Construction of a circRNA-miRNA-mRNA network based on competitive endogenous RNA reveals the key pathways and central genes of osteosarcoma *in vivo*

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Abstract

Circular RNAs (circRNAs) play important roles in a variety of pathological functions. However, the potential functions and detailed mechanisms of circRNAs in osteosarcoma (OS) have not been fully elucidated. In this study, the circRNA, micro RNA (miRNA), and messenger RNA (mRNA) expression profile of human OS was investigated based on the raw microarray data GSE140256, GSE65071 and GSE16088 in Gene Expression Omnibus (GEO) datasets, and seven differentially-expressed circRNAs (DEcircRNAs), 166 differentially-expressed miRNAs (DEmiRNAs), and 175 differentially-expressed mRNAs (DEmRNAs) were identified in total. FunRich was employed to analyze the differentially-expressed transcription factors on the basis of identified DEmiRNAs. In addition, the Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were used to further study biological functions of the DEmRNAs. Interestingly, post-translational protein modification, collagen-containing extracellular matrix, and single-stranded DNA binding were the most significant pathways enriched for DEmRNAs in GO annotation analysis. Meanwhile, in KEGG pathway enrichment analysis, complement and coagulation cascades, RNA transport and drug metabolism − other enzymes were the most significantly enriched pathways of DEmRNAs in OS. We constructed circRNA-miRNA-mRNA and protein–protein interaction (PPI) networks that may be associated with pathological processes of OS. Finally, we also revealed the pattern of tumor-infiltrating immune cells in OS and further explored the ceRNA networks we constructed in which we found that COL1A1 and RAN were significantly correlated with overall survival in patients with osteosarcoma ($p < 0.05$). To our knowledge, this study provides the first profile analysis of DEcircRNAs, DEmiRNAs, and DEmRNAs with OS *in vivo* and reveals a novel idea for understanding the pathogenesis of OS.

Introduction

Osteosarcoma (OS) is the most common primary malignant tumor of bone in young people[1]. The combined application of surgery, chemotherapy, and radiotherapy has significantly improved the survival rate of patients with OS[2], but in spite of this the prognosis of patients with bone tumors remains very poor[3]. Most patients with OS will eventually die of lung metastases[4]. Research results have proven that the occurrence and evolution of OS are closely related to gene mutations and expression levels[5–7]. Therefore, studying the potential gene molecular expression mechanism of OS and finding potential biomarkers and therapeutic targets are extremely important for the early diagnosis of the disease and the proposal of new treatment options.

Circular RNA (circRNA) is a newly-discovered non-coding form of RNA with a covalent closed loop structure[8], without a 5' cap structure or a 3' poly(A) tail, which makes circRNA resistant to exonucleases with the potential to become an ideal biomarker[9]. Pan et al. found that circ_0028171 acts as a sponge of micro RNA (miR)-218-5p to increase the expression of IKBKB which promotes the progression of OS and could be a potential novel marker for early diagnosis[10]. Li et al. found that hsa_circ_0000073 can act as a sponge to inhibit miR-145-5p- and miR-151-3p-mediated down-regulation of NRAS, and promote the

proliferation, migration, invasion and MTX resistance of OS cells[11]. Zhang et al. found that high expression of hsa_circ_0136666 predicts poor prognosis and promotes the development of OS through the miR-593-3p/ZEB2 pathway[12]. Although circRNA plays an important role in OS, the potential functions and complex mechanisms of most circRNAs in OS have not been fully elucidated.

The tumor microenvironment (TME) not only provides nutrients and growth factors for the proliferation and metastasis of tumor cells, but also limits the early detection of tumors and the efficacy of immunotherapy[13]. The bone microenvironmental cell composition of OS is complex, releasing a variety of growth factors and cytokines[14]. CIBERSORT is a new type of biological information tool. Through the deconvolution algorithm developed by Bindea et al., CIBERSORT can estimate the cell composition of complex tissues based on standardized gene expression data[15, 16]. This method has been validated by flow cytometry in breast and lung cancer, and can be applied to the analysis of gene expression profile data on a large scale[17–19]. Because the microenvironment plays an important role in tumor development, analyzing the immune microenvironment of OS can provide a better understanding of the pathogenesis of OS.

