Differentially expressed circRNA and mRNA profiles of neural stem cells with radiation irradiation

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Differentially expressed circRNA and mRNA profiles of neural stem cells with radiation irradiation

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Abstract

Background: Spine metastasis is common but highly problematic in clinical oncology practice. Radiotherapy plays an important role in the treatment of spine metastasis, but it at the same time damages the nervous tissue, especially the neural stem cell (NSC), and leads to radiation induced myelopathy. Circular RNA (circRNA) is a kind of non-coding RNA which responses to external stimulus and regulates cellular functions. However, the mechanism of radiotherapy affecting NSC and the role of circRNA in this process are still unclear.

Methods: The circRNA and mRNA of NSC treated with radiation or not were detect using next-generation sequencing. RT-PCR assays were preformed to confirm the sequencing results and the feature of differentially expressed circRNA. Bioinformation analyses were conducted to identified the critical circRNA and mRNA, as well as the enriched functions and pathways. Moreover, a circRNA-miRNA-mRNA network was constructed to investigate the possible regulatory mechanism.

Results: A total of 421 differentially expressed circRNA and 1602 differentially expressed mRNA of NSC were identified after radiotherapy. The GO and KEGG analysis of the differentially expressed mRNA as well as the host genes of the differentially expressed circRNA were performed and several key signal pathways such as MAPK signal pathway were identified. Moreover, a circRNA-miRNA-mRNA network focusing on MAPK signal pathway was shown and predicted that chr5:127160496|127165240 could be the critical circRNA in the regulatory mechanism of radiation treated NSCs.

Conclusion: Our finding showed the differentially expressed circRNA and mRNA profiles of NSC after radiotherapy, suggesting that circRNA may contribute to the pathogenesis of radiation induced myelopathy.

Key words: neural stem cell, circular RNA, radiation treatment

Introduction

Bone is one of the most common organs affected by cancer metastasis[1]. Based on the post-mortem studies, bone metastasis was found in about 70% patients with breast or prostate cancers. The bone metastasis largely reduces the living quality of the patients, leading to pain, limited mobility or even malignant spinal compression[2]. To address this highly problematic issue in clinical oncology practice, a lot of therapeutic schedules including radiotherapy have been put forward in the recent years[3].

Radiotherapy is widely used for clinical treatment of cancers as well as bone metastasis. It helps to inhibit the growth of tumor and limits the bone metastatic lesions, which in turn reduced the pain symptoms and improve the quality of life[4]. However, radiotherapy may cause radiation induced myelopathy especially used for spine bone metastasis[5]. Recently, researches have demonstrated that radiation treatment damaged the functions of neural stem cell (NSC), which was one of the key pathogenesis of radiation induced myelopathy[6]. However, the detailed mechanism is largely unclear.

NSC are self-renewing and multipotent cells in nervous tissue. NSC remain quiescent most of the time, but can differentiate into multiple cell lineages including neuron, astrocyte and oligodendrocyte after external stimulation[7]. These differentiation ability was under the regulation of various mechanisms, and regulatory dysfunction lead to abnormal NSC differentiation and the followed nervous system disorder[8].

Circular RNA (circRNA) is a member of the non-coding RNA family. CircRNA lacks both the 3` and 5` end as well as the poly A tail, which allowed it to form a special closed continuous loop covalently[9]. Many studies have demonstrated that circRNA played important roles in regulation of cellular functions in NSC[10]. However, whether circRNA contributes to the radiation induced myelopathy is still unknown.

In this study, we detected the differentially expressed profiles of circRNA and mRNA of NSC after radiation treatment. Besides, the GO and KEGG analysis were performed based on the differentially expressed mRNA and the host gene of circRNA. Focusing on the MAPK signaling pathway, a circRNA-miRNA-mRNA network was constructed to investigate the possible regulatory mechanism in radiation treated NSC.

Materials and methods

Cell isolate and culture

NSC was isolated and cultured as our previous study[11]. Briefly, the spinal cord tissues of a neonatal C57BL/6 mice were isolated and digested into single cell suspension. The cells were centrifuged and then suspended with DMEM/F12 medium supplemented with 2% B27 supplement, 5μg/ml heparin, 20ng/ml bFGF and 20ng/ml EGF. All cells were cultured at 37℃ humidified atmosphere with 5% CO_2, and the medium was changed every 3 days. The cells were used for experiments without passage. This study was approved by the Committee for the Care and Use of Laboratory Animals of Sun Yat-Sen University, Guangzhou, China.

Radiation treatment

Cultured NSC were centrifuged and seeded into a 4% poly-L-polylysine coated 6-well plates. The plates were immediately treated with radiation at a dosage of 16Gy. Cultured NSC without iradiation was used as a control. All NSC were used for experiments immediately after treatments.

CircRNA and mRNA sequencing expression profiling

Total RNA was extracted using TRIzol reagent and purified using NucleoSpin RNA cleanup kits according to the manufacturer's instructions. RNA integrity was determined by formaldehyde denaturing gel electrophoresis. Ribosomal RNA in total RNA was depleted and then digested by RNase R to remove the linear RNA. The residual RNA was reverse-transcribed into cDNA using a PrimeScript™ RT reagent Kit. End-repair and adaptor ligation were processed by using Illumina's TrueSeq Total RNA Library Prep Kit. Size of 250–300 bp cDNA fragments were separated and then PCR-amplified for about 20 cycles to build the library. After further purification and detection, the RNA-Seq was generated by using Illumina Hi-Seq 2000 sequencer. RNA-Seq short reads were aligned to the mouse reference genome (GRCm38 mm10) using TopHat (version 2.1.1). The RNA-Seq data were initially filtered through TopHat by mapping to the reference genome. The canonical splicing sites were detected and most reads were mapped. Then, the unmapped reads, were processed by BWA methods.

CircRNA identification

Total RNA was extracted and reverse-transcribed into cDNA as described above. Genomic DNA was extracted using a PureLink™ Genomic DNA Mini Kit. Two sets of primers for circRNA as well as GAPDH, including an outward-facing set and an opposite-directed set, were designed using the Primer Express software version 5.0. RT-PCR was performed with the Power SYBR® Green RNA-to-CTTM One-Step Kit using a Stratagene Mx3005P Real-Time PCR detection system. After gel purification using the QIAquick Gel Extraction Kit, the RT-PCR product was sequenced using the Sanger method to confirm the head-to-tail splicing.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted and reverse-transcribed into cDNA as described above. QRT-PCR assays were performed using SYBR Premix Ex TaqTM in the LightCycler 480 PCR System. All the data were normalized by GAPDH. The relative expression levels of circRNA and mRNA were analyzed using the $2^{-\triangle\triangle Ct}$ formula. The forward and reverse primers for each gene were present in Supplemental Table 1.

