

Analysis of exosomal circRNAs upon irradiation in pancreatic cancer cell repopulation

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Abstract

Background : Pancreatic cancer is one of most malignant tumors. However, radiotherapy can lead to tumor recurrence, which is caused by the residual surviving cells repopulation stimulated by some molecular released from dying cells. Exosomes may mediate cell-cell communication and transfer kinds of signals from the dying cells to the surviving cells for stimulating tumor repopulation. Circular RNAs (circRNAs) may be one vital kind of exosomal cargos involving in modulating cancer cell repopulation.

Methods: Next generation sequencing (NGS) and bioinformatics were performed to analyze and annotate the expression and function of exosome-derived circRNAs in pancreatic cells during radiation. 4 circRNAs were chosen for qRT-PCR analysis to validate the sequencing results.

Results: In this study, 3,580 circRNAs were annotated in literatures and circBase among 12,572 identified circRNAs. There were 196 filtered differentially expressed circRNAs (the up-regulation and down-regulation respectively is 182 and 14, fold change >2, p-value <0.05). Regulation of metabolic process and lysine degradation were the main biological processes and pathway enrichment according to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis.

Conclusions: The hsa_circ_0002130-hsa_miR_4482-3p-NBN interaction network suggested potential miRNA sponge and target mRNA. Our results provided potential functions of circRNAs to explore molecular mechanisms and therapeutic targets in pancreatic cancer cell repopulation upon irradiation.

Background

Pancreatic cancer is a highly fatal disease, which the morbidity of which is almost the same as the mortality. Focusing on the global burden of cancer worldwide, the number of new cases and deaths of pancreatic cancer respectively was 458,918 and 432,242 with 2.5% and 4.5% of its proportion severally^[1]. The high mortality of pancreatic cancer may partly ascribe to the lack of effective strategies in controlling regional unresectable disease without detectable metastasis, to which radiotherapy should be an optimal choice in most cancer. Although American Society for Radiation Oncology (ASTRO) has recently issued a guideline for treatment of pancreatic cancer patients using radiotherapy, either conventional radiotherapy or SBRT, *etc.*, the quality of evidence for recommendation using of radiotherapy is largely low^[2]. Efforts to improve the efficacy of radiotherapy in pancreatic cancer is ongoing. New radiation dose, modality, fraction size, and sequencing, as well as in combination with other treatment strategies such as immunotherapy have been largely studied^[3]. However, no significant progress has been achieved so far.

Although kinds of treatments could relieve pancreatic patients' symptoms, they failed to improve five-year survival rate. It has been found that radiotherapy could cause tumor accelerated repopulation, which is known as the main cause of treatment failure^[4, 5]. Recently, our team revealed that cancer cell repopulation after radiotherapy were related to the dying cells caused by the treatment, which could inactivate or activate relevant signalings to stimulate the residual surviving cells to fast grow. The related molecules,

such as PGE2, microRNAs and other molecules, played as a switch to promote the repopulation^[6-8]. Exosomes, one type extracellular vesicle of about 30-150 nm, contain rich cargos such as DNA, mRNA and some non-coding RNAs^[9]. Recently, it has been well reported that exosomes might transmit the key factors to the receptor cells, which promote the tumorigenesis and progression^[10, 11]. Thus, further exploring the contents of pancreatic cancer cell-derived exosomes would help to reveal the molecular mechanism of repopulation upon irradiation.

CircRNAs are predominantly in the cytoplasm, which formed a covalently closed continuous loop without 5'cap or 3' polyadenylated tail^[12]. Therefore, they are resistant towards exonucleases. Based on their biogenesis mechanism, different kinds of circRNAs could be characterized to mainly five parts, exonic circRNAs, intronic circRNAs, antisense circRNAs, sense overlapping circRNAs and intergenic circRNAs^[13]. Among them, the exonic circRNAs were the most detected^[14]. As endogenous competitive RNAs, circRNAs can compete for miRNAs through MREs (miRNA recognition elements, MREs)^[15, 16]. CircRNAs could be sorted into exosomes and participate in cancer progression^[17]. Especially, exosome-derived circRNAs could promote invasive growth through miRNA sponge in pancreatic cancer^[18]. Although sponge miRNAs are partly demonstrated during radiation, the function of circRNAs remains largely unknown.

