Original Article

Identification of novel genes and pathways in colorectal cancer exosomes: a bioinformatics study

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Background: Colorectal cancer (CRC) is a major public health problem, being the leading causes of mortality and morbidity worldwide. Exosomes are crucial to the molecular pathogenesis of CRC. It is essential to investigate the key differentially expressed genes (DEGs) in CRC exosomes.

Methods: The DEGs between CRC patients and normal participants were screened using microarray data downloaded from the Gene Expression Omnibus (GEO) and exoRBase database. Gene ontology (GO), pathway enrichment analysis, protein-protein interaction (PPI) network, module analysis, overall survival (OS) analysis of hub genes and functional annotation of DEGs were performed.

Results: A total of 149 DEGs in CRC exosomes and 1,507 DEGs in CRC tissues were identified with selecting criteria of P value <0.05. GO terms of positive regulation of gene expression, protein binding to Golgi, cellular protein metabolic process and endonuclease activity were significantly enriched in CRC exosomes. CRC exosomes were mainly enriched in pathways of RNA transport, alcoholism, viral carcinogenesis, spliceosome and Ras signaling pathway. We then selected UBC, H3F3A, HIST2H2AA3, AKT3, and HSPA1B as the hub protein in CRC exosomes according to the PPI network. OS analysis showed overexpression of HIST2H2AA3 was related to poor prognosis. Four oncogenes including *H3F3A*, *U2AF1*, *P2RY8* and *APOBEC3B* were identified in the exosomes. There was no significant difference in the ratio of tumor suppressor genes (TSGs) to oncogenes between tumor tissues and exosomes according to functional annotation of DEGs.

Conclusions: Hub genes including *UBC*, *H3F3A*, *HIST2H2AA3*, *AKT3*, and *HSPA1B* might serve as new diagnostic and therapeutic targets of CRC in the future.

Keywords: Colorectal cancer (CRC); expression profiles; exosomes; bioinformatics analysis; differentially expressed genes (DEGs)

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Introduction

Colorectal cancer (CRC) is one of the most frequent malignant tumors with 1.4 million new cases and is the fourth most common cause of oncological death throughout the world (1,2). Despite the incidence and mortality rates decreased over the past several decades, more than one third of CRC patients will develop into metastatic disease (3). Thus, mechanism underlies pathogenesis and metastasis of CRC still requires a further exploration.

Exosomes, which are secreted into the extracellular milieu by various cell types, are phospholipid bilayer

nanovesicles ranging from 30 to 100 nm in diameter (4). Tumor-derived exosomes (TDEs) can carry various molecular cargo including proteins, DNA and all types of RNA and then mediate signal transduction in neighboring cells or distant anatomic locations (5). TDEs perform crucial roles in the formation of the "pre-metastatic niches" and initiation of the epithelial-mesenchymal transition (EMT) (6,7). Soldevilla *et al.* have shown that CRC exosomes enriched in ΔNp73 mRNA can diffuse into recipient cell and promote proliferation (8).

With the development of high-throughput microarray and sequencing, pathway-based methods have been the first choice for complex disease analysis, particularly malign tumors (9). Recently, several studies using CRC tissues and matched normal tissues have found out numerous differentially expressed genes (DEGs) through microarray analysis, which contribute to explaining the underlying molecular mechanism of CRC (10-12). However, the correlation between DEGs of CRC exosomes and disease pathogenesis remains unknown. Thus, we conducted a comprehensive bioinformatics analysis of CRC exosomes microarray studies to discover key DEGs in exosomes related to pathogenesis and metastasis of CRC.

Methods

Data source

The gene expression profiles of GSE100206 and GSE100063 were downloaded from exoRBase (http://www.exoRBase.org) which collects RNA-seq data analysis of human blood exosomes (13,14). Totally 32 normal exosome specimens were enrolled in GSE100206, while 12 CRC exosome specimens were enrolled in GSE100063.

The gene expression profile of GSE32323 was downloaded from the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database (15). This dataset contains 17 pairs of cancer and non-cancerous tissues from CRC patients.

