Original Article

Use of exosome transcriptome-based analysis to identify novel biomarkers in patients with locally advanced esophageal squamous cell carcinoma undergoing neoadjuvant chemoradiotherapy

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Background: The prognosis of esophageal squamous cell carcinoma (ESCC) is improved by neoadjuvant chemoradiotherapy (nCRT), especially for patients with pathologic complete response (pCR). Despite the efforts to predict treatment response using multimodality, no molecule has proven to be a strong biomarker. This study aimed to profile the expression of exosome transcriptome that could predict pCR in ESCC before and after nCRT.

Methods: We collected paired blood samples of 15 patients with ESCC who received nCRT and radical surgery. They were divided into 3 groups: (A) residual tumor in the first clinical response evaluation (CRE-1), (B) no residual tumor in CRE-1 but with residual tumor in CRE-2 which was performed after 5–6 weeks, and (C) no residual tumor in CRE-1 or CRE-2. For each patient, the blood sample was collected before nCRT (time point 0); and then 6 weeks after nCRT, the clinical response was evaluated, and another blood sample was collected (time point 1).

Results: Using the intersection of different sets, we found 23 progression-associated messenger RNAs (mRNAs) and 67 remission-associated mRNAs. Between remission-associated mRNAs and the targets of progression-associated (carcinogenic) microRNAs (miRNAs), the intersection was acquired, and 2 miRNA-mRNA networks (*IFIT2-miR-3615-IFIT2-miR-484* and *BTN3A3-miR-6803-3p*) were identified. Among the intersection of progression-associated (carcinogenic) mRNAs and the targets of remission-associated miRNAs, there is a network with miR-132-3p (remission-associated miRNA) located at the core, matched with *DICER1*, *KLHL8*, *ANKRD12*, *ASH1L*, and *IMP4*.

Conclusions: Our findings identified altered plasma exosome RNAs among the different groups and between different time points of nCRT, as well as the corresponding enrichments and regulatory networks, which may serve as potentially predictors of treatment response for patients with ESCC after nCRT.

Keywords: Esophageal squamous cell carcinoma (ESCC); neoadjuvant chemoradiotherapy (nCRT); microRNA (miRNA); exosome transcriptome; bioinformatics analysis

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Introduction

Esophageal cancer is one of the most aggressive malignancies of the gastrointestinal tract, with a 5-year survival rate of less than 25% (1). In China, esophageal squamous cell carcinoma (ESCC) is the most commonly observed malignant type, with Chinese cases accounting for more than half of the global burden. Neoadjuvant chemoradiotherapy (nCRT) followed by surgery is currently still the major treatment strategy for locally advanced ESCC and was demonstrated to improve survival by the landmark CROSS and NEOCRTEC5010 studies (2,3). Despite the survival benefits, half of patients still relapse after nCRT and surgery, especially those without pathologic complete response (pCR). This suggests that more effective biomarkers are required to increase the accuracy of predicting pCR to identify the patients most suited to receive nCRT.

Liquid biopsy-based biomarkers in cancer diagnostics are associated with low cost and minimal invasiveness (4). Significant efforts have been made in the use of liquid biopsy for the early detection and treatment stratification of cancer, as well as residual disease and recurrence monitoring. Although circulating tumor cells and circulating tumor DNA have been used for early detection of tumors, exosomes and other extracellular vesicles have become a platform with potentially wider and complementary applications (5). The exosomes secreted by tumor cells consist of many molecules closely related to the characteristics of tumor. Analysis of exosomes is benefit to the diagnose of tumor and prediction of the tumor prognosis (6). Previous study has shown that the exosomes secreted by tumors can enter into circulatory system and be detected in the blood (7). Separating the exosomes in the blood could realize the non-invasive way to identify the characteristics of tumors at an early stage and potential therapeutic targets. Therefore, plasma exosomes are attractive as biomarkers for disease progression and risk stratification for applying nCRT in patients with ESCC.

Exosomes have been reported to promote tumorigenesis in several cancers, particularly through transfer of miRNAs. Circulating exosome-miRNAs (exo-miRNAs) have been suggested as specific and stable molecular biomarkers (8,9). Besides miRNAs, the combination of other RNA types [i.e., messenger RNA (mRNA), long noncoding RNA (lncRNA), and circulating RNA (cRNA)] can provide more comprehensive information and a reference for the molecular mechanism regarding nCRT. Exosome transcriptome could represent a more detailed and specific molecular biomarker compared with a single RNA type. The use of exosome transcriptome to predict the treatment response of nCRT has been widely used in other cancers (such as breast cancer and gastric cancer) (10,11); however, it is still in its infancy and has not been reported in ESCC. In addition, the current efficacy prediction mainly uses exo-miRNA panels, which cannot reveal the deep relationship between the three important types of exo-RNAs (mRNA, miRNA, and lncRNA). Therefore, we aimed to determine the associations between miRNA, mRNA, and lncRNA expression and nCRT response in patients with ESCC. To the best of our knowledge, this is the first study to use the exosome transcriptome with three types of RNA to examine the biomarkers in patients with ESCC who received nCRT and surgery. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-23-452/rc).

Highlight box

Key findings

- Two miRNA-mRNA networks (*IFIT2-miR-3615-IFIT2-miR-484* and *BTN3A3-miR-6803-3p*) were identified as predictive biomarkers in patients with esophageal squamous cell carcinoma who have received neoadjuvant chemoradiotherapy.

What is known and what is new?

- A profile of an exosome transcriptome in patients with esophageal cancer who have received surgery alone has been established in the existing bioinformatics database.
- This present exosome transcriptome-based analysis revealed novel biomarkers to predict treatment response in patients with locally advanced esophageal squamous cell carcinoma undergoing neoadjuvant chemoradiotherapy.

What is the implication, and what should change now?

- Our results expanded upon the expression profile of exosome transcriptome in patients with esophageal squamous cell carcinoma who have received neoadjuvant chemoradiotherapy, which could be applied to predict treatment response after further confirmation.