During the last decades, next generation sequencing (NGS) has been widely used to identify the differentially expression, functional pathways and early diagnosis of pancreatic cancer at genomic, transcriptome and epigenetic level^[19, 20]. In this study, we generated RNA sequencing data from 4 types of pancreatic cancer cells (PANC-1, SW1990, BxPC-3, [MIAPaCa-2](#)) which were treated with or without irradiation, and identified ~12570 circRNAs. The accuracy of sequencing was verified by quantitative real-time RT-PCR (qRT-PCR) of the differentially expressed circRNAs (DE-circRNAs). Bioinformatics analysis including GO and KEGG were then performed to annotate the selected DE-circRNA functions. A circRNA-miRNA-mRNA network was subsequently constructed to reveal the molecular regulatory networks.

Methods

Cell culture

4 pancreatic cancer cells were used for analysis, including PANC-1 (CRL-1469, ATCC), BxPC-3 (CRL-1687, ATCC), SW1990 (CRL-2172, ATCC) and MIA PaCa-2 (CRL-1420, ATCC). Receiving irradiation as an experimental group (group B), while no radiotherapy as a control group (group A). 10 Gy of X-ray radiation was used in experimental group as we reported to induce tumor repopulation before^[6, 7]. Fetal bovine serum (FBS) centrifuged at 120,000 g for 18 hr was used for cell culture to avoid the interference with experimental results^[21].

Exosome isolation and identification

Differential centrifugation is used for exosome purification. Briefly, low-speed centrifugation and high-speed centrifugation are used alternately to separate vesicular particles of similar size. Centrifugation was performed at 300, 2,000 and 10,000 g to remove the live cells, dead cells and cell debris, respectively. The crude extract of exosomes was obtained by ultra-high speed centrifugation (>100,000 g), and the operation was repeated twice to remove the contaminated protein for collecting the purified exosome^[22].

RNA extraction and purification

Total RNAs from two groups (group A and B) were extracted using TRIzol reagent (Life Technologies, USA), and then treated with DNase I (Takara, China) to reduce interference with genomic DNA, according to manufacturer's instructions. The quantity and purity of total RNAs was determined by NanoDrop ND-100 (Thermo Fisher Scientific, USA). Well qualified RNA was further studied in the following experiments.

circRNA library construction and sequencing

rRNAs were removed by Ribo-Zero rRNA Removal Kits (Illumina, USA) from total RNAs. TruSeq Stranded Total RNA Library Prep Kit (Illumina, USA) was used for constructing RNA libraries by following the manufacturer's instructions. The quality and quantification of libraries was detected by BioAnalyzer 2100 system (Agilent Technologies, USA). 10 pM libraries was denatured to single-stranded DNA molecules, which amplified *in situ* as clusters and sequenced for 150 cycles on Illumina HiSeq Sequencer according to the manufacturer's instructions.

Sequence mapping and circRNA annotation

Paired-end reads were obtained from Illumina HiSeq 4000 sequencer for quality control by Q30. After 3' adaptor-trimming, [Cutadapt](#) software (v1.9.3) was used to remove low quality reads, and STAR software (v2.5.1b) was used to compare high quality reads with hg19 human reference genome. CircRNAs were detected and identified using DCC software (v0.4.4). EdgeR software (v3.16.5) was used for data normalization and DE-circRNA analysis. GO and KEGG analysis were performed to predict the function of associated genes of candidate circRNAs.

Identification of DE-circRNAs

DE-circRNAs in the two groups were identified using the edgeR software with quasi-likelihood F test (fold change >2 and p-value <0.05). The DE-circRNAs were log₂ transformed, gene mean centred and

visualized as a heatmap. Volcano plots were generated using ggplot2 in R (<https://cran.r-project.org/web/packages/ggplot2/index.html>).