Date processing and screening for DEGs

For the process of GSE100206 and GSE100063, Trimmomatic was used to remove adapters and low-quality bases from the raw sequencing reads. Read counts for each gene were normalized to TPM values (transcript per million). Detailed methods can be found in this paper (14). We used affy package in R to normalize the raw data of GSE32323(16).

The DEGs between CRC group and normal group were estimated using limma package in R statistical software (17). Only genes met the cut-off criteria of $P<0.05$ and $|\log_2$ fold change (FC)$| \geq 1.0$ were regarded as DEGs.

Enrichment analysis of DEGs

DAVID (The Database for Annotation, Visualization and Integrated Discovery, https://david.ncifcrf.gov/), as a program used for genes or proteins functional annotation, was adopted for functional and pathway enrichment analysis (18). Based on selected DEGs, we performed gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis with the thresholds of $P<0.05$ (19,20).

Protein-protein interaction (PPI) network construction

We used Search Tool for the Retrieval of Interacting Genes (STRING) database to recognize potential interactions among DEGs with a combined score >0.4 (21). Then the PPI network was visualized by Cytoscape software (22). To find dense clique-like structures within a network, Molecular Complex Detection (MCODE) was conducted with MCODE score >3 and number of nodes >4. The hub proteins were identified on the basis of degree and betweenness value using cytoHubba (23,24).

Functional annotation of DEGs

Catalogue Of Somatic Mutations In Cancer (COSMIC, http://cancer.sanger.ac.uk/census) database provides detailed annotation for each tumor-associated gene (TAG) including oncogenes and tumor suppressor genes (TSGs) (25). Functional annotation of DEGs was performed to detect oncogenes and TSGs among DEGs. Up-regulated oncogenes and down-regulated TSGs were regarded as positive genes for tumor formation, otherwise, TAGs were defined as negative genes.

Overall survival (OS) analysis

LinkedOmics (http://www.linkedomics.org) is a publicly available portal that includes multi-omics data from all 32 TCGA cancer types (26). We used cox regression analysis to evaluate the relationship between selected DEGs expression and CRC patients' OS. Then, the CRC patients were

