Dynamic plasma extracellular vesicle long RNA profiling changes during the peri-operative period

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Primary research

Keywords: extracellular vesicles, long RNA sequencing, peri-operative period, colorectal cancer

Posted Date: August 4th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-49983/v1

Abstract

Background: Surgery remains the most important treatment strategy for solid tumors, such as colorectal cancer (CRC); However, a number of studies have suggested that surgical stress contributes to tumor recurrence or distant metastases. Extracellular vesicles (EVs), which contain a rich variety of RNAs with specialized functions and clinical applications, have been shown to be an indicator for diagnosis and prognosis of cancers. The effect of surgical stress on the landscape and characteristics of EV long RNA (exLR) in human blood, however, remains largely unknown.

Methods: We present an optimized strategy for exLR sequencing (exLR-seq) the plasma from three patients with CRC at 4 time points (before surgery [T0], after extubation [T1], 1 day after surgery [T2], and 3 days after surgery [T4]). The "Limma" R package was used to evaluate the dynamic changes of mRNAs and long non-coding (lnc)RNAs from EVs. We also constructed a protein–protein interaction (PPI) network of hub genes and predicted biological processes, cellular components, and molecular functions of gene ontology (GO) functional analysis and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway.

Results: We observed a sufficient number of exLRs, including 12,924 mRNAs and 2196 lncRNAs. Both mRNAs and lncRNAs underwent dynamic changes during the peri-operative period. Compared with T0, there were 110 mRNAs differentially expressed after extubation, 60 differentially expressed genes□DEGs□ 1 day after surgery, and 50 DEGs 3 days after surgery. A total of 11 genes changed at all 3 time points and were related to regulation of the membrane potential, receptor complex, and passive transmembrane transporter activity. In addition, 22 lncRNAs were differentially expressed after extubation (T1). Nineteen lncRNAs were differentially expressed between T0 and T2, and 38 lncRNAs were differentially expressed between T0 and T3. In addition, we found that only 3 lncRNAs changed at 3 time points. Interestingly, blood exLRs reflected the tissue origins and relative fractions of different immune cell types. EVs from CD8+ T□CD4+ memory T, and NK cells decreased after surgery and the absolute quality of EVs from immune cells decreased as well.

Conclusion: In summary, this study demonstrated abundant exLRs in human plasma and the dynamic changes of these exLRs and exLRs originating from CD8+ T and CD4+ memory T cells were reduced during the peri-operative period.

Introduction

Colorectal cancer (CRC) has a high prevalence and mortality,

accounting for 861,663 deaths worldwide in 2018[1]. According to statistics from 2020[2], approximately 147,950 new cases of CRC will be diagnosed in the United States in 2020, making CRC the third most common malignancy in the world.

Despite the rapid development of advanced chemotherapy and targeted therapy and immunotherapy during the past 2 decades, surgical resection remains the mainstay for the treatment of CRC[3-5]. Previous studies have demonstrated that surgical trauma influences long-term oncologic outcome by facilitating metastasis and recurrence of tumors[6, 7]. A variety of factors participate in the metastasis and recurrence of primary tumors after surgery, such as dissemination of tumor cells, drugs used in anesthetic and analgesic procedures, destruction of the extracellular matrix, release of vascular endothelial growth factor (VEGF), and post-operative immunosuppression[8].

Extracellular vesicles (EVs [exosomes and microvesicles]) are lipid bilayer-enclosed, nanosized endocytic vesicles that are secreted by most cell types[9, 10]. EVs contain various bioactive molecules, such as proteins (enzymes, extracellular matrix proteins, transcription factors, and receptors), nucleic acids (DNA and RNA), and lipids which can modify the function of a recipient cell[11]. Recent studies have begun to characterize the abundant EV cargoes.

It has been shown that EV long RNAs (exLRs), including circular RNA (circRNA), long non-coding RNA (lncRNA), and messenger RNA(mRNA), are in human plasma EVs and play a vital role in the progression of tumor development[12-15]. For example, 12.Nabet *et al.[14]* reported that an unshielded exosome (RNA RN7SL1) acts as a damage-associated molecular pattern (DAMP) to activate the pattern recognition receptor, RIG-I, to drive anti-viral signaling when transferred to recipient breast cancer cells via an exosome that ultimately leads to tumor growth and therapy resistance. The CD274 mRNA in plasma-derived EVs is related to the response to anti-PD-1 antibodies in melanoma and non-small cell lung cancer[15]. These results reveal the potential of exLRs to be applied in the diagnosis of human cancers and cell-to-cell mediators of metastasis.

EVs contain a large number of valuable and functional exLRs[16, 17]. In the current study, exLRs sequencing (exLR-seq) was performed on plasma samples collected from 3 CRC patients at 4 specific time points (before surgery [T1], after extubation [T2], 1 day after surgery [T3], and 3 days after surgery [T4]) and produced a catalog of blood exLRs, which includes sufficient species of mRNA and lncRNA to investigate the potential role for the progression of CRC to detect the effects of surgical stress on the exLR expression profile.