Bioinformatics analysis

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery, version 6.8)[12, 13]. Differentially expressed mRNA and host gene of differentially expressed circRNA were divided into three subgroups by GO analysis, including biological process (BP), cellular component (CC) and molecular function (MF). The related signaling pathways of these genes were enriched by KEGG analysis to determine the primary functions of the differentially expressed genes. Pathway analysis of the differentially expressed genes was also carried out based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Further, coding-non-coding gene co-expression (CNC) networks were constructed based on the results of correlation analyses between differentially expressed mRNA and circRNA (Pearson correlation coefficients > 0.99 or < 0.99). P values < 0.05 were considered statistically significant. We utilized miRanda and RNAhybrid as tools to predict the related target miRNAs and mRNAs. Ultimately, the graphs of circRNA-miRNA-mRNA sharing

meaningful correlation interaction networks were drawn using Cytoscape (version 3.3).

Statistical analysis

Statistical analysis was performed with SPSS software. All data are expressed as means ± SD. P values < 0.05 were considered to indicate a statistically significant difference. CircRNA demonstrating Log_2Ratio(radiation treated NSC/NSC) of ≥|1| and P-values of ≤0.05 were regarded as significantly differentially expressed.

Results

Differential expression profiling of circRNA and mRNA of NSC treated with radiation

The expression profiles of circRNA and mRNA of NSC before and after radiation treatment were analyzed by next-generation sequencing. Results showed that the expression profiles of both circRNA and mRNA were dramatically altered by radiation treatment (Figure 1). A total of 421 differentially expressed circRNA of NSC treated with radiation were identified compared to NSC without radiation treatment. Among these differentially expressed circRNA, there were 21 up-regulation circRNA and 400 down-regulated circRNA. The top 10 differentially expressed circRNA were shown in Table 1. Besides, a total of 1602 differentially expressed mRNA were also identified, with 641 up-regulated mRNA and 961 down-regulated mRNA. The top 10 differentially expressed mRNA were shown in Table 1.

GO and KEGG analysis of the host gene of differentially expressed circRNA

Under the assumption that circRNA function would be related to the known function of the host linear transcripts, we analyzed the host gene of differentially expressed circRNA using DAVID bioinformatics resources to investigate the possible mechanism of NSC affected by radiation treatment. GO analysis was performed to classify these host genes of differentially expressed circRNA into 3 domains, including biological process (BP), molecular function (MF), and cellular component (CC). The GO terms of BP, MF and CC with significant difference were shown in Figure 2A, B and C. Moreover, the functional pathways of these host genes were enriched by KEGG analysis, indicating that several key pathways, such as MAPK signal pathway and miRNA in cancer signaling, were involved in reaction of NSC treated by radiation

(Figure 2D).

GO and KEGG analysis of differentially expressed mRNA

A cell's mRNA expression profile reflects its status and function. Then we studied the change of NSC treated with radiation through analyzing the GO terms of those differentially expressed mRNA. The GO terms of BP, MF and CC with significant difference from the differentially expressed mRNA of radiation treated NSC were shown in Figure 3A-C. Besides, KEGG analysis was also performed to study the involved signaling pathways. Several related pathways including MAPK signaling pathway and cancer pathway were enriched in the top 20 signaling pathways (Figure 3D).

CircRNA-miRNA-mRNA analysis of differentially expressed circRNA and mRNA of MAPK signaling pathway

Many researches have demonstrated that MAPK signaling pathway was the critical pathway in regulating the function and differentiation of NSC. Shown by KEGG analysis, MAPK signaling pathway was enriched as one of the most significant functional pathways in both the differentially expressed mRNA and the host gene of the differentially expressed circRNA, indicating the importance of MAPK signaling pathway in radiation treated NSC (Figure 2 and 3). Therefore, a circRNA-miRNA-mRNA network was constructed based on the differentially expressed mRNA in MAPK signaling pathway (Figure 4). This network included six circRNA, 46 miRNA and 13 mRNA, and the top 5 circRNA-miRNA-mRNA connections in MAPK signaling pathway were shown in Table 3. Among this network, chr5:127160496|127165240 possessed more binding site and target miRNA, which had a relationship with all the mRNA in MAPK signaling pathway, emphasizing its role on the NSC with radiation treatment.

Validation of differentially expressed circRNA and mRNA of MAPK signaling pathway

In order to confirm the differential expression of circRNA and mRNA in the circRNA-miRNA-mRNA network of MAPK signaling pathway, PCR assays with sanger sequencing were performed. As shown in Figure 5A, all 6 differentially expressed

circRNA in the network were amplified using the outward-facing primers and cDNA as templates, which it could not be amplified using genomic DNA. These results confirmed the circular form of the 6 circRNA. Besides, the conjunctive site was identified by the sanger sequencing. Besides, the differential expression levels of these circRNA and mRNA were also confirmed by the qRT-PCR assays, which were consistent with results of the sequencing (Figure 5B and C).

Discussion

In this study, we performed the expression profiling of circRNA and mRNA of NSC treated with radiation. Hundreds of differentially expressed circRNA and mRNA related to radiation treatment were identified in NSC. Besides, the function and signaling pathways of these differentially expression circRNA and mRNA were enriched using the GO and KEGG analysis, from which MAPK signaling pathway was identified as the key pathway in NSC treated with radiation. Moreover, a circRNA-miRNA-mRNA network based on the MAPK signaling pathway was constructed to investigate the possible regulatory mechanism in NSC after radiation treatment.

Bone metastasis, especially spine metastasis, is one of the commonest and most thorny problems in orthopedic clinics. The therapeutic schedule for spine metastasis is the focus of clinical research, but several controversies still need to be addressed[14]. Previously, we demonstrated that intraoperative radiotherapy could effectively relieve pain, achieve good local control of spine metastasis and improve the quality of life[4]. However, radiation treatment may cause radiation induced myelopathy through damaging NSC[6]. NSC is a kind of undifferentiated stem cell that are defined by their replicative potential and long-term self-renewal, as well as the ability to differentiate into multiple neuronal and glial cell types[15]. Several studies have demonstrated that radiation treatment increased the apoptosis of NSC and inhibited the self-renewal and differentiation potential of NSC[16, 17]. However, the mechanism of radiation treatment affecting NSC is largely unknown.

