs were treated with monoclonal antibody CD3 and CD28 in 1 ml of RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) for 48 h. The media was collected and defined as conditioned media. Control experiments were performed in the same manner except that isotype control antibodies were used. For detailed experimental design and treatment procedures, please refer to paper [5] and Table S1.

2.2. Gene level expression analyses

Paired-end reads were mapped to the human genome primary assembly (GRCh37) [18], and the Ensembl human gene annotation for GRCh37 gene build was used to improve the accuracy of the mapping with STAR software (version 2.4.2a) [19]. FeatureCounts (version 1.4.6-p5) [20] was performed to assign sequence reads to genes. Mitochondrial genes, ribosomal genes, and genes possessing less than five raw reads in half the samples were removed. Differential expression analyses were realized using the Bioconductor edgeR package 1.6 [21]. Significant genes were determined by an adjusted *P*-value of <0.05 after the Benjamini and Hochberg multiple testing corrections and a log2 transformed fold change >2 or <−82.

2.3. Alternative splicing (AS) analyses

The recommend pipeline of Mixture of Isoforms (MISO) (version 0.5.4) [22] was used to process mRNA-Seq data and estimate the percentage of splicing isoforms (Ψ values, for "percent spliced isoform"). Significantly different AS events between samples were determined by Bayes factor >6 and $|\Delta PSI| > 0.2$.

2.4. Principal component analyses (PCA)

PCA was separately performed on two different levels of profiles: gene expression level and AS level. For the first one, the gene expression profile comprising of 18,984 genes of each sample was accounted in PCA. Gene expression was normalized across samples with the method of reads per million mapped reads (RPM). For the second analysis, a total of 31,323 AS events that possess values across 12 samples were accounted in the PCA. The prcomp package from R was used to perform PCA and the default parameters were used.

2.5. Functional enrichment analyses

Functional enrichment of Gene Ontology (GO) terms was conducted with web server DAVID 6.8 [23]. $P < 0.05$ was set to identify significantly enriched biological processes.

2.6. Protein–protein interaction analyses

Web server STRING [24] was utilized to explore the protein–protein interactions. Default parameters were applied. We established AS factor–AS events association method to weight the importance of AS factors in the regulation of AS events. To isolate the key AS factor responsible for the altered AS profiles in TNBC when co-culturing with activated T cells, we established AS factor–AS events association method to weight the importance of AS factors in the regulation of the AS events. Firstly, we adopted the comprehensive list of AS factors in our previous study [25], which comprises of a total of 222 putative and experimentally supported splicing factors. We further identified 12 AS factors out of 222 AS factors who showed significant expression alteration after activated-T cell treatment in TNBC (fold change > 2 and student's *t*-test $P < 0.05$). Secondly, we adopted one previous study with 44 transcriptomic profiles from breast cancer cell lines (accession: GSE96860). Then, we used the number of significant association events, which showed significant correlation with the expression of AS factor ($|R| > 0.2$ and $P < 0.0001$), to weight the importance of each AS factor in the regulation of AS profiles.

3. Results

3.1. Data quality confirmation

In the past decade, we have witnessed that the immunotherapy has revolutionized the research and treatment of many types of cancers and making tumor–immune interactions come into focus. To increase our understanding of tumor–immune cell interactions, we re-explored one previous study [5], which compared

immunophenotype between the TNBC cell line (MDA-MB-231) and the ER$^+$ breast cancer cell line (MCF7) under various treatments (co-cultured with activated-T cells or conditioned media from activated-T cells or a combination of both) (Table S1). Here, we first evaluated the data quality by principal component analysis (PCA) based on the assumption that samples from the same cell type under the same treatment should distribute closely to each other. PCA was carried out with 18,984 genes and our results showed that samples were divided into different groups according to the cell types and treatments (Fig. 1B). Also, recapitulating the result from PCA results in that samples from the same cell type under the same treatment distribute closely to each other (Fig. 1C). Thus, both PCA and unsupervised clustering results preliminarily confirmed the dataset quality.