Materials And Methods

Patient specimens and clinical assessments

The present study recruited 3 paired CRC patients, all of whom underwent surgery at Fudan University Shanghai Cancer Center by the same surgeon. All the participants were histologically confirmed to have colorectal adenocarcinoma (stage II [AJCC Cancer Staging Manual]) and did not receive any other forms of therapy on the time of enrollment. This study was pre-approved by the Ethics Committee of Fudan University Shanghai Cancer Center and informed written consent was obtained from all patients.

Isolation of plasma from blood

Peripheral blood samples were collected from 3 CRC patients at 4 times (before surgery, after extubation, 1 day after surgery, and 3 days after surgery) in 10 mL of EDTA-coated vacutainer tubes. Plasma was then separated by centrifugation at 3000 rpm (~800 × g) for 10 min at 25°C within 2 h after blood collection, and then centrifuged at 13,000 rpm (~16 000 × g) for 10 min at 4°C to remove debris. The plasma samples were then stored at −80°C until use.

Isolation of EVs and EV RNA

For every patient, 1 mL of plasma was used, and EVs were isolated by affinity-based binding to spin columns via an exoRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Briefly, melted plasma was mixed with binding buffer and added to the exoEasy membrane affinity spin column. For transmission electron microscopy (TEM), size distribution measurement and western blotting (see online Supplementary Methods), the EVs were eluted with 400 µL of XE elution buffer. To reduce the eluate volume to 50 µL and exchange buffer with phosphate buffer saline (PBS), samples were subjected to ultrafiltration using Amicon Ultra-0.5 Centrifugal Filter 10 kDa (Merck Millipore,Germany). For EV RNA isolation, EVs were lysed on the column using QIAzol (Qiagen), and total RNA was then eluted and purified.

ExLR-seq analysis

The strategy for exLR sequencing (exLR-seq) analysis includes plasma preparation, isolation of EV and EV RNAs, RNA-seq library construction, sequencing, and data analysis. Briefly, to remove DNA, total EV RNA isolated from 1 mL of plasma was treated with DNase I (NEB; Ipswich, Massachusetts, USA). RNA-seq libraries were generated using SMART technology (Clontech). The sequencing reads were aligned using HISAT2. Annotations of mRNA and lncRNA were retrieved from the GENCODE database.

Identification of differentially expressed mRNAs and lncRNAs

Transcriptional profiles of EVs from 3 CRC patients were evaluated during the peri-operative period (before surgery [T0], after extubation [T1], 1 day after surgery [T2], and 3 days after surgery [T3]). Significant differentially expressed genes (DEGs) and differentially-expressed lncRNAs (DELRs) were identified using the Limma R package (version 3.6.3) with a FDR < 0.05 and a |logFC| > 0.5.

Functional enrichment analysis of DEGs and DELRs

The intersective hub genes during the peri-operative period were selected for further analyses using a Venn diagram. A protein–protein interaction (PPI) network of hub genes was constructed using GeneMANIA (http://genemania.org/). Biological processes, cellular components, and molecular function of gene ontology (GO) functional analysis and Kyoto encyclopedia genes and genomes (KEGG) pathway were predicted using the Web-based Gene seT AnaLysis Toolkit (WebGestalt [http://www.webgestalt.org/]) and visualized using R software.

Data and statistical analyses

All statistical analyses were 2-sided. A |logFC| > 0.5 and p < 0.05 were considered statistically significant. The following R software packages were used in this study: e1071, glmnet, varSelRF, pROC, and caret.

Results

Human EV isolation

As shown in Figure 1, the isolated vesicles in plasma were cup-shaped, rounded, double membrane-bound vesicle-like (Fig. 1A), and flow cytometry exhibited a heterogeneous population of spherical nanoparticles, with abundant peaks ranging from 50–200 nm (Fig. 1B). In addition, western blot analysis revealed characteristic exosomal marker (CD63 and TSG101) expression in isolated vesicles, but not in peripheral blood mononuclear cells (PBMCs). Calnexin, which is an intracellularly enriched protein in PBMCs and often used as a negative-control protein marker for EV identification, was detected in PBMCs, but not in isolated vesicles (Fig. 1C).

Dynamic changes of mRNAs in EVs before and after surgery

ExLR-seq was conducted using plasma samples from 3 CRC patients.

Approximately 12,924 mRNAs and 2196 lncRNAs were reliably detected in each sample. The exLR profiles of CRC patients after surgery generally differed from the exLR profiles before surgery. We identified 110 mRNAs that were differentially expressed after extubation compared with before surgery using the “Limma” R package (|logFC|>0.5, p<0.05; Fig. 2A). In addition, compared with before surgery, there were 60 differentially expressed genes) DEGs 1 day after surgery（Fig. 2B） and 50 DEGs（Fig. 2C） 3 days after surgery. Taking the intersection of the 3 groups of DEGs revealed that a total of 11 genes changed at 3 time points（Fig. 2D).