Functional annotation of target miRNAs and prediction of interaction networks

In terms of functional annotation of candidate circRNAs, the genes of DE-circRNAs were clustered in GO annotations (<http://www.geneontology.org>) and KEGG pathways (<http://www.genome.jp/kegg/>) by DAVID Bioinformatics Resources (<http://david.ncifcrf.gov/home.jsp>). P-value was calculated by fisher test and adjusted by Benjamini& Hochberg. The result of prediction of potential circRNA-miRNA binding sites was obtained through TargetScan (<http://www.targetscan.org>) and miRanda (<http://www.microrna.org/microrna/home.do>). The mRNA-miRNA-circRNA interaction analysis was performed for top 3 expression quantity by using TargetScan, miRanda, Circinteractome (<https://circinteractome.nia.nih.gov/>) and Circbank (<http://www.circbank.cn/>). The enrichment score was comprehensive considered to the score from TargetScan and the thermodynamic properties of the binding site from miRanda. The predicted target genes of the DE-circRNAs were further subjected to GO term and KEGG pathway analyses. The gene network analysis was performed using Cytoscape.

qRT-PCR

Total RNA was reverse transcribed using random primers with the PrimeScript RT reagent kit (RR037A, Takara), following the manufacturer's protocol. We randomly selected 4 DE-circRNAs, including 3 up-regulated and 1 down-regulated circRNAs. Details of the primer sequences are summarized in Supplementary Table 4. Only primers whose PCR products showed a single peak in the melting curve were considered for qRT-PCR validation. Then, qRT-PCR was performed using SYBR Premix Ex Taq II (Takara, Dalian, China) on a QuantStudio 6 Flex (Life technologies, USA) according to the manufacturer's instructions. The expression level of candidate circRNAs was normalized to β -actin and then calculated using the $2^{-\Delta\Delta Ct}$ method. All experiments were repeated in triplicate and presented as the means \pm SD.

RNase R treatment

DNase-treated total RNAs were incubated for 1 hr at 37°C with or without RNase R (Epicentre). We used linear RNA FBXW7, GAPDH, β -actin and 18S RNA (which is poly(A)-tailed and must be degraded by RNase R treatment) and circRNA FBXW7 as controls.

Patient-derived xenograft mouse model

Clinical surgical specimens from tumor patients were cut into pieces and subcutaneously implanted into BALB/c nude mice (about 6-week-old) at right groin to build patient-derived xenograft (PDX) mouse model. When PDX tumor grew into about 500 mm³, mice were subjected to 10 Gy radiation. One week after radiation, blood was collected using EDTA as the anticoagulation agent. Plasma was separated by centrifuge at 3,000 rpm for 15 min. Exosomes were collected from plasma by ultracentrifugation.

Western blot

Protein was isolated by 10% SDS-polyacrylamide gel electrophoresis (SDS-page) and then transferred to a nitrocellulose membrane (Sigma-Aldrich, USA). After 90 min, membranes were incubated with TSG101 antibody (1:1000; Abcam, UK) at 4°C overnight. Membranes were washed and incubated for 1.5 hr with secondary antibodies (1:5000; Goat Anti-Rabbit IgG (H+L) Dylight 800, Odyssey, LI-COR, USA). The band intensity was measured using Empiria Studio. GAPDH was used as a loading control.

Statistical analysis

GraphPad Prism 7.0 was performed to Statistical calculation (version 7.0c; GraphPad Software, San Diego, USA). All the data were expressed as \pm SD of the mean value for triplicate independent measurements. t test was used to evaluate the differences between groups. p-value <0.05 were considered statistically significant.

Results

Exosome characterization and RNA-seq library construction

Exosomes secreted by 4 kinds of pancreatic cancer cells (PANC-1, SW1990, MIA Paca-2, BxPC-3) which were treated with (group B) or without (group A) irradiation were obtained by ultracentrifugation. Morphology and grain size observation and western blot analysis were used to identify the exosomes. Under transmission electron microscope (TEM), exosomes were irregular spheres ranging of 30-100 nm in diameter (Figure 1A, B). The irradiation group was denser than the control one. Grain size of exosomes was shown at 30-150 nm, which agreed well with the references^[9-11]. Accordingly, the concentration of irradiation group was higher than the control one (Figure 1C, D). Western blot was performed to analyze TSG101 (Figure 1E), a common biomarker of exosome^[22]. However, there was no expression inside the cells.