CircRNA, a kind of non-coding RNA, is recently recognized as a new class of functional molecule. Due to its circular configuration through a typical 5′ to 3′-phosphodiester bond, circRNA keeps more stable in cells and take an active part in

cells` function regulation transcriptionally and post-transcriptionally[18]. Particularly, circRNA contribute greatly to the self-renewal and differentiation capacities of stem cells[19]. The circRNA profile of NSC during differentiation has been detected[20]. Besides, a circRNA HIPK2 has been shown to regulate NSC differentiation to neurons[21]. As shown by previous study that radiation altered the circRNA profile[22], we speculate that radiation treatment may damage the NSC function through their intracellular circRNA. In this study, for the first time to our knowledgement we determined that the circRNA profile of NSC was significantly changed by radiation treatment. A total of 421 circRNA were differentially expressed in NSC after radiation treatment compared to those without radiation treatment. These results indicated that radiation treatment may affect NSC through altering its circRNA profiling, and these 421 differentially expressed circRNA play important roles in regulating NSC function during radiation treatment.

NSC`s status and function were represented by their gene expression profiles[23]. In order to investigate the altered function and possible mechanism of NSC treated with radiation, we analyzed the differentially expressed mRNA and host gene of differentially expressed circRNA using GO and KEGG database. Specifically, we found that MAPK signaling pathway was enriched as the key pathway by both the differentially expressed mRNA and the host gene of differentially expressed circRNA, indicating that MAPK signal pathway play an important role of NSC after radiation treatment. MAPK signal pathways was widely involved in cell function regulation including NSC[24, 25]. Previous researches have demonstrated that radiation therapy upregulated the gene expression of MAPK signal pathway(26). Moreover, the activation level of MAPK signal pathway has been reported to be under the control of large numbers of circRNA[27-29]. From these results we suggest that radiation therapy may alter the circRNA expression profile, which in turn regulated the activation level of MAPK signal pathway and affect the function of NSC.

The amounting evidences have indicated that circRNA might regulate the function of miRNA acting as competing endogenous RNA (ceRNA)[30]. In order to study the detailed mechanism of circRNA to regulate the MAPK signal pathway of radiation

treated NSC, a circRNA-miRNA-mRNA was constructed using the differentially expressed circRNA and the differentially expressed mRNA in the MAPK signal pathway. Several miRNA in this network, such as miRNA204, has been demonstrated to be the key regulator of NSC function[31]. Besides, we found in this network that chr5:127160496|127165240 have the highest numbers of target per 100b. Besides, it may bind to a total of 34 predicted miRNA and regulate all the 13 differentially expressed mRNA in MAPK signal pathway of radiation treated NSC. Therefore, we suggest that chr5:127160496|127165240 may be the critical circRNA of NSC after radiation therapy. Whether and how circRNA regulate the expression of these differentially expressed mRNA in MAPK through miRNA merits further study.

Conclusions

In this study, we investigate the differentially expressed profile of circRNA and mRNA of NSC treated with radiation. The possible affected function and mechanism were analyzed using bioinformatics methods. However, some limitations still exist in this study. For example, which circRNA exactly play the key role during radiation therapy still need to confirm. Besides, how the key circRNA function and regulate the NSC function is also unknown. Further studies should be addressed to elucidate these questions.

Declarations

Ethics approval and consent to participate

This study was approved by the Committee for the Care and Use of Laboratory Animals of Sun Yat-Sen University, Guangzhou, China.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Zhaopeng Cai, Shan Wang and Zhongyu Xie designed the study and performed the experiments; Keng Chen and Huiyong Shen collected the data and wrote the manuscript. Peng Wang analysed the data. All authors read and approved the final manuscript.