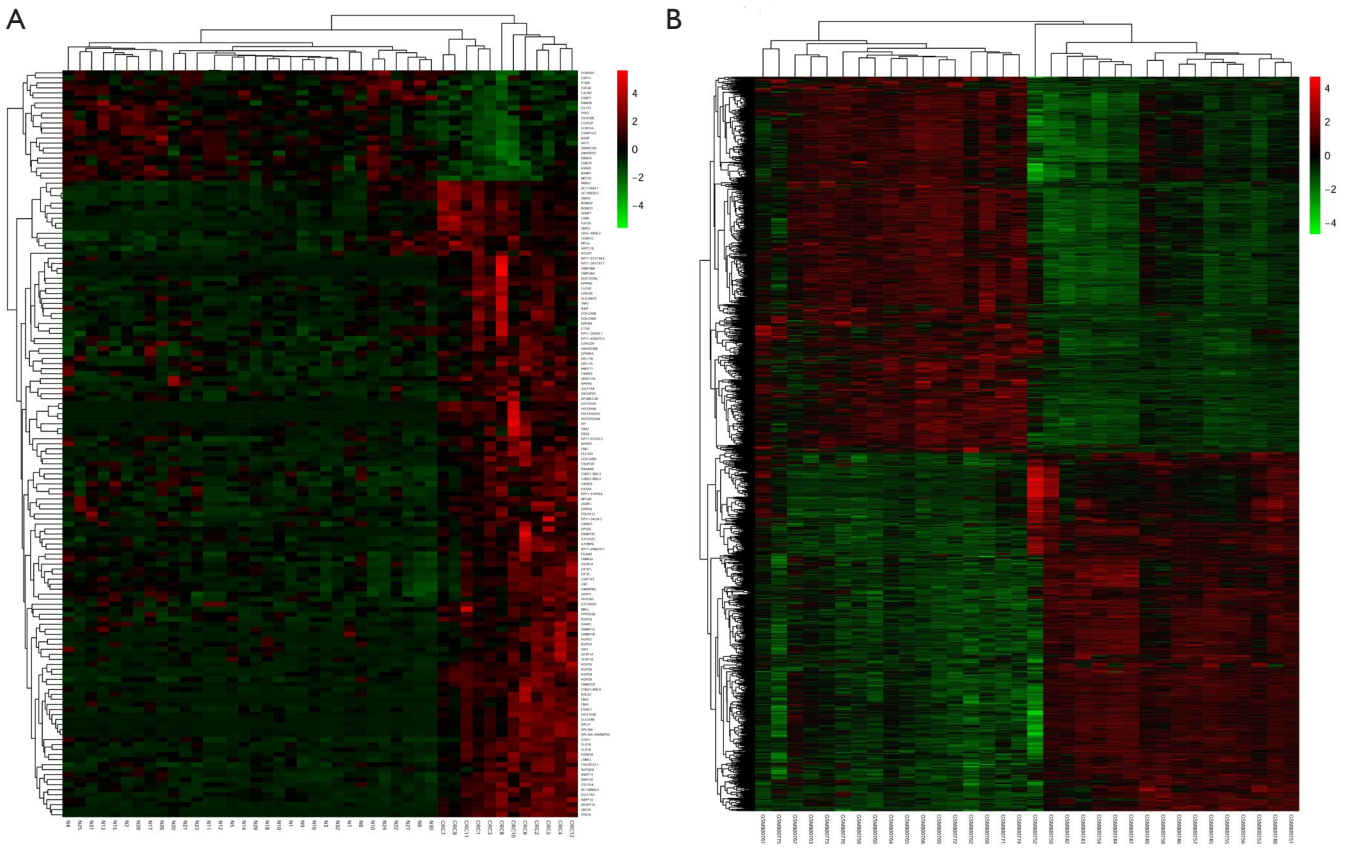

Figure 1 The hierarchical clustering heat-map of DEGs in CRC exosomes *vs.* control group (A) and CRC tissues *vs.* control group (B). The colour represents the expression of the gene; green means down-regulated genes and red means up-regulated genes. N, normal exosomes; CRC, colorectal cancer exosomes; DEGs, differentially expressed genes.

divided into high- and low-expression groups according to the median mRNAs expression level. Log-rank P value was calculated to compare the survival distribution between two groups.

Statement of ethics approval

This article does not contain any studies with human participants or animals performed by any of the authors. Ethics approval is not required for this study.

Statistical analysis

Chi-square test was used to determine the statistical significance of TAGs number. All statistical analysis was performed using STATA12 (StataCorp, College Station, TX, USA), and a P value of <0.05 was considered to be statistically significant.

Results

Identification of DEGs

A total of 149 DEGs (127 up-regulated and 22 down-regulated) were obtained in the tumor exosomes group according to the cut-off value for screening. We also identified 1,507 DEGs from the tumor tissues group, which included 771 up-regulated and 736 down-regulated DEGs. The representative heatmap of DEGs in tumor exosomes and tissues were illustrated in *Figure 1A*,*B* respectively.

GO and pathway enrichment analysis

To annotate the biological function of the DEGs in the tumor exosomes, we performed GO and KEGG pathway analysis. The top 10 terms of DEGs were displayed in *Figure S1A*. GO analysis revealed that the DEGs were significantly involved in biological processes (BP) of