Methods

Patients and specimens

The patient population consisted of 15 patients with ESCC who received nCRT and surgery in Shanghai Chest Hospital. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) (12). This study was approved by the institution ethics committee of Shanghai Chest Hospital (No. KS2160). All patients provided written informed consent for the use of blood and specimens. The study flowchart is shown in

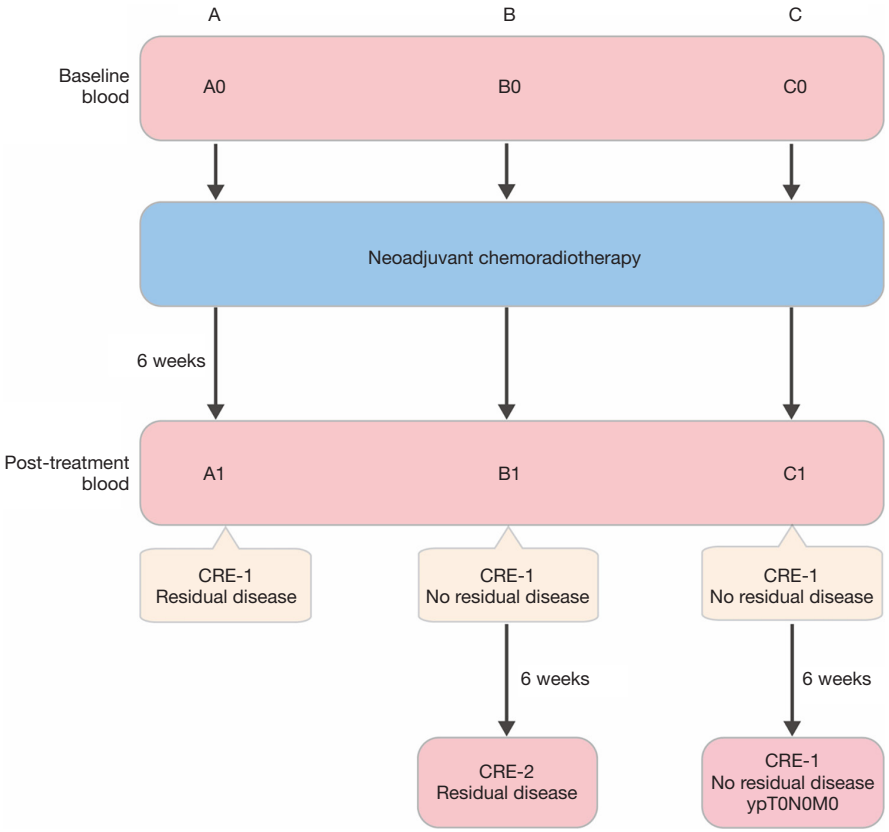

Figure 1 Study flowchart. For each patient, a blood sample was collected (time point 0) before nCRT. Clinical response was evaluated at 6 weeks after nCRT, and another blood sample was collected (time point 1). Subsequently, radical esophagectomy was performed. Three groups were created according to the pathologic results. For group A, the clinical assessment observed residual tumor, which was confirmed by the surgery. For group B, no residual tumor was observed after nCRT, but tumor progression was discovered after 6-week follow-up and was confirmed by surgery. For group C, complete tumor remission was achieved (ypT0N0M0). Five patients in each group were selected (with similar baseline information), and for each patient, 2 blood samples at different time points of the same patient were sent for exosome purification and sequencing. nCRT, neoadjuvant chemoradiotherapy; CRE, clinical response evaluation.

Figure 1. The CROSS regimen (i.e., 5 weekly cycles of carboplatin and paclitaxel with concurrent radiation) was used in nCRT. Previous study showed that roughly 40% of patients with ESCC have a pathologically complete response (pCR) in the resection specimen after nCRT (3). For these patients surgical resection might not be necessary. Hence, an active surveillance strategy has been proposed in which patients will undergo frequent clinical response evaluations. We conducted a second response to increase the diagnostic accuracy and therefore patients will be divided into three groups. For each patient, a blood sample was collected (time point 0) before nCRT. The clinical response was evaluated at 6 weeks after nCRT, and another blood sample was collected (time point 1). Subsequently, radical esophagectomy was performed. According to the pathologic results, patients were divided into 3 groups. For group A, the clinical assessment observed residual tumor, and this was confirmed by the surgery. For group B, no residual disease was observed after nCRT, but tumor progression was discovered after 6-week follow-up. For group C, complete tumor remission was achieved (ypT0N0M0). Five patients in each group were selected (with similar baseline information), and for each patient, 2 blood samples at different time points of the same patient were sent for exosome purification and sequencing.

Exosomal RNA purification

The exosomes were isolated from the blood plasma samples. Quality control were indicated with representative images of

transmission electron microscopy (Figure S1) and western blotting for positive markers of exosomes (Figure S2). The diluted serum specimens were centrifuged (500 g for 5 min and 2,000 g for 10 min). The supernatant was collected and centrifuged (10,000 g) for another 30 min. The pellet was filtered with a 0.22-μm filter and sent for ultracentrifugation (110,000 g for 90 min). The resuspended solution was later ultracentrifuged for another 90 min at 110,000 g. After removal of the supernatant, resuspension of the final pellet (with phosphate-buffered saline (PBS)] was later concentrated at 4,000 g using the MilliporeAmicon Ultra-15 Centrifugal Filter Unit. When the volume reached 100 μL, the process was stopped. The concentrated exosomes were placed at −80 ℃. During exosome analysis, the particle size of nanoparticle suspension was examined using scattered light detection. Then, the size and structure of exosomes were directly observed under transmission electron microscopy. Plasma exosomes were further confirmed with following protein markers under Western blotting: positive for CD9/TSG101/HSP70 and negative for calnexin. RNA was isolated using the RecoverAll Total Nucleic Acid Isolation Kit (Thermo Fisher, Inc. Waltham, MA, USA). Amplification and complement DNA (cDNA) labeling were performed using the SensationPlus FFPE Amplification Kit (Thermo Fisher Scientific) according to manufacturer's instructions.

Gene expression analysis

Raw reads in FastQ format were acquired using the Illumina HiSeq2500 platform. Clean data were obtained by removing low-quality reads and those containing adapter or ploy-N. For miRNA analysis, the filtered ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), and other ncRNA and repeats were removed. The remaining reads were used to detect known miRNAs and novel miRNAs as predicted via the comparison with the known miRNAs from miRBase. For lncRNA analysis, the HISAT2 software was used. Sequence alignment and subsequent analysis were performed based on the GRCh38 reference. After alignment, the reads were assembled using StringTie software. The mRNA, miRNA, and lncRNA expression profile were analyzed for differentially expressed genes (DEGs). According to the grouping information, the sample expression data were imported into the "DEGseq" R package (The R Foundation for Statistical Computing) to compare the expression differences between samples. The fold change (FC) and adjusted P value were obtained for each transcript. Then, according to the threshold of P value <0.05 and $|\log2(FC)| \geq 0.585$, the DEGs of each data set were acquired. Volcano plots were produced for DEGs and DE transcripts using the "ggplot2" R package. In particular, we paid attention to the DE mRNAs/lncRNAs and miRNAs with opposite change directions. The intersection of different sets was visualized in a Venn diagram. For each comparison, a heatmap of DEGs between 2 groups was drawn.