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Figure Legend

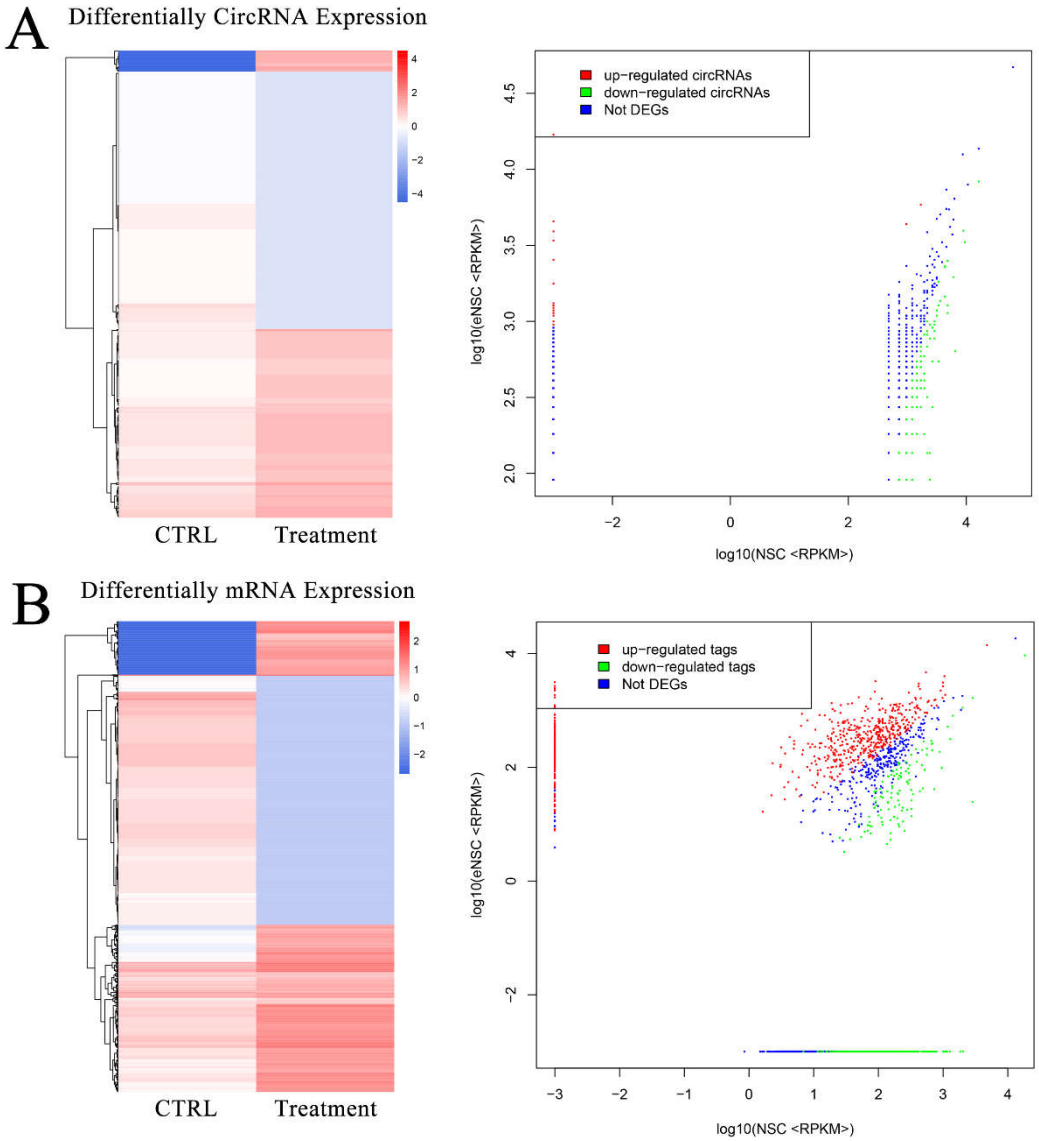


Figure 1 Differentially expressed profiles of circRNA and mRNA. (A) A total of 421 circRNA were differentially expressed in NSC treated with radiation (Treatment group) compared to NSC without treatment (CTRL group). Hierarchical clustering and volcano figure showed a distinguishable circRNA expression profile. **(B)** A total of 1602 mRNA were differentially expressed in NSC treated with radiation (Treatment group) compared to NSC without treatment (CTRL group). Hierarchical clustering and volcano figure showed a distinguishable mRNA expression profile.

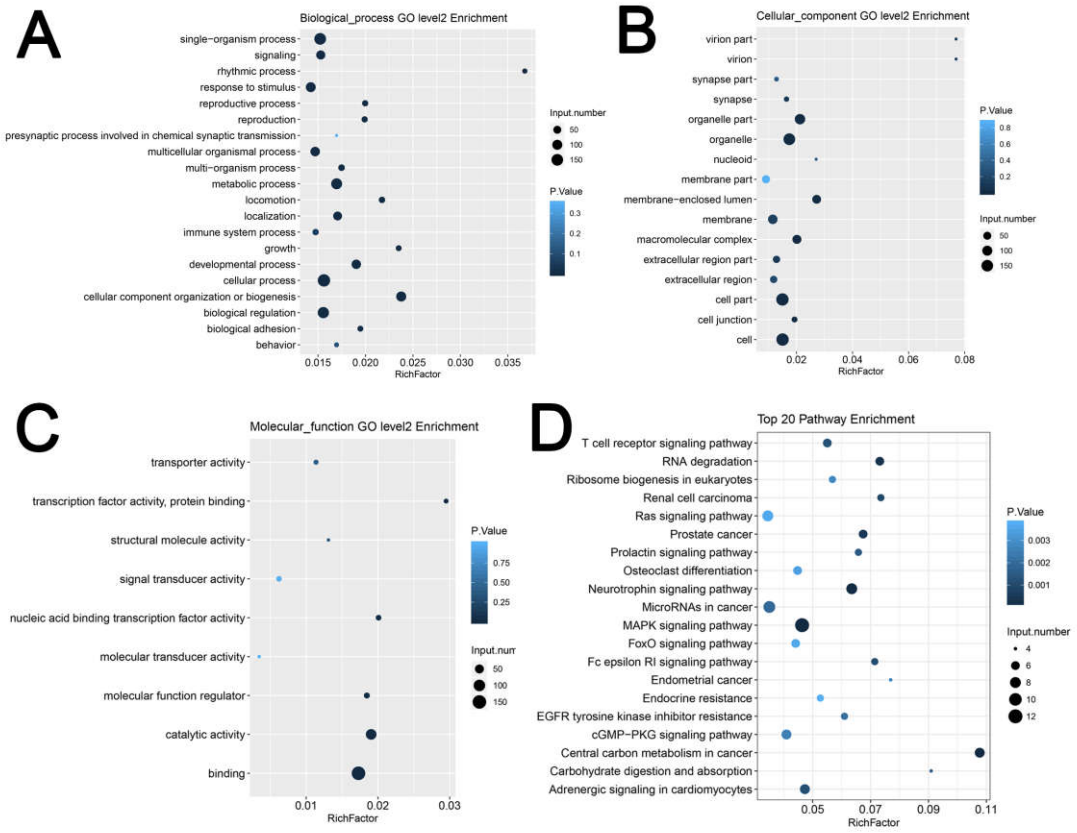


Figure 2 GO and KEGG analysis of the host genes of differentially expressed circRNA. (A) The top 20 biological process terms of GO analysis were shown. **(B)** The molecular function terms with significant difference of GO analysis were shown. **(C)** The cellular component terms with significant difference of GO analysis were shown. **(D)** The top 20 functional pathways of these host genes were enriched by KEGG analysis.