Functional annotations of mRNAs in EVs

Next, we determined the functional annotations of mRNAs in EVs. As shown in Figure 3A, 9 of the 11 DEGs were involved in the biological regulation process, 9 were membrane components, and 6 were protein-binding mRNAs (Fig. 3A). To further determine the interactions among the 11 DEGs, the protein–protein interaction network was used (Fig. 3B). In addition, the 11 hub genes most significantly involved in changed GO functions, including regulation of the membrane potential, receptor complex, and passive transmembrane transporter activity (Fig. 3C). KEGG pathway analysis revealed that differentially-expressed mRNAs were enriched for some pathways involved in nicotinic acetylcholine receptors activities, Tie2 signaling, other semaphoring interactions, and choline and glycerolipid metabolism (Fig. 3D).

Dynamic changes of lncRNAs in EVs before and after surgery

Except for mRNAs, we detected approximately 2200 lncRNAs. As shown in Figure 4A, we identified 22 lncRNAs that were differentially expressed after extubation (T1) compared with before surgery (T0;

|logFC| > 0.5; p < 0.05). In addition, 19 lncRNAs differentially expressed between T0 and T2 (Fig. 4B) and 38 lncRNAs were differentially expressed between T0 and T3 (Fig. 4C). In addition, we found that only 3 lncRNAs (C15orf54, RP11-446N19.1, and RP11-87H9.4) changed at all 3 time points (Fig. 4D).

Cell source analysis of EVs

Because blood EVs are derived from a variety of tissues, we used the xCell tool (http://xcell.ucsf.edu) to characterize the proportions of cell types derived from EVs. We identified 67 immune and stroma cell types and evaluated the correlation of immune and stroma cell expression (Fig. 5A). We also compared the dynamic changes of the origination of EVs (Fig. 5B). Only a few cell types changed during the peri-operative period. Specifically, EVs derived from platelets were gradually reduced after surgery. Clinically, the immunosuppressive microenvironment caused by surgery can lead to tumor metastases and recurrence and the role of EVs in immune regulation has been intensively studied [18, 19]. The release of EVs carrying immunomodulatory molecules by various immune cells upon infection can influence primary and secondary immune responses. Therefore, we further investigated the changes in immune cells derived from EVs before and after surgery. As shown in Figure 5C, the total number of immune cells derived from EVs decreased after surgery. EVs derived from CD8+ T、CD4+ memory T, and natural killer (NK) cells decreased as well (Fig. 5C).

Discussion

Recently, EVs have attracted more and more interest as a means to analyze the molecular pathways involved in cancer diagnosis and progression. Studies of EVs have expanded rapidly, but few studies have compared changes in EVs before and after surgery. Our results showed a large variety of exLRs in human blood. Given the high abundance and heterogeneity of blood exLRs, we intended to find the differences in exLRs before and after surgery and the influence on CRC progression and prognosis.

To obtain reliable exLR-seq data, it is critical to pay attention to the

isolation of plasma, EV purification, preparation of EV RNA, and construction of the RNA-seq library. We confirmed that the isolated EVs consisted mostly of exosomes from the aspect of morphology, particle size, and characteristic exosomal markers (CD63 and TSG101). In this study, we detected ample exLRs from each exLR-seq analysis of human plasma; mRNAs still made up a majority of the total mapped reads and 11 mRNAs (DGKI, GRB14, KIAA1549, WT1, ACKR4, PLXNB3, KCNH8, TCTEX1D1, ILDR2, DYTN, and CHRNA2) had the most significant changes. These mRNAs may be related to the progression and prognosis of CRC. WT1 is an appropriate a marker for CRC. WT1 expression in CRC primary tumors could be a novel independent marker for prognosis and tumor progression[20, 21]. Growth factor receptor-bound protein 14 (GRB14), which belongs to a small family of adapter proteins, could encode a growth factor receptor-binding protein that interacts with insulin receptors and insulin-like growth factor receptors (IGF-Rs)[22]. IGF-1R expression is associated with tumor progression and poor prognosis in several cancer types, including gastrointestinal malignancies[23]. KIAA1549 belongs to the UPF0606 family and is related to oncogenic MAPK signaling[24]. Atypical chemokine receptor 4 (ACKR4), which is a receptor

for C-C type chemokines, has been shown to bind T cells and dendritic cell-activated chemokines and plays a significant role in controlling the migration of immune and cancer cells[25]. These findings indicated that a number of mRNAs have changed during the peri-operative period that may play a role in the development of CRC. However, the exact effect of these mRNAs and the underlying mechanisms require further investigation.