To further investigate the biological pathways disturbed by different treatments in MDA-MB-231 and MCF7 cells, we identified co-cultured activated T cell-specific response genes and conditional-media-specific response genes in MDA-MB-231 and MCF7 cells. Specifically, we identified co-cultured activated T cell response genes by intersecting the differentially expressed genes among co-cultured treatment and conditional-media treatment. Finally, 1218 and 551 differentially expressed genes were found when treated with conditional-media from activated T cells and co-cultured with activated T cells in MDA-MB-231. 1095 and 1245 differentially expressed genes were found when treated with conditional-media and co-cultured in MCF7. The number of genes responding to conditional-media and co-cultured treatment in MDA-MB-231 and MCF7 cells were shown in Fig. 1D–E, respectively, and there were 221 common genes across four conditions (Fig. 1F). The corresponding biological processes disturbed by each treatment were showed in Fig. 1G–J. Generally, immune response, interferon-gamma-mediated signaling pathway, inflammatory response, and type I interferon signaling pathway were significantly enriched in the above four conditions. Because activated-media-treatment and activated-cell-treatment correspond to specific gene sets alterations in both MDA-MB-231 and MCF7 cell lines (Fig. 1D and 1E). These results showed that activated T cells exert its functions through cell-specific and soluble media-specific regulations and these results also confirmed the reliability of the laboratory experiment. To show the disturbed pathways enriched from up-regulated and down-regulated genes, we also provided the Table S2 which containing DEGs and GO termed from up-regulated and down-regulated genes from each condition.

3.2. ER$^+$ and TNBC cells showed remarkably different responses to activated T cells at the AS level

To further investigate how activated T cells educate different breast cancer cells at the AS level, we derived AS profiles from each sample by performing Mixture of Isoforms pipeline (MISO) [22] according to its recommended procedure (https://miso.readthedocs.io/en/fastmiso/). Delta PSI > 0.2 and Bayes factor > 6 were applied to identify statistically different splicing events between control samples and treatment samples. Finally, a total of 31,323 splicing events with values across 12 samples were isolated for subsequent analyses. Firstly, PCA was used to characterize the genetic distances among samples. Our results showed the remarkable difference between MCF7 samples and MDA-MB-231 samples. MCF7 samples of different treatments distribute tightly together and the result suggested MCF7 cells negatively respond to co-cultured activated-T cell and conditional-media treatments on AS levels.

However, control MDA-MB-231 samples and those with different treatments including activated T cells and activated T media were notably separated, which indicated that AS profiles of TNBC cells responded positively to T cell immune surveillance (Fig. 2A). Specifically, when focusing on MDA-MB-231, we can see that MDA-MB-231 under treatment of conditional-media from activated T cell (MDA-MB-231_activated_media), co-cultured with activated T cells (MDA-MB-231_activated_cell) or both (MDA-MB-231_activated_both) were notably detached from treatment of conditional-media from the deactivated T cell (MDA-MB-231_deactivated_media), co-cultured with deactivated T cell (MDA-MB-231_deactivated_cell) or both (MDA-MB-231_deactivated_both). When focusing on the activated treatments, we can see that the AS profiles from treatment of co-cultured activated T cell (MDA-MB-231_activated_cell) and both (MDA-MB-231_activated_both) were similar, which was different from treatment with conditional-media from activated T cell (MDA-MB-231_activated_media), which indicated that cell contact imposed greater effects on the AS regulation than activated-T-media (Fig. 2A). Unsupervised clustering results also supported that breast cancer cells were separated into different groups from those of control groups based on AS profiles both in MDA-MB-231 and MCF7 cells (Fig. 2B).

Above results suggested that the AS profiles in TNBC breast cancer were dramatically disturbed by treating with activated cells or media, whereas the AS profiles in MCF7 were slightly altered (Fig. 2A). Thus, we further identified which AS events and the corresponding biological pathways were affected in MDA-MB-231 by activated T cell treatment. We identified cell-specific responding AS events by intersecting the altered AS events in MDA-MB-231_activated_cell and MDA-MB-231_activated_both. 526 cell-specific altered splicing events were isolated (Table S3). Similarly, 37 media-specific altered splicing events were identified (Table S4). Interestingly, there were no media-specific altered events after intersecting with cell-specific altered events (Fig. 2C). Venn plot of Fig. 2E showed the altered AS events among 4 conditions (MCF7_media_AS, MCF7_cell_AS, MDA-MB-231_media_AS, and MDA-MB-231_cell_AS).

Next, we performed GO enrichment analyses with the altered cell-specific AS events in MDA-MB-231 to explore the disturbing biological pathways. GO terms such as translational initiation, tRNA aminoacylation for protein translation, NF-KB signaling pathway, and T cell receptor signaling pathway were significantly enriched in cell-specific altered AS events (Fig. 2D). KEGG pathway results showed that RNA transport, Shigellosis, and Aminoacyl-tRNA biosynthesis pathways were enriched in cell-specific altered AS events (Fig. 2F). Briefly, RNA transport and aminoacyl-tRNA biosynthesis-related pathways were significantly enriched in T cell-specific altered AS events.