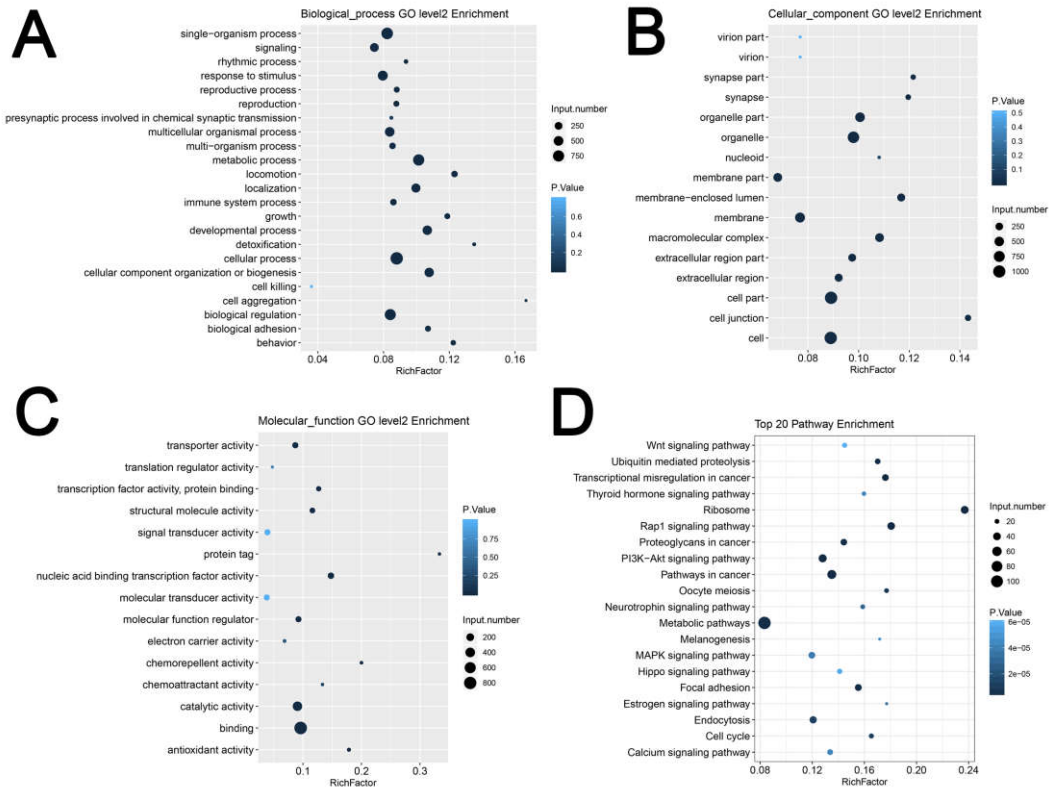


Figure 3 GO and KEGG analysis of differentially expressed mRNA. (A) The significantly different 23 biological process terms of GO analysis were shown. **(B)** The 16 molecular function terms with significant difference of GO analysis were shown. **(C)** The 15 cellular component terms with significant difference of GO analysis were shown. **(D)** The top 20 functional pathways of these host gene were enriched by KEGG analysis.

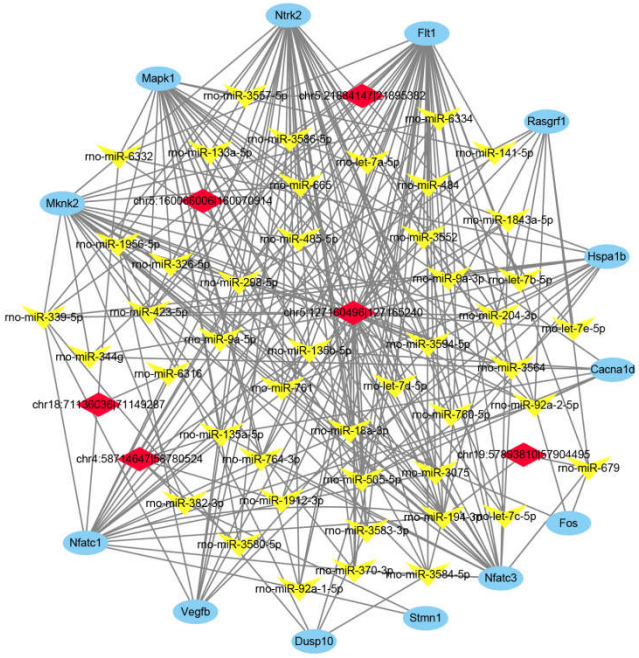


Figure 4 CircRNA-miRNA-mRNA network of differentially expressed circRNA and mRNA in MAPK signaling pathway. CircRNA-miRNA-mRNA network were constructed based on the MAPK signaling pathway. Six circRNA, 46 miRNA and 13 mRNA were included.