To systematically identify circRNAs from exosomes of the pancreatic cancer cells suffering irradiation, approximately 380 million reads for 4 samples were sequenced using quality-controlled total RNA-seq (rRNA depleted). The quality of RNA was shown in Supplementary Table 1. Libraries were controlled for quality and quantified showed in Supplementary Table 2. More than 86% of 10,096 circRNAs were

aligned by UCSC hg19 with STAR software (v2.5.1b) (Supplementary Table 3). CircRNAs were detected and identified. Whereafter, the data was normalized and analyzed to identify differentially expressed circRNA analyzed (Figure 1F).

Expression profiling of circRNAs

12572 circRNAs were quantitated the expression of circRNAs in whole samples, of which 8992 were novel based on comparison with circBase and published literatures. The majority of circRNAs were exonic circRNAs in all samples, whereas the sense overlapping circRNAs were just taken up a small proportion. Differently, among different expressed circRNAs (DE-circRNAs, fold change >2, p-value <0.05), the intergenic circRNAs were taken up the least (Figure 2A). The details of the newly and known circRNAs in 5 isoforms were shown respectively in Figure 2B. The further proportion rate among 5 isoforms of DE-circRNAs was shown in Supplementary Figure 1A. Among the exonic circRNAs, the mean length of known circRNAs were majorly concentrated on 200-300 nt (254 nt, Figure 2C). On the basis of the expression, known circRNAs were distributed across all chromosomes among the whole circRNAs, while except for chromosome 22 and chromosome Y among novel circRNAs. The distribution of circRNAs across chromosome 1 and 2 was more significant than other chromosomes (Figure 2D). Similarly, the chromosome distribution of DE-circRNAs was also exhibited (Supplementary Figure 1B). Furthermore, the details of expression files of all circRNAs quantitated in our study were presented in Supplementary Data 1 and 2. And the data of chromosomal location of the DE-circRNAs were also shown in Supplementary Data 3.

Annotation, visualization and functional clustering of DE-circRNAs

CircRNA expression levels changed in these exosomes. 196 of DE-circRNAs were identified and shown in heatmap, consisting of 182 up-regulated and 14 down-regulated circRNAs (Figure 3A, fold change >2, p-value <0.05). Especially, all the down-regulated circRNAs were located on the chromosome M. We noted that 174 circRNAs were 6 folds significantly up-regulated, 7 circRNAs were 8 folds significantly up-regulated, and 1 circRNA (hsa_circ_000284) were more than 8 folds significantly up-regulated. Moreover, 12 circRNAs were 6 folds significantly down-regulated, and 2 novel circRNAs (chrM:14131-15754- and chrM: 14131-115754+) were 8 folds significantly down-regulated (Figure 3B). Based on the expression, the volcano plot also revealed the dysregulated circRNAs clustered in red square (Figure 3C).

586 GO terms were significantly enriched for the host genes of the up-regulated circRNAs, which contained biological process (BP), cellular component (CC) and molecular function (MF). As shown, the top 10 significantly terms enriched in BP, CC and MF (Figure 3D). As can be seen from the results, genes on the regulation of metabolic process, binding and intracellular were most significant respectively in GO analysis. However, there was no term enriched for the genes of the down-regulated circRNAs. 26 KEGG

pathways were associated with the DE-circRNAs, which the top 10 enriched pathways highly associated with lysine degradation, phagosome, axon guidance, allograft rejection, HTLV-I infection, endocytosis, Type I diabetes mellitus and Graft-versus-host disease (Figure 3E). Furthermore, the details of all terms of GO and KEGG were shown in Supplementary Data 4 and 5.