Target prediction of key miRNAs and screening of key oncogenes and tumor-suppressor genes

The mRNAs potentially targeted by key miRNAs were predicted using the miRWalk online tool (http://mirwalk.umm.uni-heidelberg.de/). The following pairs were specifically scrutinized: tumor-promoting miRNAs, tumor-suppressor mRNAs, tumor-suppressor miRNAs, and tumor-promoting mRNAs.

Enrichment analysis

Based on key oncogenes and tumor-suppressor genes, we used the Metascape tool to explore the enriched Gene Ontology (GO) terms, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, Reactome sets, canonical pathways, TRRUST sets, and enriched transcription factor-targets. Terms with a P value <0.05, a minimum count of 3, and an enrichment factor >1.5 were collected.

Protein-protein interaction (PPI) analysis

Using all the key genes, PPI enrichment analysis was conducted based on Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; https://string-db.org/). All interactions except text mining (physical score >0.14) were used to establish the PPI network.

Results

Remission- and progression-associated DEGs (mRNAs)

First, we compared the exosome transcriptome at 2 time points (before and after nCRT) in 3 cohorts (n=5 per group). In group A, there were 73 upregulated mRNAs and 184 downregulated mRNAs (*Figure 2A*); in group B, there were 68 upregulated and 26 downregulated ones (*Figure 2B*); in group C, there were 118 upregulated and 26 down-regulated ones (*Figure 2C*). The intersection of

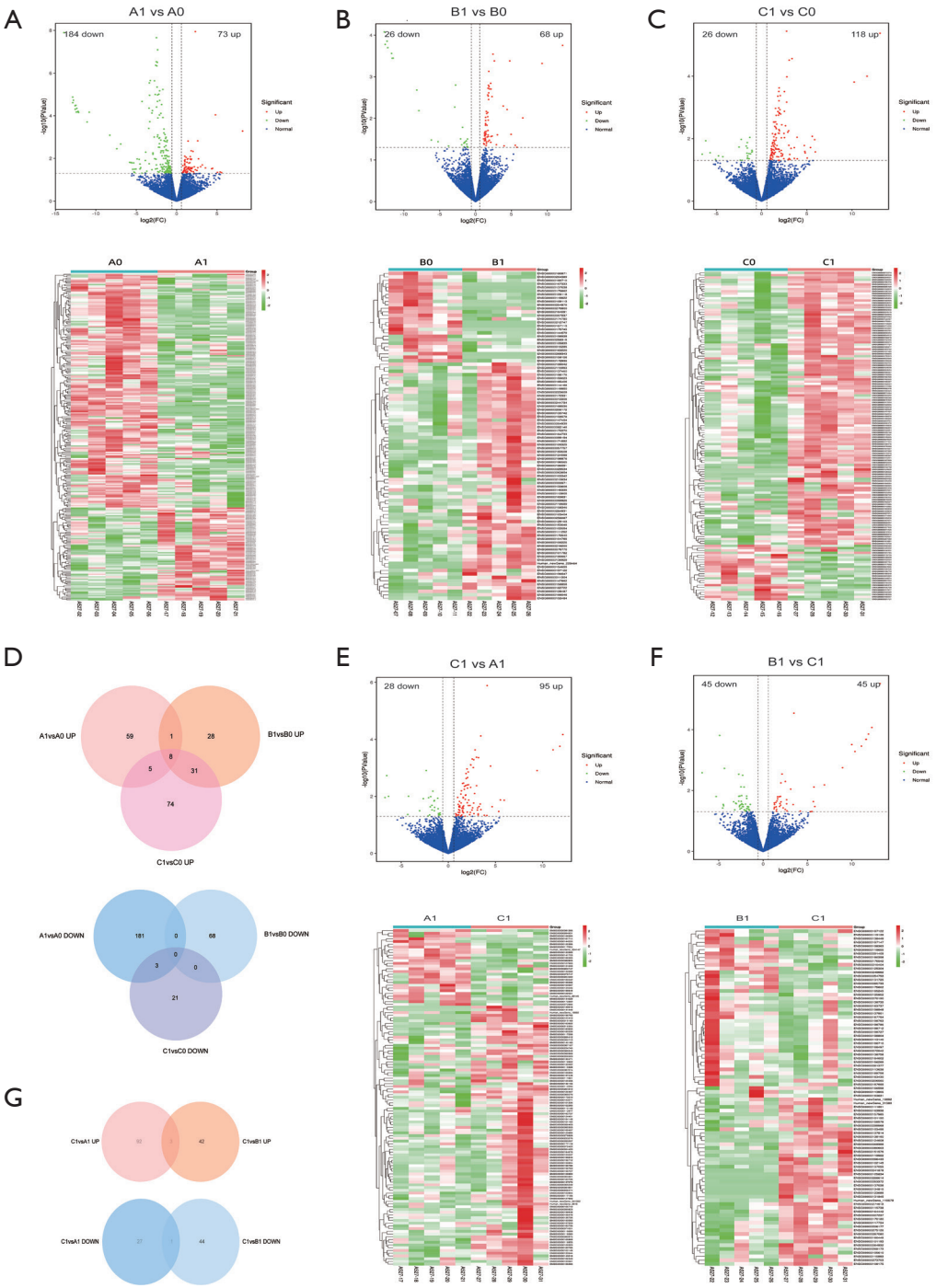

Figure 2 Remission- and progression-associated DEGs (mRNAs) compared in 2 time points and 2 groups. (A) The volcano plots and heatmap of DE-mRNAs in group A. (B) The volcano plots and heatmap of DE-mRNAs in group B. (C) The volcano plots and heatmap of DE-mRNAs in group C. (D) Venn diagram of the intersection of upregulated and downregulated genes. (E) The volcano plots and heatmap of DE-mRNAs in the comparison of C1 *vs.* A1. (F) The volcano plots and heatmap of DE-mRNAs in the comparison of C1 *vs.* B1. (G) Left: The intersection of C1-A1 upregulation and C1-B1 upregulation included 3 genes (named CvAB-Up) which may be associated with remission; Right: The intersection of C1-A1 downregulation and C1-B1 downregulation included 1 gene (named CvAB-Down), which may be associated with progression. FC, fold change; DEG, differentially expressed gene; mRNA, messenger RNA; DE-mRNA, differentially expressed mRNA.