Our data also showed that lncRNAs are enriched in EVs; however, we only detected 3 common lncRNAs at 3 time points compared with T0. This finding may reflect our small sample size. In a corollary study, we will expand our samples to further explore the exact effect of surgery on EVs and the underlying mechanism.

Innate immune cells, such as NK cells, neutrophils, mast cells (MCs), macrophages, and eosinophils, and adaptive immune cells, including DCs, T cells, and B cells, derived from exosomes can directly interact with cancer cells and uptake by tumor cells can induce different types of immune responses[26, 27]. Accumulating evidence has shown that EVs derived from immune cells can promote pro-tumor and anti-tumor immunity, which suggests a complex relationship between the immune cell-derived EVs and the immune system[28-31]. NK cells that were previously exposed to neuroblastoma (NB) can secrete exosomes containing NK cell receptors, such as CD56, KIR2DL2, and NKG2D receptors, which can subsequently stimulate normal NK cells, generating greater and more efficient cytotoxicity against NB tumor cells[30]. CD8+ T cell-derived exosomes with membrane expression of Fas ligand (FasL) can promote invasion and metastasis of Fas+ tumor cells through matrix metalloproteinase-9 (MMP-9)-mediated degradation of extracellular matrix proteins[28]. In our study, we found that total EVs derived from immune cells, especially from CD8+ T, CD4+ memory T, and NK cells, decreased after surgery. Further studies are warranted to fully characterize EVs derived from immune cells, and to learn how to precisely engineer exosomes for therapeutic antitumor treatment.

In short, our study established a blood exLR atlas of CRC patients at 4 time points before and after surgery and showed the dynamic changes of exLRs during the peri-operative period. These findings open an avenue for the investigation of EVs at different time points. Further exploration will focus on the influence of peri-operative EV dynamic changes on tumor occurrence and development and the potential underlying mechanism.

Conclusion

To the best of our knowledge, this report presents abundant exLRs in human plasma and the dynamic changes of these exLRs and exLRs originating from CD8+ T and CD4+ memory T cells were reduced during the peri-operative period. Future studies will focus on the particular exLRs and their origination to explore their impact on the occurrence and progression of CRC.

Abbreviations

colorectal cancer	CRC
Extracellular vesicles	EVs
EV long RNA	exLR
long non-coding RNAs	lncRNA
protein–protein interaction	PPI
gene ontology	GO
Kyoto Encyclopedia of Genes and Genomes	KEGG
differentially expressed genes	DEGs
vascular endothelial growth factor	VEGF
circular RNA	circRNA
messenger RNA	mRNA
damage-associated molecular pattern	DAMP
exLRs sequencing	exLR-seq
peripheral blood mononuclear cells	PBMC
natural killer cells	NK cells
Growth factor receptor-bound protein 14	GRB14
growth factor receptors	IGF-Rs
Atypical chemokine receptor 4	ACKR4
mast cells	MCs
neuroblastoma	NB
Fas ligand	FasL
matrix metalloproteinase-9	MMP-9
phosphate buffer saline	PBS
differentially-expressed lncRNAs	DELRs