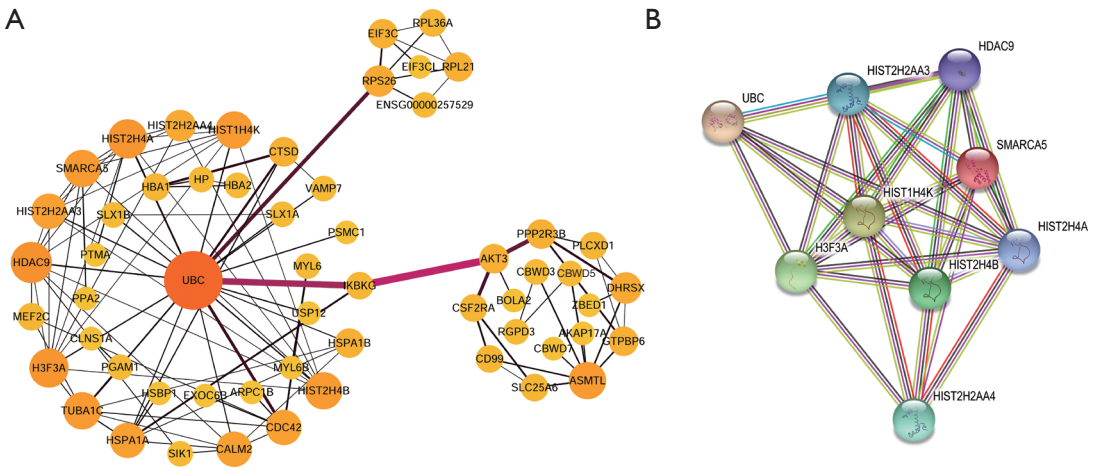

Figure 2 Protein-protein interaction network and a significant module. (A) Protein-protein interaction network of DEGs in CRC exosomes constructed using Cytoscape. Each node is representative of a gene. The node size represents the connectivity degree. The thickness of the edges stands for the credibility. (B) A significant module identified from protein-protein interaction network. CRC, colorectal cancer exosomes; DEGs, differentially expressed genes.

Table 1 The KEGG pathway enrichment analysis of genes in the module

KEGG pathway term	Genes	Count	P value
Alcoholism	*HIST2H2AA3, HIST2H2AA4, HIST1H4K, H3F3A, HIST2H4A, HDAC9, HIST2H4B*	7	1.78E–9
Systemic lupus erythematosus	*HIST2H2AA3, HIST2H2AA4, HIST1H4K, H3F3A, HIST2H4A, HIST2H4B*	6	5.18E–8
Viral carcinogenesis	*HIST1H4K, HIST2H4A, HDAC9, HIST2H4B*	4	8.25E–4

KEGG, Kyoto Encyclopedia of Genes and Genomes.

response to protein targeting, protein metabolism and gene expression. As for molecular function (MF), enrichment indicate protein and histone binding, endonuclease activity and pre-mRNA splice site binding. Besides, cell component (CC) enrichment displayed endocytic vesicle lumen, cytoplasm and blood microparticle, which hinted that DEGs might play a critical role in exosomes formation.

KEGG pathway analysis suggested that the DEGs were enriched in 5 pathways including RNA transport, alcoholism, viral carcinogenesis, spliceosome and Ras signaling pathway (*Figure S1B*).

PPI network construction

A total of 116 PPI relationships were constructed on the basis of STRING database (*Figure 2A*). In the network, 5 node proteins, including UBC, H3F3A, HIST2H2AA3, AKT3, and HSPA1B, which showed a strong association with other node proteins, were selected as hub proteins. As shown in *Figure 2B*, one significant module was selected with the criteria of number of nodes >4. The key module showed functions enriched in pathways such as alcoholism and viral carcinogenesis (*Table 1*).

Functional annotation of DEGs

As shown in *Figure 3A*, 21 TSGs and 25 oncogenes were found in tumor tissues with 4 oncogenes (*H3F3A*, *U2AF1*, *P2RY8* and *APOBEC3B*) in tumor exosomes. However, there was no significant difference in the ratio of TSGs to oncogenes among DEGs of tumor tissues or exosomes (P=0.21). Subgroup analysis was conducted in up-regulated and down-regulated DEGs (*Figure 3B*, *Table 2*). According to our definition of positive genes (up-regulated oncogenes and down-regulated TSGs) and negative genes (up-regulated TSGs and down-regulated oncogenes), we found 22 positive