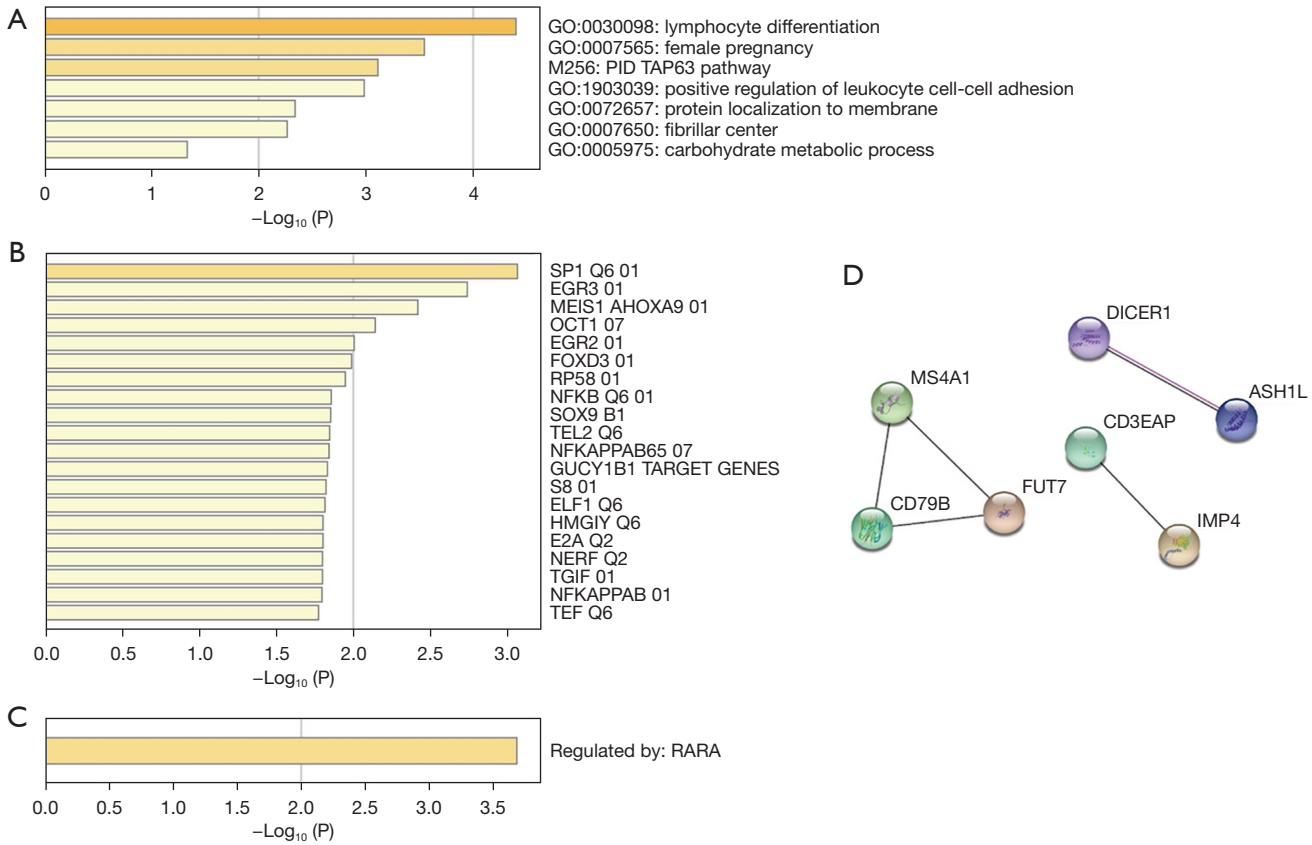

Figure 3 The enrichment analysis of ESCC progression-associated genes. (A) Top enriched GO/KEGG/Reactome terms. (B) The transcription factor-target enrichment. (C) The TRRUST enrichment. (D) The PPI network. ESCC, esophageal squamous cell carcinoma; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction.

upregulated and downregulated genes was visualized in a Venn diagram (*Figure 2D*). Theoretically, the 74 unique upregulated genes in the C1 *vs.* C0 comparison (but not B1 *vs.* B0 or A1 *vs.* A0) were associated with nCRT response or tumor remission (*Figure 2D*, named only-C-Up set); the unique downregulated genes in the C1 *vs.* C0 comparison (but not B1 *vs.* B0 or A1 *vs.* A0) were associated with ESCC progression (*Figure 2D*, named only-C-Down set). Among the common genes between A1-A0 DEGs and B1-B0 DEGs, the unique upregulated gene (not among C1-C0 DEGs) appeared to be associated with ESCC progression (*Figure 2D*, named only-AB), while the unique downregulated gene (not among C1-C0 DEGs) appeared to be associated with ESCC remission (*Figure 2D*, named only-AB-Down set). The significance of these 4 sets is particularly important in nCRT treatment.

Next, at the second time point, the DEGs of C1 *vs.* A1 and C1 *vs.* B1 were acquired, including 95 C1-A1 upregulated (*Figure 2E*) and 45 C1-B1 upregulated (*Figure 2F*) DEGs. The intersection of C1-A1 upregulation and C1-B1 upregulation included 3 genes (*Figure 2G*, named CvsAB-Up) potentially associated with remission, and the intersection of C1-A1 downregulation and C1-B1 downregulation included 1 gene (*Figure 2G*, named CvsAB-Down) potentially associated with progression. Overall, ESCC remission was associated with 3 sets: only-C-Up, only-AB-Down, and CvAB-Up, while ESCC progression was associated with another 3 sets: only-C-Down, only-AB-Up, and CvAB-Down.