Considering that translation initiation, tRNA aminoacylation, and T cell receptor signaling pathways were comprehensively affected by T cells at the AS profile. Then, we further analyzed the three most affected pathways including the translational initiation, tRNA aminoacylation for protein translation, and T cell receptor signaling at the AS level. Protein–protein interaction analyses showed that proteins from each pathway have extensive interactions, which suggested the AS may exert comprehensive affections to these biological pathways (Fig. 3A–C). Three examples of altered splicing events were shown in Fig. 3D–3F. AS event of PSMA4 from T cell receptor signaling pathway was significantly altered (delta PSI = 0.17), which suggested that third exon were more included after active-cell treatment. LARS from the tRNA aminoacylation pathway showed the similar trend. The second exon in figure was dramatically included after activate-cell treatment. The exon inclusion/exclusion events affected the function of genes and further disturbed the pathways through protein–protein interaction networks (Fig. 3A–C).

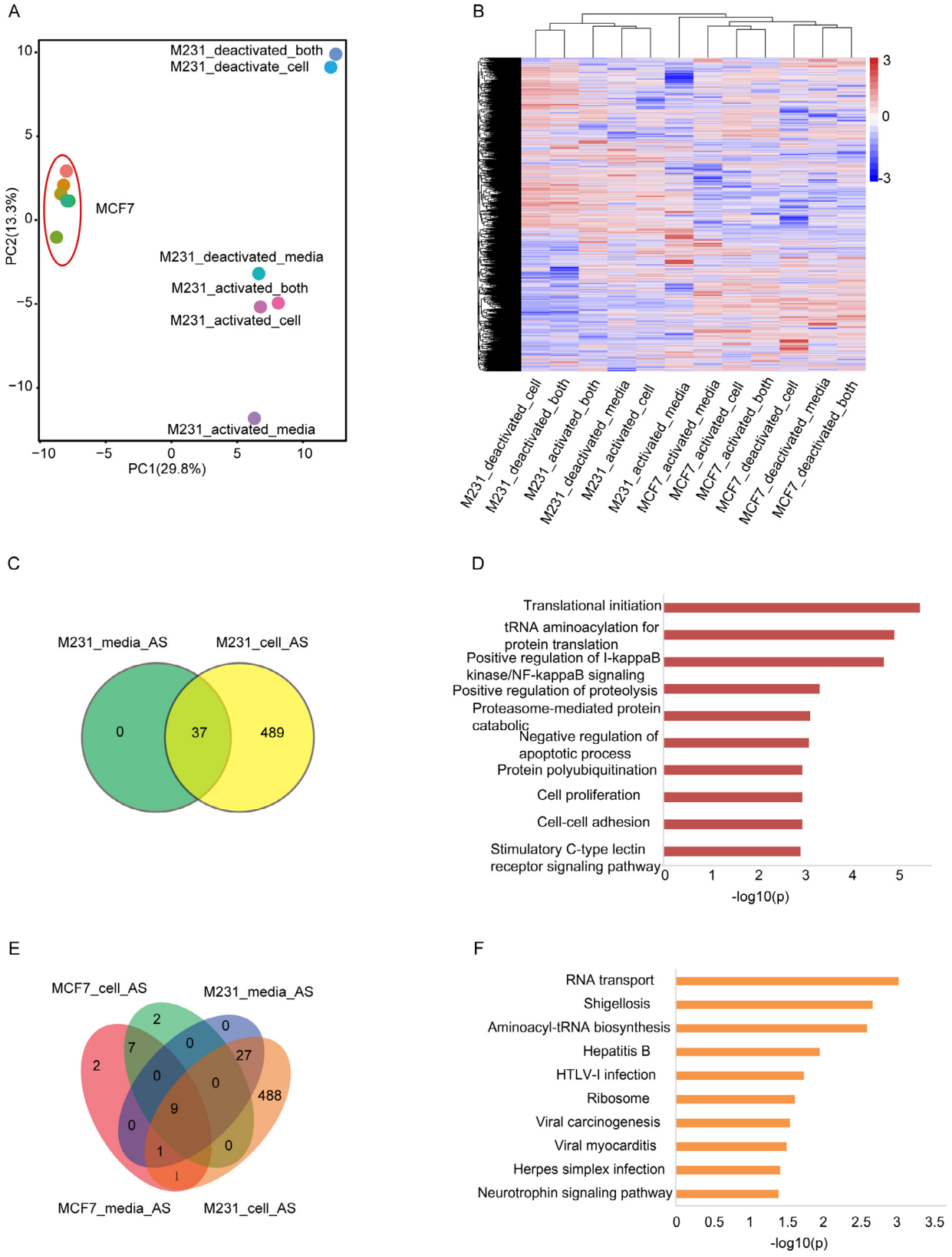


Fig. 2. ER$^+$ and TNBC cells showed remarkably different responses to activated T cells and biological processes enrichment analyses of genes at the AS level. PCA analysis of AS profiles of MCF7 cells and MDA-MB-231 cells under different treatment. (B) Unsupervised clustering of AS profiles of MCF7 cells and MDA-MB-231 cells under different treatment. (C) The overlap of altered AS events in TNBC under activated-T cells treatment and activated-T media treatment. (D) The overlap of altered AS events in TNBC and ER$^+$ cells under activated-T cell treatment and activated-T-media treatment. (E) GO analyses of genes in altered AS events in TNBC cells. (F) KEGG analyses of genes in altered AS events in TNBC cells.