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Figures

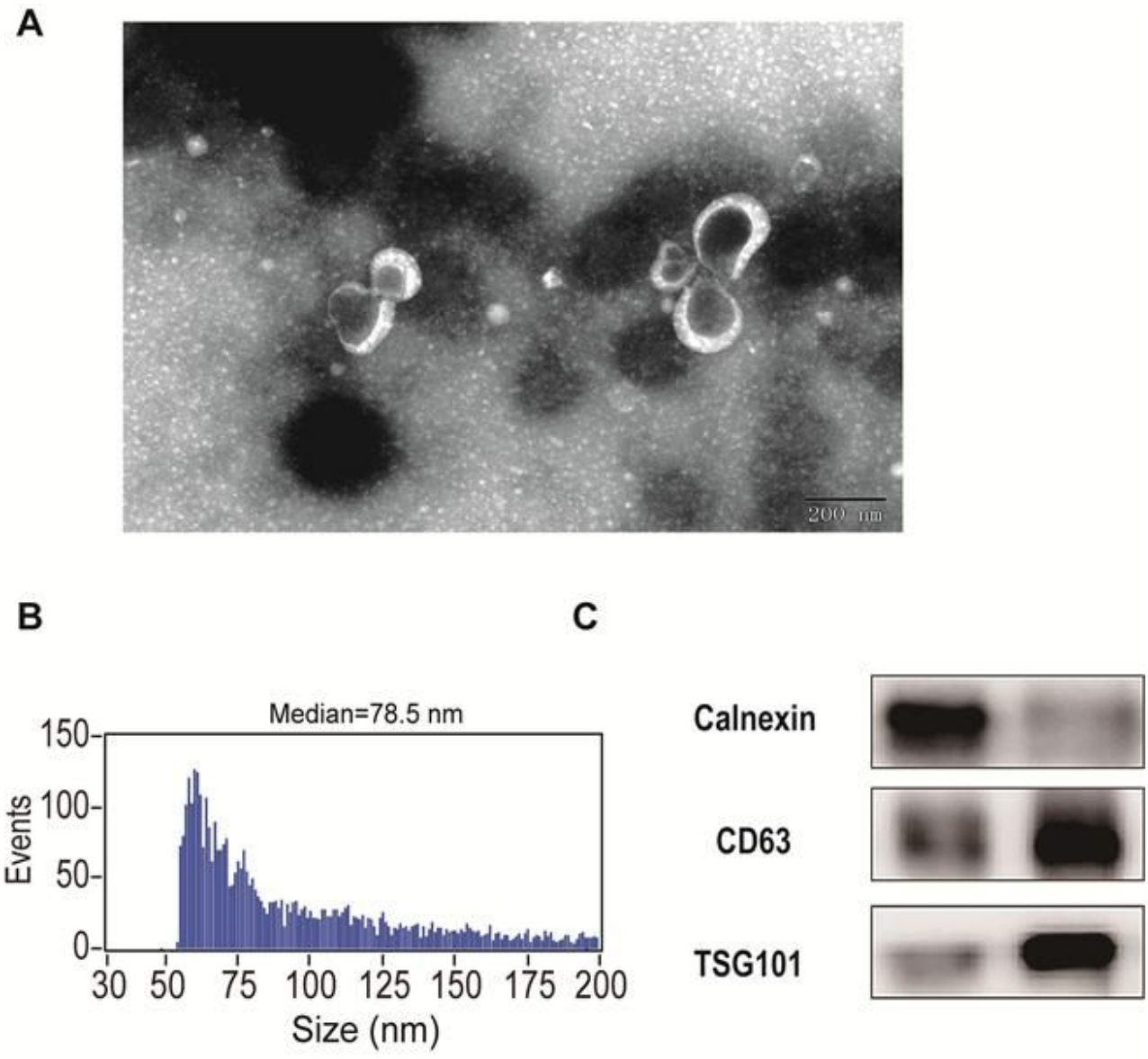

Figure 1

Human blood EV confirmation. EVs were isolated and purified from plasma using membrane affinity spin columns. (A) Electron microscopy image of isolated vesicles. (B) Size distribution measurements of isolated vesicles. (C) Western blots of calnexin, which can be detected in PBMCs, but not in isolated vesicles, was used as a control. EV markers (TSG101 and TCD63) in isolated vesicles were detected in EVs, but not in PBMCs.