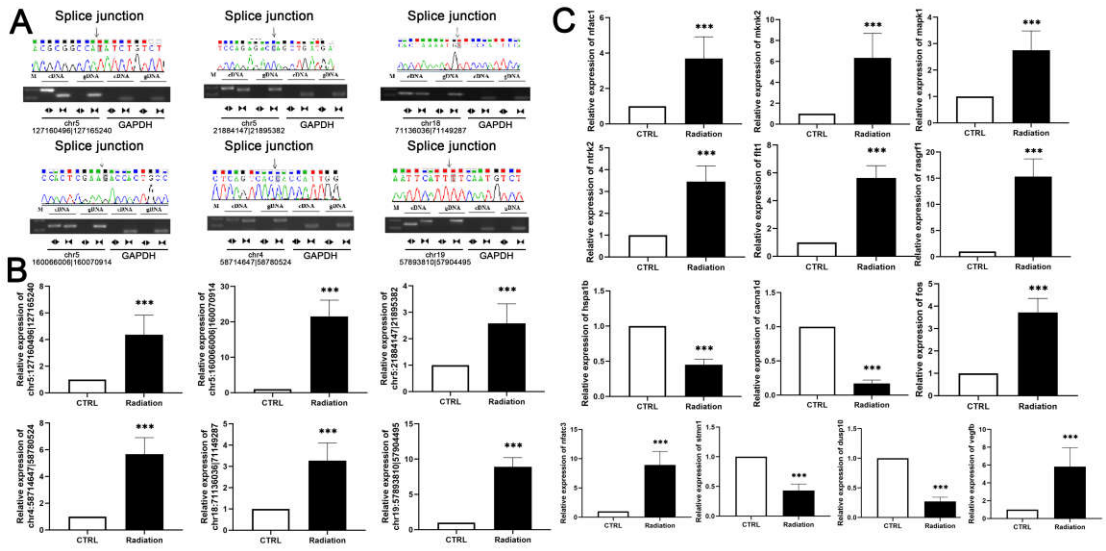
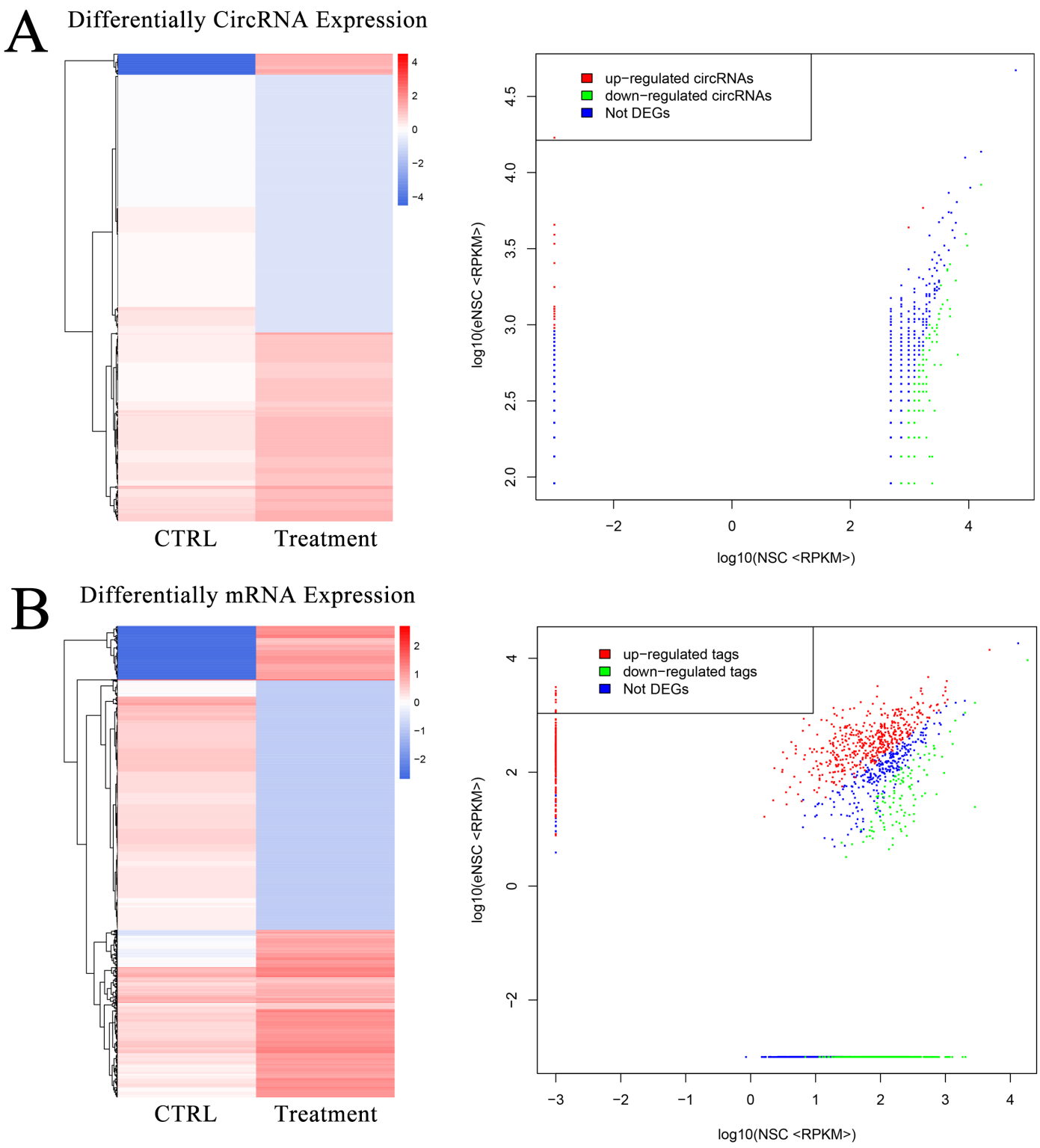


Figure 5 Validation of differentially expressed circRNA and mRNA of MAPK signaling pathway. (A) RT-PCR and sanger sequencing assays confirmed the circular form and conjunctive site of 6 circRNA in the circRNA-miRNA-mRNA network. **(B)** QRT-PCR confirmed the differential expression of 6 circRNA in the circRNA-miRNA-mRNA network. **(C)** QRT-PCR confirmed the differential expression of 13 mRNA in the circRNA-miRNA-mRNA network. *** indicates P<0.001.

Figures



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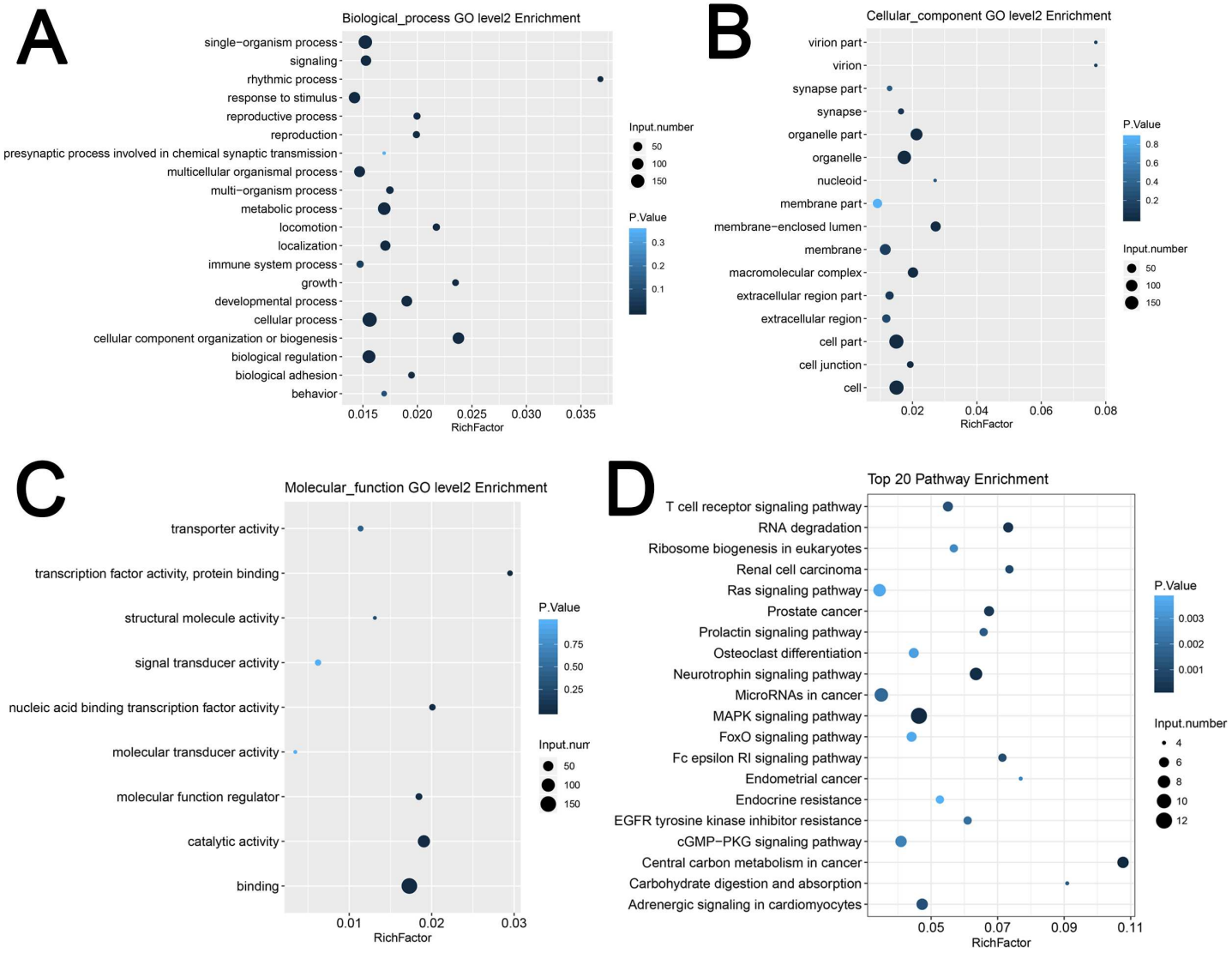