In this study, we collected circRNA, miRNA and mRNA expression profiles of OS *in vivo* obtained from OS patient tissue samples and normal tissue samples from the Gene Expression Omnibus (GEO) dataset. We used R language to identify differentially-expressed circRNAs, miRNAs and mRNAs (DEcircRNAs, DEmiRNAs and DEmRNAs) in OS, predict the upstream and downstream connections of differentially-expressed genes (DEGs), and compare the predictions with the results we identified to construct a competing endogenous RNA (ceRNA) network and protein–protein interaction network. In order to understand the functional mechanism of OS *in vivo*, we used Gene Ontology (GO) annotations and the "Kyoto Encyclopedia of Genes and Genomes" (KEGG) to annotate the functions of DEmRNAs. Using Cytoscape software, a protein–protein interaction (PPI) network was constructed, and important modules and key genes were screened. Finally, we also revealed the pattern of tumor-infiltrating immune cells in OS.

Materials And Methods

Microarray data

The raw microarray datasets GSE140256, GSE65071 and GSE16088 were obtained from the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) which is an online public gene data repository for high-throughput sequencing research. GSE140256, a circRNA expression profile, included three cancer tissue samples and three paracancer samples, and miRNA sequencing data obtained from GSE65071 included 20 serum samples from patients with OS and 15 normal serum samples. In the mRNA expression profile data, GSE16088 included 17 cancer tissue samples and six normal tissue samples. The above raw microarray data were extracted from the GEO database. Consequently, this research did not require any ethical review or informed consent because of the public availability of GEO data.

Identification of DEcircRNAs, DEmiRNAs and DEmRNAs

The DEcircRNAs, DE micro RNAs (miRNAs) and DE messenger RNAs (mRNAs) were identified by the difference in expression between the normal and OS samples in microarray data. The adjusted *P*-value (adj.p) and the absolute log value of fold-change (log|FC|) were analyzed in R language by the limma package. The log|FC|> 1.0 and adj.p < 0.05 were the selection criteria to define the DEGs.

Analysis of differential expression transcription factors

Based on the DEmiRNAs identified, FunRich (Version 3.1.3) was used to analyze and visualize the differentially-expressed transcription factors. FunRich is a stand-alone software tool used mainly for functional enrichment and interaction network analysis of genes and proteins.

Pathway enrichment analysis

We used GO annotation (http://www.geneontology.org) and KEGG pathway analysis to determine the functions of the DEmRNAs. The adj.p < 0.05, as a screening condition, was considered highly credible by using R language of the clusterProfiler package.

Construction of the ceRNA network

In the results of the differential analysis of the microarray data information, the DEcircRNAs were predicted by TSCD (tissue-specific circRNA database) (http://gb.whu.edu.cn/TSCD) and DEmiRNA target genes were predicted by the databases miRDB, miRTarBase and TargetScan. In this study, downstream molecules predicted in all three databases were identified as target genes of DEcircRNAs and DEmiRNAs.

We used the prediction of the DEcircRNAs and DEmiRNAs target genes to intersect with identified differentially-expressed downstream genes to further screen the prediction results. Finally, we used the prediction results to construct a ceRNA interaction network and visualize it by employing cytoscape (Version 3.8.0).

Survival analysis

In order to further verify the value of the ceRNA network we constructed in osteosarcoma, mRNA expression data and prognostic information of osteosarcoma patients were obtained from Therapeutically Applicable Research To Generate Effective Treatments (TARGET) database. TARGET database provides genetic data and clinical information of a variety of tumors, including acute lymphoblastic leukemia, kidney tumors and osteosarcoma, etc. The survival analyses of genes in ceRNA network by survival and survminer package in R language under the condition of P <0.05.