Experimental validation of circRNA-sequencing

4 circRNAs (hsa_circ_0000419, hsa_circ_0001523, hsa_circ_0000825, and chrM:14131-15754-) identified as DE-circRNAs by high-throughput sequencing were randomly selected for validation (Figure 4A). qRT-PCR was performed to detect the expression of these circRNAs in Group A and B, which used for RNA-seq. We also selected the above mentioned circRNA, circFBXW7, and the paired linear RNA^[23], mRNA of common housekeeping genes (β -actin, GAPDH, and 18S RNA) as controls for RNase R resistance experiment. The results of qRT-PCR revealed that the expression of all linear RNAs (FBXW7, β -actin, GAPDH and 18S RNA) significantly decreased after 1 hr of RNase R digestion (** p-value <0.001). This indicated that circRNAs were resistant to RNase R digestion, whereas linear RNA was sensitive to R treatment (Figure 4B).

Prediction of circRNA-miRNA interaction network

To further clarify the potential roles of circRNAs, we showed most significant circRNAs in two samples of each group (p-value <0.0001) to build a circRNA-miRNA interaction network using bioinformatics prediction software. Top 5 significant miRNAs were chosen as the circRNA targets (Figure 5). The circRNA-miRNA interaction analysis for all DE-circRNAs was shown in Supplementary Data 6.

hsa_circ_0002130/hsa_miR_4482-3p/NBN interaction axis

According to the expression quantity of the bioinformatics prediction, 3 top known circRNAs (hsa_circ_0002130, hsa_circ_0000825 and hsa_circ_0005882, p-value <0.001) were chosen to construct the mRNA-miRNA-circRNA interaction network axis (Figure 6A), and the newly circRNAs were shown in Supplementary Figure 2.

CircRNAs could act as “miRNA sponge” to regulate miRNA level^[19,20]. To explore the function of circRNAs, the binding sites between DE-circRNAs and miRNAs were conducted by online databases. With the gene sequence of DE-circRNAs and the predicted binding sites from miRNA, a circular RNA-miRNA network diagram was constructed using cytoscape software. Similarly, the miRNA paired with their target mRNAs. Therefore, their interactions formed the circRNA-miRNA-mRNA axis.

For the binding sites of miRNAs on the 3'UTR of differentially expressed genes, top 5 miRNAs and top 3 genes with the highest binding force were chosen. The most significant circRNA, hsa_circ_0002130, as to further observed the influence of target genes. For example, the irradiation-induced cellular damage includes the modification of DNA bases resulting in DNA strand breaks^[24]. Nibrin (NBN) is the gene whose product is associated with DNA double-strand break repair and DNA damage-induced check point activation^[25]. As shown, NBN could be regulated by hsa_miR_4482-3p. The predicted binding sites between hsa_circ_0002130, hsa_miR_4482-3p and NBN were demonstrated in Figure 6B. And it showed two possible binding sites between hsa_miR_4482-3p and NBN. The expression level was shown in Figure 6C (Left). RNase R experiments were shown in the Supplementary Figure 1C. However, to validate the change of hsa_circ_0002130, the vital part of the axis, *in vivo* is of great value. Thus, we performed qPCR assay in exosomes derived from plasma of the irradiated/unirradiated PDX tumor, which was generated in our previous work^[26]. We identified that exosomal hsa_circ_0002130 was apparently higher in plasma from the irradiated mice compared with that in the untreated group (Figure 6C (Right)). Furthermore, mRNA level of NBN was up-regulated in exosomes during irradiation (Figure 6D). And according to TCGA database, the survival curve for NBN was shown in Figure 6E (Log rank p-value=0.0234). The survival probability of the high expression of NBN was lower than that of the low one.

Discussion

Tumor repopulation usually occurs after a lag phase of cancer cytotoxic therapies, which causes the treatment failure of cancers. Our previous studies revealed that chemoradiation-induced dying tumor cells could stimulate tumor repopulation^[4, 6-8]. Exosomes are one type of extracellular vesicles containing rich cargos to mediate cell-cell communication. Several circRNAs such as circ-PDE8A^[18], circ-IARS^[27] have already been suggested to promote pancreatic cancer progression and might be the potential indicator for early diagnosis and prognostic prediction in pancreatic cancer.