Enrichment analysis of ESCC progression and remission genes

In the progression gene enrichment, we removed unknown genes and input the remaining 23 genes. In GO enrichment (*Figure 3A*), the following terms are enriched: lymphocyte differentiation, female pregnancy, and positive regulation of leukocyte cell-cell adhesion, among others. In enriched

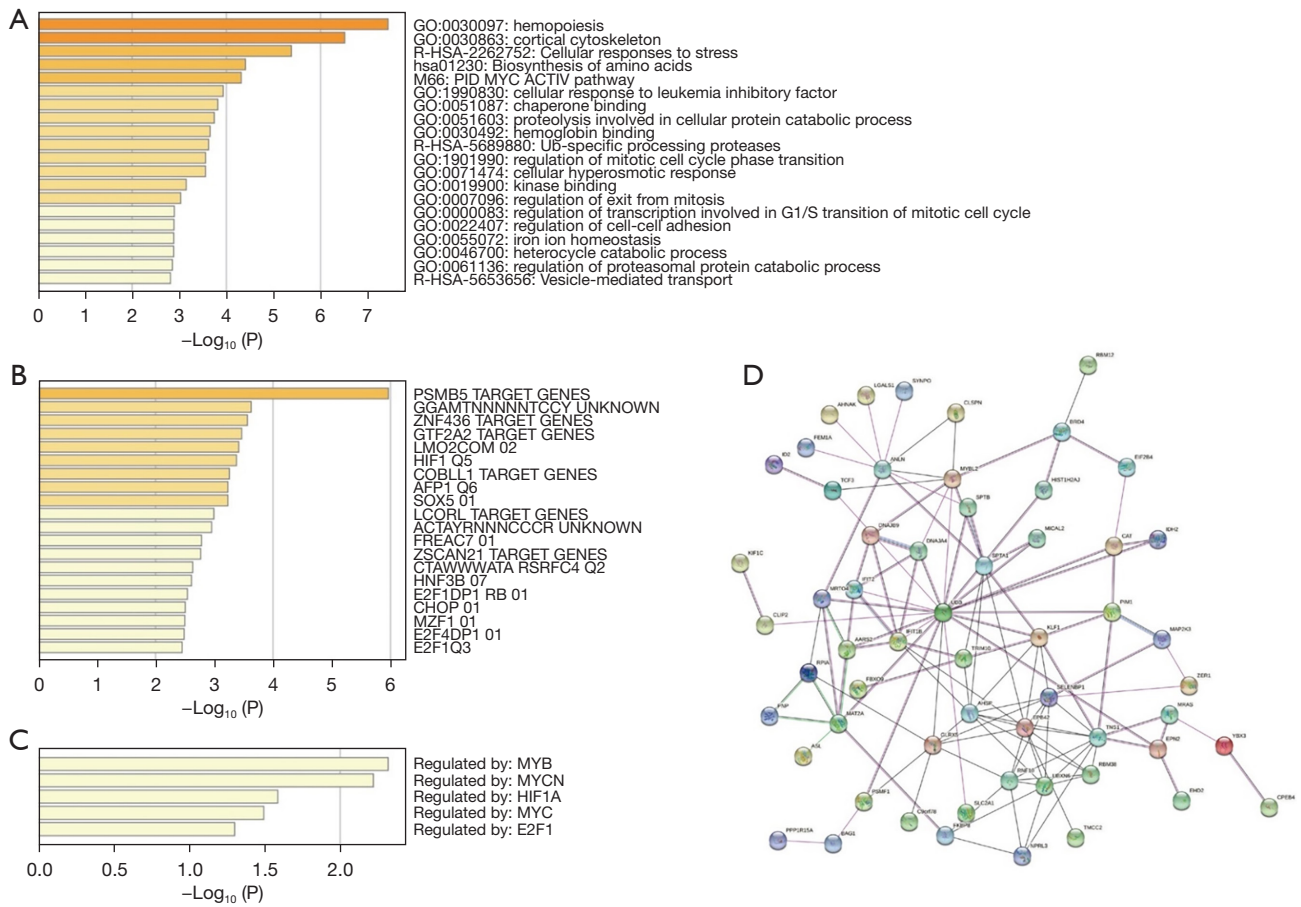

Figure 4 The enrichment analysis of ESCC remission-associated genes. (A) The top enriched GO, KEGG, and Reactome terms. (B) The transcription factor-target enrichment. (C) The TRRUST enrichment. (D) The PPI network. ESCC, esophageal squamous cell carcinoma; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction.

the canonical pathways, the *TAP63* pathway was enriched. In the transcription-factor targets, the following factors appeared enriched: *SP1*, *EGR3*, *MEIS1AHOXA9*, *OCT1*, *EGR2*, *FOXD3*, *RP58*, *NFKB*, and *SOX9*, among others (*Figure 3B*). The TRRUST enrichment showed that *RARA* may modulate these progression genes (*Figure 3C*). Based on these 23 genes, a PPI network showed that *MS4A1*, *CD79B*, *FUT7*, *CD3EAP*, *IMP4*, *DICER1*, and *ASH1L* were located at the key positions, which indicated the crucial role of these genes in ESCC progression (*Figure 3D*).

Similarly, 76 remission-associated genes were enriched in many GO terms, including in hemopoiesis, cortical cytoskeleton, and cellular response to leukemia inhibitory factor (*Figure 4A*). Reactome analysis also indicated that up-specific processing proteases, *RUNX1* regulates transcription of genes involved in differentiation of hematopoietic stem cells (HSCs), vesicle-mediated transport, and others, were enriched. Meanwhile, biosynthesis of amino acids and carbon metabolism were the enriched KEGG pathways. In the transcription factor-target analysis, the following factors were enriched: *PSMB5*, *ZNF436*, *GTF2A2*, *COBLL1*, *AFP1*, and *SOX5*, among others (*Figure 4B*); and remission associated genes appeared to be largely regulated by *MYB*, *MYCN*, *HIF1A*, *MYC*, and *E2F1* (*Figure 4C*). The PPI network of the remission-associated genes is shown as *Figure 4D*, in which *UBB*, *IFIT1B*, *AHSP*, *MAT2A*, *SELENBP1*, *SPTA1*, and *TNS1* appear as the hub genes. Taken together, these results showed that the filtered hub genes play an important role in tumorigenesis and development.