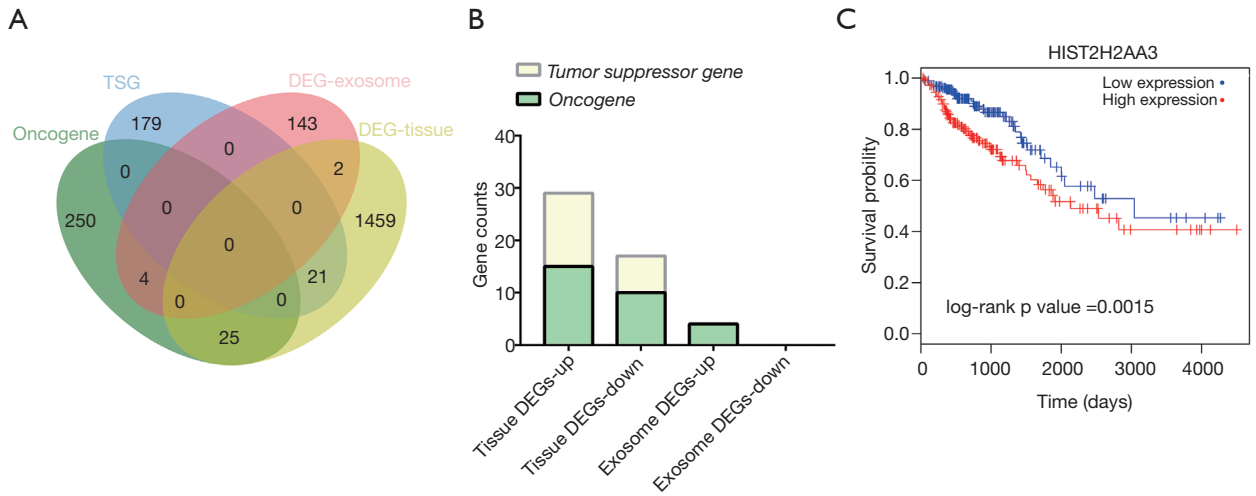

Figure 3 Functional annotation of DEGs. (A) Venn-diagram presenting DEGs in CRC exosomes, CRC tissues, tumor suppressor genes and oncogenes. (B) Different expression of tumor suppressor genes and oncogenes in CRC exosomes and CRC tissues. Up, up-regulated genes; down, down-regulated genes. (C) The Kaplan-Meier curve for overall survival of CRC patients with high HIST2H2AA3 expression. The significant differences were examined using the two-sided log-rank test. CRC, colorectal cancer exosomes; DEGs, differentially expressed genes.

Table 2 The functional annotation of DEGs in tissues and exosomes

Categories of DEGs	DEGs of tumor exosomes		DEGs of tumor tissues	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Oncogenes	*H3F3A, U2AF1, P2RY8, APOBEC3B*	–	*CDK4, SF3B1, BCL11A, CCND1, CCND2, ETV4, MYC, NPM1, PLAG1, STIL, TRIM27, EZH2, RAD21, TRIM24, WT1*	*GNA11, GNAQ, KIT, PIM1, RSPO3, SETBP1, TCL1A, TNFRSF17, KLF4, BIRC3*
TSGs	–	–	*ATR, ATRX, BUB1B, CHEK2, FANCF, MSH2, NBN, PALB2, POT1, RNF43, CCNB1IP1, EIF3E, RMI2, WIF1*	*DNM2, FAM46C, FAS, KLF6, BCL10, CBFA2T3, ZBTB16*

DEGs, differentially expressed genes; TSGs, tumor suppressor genes.

genes and 24 negative genes in tumor tissues with 4 positive genes in tumor exosomes. We did not find any significant difference either (P=0.14). Subsequently, OS analysis showed that higher HIST2H2AA3 expression level was significantly associated with lower OS (P=0.0015, *Figure 3C*).

However, no significant differences in OS for H3F3A, U2AF1, P2RY8, APOBEC3B, UBC, AKT3, and HSPA1B were observed (*Figure S2*).

Discussion

Identifying abnormally expressed genes associated with CRC would greatly benefit the diagnosis and therapies of this disease. In this study, a total of 149 DEGs were identified in CRC exosomes relative to normal exosomes through analyzing gene expression profile of GSE100206 and GSE100063. Enrichment analysis of DEGs might provide novel insights for unraveling mechanism of CRC development and progression.

As was suggested by DAVID analysis, the significant GO terms were related to positive regulation of gene expression, protein binding to Golgi, cellular protein metabolic process and endonuclease activity. It is reasonable that sustaining proliferative signaling and deregulating cellular energetics are hallmarks of cancers including CRC (27). Regard to the signaling pathway, we found that RNA transport,

alcoholism, viral carcinogenesis, spliceosome and Ras signaling pathway were highly enriched. It is consistent with the fact that carcinogens induce long-lasting increased in insoluble NTPase may alter the RNA transport associated with cancer and carcinogenesis (28,29). Dysregulation of spliceosome gene expression including U2AF1 is also associated with tumor formation and cell survival (30). Viral pathogens and alcoholism have also been found to increase risk of CRC development (31,32). Ras signaling pathway enriched in AKT3 is activated in many malignancies and has become a major focus of drug targeting efforts (33).