CircRNAs are one class of RNA regulatory molecules, which were ubiquitous in the transcriptomes of different organisms such as humans, mice, flies worms and other organisms^[28, 29]. In our study, different circRNAs were produced by selecting different binding sites and existed mainly in exons. 12,572 circRNAs were identified, and 3,580 circRNAs were annotated as the known circRNAs. We obtained the reads of circRNAs from 5 parts depending on the location of the chromosome, including exonic, intronic, sense overlapping, intergenic and antisense, which were similar to the publication^[13]. Importantly, most of the identified circRNAs (71.52%) are novel, which mainly concentrated on four parts except the exon (Figure 2C). Besides, the mean length of these identified exonic circRNAs is focus on 200-300 nt. Interestingly, it was reported recently that circRNAs releasing could be influenced by their own length, which indicated that the length of circRNAs might influence its formation^[30]. Furthermore, the unique structure of circRNA without a poly(A)-tail rendered themselves highly insensitive to ribonuclease. We showed that circRNAs were more stable than linear RNAs through RNase R digestion. Alternatively, Ribo-zero could also be used to circRNA validation^[31].

As known, GO and KEGG pathway analysis could predict the potential molecules participated in several **biological behaviors**. In our study, the circRNA profiles were investigated and the potential targets of circRNAs were accordingly predicted. The results in BP indicated that the highly expressed target genes might be involved in regulation of metabolic process, RNA biosynthetic and metabolic process, and nucleic acid-templated transcription. These GO terms might be associated with nucleotide metabolism, which was involved in radiotherapy^[32]. Refer to MF, “Binding”, “protein methyltransferase activity”, “histone methyltransferase activity”, “histone methyltransferase activity (H3-K4 specific)” and “histone-lysine N-methyltransferase activity” were highly enriched. As reported, VDR and p53 in irradiated HEK 293T was related to various biological effects, which mediated gene transcription^[33]. Moreover, aberrant methylation could lead to dysregulation of related transcription-factor genes, which might be closely bound up to irradiation^[34, 35]. KEGG analysis indicated that the lysine degradation was important, which was consistent with the results of GO analysis. Lysine degradation mainly participated in the control of gene expression and transcription. These findings indicated the cargos in exosomes could regulate different pathway and biological processes concerning cell proliferation and irradiation. However, further experimental verification is still needed, especially onto tumor repopulation owing to radiotherapy.

In studies, miRNA sponge acts as an important molecular strategy to modulate expression of proteins^[36]. According to our results, numerous miRNA binding sites were predicted by Circinteractome, Circbank, Miranda and TargetScan, which revealed circRNAs could interact with miRNAs. Based on bioinformatics, mRNA-miRNA-circRNA regulatory network was constructed to clarify the interactions between molecules. As shown in Figure 6A, the hsa_circ_0002130/hsa_miR_4482-3p/NBN interaction axis was involved in the regulatory network. NBN gene is a member of the MRE11/RAD50 double-strand break repair complex. Several studies confirmed that NBN is crucially involves in DNA double strand fracture repairing and DNA damage-induced checkpoint activation^[37]. And the expression level of hsa_circ_0002130 was also up-regulated during irradiation, consistent with the expression pattern of NBN. Given the potential binding sites among hsa_circ_0002130/hsa_miR_4482-3p/NBN, hsa_circ_0002130 was considered to promote the expression of NBN through the absorption of hsa-miR-4482-3p. More remarkably, some circRNAs were also shown to be translated into proteins^[38, 39]. Thus, the underlying mechanisms still needed further investigations.

In recent years, expression of circRNAs in pancreatic cancer is widely identified in several studies. However, few studies focused on the pancreatic cancer cells derived-exosome after irradiation. Here, circRNA profiles of exosome-derived circRNAs were shown, and the top 5 putative target miRNAs for DE-circRNAs were identified in pancreatic cancer during irradiation. The construction of a mRNA-miRNA-circRNA interaction network will provide a better understanding of the regulatory relationships and mechanisms between multiple kinds of RNAs of irradiation resistant. The axis of hsa_circ_0002130/hsa_miR_4482-3p/NBN might play vital roles in pancreatic cancer radioresistance and repopulation. As we recently found that irradiated dying tumor cells-derived exosomes promoted DNA damage repair and the survival of damaged tumor repopulating cells^[26], the exosomal hsa_circ_0002130 might also mediate the vital role of the exosomes. Thus, further works could be directed to identify the

role of dying tumor cell-derived exosomal hsa_circ_0002130 in potentiating tumor repopulation via promoting DNA damage repair, which is deduced to be mediated by the hsa_circ_0002130/hsa_miR_4482-3p/NBN axis.