Remission and progression associated miRNAs

The DE miRNAs were analyzed in the same protocol. The altered miRNAs in A1 *vs.* A0, B1 *vs.* B0, and C1 *vs.* C0

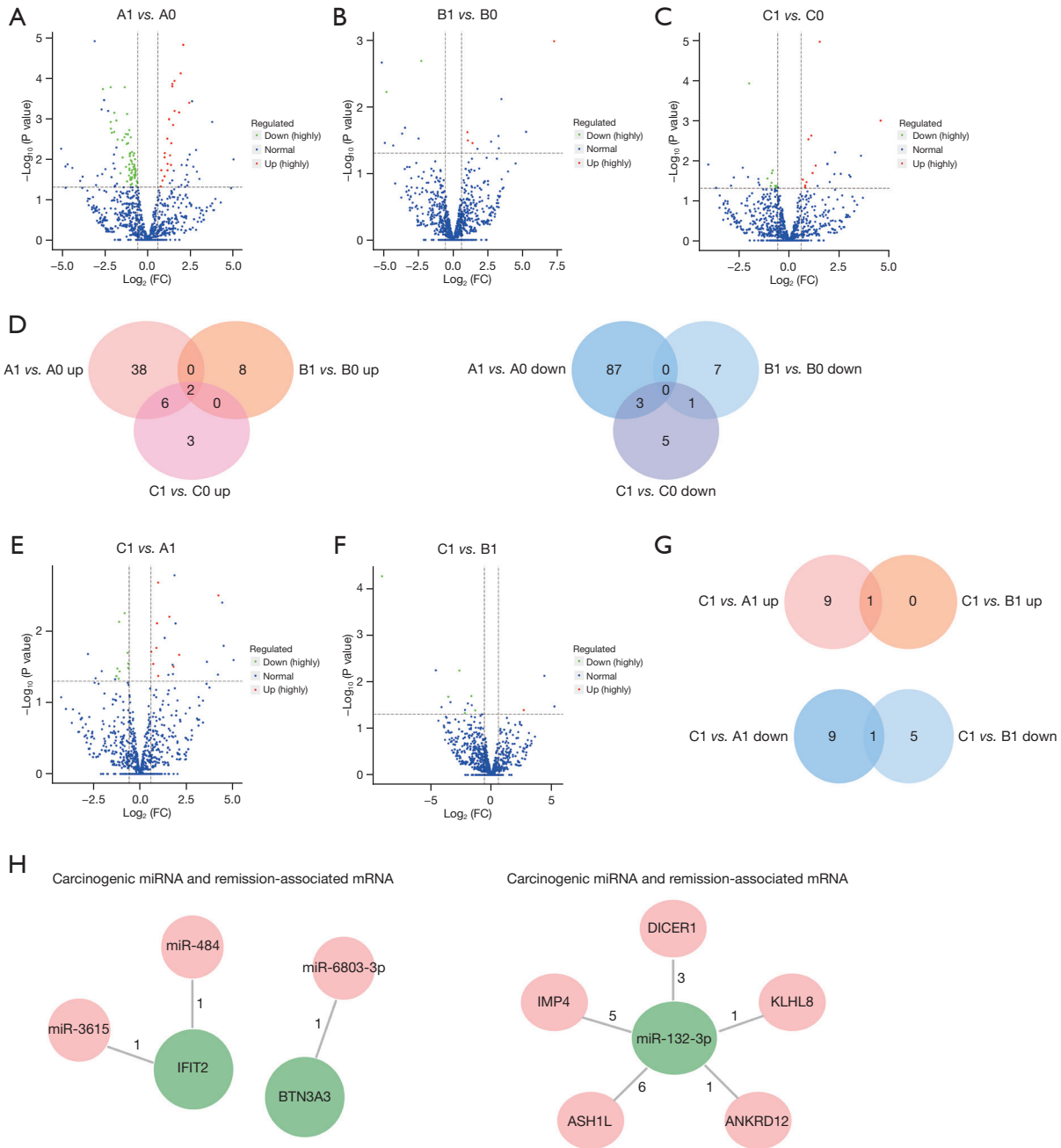

Figure 5 The remission- and progression-associated DE-miRNAs and the key miRNA-mRNA pairs. (A) The volcano plots of DE-miRNAs in group A. (B) The volcano plots of DE-miRNAs in group B. (C) The volcano plots of DE-miRNAs in group C. (D) Left: The intersection of upregulated genes in time point 1 *vs.* time point 0; Right: The intersection of downregulated genes in time point 1 *vs.* time point 0. (E) The volcano plots of DE-miRNAs in the comparison of C1 *vs.* A1. (F) The volcano plots of DE-miRNAs in the comparison of C1 *vs.* B1. (G) Upper: the intersection of the C1-A1 upregulation and C1-B1 upregulation sets; Lower: the intersection of the C1-A1 downregulation and C1-B1 downregulation sets. (H) The key miRNA-mRNA pairs. Left: the intersection between the remission-associated mRNAs and the targets of progression-associated (carcinogenic) miRNAs. Two miRNA-mRNA networks were identified: IFIT2-miR-3615-IFIT2-miR-484 and BTN3A3-miR-6803-3p. Right: based on the intersection of progression-associated (carcinogenic) mRNAs and the targets of remission associated miRNAs, a network with miR-132-3p (remission-associated miRNA) was located at the core. FC, fold change; miRNA, microRNA; DE-miRNA, differentially expressed miRNA.

are shown in *Figure 5A-5D*. In *Figure 5E-5G*, there are 3 miRNAs in the only-C-Up set and 5 miRNAs in the only-C-Down set (hsa-miR-92b-3p, hsa-miR-6803-3p, hsa-miR-484, hsa-miR-941, and hsa-miR-3615 are within the only-C-Down set; hsa-miR-122-5p is within the CvsAB-Down set; hsa-miR-335-5p and hsa-miR-132-3p are within the only-C-Up set; and there are no known miRNAs in the only-AB-Up, only-AB-Down, or CvsAB-Up sets).

Key miRNA-mRNA pairs

The targets of remission- and progression-associated miRNAs were then screened. Between the remission-associated mRNA and the targets of progression-associated (carcinogenic) miRNAs, the intersection was acquired, and 2 miRNA-mRNA networks were identified (*Figure 5H*): *IFIT2*-miR-3615-*IFIT2*-miR-484 and *BTN3A3*-miR-6803-3p. Meanwhile, we screened the intersection of progression-associated (carcinogenic) mRNAs and the targets of remission-associated miRNAs. Based on network analysis, miR-132-3p (remission-associated miRNA) was located at the core and matched with *DICER1*, *KLHL8*, *ANKRD12*, *ASH1L*, and *IMP4*.

Remission- and progression-associated lncRNAs

Finally, the DE lncRNAs were analyzed. There were 80 lncRNAs in the only-C-Up set, 2 lncRNAs in the only-AB-Down set, and 53 lncRNAs in the only-C-Down set (*Figure 6A-6E*). Additionally, there were 61 C1-A1 upregulated lncRNAs and 60 C1-B1 upregulated lncRNAs, between which 7 DE lncRNAs were shared (*Figure 6F,6G*). Meanwhile, we discovered 76 C1-A1 downregulated lncRNAs and 61 C1-B1 downregulated lncRNAs, between which 12 lncRNAs were shared (*Figure 6H,6I*). For these remission- and progression-associated lncRNAs, we screened all the cis-target genes and trans-target genes, but no common ones were found with the remission- and progression-associated mRNAs. Finally, the lncRNA targets of key miRNAs (as mentioned above) were collected, but no common ones were shared with these remission- and progression-associated lncRNAs.