In PPI network analysis, top 5 hub genes appeared to be *UBC*, *H3F3A*, *HIST2H2AA3*, *AKT3*, and *HSPA1B*. As the most significant hub gene, the detailed function of UBC for CRC has yet been reported to data. Due to the lowest levels of expression variability and constitution in CRC and other tissues, UBC, a ubiquitin gene, is used as the housekeeping genes (34). When compared the expression of UBC between CRC tissues and matched normal tissues, there was no significant difference at protein level (35). However, ubiquitination has been associated with CRC (36). Thus, further molecular research may focus on the role of UBC in exosomes instead of tumor tissues. *H3F3A* and *HIST2H2AA3* are histone genes belonging to the H3A and H2A family respectively. They constitute the octamer of core histone proteins with H2B, H3 and H4. Histones are basic nuclear proteins that are responsible for the nucleosome structure (37). *H3F3A* mutations often occur in gliomas especially high-grade gliomas and reprogram epigenetic landscape (38). Decreased expression of HIST2H2AA3 at the protein and mRNA levels has been reported in the hepatocellular carcinoma (39). *AKT3* gene encodes a protein belonging to Akt kinase family, which exert functions in cell proliferation, differentiation, apoptosis, tumorigenesis, as well as glycogen synthesis and glucose uptake. Compared with ubiquitous expression Akt1 and Akt2, AKT3 has a more limited tissue distribution. Overexpression of AKT3 occurs in breast cancer and prostate cancer, suggesting that Akt3 is strongly oncogenic (40). Although the role of AKT2 and AKT1 in CRC has been well elucidated, limited information of AKT3 in CRC can be found (41,42). Heat shock protein encoded by HSPA1B is a member of the heat shock protein 70 family. Elevated expression of HSPA1B promotes breast cancer cell growth by arresting cancer cells in G1 (43). A recent meta-analysis suggests HSPA1B ± 1267A/G polymorphism increases risk of hepatocellular carcinoma (44). The roles of all above genes in CRC require further explorations to gain an insight into the function of CRC exosomes.

When we compared the DEGs in tumor exosomes with DEGs in tumor tissues, it was surprised to find similar ratios of TSGs to oncogenes existed in exosomes and tissues. Previous have proven that exosomes mediate the metastasis of cancer (7). Thus, tumor exosomes were expected to contain more oncogenes instead of TSGs. Although we did not find any TSGs in the CRC exosomes, the research conducted by Teng *et al.* may offer a novel explanation of TSGs enriched in exosomes if they do exist in exosomes (45). Selective sorting of TSGs into exosomes with more oncogenes left in tumor cells can also promote primary tumor progression. Moreover, it should be remembered that exosomes used in GSE100206 and GSE100063 were not tumor-specific exosomes. Therefore, the results can be affected by the mixed exosomes in blood (46).

Although some key genes and pathways were identified, several limitations should be acknowledged in this study. The clinical prognosis analyzed in our study used the date from tumor tissues but not the exosomes due to the availability of data. Besides, we could not define the biological function of tumor derived exosome without a cancer exosome-specific biomarker. Further molecular experiments should be conducted to better confirm our findings of the important genes and pathways in CRC.

In conclusion, our study revealed a number of DEGs in the CRC exosomes by bioinformatics analysis. The data from this study may contribute to future translational medicine studies in identifying new approaches for the diagnosis and treatment of CRC.

Acknowledgments

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2018.05.32). 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 article does not contain any studies with human participants or animals performed by any of the authors. Ethics approval is not required for this study and informed consent was waived.