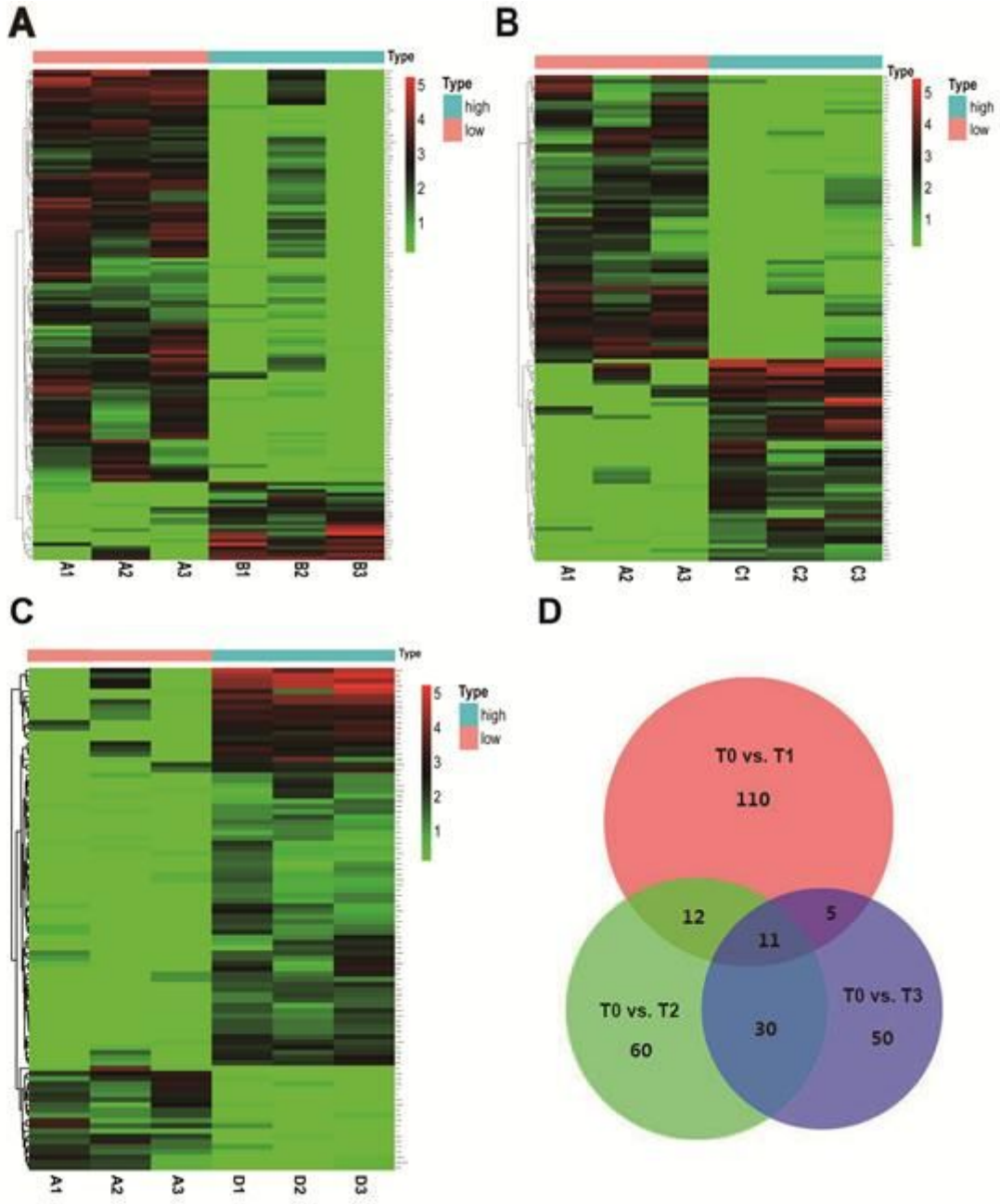

Figure 2

Comprehensive mRNAs in extracellular vesicles and functional annotations before and after surgery. (A-C) Significant DEGs in extracellular vesicles were screened and identified using the “Limma” R package between samples before surgery and after extubation (Fig. A), or 1 day after surgery (Fig. B), or 3 days

after surgery (Fig. C). (D) A total of 11 common DEGs were obtained in extracellular vesicles before and different time points after surgery using a Venn diagram.