Discussion

The significant transcriptomes associated with ESCC have been widely investigated through high-throughput approaches. For example, known ESCC-related miRNAs include miR-23b-5p, miR-877-3p, and miR-17-92, among others (13-16). Several published works demonstrated that chemotherapy resistance can be affected by mRNAs like *TUG1*, *ZFX*, and *NRF2*, and miRNAs like miR-130a-3p, miR-125a-5p, and miR-224 (17-21). However, the key prognostic molecules in the therapeutic mechanism of ESCC, especially in nCRT, still need to be investigated. In this study, we used transcriptome analysis to assess the global signature of exo-RNAs and identified distinct exo-RNA signatures that may effectively predict treatment outcomes of nCRT. Here, *CCDC85B* (only-AB-Up) and stearoyl-CoA desaturase (*SCD*) (CvsAB-Down) were found to be 2 important progression-associated mRNAs, while *IFIT2* was found to be a key remission-associated mRNA (CvsAB-Up). Additionally, several important miRNA-mRNA networks were identified: miR-484-*IFIT2*, miR-3615-*IFIT2*, miR-6803-3p-*BTN3A3*, and miR-132-3p-*DICER1-KLHL8-ANKRD12-ASH1L-IMP4*. All these findings are highly novel, as the roles of these molecules in the development of ESCC and treatment response to nCRT are completely unknown or not well studied.

In colorectal cancer, *CCDC85B* (also known as *DIPA*) is a carcinogenetic gene (22). It could be found on the centrosome and directly interacts with p78 and influences malignant transformation by modulating gene transcription (23). It has been reported that *CCDC85B* is a master regulator that can transform the cellular state of fast-growing subtype cells into the slow-growing subtype (24). *SCD* is a member of the de novo fatty-acid synthesis pathway (25), and it has been associated with esophageal cancer (26). Additionally, we found *IFIT2* to be a key remission-associated mRNA, and the consistent result of the miR-484-*IFIT2* axis was a particularly striking finding in this work. Currently, the direct association between ESCC and *IFIT2* has not been clearly reported. *IFIT2* (interferon-induced protein with tetratricopeptide repeats 2) enables RNA-binding activity, and it is involved in the negative regulation of protein-binding activity, positive regulation of apoptotic process, and response to virus. In osteosarcoma cells, it is a cell death-promoting factor and can augment cisplatin-induced apoptosis (27), which is in line with our result. Similarly, lncRNA LINC00161 was reported to sensitize osteosarcoma cells to cisplatin-induced apoptosis by regulating the miR-645-*IFIT2* axis (27). Indeed, interferon signaling is generally associated with tumor apoptosis or remission (28). A microarray study showed that the expression of *IFIT2* is upregulated in ESCC (29). Therefore, it is reasonable to hypothesize that a high expression of *IFIT2* plays a role in the anti-ESCC process. The role of *BTN3A3*