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Supplementary

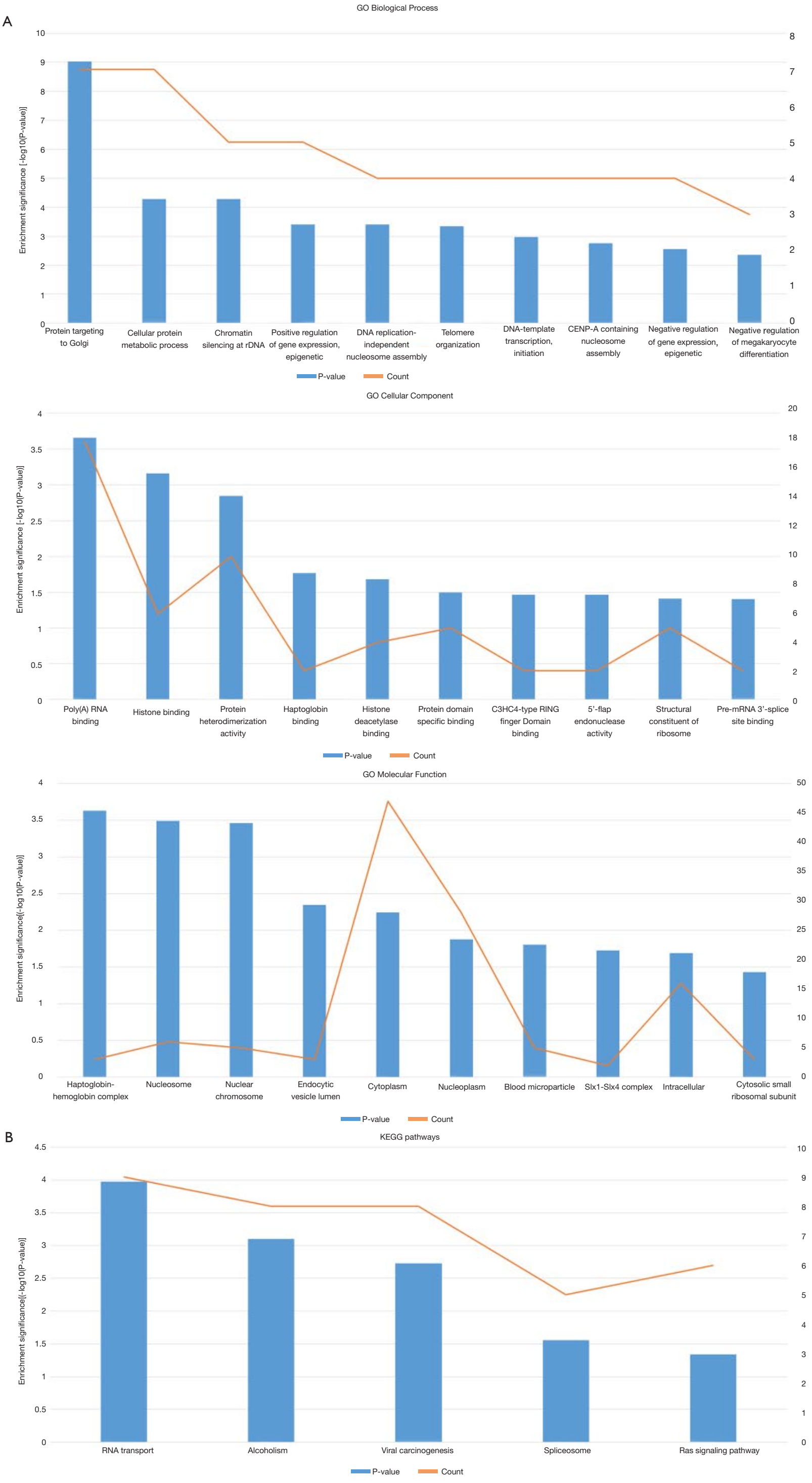

Figure S1 Enrichment analysis result of DEGs. (A) The top 10 most significantly enriched GO terms of DEGs in CRC exosomes compared to control group. (B) The top 5 most significantly enriched KEGG pathways of DEGs in CRC exosomes compared to control group. Count, number of DEGs enriched in GO term and KEGG pathways; orange trend line, log10 (P value). CRC, colorectal cancer exosomes; DEGs, differentially expressed genes; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