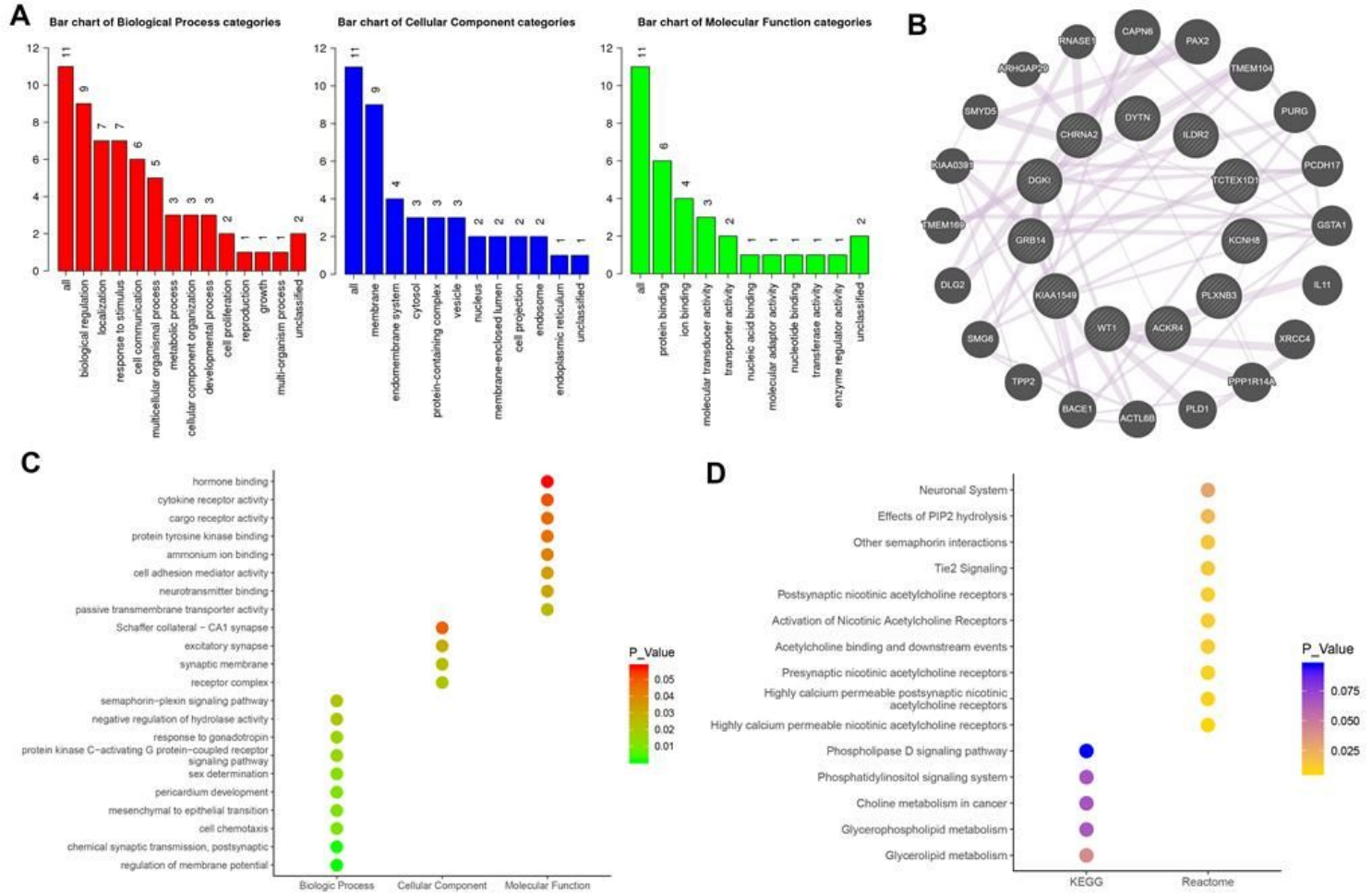

Figure 3

The functional annotations of mRNAs in EVs. (A) Biological processes, cellular components, and molecular function analysis from GO items of 11 hub genes were evaluated. (B) The protein–protein interaction network was used, showing direct interactions and potential associations between proteins. (C) The 11 hub genes most significantly involved in changed GO functions. (D) Significantly-altered KEGG and Reactome pathways were predicted.

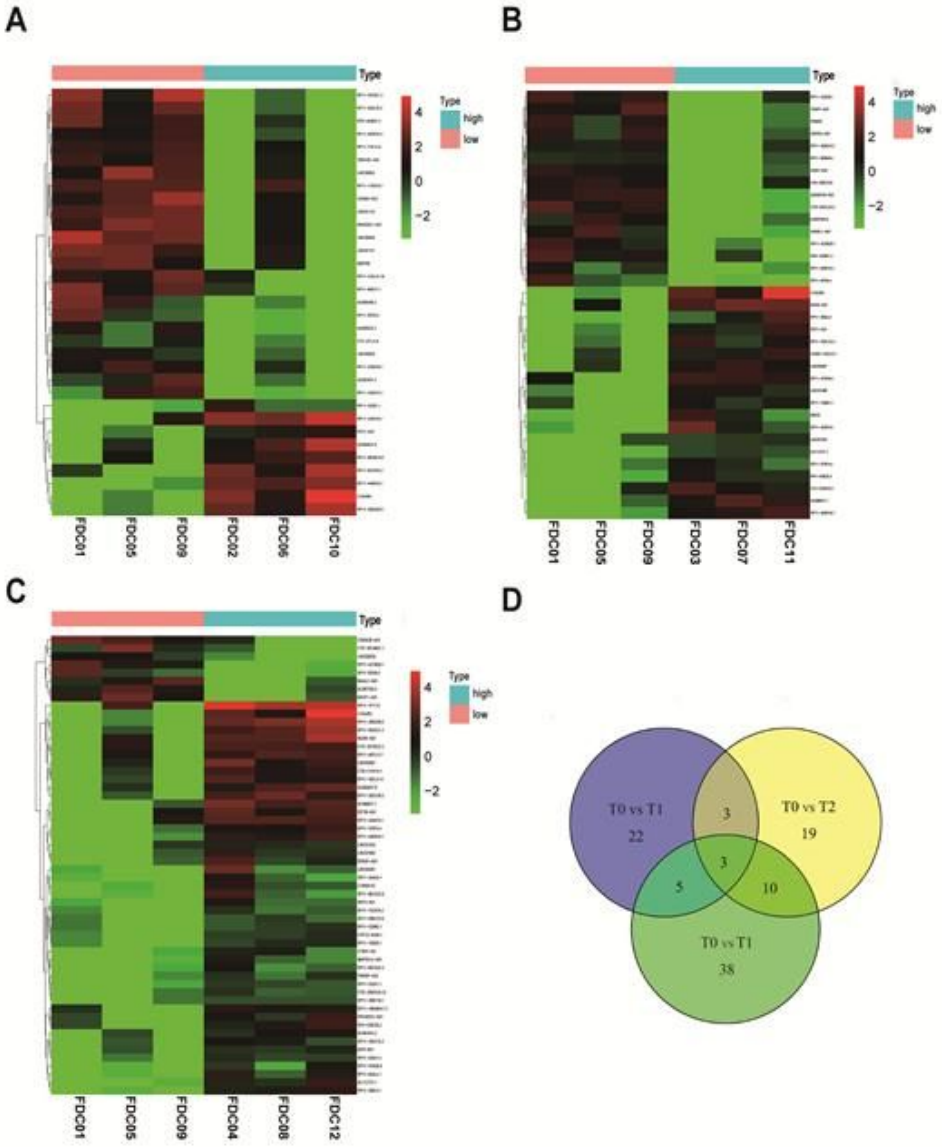

Figure 4

Comprehensive lncRNAs in EVs before and after surgery. (A-C) Significant lncRNAs in EVs were also screened and identified using the “Limma” R package between samples before surgery and after extubation (Fig. A), or 1 day after surgery (Fig. B), or 3 days after surgery (Fig. C), |logFC|>0.5, p<0.05. (D) A total of 3 common DEGs were obtained in EVs before and different time points after surgery using a Venn diagram.