PPI network and clustered subnetworks construction

The exploration of protein interactions helps to reveal the underlying pathological mechanism of OS. In this study, we used the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database

(https://string-db.org/) to construct a protein–protein interaction network. On this basis, the clustered subnetworks and hub genes were identified by employing Molecular Complex Detection (MCODE) and cytohhuba in cytoscape (Version 3.8.0).

Tumor-infiltrating immune cell analysis

We used Cibersort software to analyze DEmRNAs differentially expressed between OS tissues and normal tissues. The distribution characteristics of 22 immune cells were calculated by the deconvolution method of Cibersort software. The 22 immune cell types included activated dendritic cells (DCs), resting DCs, activated mast cells, resting mast cells, activated natural killer cells (NKs), resting NKs, activated memory CD4+ T cells, resting CD4+ T cells, naïve CD4+ T cells, regulatory T cells (Tregs), T follicular helper cells (Tfhs), gamma delta T cells (Tgds), CD8+ T cells, eosinophils, neutrophils, monocytes, macrophages (M0s), type 1 macrophages (M1), type 2 macrophages (M2), memory B cells, naïve B cells, and plasma cells. Values of $P < 0.05$ were considered as statistically significant.

Results

Identification of DEcircRNAs, DEmiRNAs and DEmRNAs

Values of log|FC|> 1.0 and adj.p <0.05 were the selection criteria to define the DEGs. In the GSE140256 dataset, four upregulated circRNAs and three downregulated circRNAs were identified, which included three cancer tissue samples and three paracancer samples (Figure 1A, 1B). The GSE65071 dataset, including 20 serum samples from patients with OS and 15 normal serum samples, revealed a total of 78 upregulated miRNAs and 88 downregulated miRNAs (Figure 1C, 1D). In the mRNA expression profile data, which included 17 cancer tissue samples and six normal samples, the GSE16088 dataset, we identified 175 DEmRNAs, of which 149 mRNAs were up-regulated and 26 mRNAs were down-regulated (Figure 1E, 1F).

Analysis of differentially-expressed transcription factors

Transcription factors are important molecules that regulate gene expression. We analyzed differentially-expressed transcription factors associated with the 166 identified DEmiRNAs, and found 185 statistically-significant transcription factors. Among these results, EGR1, SP1, SP4, POU2F1 and NFIC are the most significant molecules in OS (Figure 2).

Pathway enrichment analysis

To better understand the potential biological functions of the identified DEmRNAs in OS, GO annotation and KEGG pathway analysis were performed. In GO annotation analysis, we found that the DEmRNAs were significantly enriched in the terms of post-translational protein modification, RNA localization and extracellular structure organization in the biological process (BP) subgroup. Collagen-containing extracellular matrix, endoplasmic reticulum lumen and blood microparticle were the most significant GO terms in the cellular component (CC) subgroup. The top three GO processes were single-stranded DNA

binding, heat shock protein binding and extracellular matrix structural constituent in the molecular function (MF) subgroup for DEmRNAs (Figure 3A).

In KEGG pathway enrichment analysis, complement and coagulation cascades, RNA transport and drug metabolism–other enzymes were the most significant pathways enriched of DEmRNAs in OS (Figure 3B).

Construction of the ceRNA network

In order to better understand the endogenous regulatory mechanism of DEGs in OS, we predicted the downstream target genes of DEcircRNAs by employing the TSCD databases. At the circRNA level, a total of seven circRNAs were predicted downstream miRNAs. We used the predicted miRNAs as candidate miRNAs. Candidate miRNAs were cross-compared with DEmiRNAs identified in the microarray. In the analysis results, four intersecting miRNAs were obtained in data of the identified DEmiRNAs. At the miRNA level, a total of 153 miRNAs were predicted, and a total of 7,580 mRNAs were involved. In the results after comparison, five intersecting mRNAs were identified. Based on the predicted results of circRNA–miRNA and miRNA–mRNA pairs, we constructed a ceRNA network. In the ceRNA network, there were a total of one circRNA, three miRNAs and four mRNAs involved in the network construction (Figure 4).