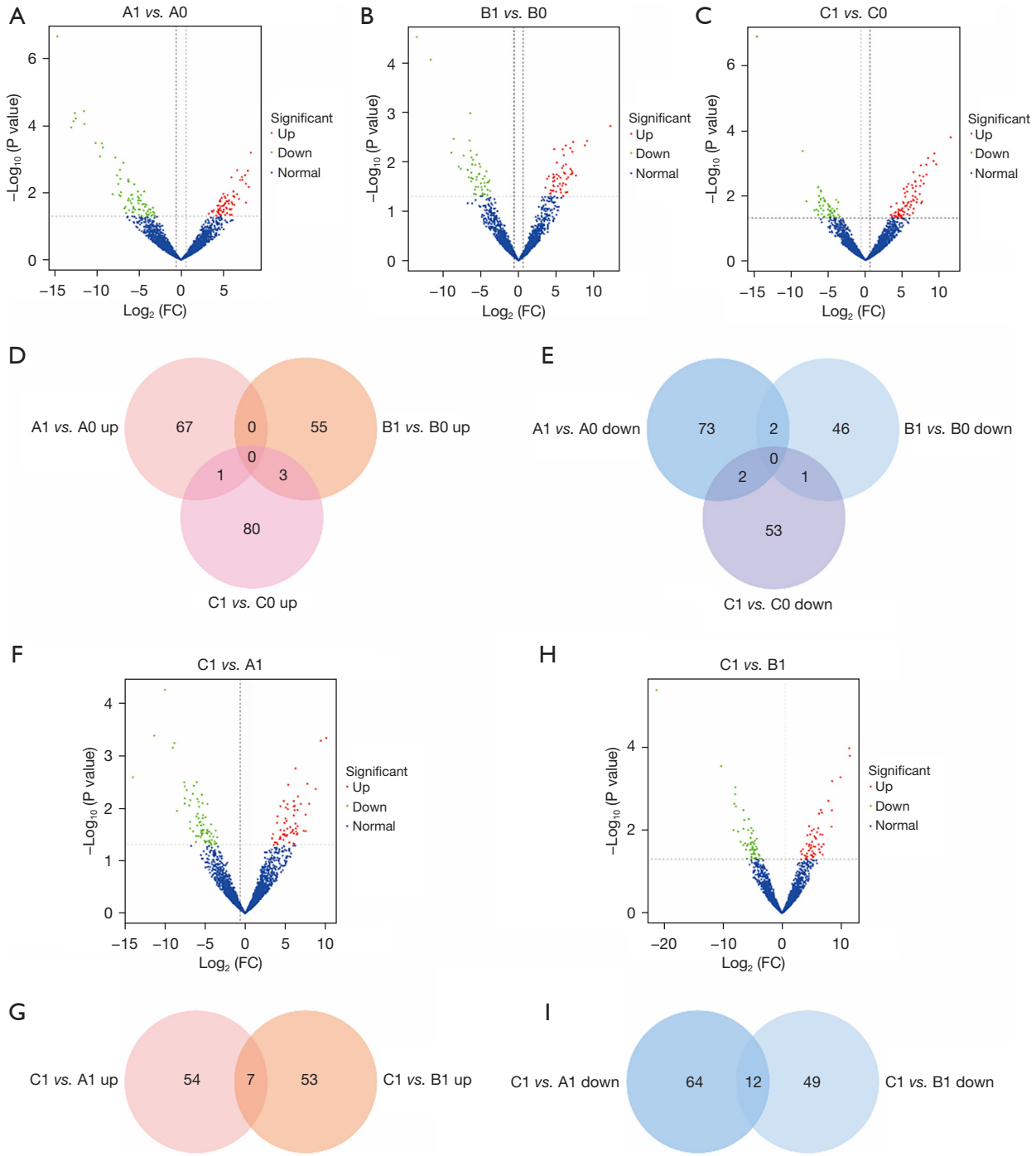

Figure 6 Remission- and progression-associated DE lncRNAs. (A) The volcano plots of DE lncRNAs in group A. (B) The volcano plots of DE lncRNAs in group B. (C) The volcano plots of DE lncRNAs in group C. (D) The intersection of upregulated genes in time point 1 *vs.* time point 0. (E) The intersection of downregulated genes in time point 1 *vs.* time point 0. (F) The volcano plots of DE lncRNAs in the comparison of C1 *vs.* A1. (G) The intersection of the C1-A1 upregulation and C1-B1 upregulation sets. (H) The volcano plots of DE lncRNAs in the comparison of C1 *vs.* B1. (I) The intersection of the C1-A1 downregulation and C1-B1 downregulation sets. FC, fold change; DE lncRNA, differentially expressed lncRNA; lncRNA, long noncoding RNA.

as a remission-associated mRNA in ESCC was another novel finding, and similar roles in tumor research have been reported. *BTN3A3* is expected to become a potential therapeutic target for breast cancer (30), and the upregulation of *BTN3A3* has been significantly correlated with better overall and relapse-free survival (31). Moreover, it has been noted to be a novel prognostic factor for hepatocellular carcinoma (32). In non-small cell lung cancer, knocking down *BTN3A3* was shown to promote cell proliferation, migration, and invasion, and patients with a low expression of *BTN3A3* have poor survival (33). A potential mechanism of its anticancer effect is that the *BTN3A* family proteins might be involved in the activation and proliferation of γδ T cells in the tumor microenvironment (34). Collectively, *IFIT2*, *BTN3A*, *CCDC85B*, *SCD*, and the hub genes in the remission/progression PPI networks (i.e., *PSMB5*, *ZNF436*, *GTF2A2*, *COBLL1*, *AFP1*, *SOX5*, *MS4A1*, *CD79B*, *FUT7*, *CD3EAP*, *IMP4*, *DICER1*, and *ASH1L*) are promising targets for ESCC treatment.

In this study, 4 miRNAs were found to be especially important: miR-484, miR-3615, miR-6803-3p, and miR-132-3p. Recently, miRNA expression profiling for the prediction of resistance to nCRT in ESCC has been reported (35), with miR-484 being reported to be an anti-ESCC marker. However, it is a carcinogenic miRNA, as shown in *Figure 5H*. Another study based on The Cancer Genome Atlas (TCGA) samples established a novel lncRNA panel for the prognosis of ESCC according to the competing endogenous RNA (ceRNA) mechanism (36). It was found that miR-3615 (another carcinogenic miRNA in our study) was positively correlated with patients' overall survival. However, the association between miR-6803-3p or miR-132-3p and ESCC has not thus far been established. However, miR-132-3p does play an antitumoral role in bladder cancer, colorectal cancer, and osteosarcoma progression (37-39). In subsequent studies on ESCC, the roles of these 2 miRNAs warrant further attention.

This work has some limitations. First, we included only 15 patients and 3 groups, which may represent an insufficiently large sample to construct a prognostic model based on the correlations found in this study. Future studies will focus on the above key progression and remission markers in exo-RNAs and develop novel models to predict the treatment response to nCRT. Second, the lncRNA results showed no meaningful links to the miRNA-mRNA axes, either in the ceRNA networks or cis-regulatory networks. Our subsequent research will endeavor to clarify the molecular mechanisms, specifically, the miRNA precursor mechanism. Third, the difference in findings concerning the functions related to miR-484 and miR-3615 between our study and other works needs be further examined. Finally, *in vitro* and *in vivo* experiments are needed in the next research plan to validate the representative genes.

Conclusions

We identified the significantly altered plasma exo-RNAs among outcome groups and between different time points of nCRT, and clarified the corresponding enrichment and regulatory networks. They may serve as minimally invasive predictors of the progression and remission of patients with ESCC. In particular, several genes were found to have previously unknown roles in ESCC treatment, including *IFIT2*, *BTN3A*, *CCDC85B*, *SCD*, miR-484, miR-3615, miR-6803-3p, and miR-132-3p. The novel candidates in this study merit further investigation and may provide a deep understanding of the molecular mechanisms. Studies with a larger cohort of patients are needed to confirm the potential use of exosome transcriptome miRNAs as predictive biomarkers of the treatment response to nCRT.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-23-452/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-23-452/dss

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-23-452/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the institution ethics committee of Shanghai Chest Hospital (No. KS2160). All patients provided written informed consent for the use of blood and specimens.

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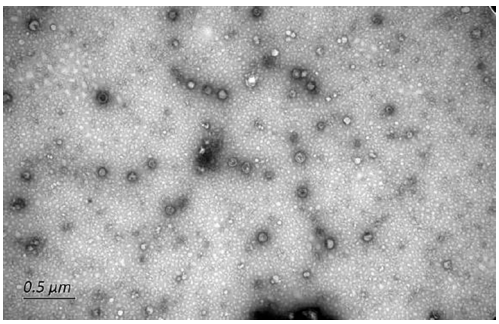

Figure S1 Representative image of transmission electron microscopy.

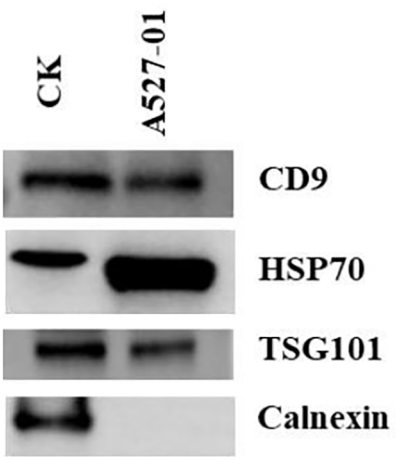

Figure S2 Representative image of western blotting. CD9, TSG101 and HSP70 are positive markers for exosomes, while Calnexin is considered as negative marker.

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