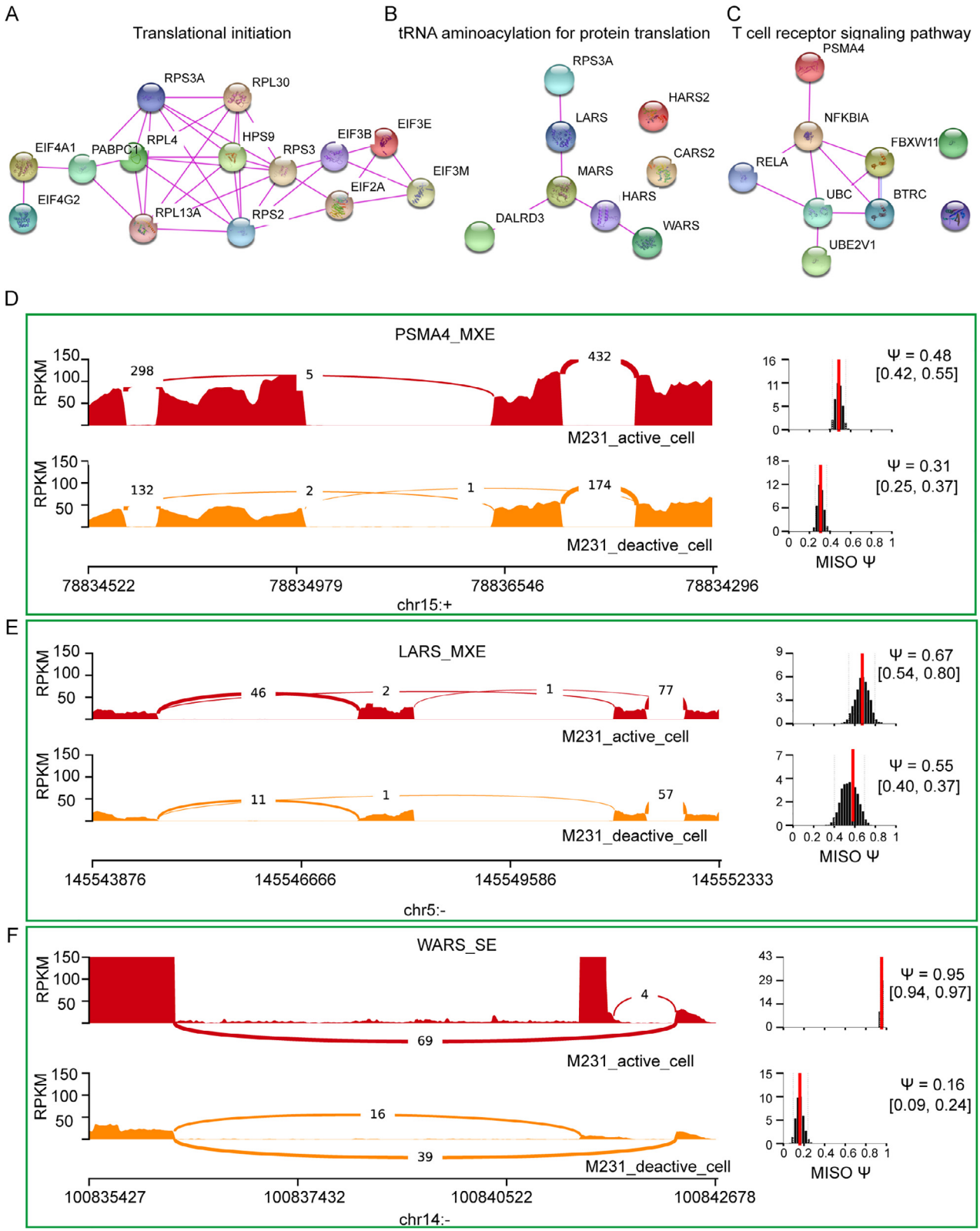


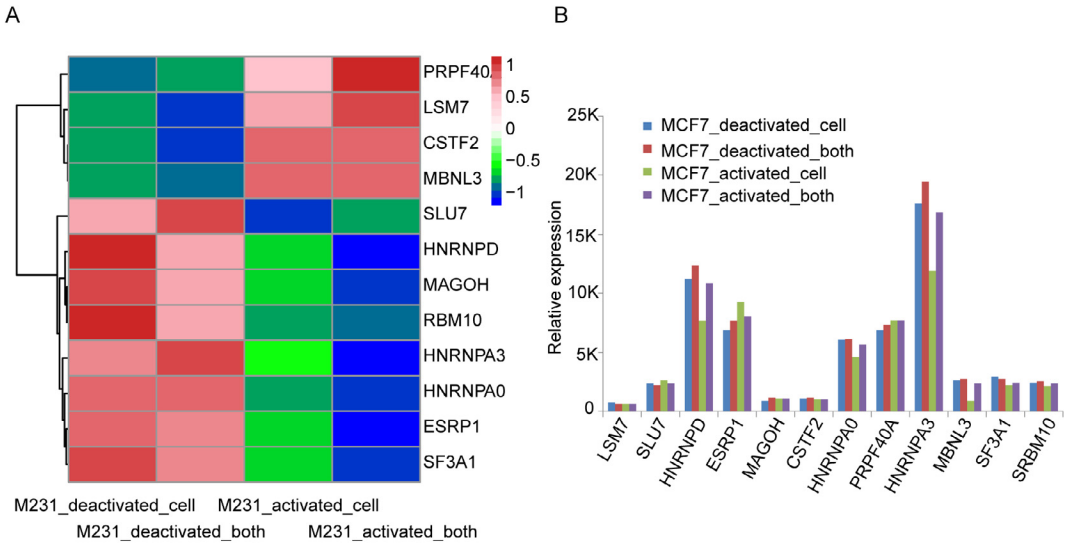
Fig. 3. Protein–protein interaction network analyses of genes from the most affected three pathways in TNBC. Protein–protein interaction networks from translation initiation pathway (A), tRNA aminoacylation pathway (B), and T cell receptor signaling pathway (C). Sashimi plots showing three representative AS events between control and treatment samples from translation initiation pathway (D), tRNA aminoacylation pathway (E) and T cell receptor signaling pathway (F).

3.3. The expression of LSM7 explained the major part of AS profile alteration in TNBC

Considering AS profiles are largely regulated by AS factors, then we established an approach to identify the splicing factors responsible for the AS profile altered in TNBC co-cultured with activated T cells (Section 2). Firstly, we identified 12 AS factors out of 222 AS factors which showed significant expression alteration after activated T cell treatment in TNBC (fold change >2 and student's *t*-test $P < 0.05$) (Fig. 4A). Meanwhile, we also detected the expression of 12 AS factors in MCF7 cell samples to investigate whether the expression alteration during T cell treatment was specific to TNBC cells. Our results showed that the expression levels of these 12 AS factors were not obviously changed between control samples and treatment samples in MCF7 cells, which suggested that these AS factors' expression changes were specific to TNBC cell line (Fig. 4B). Secondly, the number of AS events associated with these splicing factors in MDA-MB-231 was calculated (Fig. 4C). The top splicing factors which explained the most MDA-MB-231 cell AS events were LSM7, which explained 143 splicing events. Survival analysis showed that patients with high expression of LSM7 had poor survival which was consistent with the higher expression in breast cancer cells co-cultured with activated T cells (Fig. 4D).