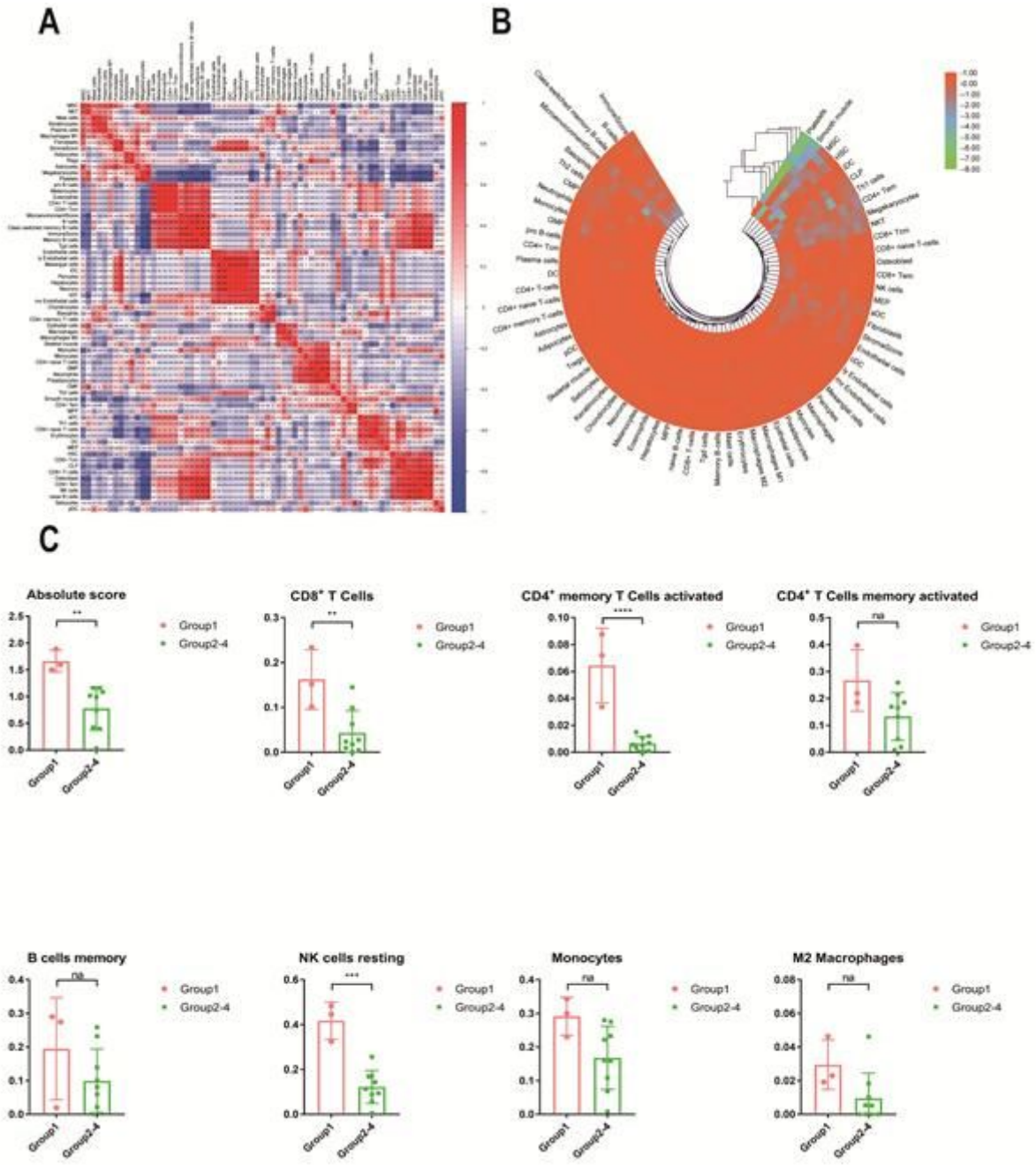

Figure 5

Cell source analysis of EVs. (A) The correlation of immune and stroma cell expression derived from EVs. (B) Dynamic changes of cells derived from EVs before and after surgery. (C) Comparison of immune cells producing EVs before and after surgery. |logFC| > 0.5; p<0.05

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