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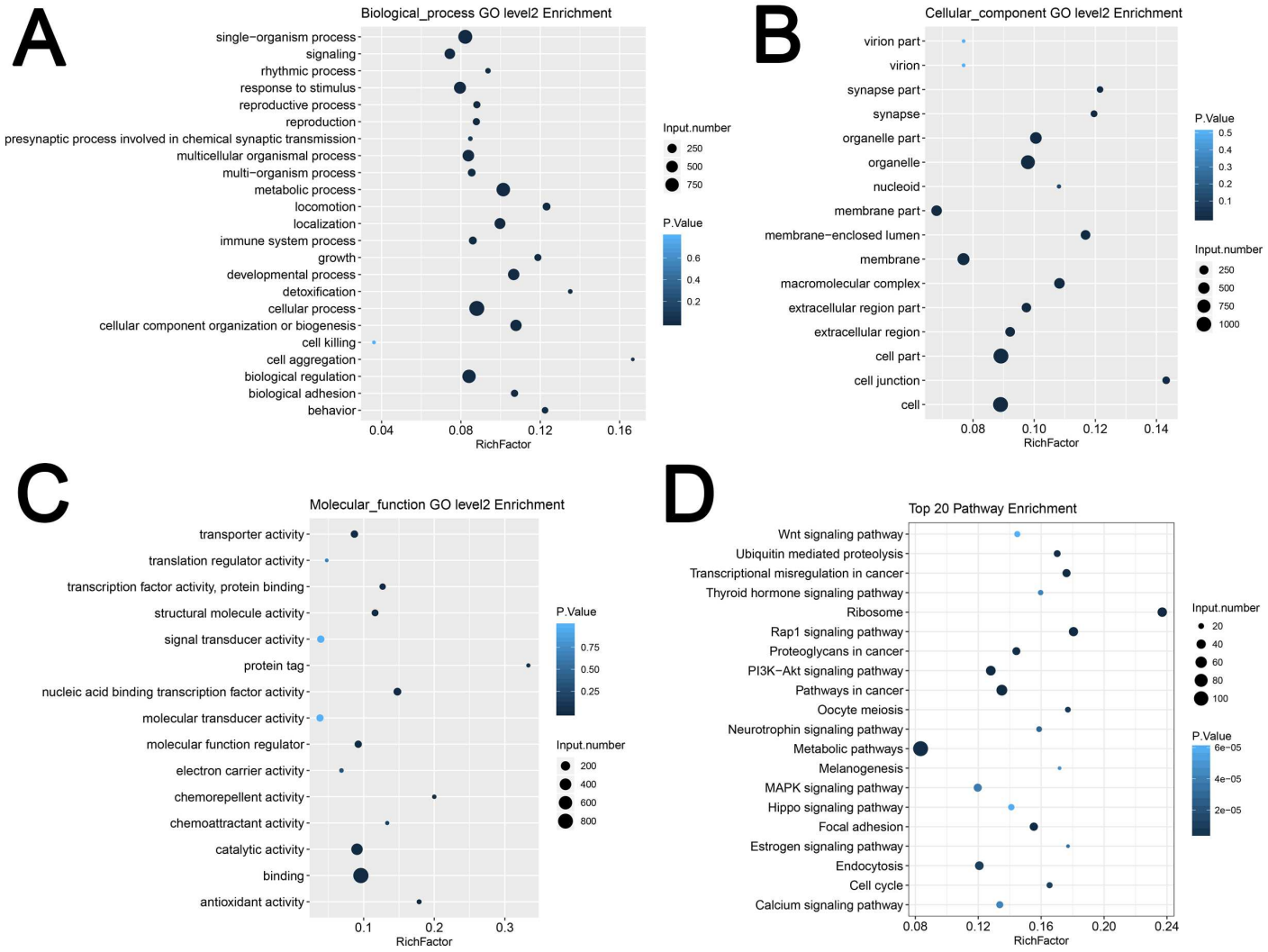

Figure 3

GO and KEGG analysis of differentially expressed mRNA. (A) The significantly different 23 biological process terms of GO analysis were shown. (B) The 16 molecular function terms with significant difference of GO analysis were shown. (C) The 15 cellular component terms with significant difference of GO analysis were shown. (D) The top 20 functional pathways of these host gene were enriched by KEGG analysis.