Nevertheless, there are still several drawbacks inherent to our study. The small sample size was for RNA-sequencing, and samples from the patients were hard for us to collect. Therefore, the influence from the patients should be further validated. On the other hand, even if we believe that they worked, we did not perform further experiments to confirm the regulatory relationship among circRNAs, miRNAs and mRNAs, as well as other functions of circRNAs.

Conclusions

We herein above first identified and annotated 12572 circRNAs in the exosomes of human pancreatic cancer cells upon irradiation using RNA-seq analysis. Most of these circRNAs were composed of exons. Some circRNAs were significantly changed during irradiation treatment. Among them, the DE-circRNAs were associated with methylation, which was in accord with the signaling pathways. 4 circRNAs were chosen to verify the reliability of RNA-sequencing by using a series of biological experiments. Most of them are consistent with the expectation, but more research on the reliability of results is also needed. Furthermore, mRNA-miRNA-circRNA interaction network axis provided a line of inquiry to understand of the relationship of different molecules. We demonstrated that hsa_circ_0002130 could target NBN by combing with the hsa_miR_4482-3p. Based on TCGA database, high expression of NBN revealed a worse survival. This indicated that hsa_circ_0002130 may play an important role in pancreatic cancer during radiotherapy.

Abbreviations

circRNAs: circular RNAs

NGS: next generation sequencing

qRT-PCR: quantitative real-time reverse transcription PCR

miRNA: microRNA

lncRNA: long non-coding RNA

MREs: miRNA recognition elements

GO: gene ontology

KEGG: kyotoencyclopedia of genes and genomes

DE-circRNAs: differentially expressed circRNAs

TEM: transmission electron microscope

BP: biological process

CC: cellular component

MF: molecular function

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

RNA-Seq data has been submitted to GEO database; accession GSE132678 while it remains in private status: ybkzeiuqjpixhkd. All other relevant data is available within the manuscript or its accompanying supplementary material.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YC and MJ performed the experiments, analyzes the data and wrote the manuscript. LT designed the protocols and revised the manuscript. ZC provided some helpful supports. All authors have read and approved the manuscript.

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Supplementary Material

Supplementary Figure 1. Different circRNAs in samples. (A) The further proportion rate among 5 isoforms of DE-circRNAs. (B) The further dysregulated chromosomal distribution of DE-circRNAs. (C) RNase experiment in samples were examined by qRT-PCR.

Supplementary Figure 2. The predicted mRNA-miRNA-circRNA interaction network. The interaction network of mRNA-miRNA-circRNA was predicted using bioinformatics online programs ([CircInteractome](#), [circBank](#), TargetScan, and miRBase). The red circle indicates up-regulated circRNA, the green indicates down-regulated, the arrow and the hexagon indicate miRNA and target gene respectively.

Supplementary Table 1. Quality control (QC) of RNA experiment.

Supplementary Table 2. Quality assessment of sequencing library.

Supplementary Table 3. Analysis of sequencing reads.

Supplementary Table 4. Primer sequences used in this study.

Supplementary Data 1. Detail information of all the circRNAs identified in this study – see Full Excel version

Supplementary Data 2. Detail information of the differentially expressed circRNAs identified in this study – see Full Excel version

Supplementary Data 3. The sequence of DE-circRNAs in this study - see Full FASTA version

Supplementary Data 4. Details of the GO enrichment with statistical significance – see Full Excel version

Supplementary Data 5. Details of the KEGG enrichment with statistical significance – see Full Excel version

Supplementary Data 6. The mRNA-miRNA-circRNA interaction analysis for all DE-circRNAs – see Full Excel version