Survival analysis

The four mRNAs in the ceRNA network and the overall survival of osteosarcoma patient from TRAGET database were analyzed by R language. Kaplan-Meier (K-M) survival curves were performed by dividing patients with osteosarcoma into two groups with best-separation cutoff value of mRNA. The results revealed that the mRNA expression level of COL1A1 and RAN were significantly correlated with overall survival in patients with osteosarcoma (Figure 5A 5B).

Construction of the protein–protein interaction (PPI) network

We used the identified DEmRNAs to construct a PPI network. This network included 146 nodes and 687 edges, under the conditions that the comprehensive Gt score > 0.4 and unconnected points were removed. Among the 146 genes, 67 genes had a score > 100 when analyzed by the Maximal Clique Centrality (MCC) method in cytohubba. The top five hub genes were CDC20, MAD2L1, PCNA, KPNA2 and PRB1 (Figure 6A). In this study, we also defined the most closely-clustered subnetwork by employing the MCODE plug-in in Cytoscape. Using this, the most closely-clustered subnetwork was found to consist of 14 nodes and 90 edges (Figure 6B). In addition, we also identified two other clustered subnetworks, which contained 20 nodes and 97 edges, and 13 nodes and 35 edges, respectively (Figure 6C 6D). These groups were all composed of up-regulated mRNAs.

Tumor-infiltrating immune cell analysis

We used Cibersort to evaluate the distribution of immune cells from the data of 17 OS patient tissues and six normal human tissues. Among them, we found that the immune cell types with the highest level of

infiltration in OS patients were M0 macrophages, M2 macrophages and CD8+ T cells (Figure 7A). Compared with normal tissues, M0 macrophages (P = 0.010), M2 macrophages (P = 0.010), CD8+ T cells (P = 0.020), memory B cells (P = 0.021), plasma cells (P = 0.036) and activated NK cells (P = 0.033) showed statistically-significant differences in OS patient tissues (P < 0.05) (Figure 7B). In the tissues of OS patients, immune cells with a larger positive correlation coefficient included CD8+ T cells and plasma cells (0.66), memory B cells and naïve B cells (0.59), plasma cells and naïve CD4+ T cells (0.58). In addition, immune cells with larger negative correlation coefficients included M0 macrophages and CD8+ T cells (0.83), resting mast cells and activated mast cells (0.62), plasma cells and M0 macrophages (0.61) (Figure 7C). Results of Principal Component Analysis (PCA) showed that immune cell infiltration patterns of 22 types of immune cell can effectively distinguish OS patients from healthy controls (Figure 7D).

Discussion

CircRNA has long been considered to be non-functional and the product of abnormal gene splicing[20]. In recent years, more and more studies have found that circRNA is related to the occurrence and development of a variety of cancers[21–23]. OS is a common malignant tumor in orthopedics. Although the combined application of surgery, chemotherapy, and radiotherapy significantly improves the survival rate of patients with OS, their prognosis is still very poor[3]. Related studies have described the functional mechanisms of some circRNA in OS[24, 25], but the potential functions and complex mechanisms of most circRNAs in OS have not been fully elucidated.