4. Discussion

There is a rapid pace of development of immunotherapies, making AS, an important mechanism providing neoantigens or immune targets came into focus [26]. Here, we characterized the AS responses of TNBC cells and ER$^+$ breast cancer cells to activated T cells. We carried out PCA at two different levels: gene expression level and alternative splicing level. Both TNBC and ER$^+$ cells respond to activated T cells similarly at the gene expression level. However, TNBC and ER$^+$ cells show a remarkably different response to activated T cells at the AS level. Mainly, we found that ER$^+$ breast cancer cells showed a slight response, whereas TNBC cells showed dramatic response to activated T cells. GO analysis showed that amino-tRNA pathway was significantly enriched within the altered AS events. These results suggested the existence of different AS regulation mechanisms between TNBC and ER$^+$ breast cancer cells which is supported with our previous study showing that TNBC possesses significant different AS profiles comparing to other three breast cancer subtypes [25].



C

Splicing factors	Number of events associated with the splicing factor
LSM7	143
SLU7	136
HNRNPD	109
ESRP1	105
MAGOH	103
CSTF2	102
HNRNPA0	102
PRPF40A	101
HNRNPA3	99
MBNL3	98
SF3A1	87
RBM10	73

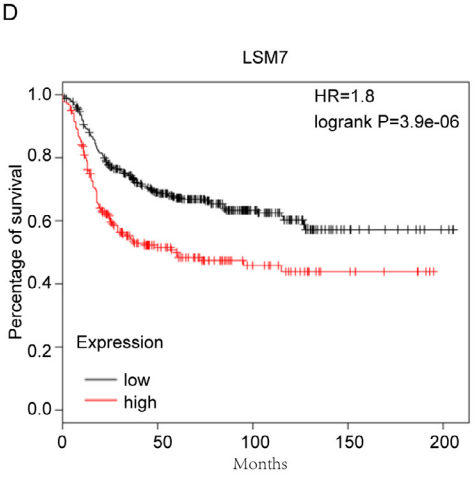


Fig. 4. Identification of putative splicing factors responsible for the altered AS events in TNBC co-cultured with activated T cells. (A) 12 AS factors were identified as significantly differentially expressed genes after activated T cell treatment in TNBC. (B) Expression of the 12 AS factors in ER$^+$ cells after activated T cell treatment. (C) The number of significant associated altered AS events in T cell treatment with the 12 AS factors in TNBC cells. (D) The expression of LSM7 associated with overall survival in breast cancer patients.

We also identified the putative responsible splicing factor LSM7, which explained the major part of altered AS events in TNBC co-culturing with T cells.

Through a combination of differentially expressed AS factors and AS events, we identified 12 AS factors, which explains the most altered splicing events in TNBC co-culturing with activated T cells (Fig. 4C). Meanwhile, we found that these 12 AS factors showed no significant differences in MCF7 cells when co-cultured with activated T cells (Fig. 4B). This may explain why MCF7 showed slight response to activated T cells.

tRNA aminoacylation pathway may serve as the effector of T cell treatment at the AS level in TNBC. Another interesting phenomenon was that amino-tRNA pathway was extensively affected during T cell co-culture treatment. As we have known, transfer RNA (tRNA), considered as a housekeeping molecule [27], mainly participates in protein translation by transporting amino acids to the ribosome. Accumulating evidence has shown that tRNAs are closely related to various physiological and pathological processes including regulation of the proper functioning of the immune system [28]. Indoleamine 2,3-dioxygenase (IDO) is a rate-limiting enzyme that catabolizes tryptophan to produce bioactive catabolites called "kynurenines" and is involved in inflammation, autoimmunity, and tumorigenesis [29]. Munn et al., [30] observed that IDO overactivation could lead to an increase in the level of uncharged tRNATrp. These uncharged tRNAs then activated general control nonderepressible 2 (GCN2) kinase, which mediated cell cycle arrest and anergy induction in response to IDO in T cells. Noonepalle et al., [5] showed that MDA-MD-231 cells were able to transcribe more IDO1 than MCF7 cells when co-culturing with activated T cells. Thus, taken together, the tRNA aminoacylation pathway is at least regulated by two factors: IDO1 and AS. But whether IDO1 and AS act alone or interact mutually and how they confer to the T cell response deserve more investigation. There are some deficiencies in our study, for example, experimental knockdown of LSM7 under activated T cells would more strongly support the importance of its roles in AS regulation and T cell response.

In summary, our data revealed an important distinguishing attribute of TNBC at the AS level comparing to ER^+ breast cancer cells, which has significant implications for immunotherapy.

Conflicts of Interest

The authors declare no conflict of interest.

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Code availability

All inhouse codes used in this study have been deposited in https://github.com/Hongleigithub/Triple-negative-breast-cancer-cells-respond-to-T-cells-severely-at-the-alternative-splicing-layer.

Supplementary material

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