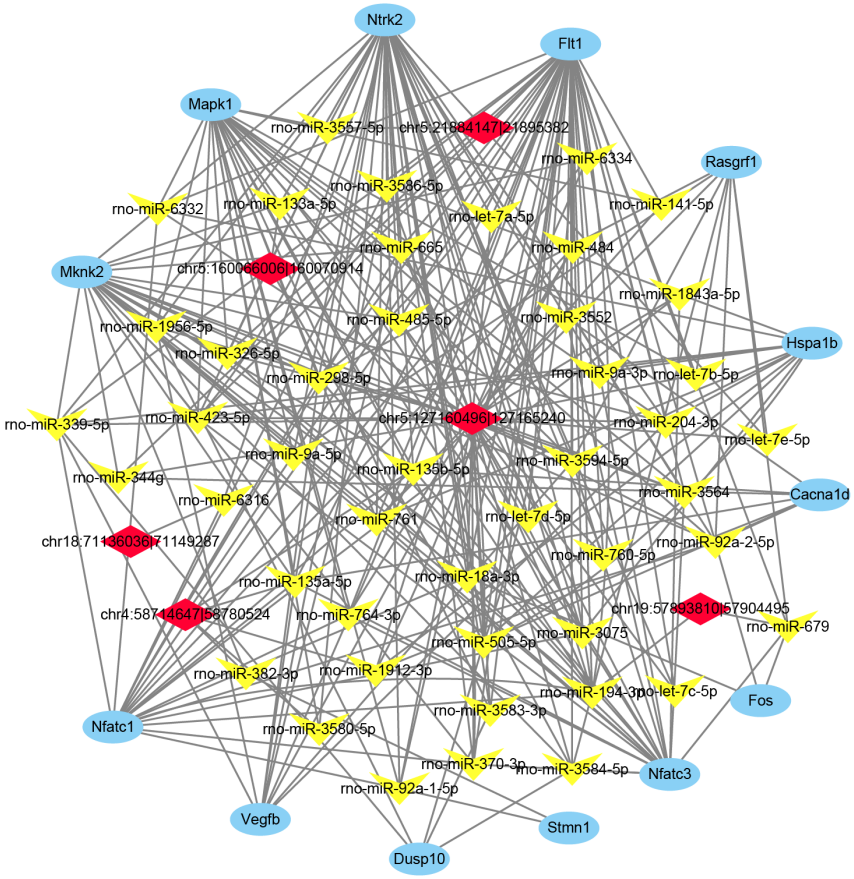

Figure 4

CircRNA-miRNA-mRNA network of differentially expressed circRNA and mRNA in MAPK signaling pathway. CircRNA-miRNA-mRNA network were constructed based on the MAPK signaling pathway. Six circRNA, 46 miRNA and 13 mRNA were included.

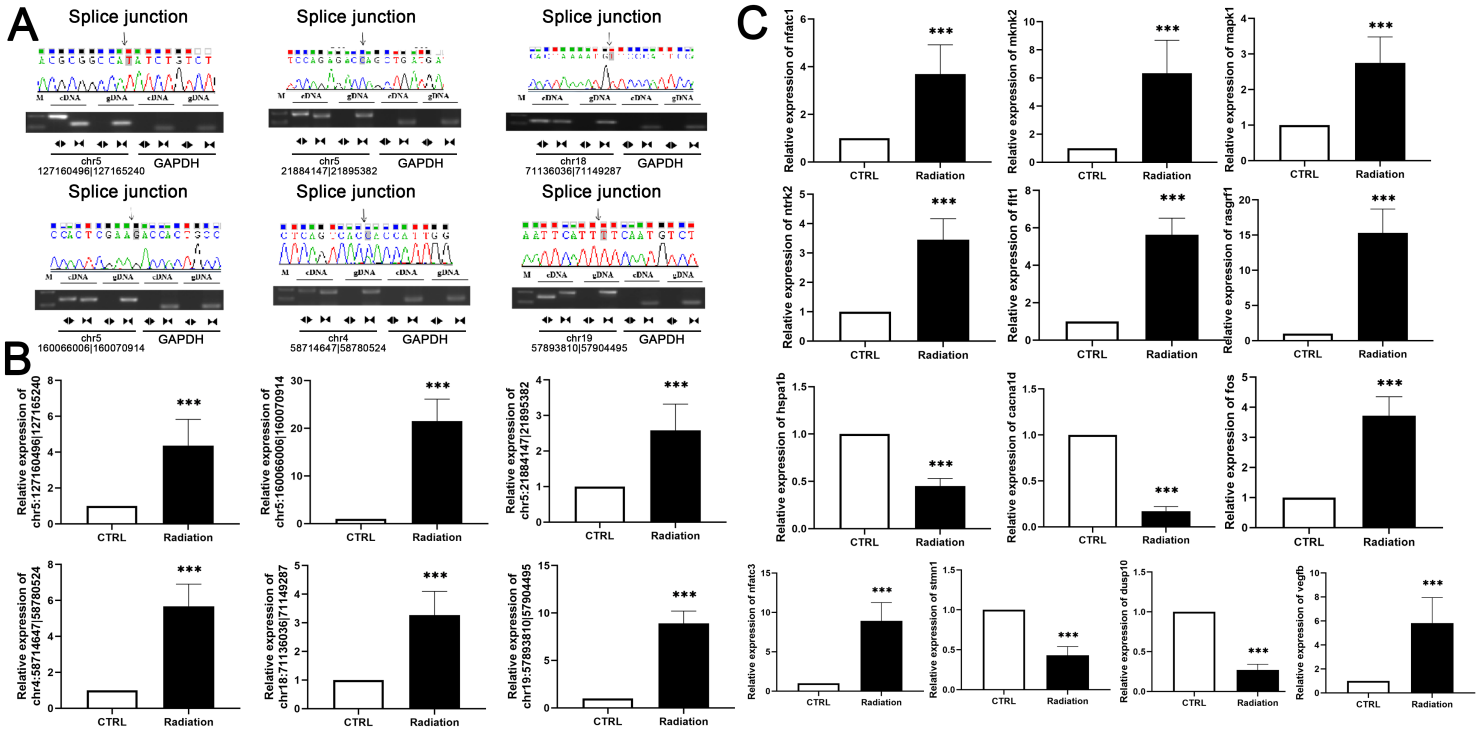

Figure 5

Validation of differentially expressed circRNA and mRNA of MAPK signaling pathway. (A) RT-PCR and sanger sequencing assays confirmed the circular form and conjunctive site of 6 circRNA in the circRNA-miRNA-mRNA network. (B) QRT-PCR confirmed the differential expression of 6 circRNA in the circRNA-miRNAmRNA network. (C) QRT-PCR confirmed the differential expression of 13 mRNA in the circRNA-miRNA-mRNA network. *** indicates P<0.001.

Supplementary Files

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- Supplementaltable1.docx