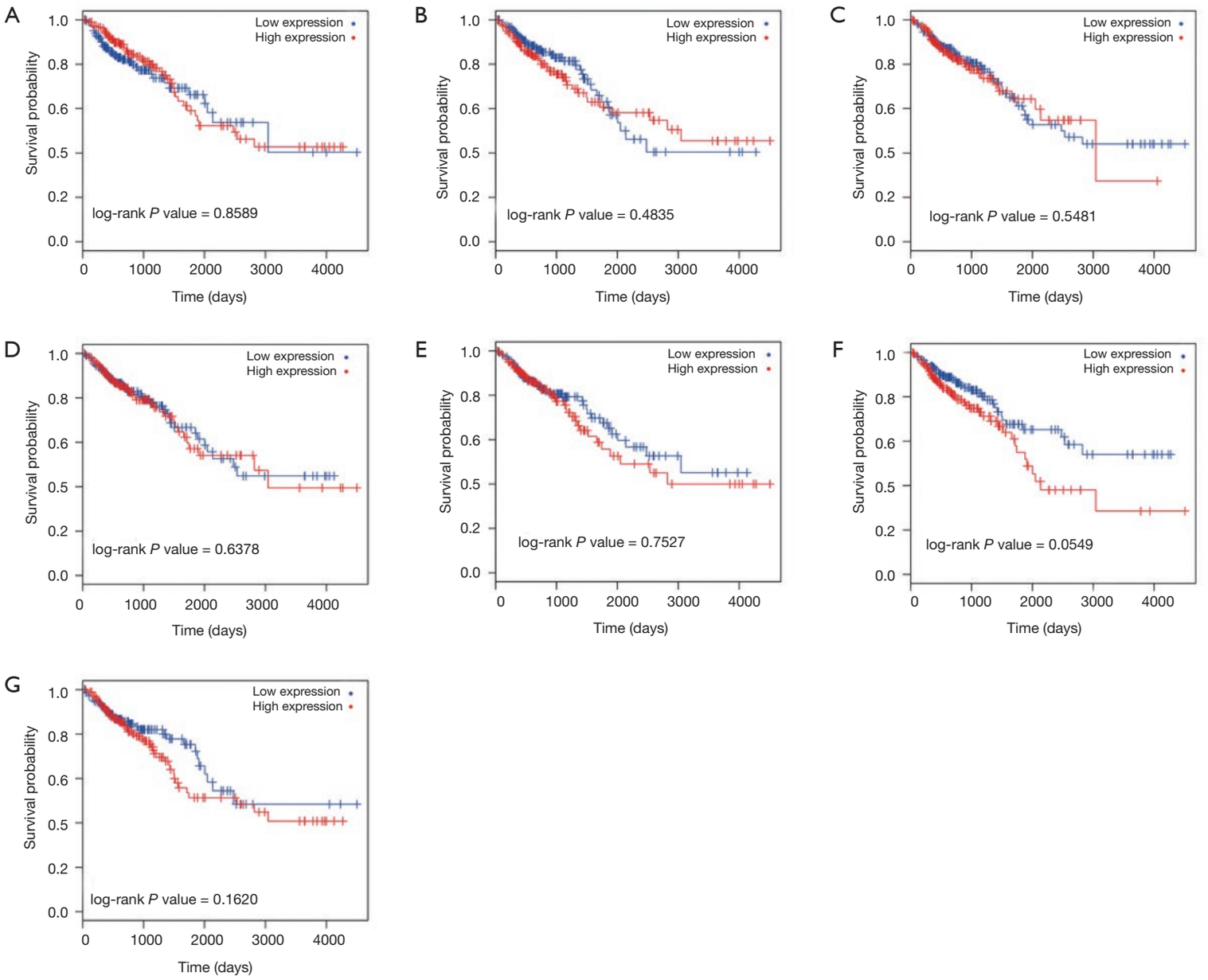

Figure S2 Prognostic assessment of the DEGS. Kaplan-Meier survival. curves of overall survival for CRC patients with high H3F3A (A), U2AF1 (B), P2RY8 (C), APOBEC3B (D), UBC (E), AKT3 (F), and HSPA1B (G) expression. DEGS, differentially expressed genes; CRC, colorectal cancer exosomes.