In this study, we used bioinformatics technology and the OS genome data in the GEO database to identify differentially-expressed circRNA, miRNA and mRNA expression profiles in OS. According to the differential gene expression profile of OS, we conducted a biological analysis of transcription factors, functional enrichment, immune cell infiltration, ceRNA interaction network and PPI network in OS. We identified seven DEcircRNAs, 166 DEmiRNAs, and 175 DEmRNAs. We found 185 differentially-expressed transcription factors, of which EGR1, SP1, and SP4 were the top three most significant transcription factors. In the GO annotation analysis, the most enriched pathways in the BP, CC, and MF subgroups were post-translational protein modification, collagen-containing extracellular matrix and single-stranded DNA binding. In KEGG pathway enrichment analysis, complement and coagulation cascades, RNA transport, and drug metabolism – other enzymes were the most significant enrichment terms of DEmRNAs in OS. In the constructed PPI network, 146 DEmRNAs were involved. Meanwhile, CDC20, MAD2L1, and PCNA were the top three most important hub genes which were revealed by cytohubba in cytoscape software. On the other hand, we studied the immune cell infiltration of OS and found that the immune cell infiltration pattern can effectively distinguish OS patients from normal people. M0 macrophages, M2 macrophages, CD8 + T cells, memory B cells, plasma cells and NK cell-activated immune cell infiltration differed in the tissues of OS patients compared with normal people (P < 0.05). In OS patients, CD8 + T cells and plasma cells, M0 macrophages and CD8 + T cells, plasma cells and M0 macrophages exhibited strong correlation

coefficients among the above immune cells. Our research results provide the feasibility for identifying patients with osteosarcoma based on the characteristics of immune infiltrating cells.

In the ceRNA network we constructed, one circRNA, three miRNAs and four mRNAs were involved. In the ceRNA network, COL1A1 and RAN were in the same clustered subnetworks in the PPI network. Our research is the first report of a ceRNA network based on circRNA of osteosarcoma *in vivo.*

Among the miRNAs in the ceRNA network, miR-324-5p and miR-338-3p were first reported in OS, and they have been found to be involved in other biological mechanisms or diseases. MiR-324-5p inhibits H5N1 virus replication by targeting virus PB1 and host CUEDC2[26]. Zhao et al. reported the involvement of miR-338-3p in salivary adenoid cystic carcinoma. The sponge of miR-338-3p is hsa_circRNA_0059655 which regulates cancer cell proliferation, migration and invasion in salivary adenoid cystic carcinoma[27]. Meanwhile, Xue et al. also reported on miR-338-3p, stating that the overexpression of miRNA-338-3p inhibited the expression of smoothened protein in colorectal cancer cells, showing significant inhibition of colorectal cancer cell invasion and migration[28]. A role of miR-326 in OS has been reported, with the long non-coding RNA SNHG1 acting as a sponge of miR-326 to regulate the expression of human NIN1 binding protein (NOB1) and affect the growth, migration and invasion of OS[29].

At the mRNA level of the ceRNA network, Wang et al. found that in hFOB1.19 cells which were heterozygous for the c.3781A allele and the c.3781C allele, the expression of COL1A1 was regulated by hsa-miR-345-5p and affected alkaline phosphatase activity and substrate mineralization level[30]. Research by Hawkins et al. on Ewing's sarcoma found that activation of Wnt/beta-catenin signaling affects the expression level of COL1A1[31]. In addition, studies have found that COL1A1 polymorphism is associated with risks of OS transfer and patient death in which COL1A1 polymorphism at rs1061970 has value for overall survival in Chinese OS patients[32]. Research by Li et al. found that high expression of HNRNPA2B1 is associated with poor prognosis of OS, and Cox regression analysis showed that HNRNPA2B1 is an independent risk factor for OS[33]. But in our study, the expression level of HNRNPA2B1 does not shown the significantly correlated with overall survival in patients with osteosarcoma. The role of HNRNPA2B1 in osteosarcoma deserves further study. In addition, there have been no research reports on the mechanism involved in FAM98A and RAN in OS.

In conclusion, the above research verifies the great potential value of this research result in OS. This means that the differentially-expressed RNAs involved in this network may play a key role in the occurrence and development of OS. These RNAs deserve further research in the future.

Declarations

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Conflict of interests

The authors declare no conflict of interests.

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