Figures

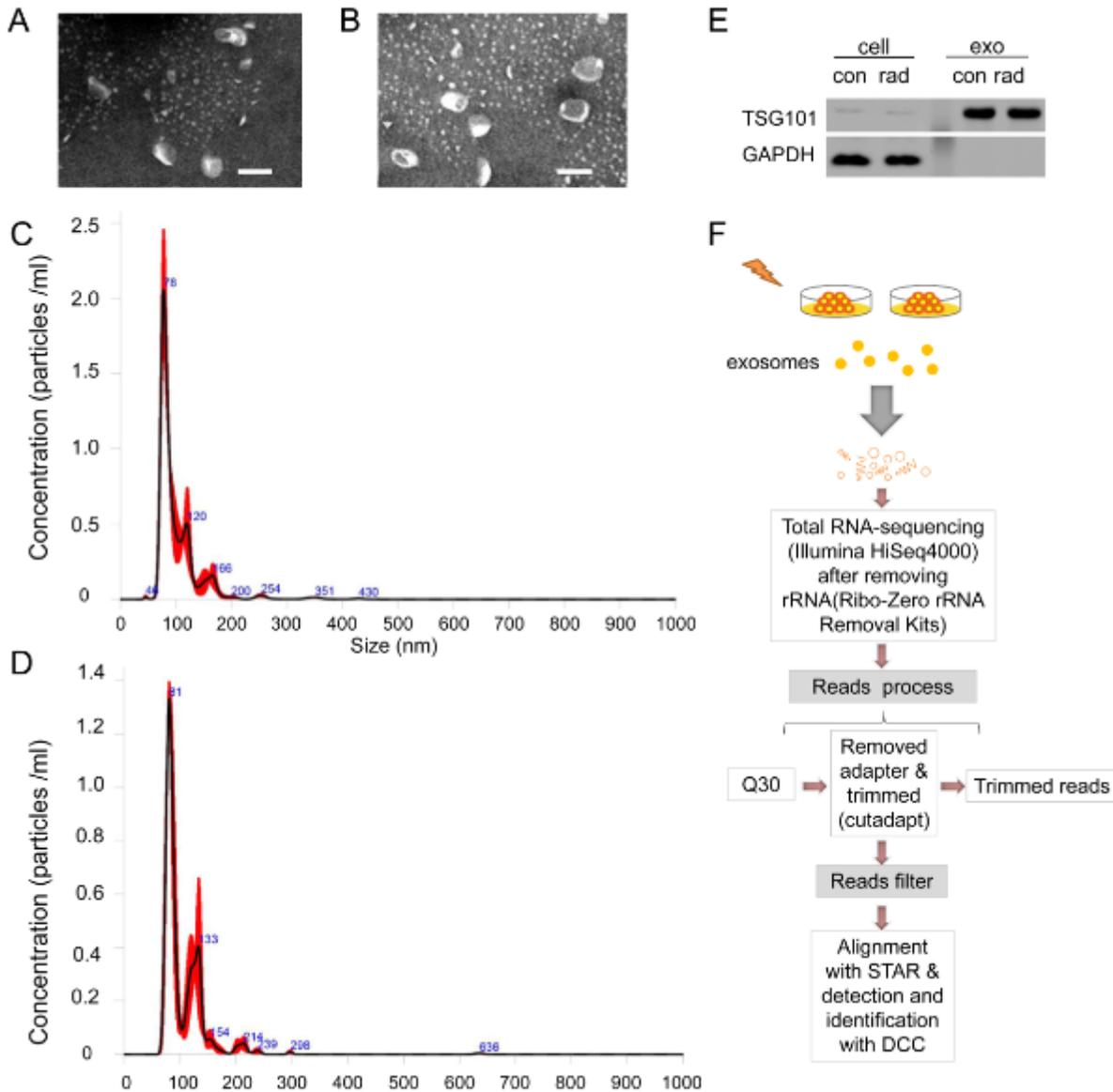


Figure 1

Exosomes identification and RNA-seq libraries construction. (A, B) Representative TEM image of exosomes in samples. Negative staining was used to enhance the view of membrane structures (bar = 100 nm). (C, D) Representative grain-size graph of exosomes in samples. (E) Representative western blot images of the exosomes and cells. (F) Overview of the comprehensive computational scheme for systematic identification of circRNAs in samples.

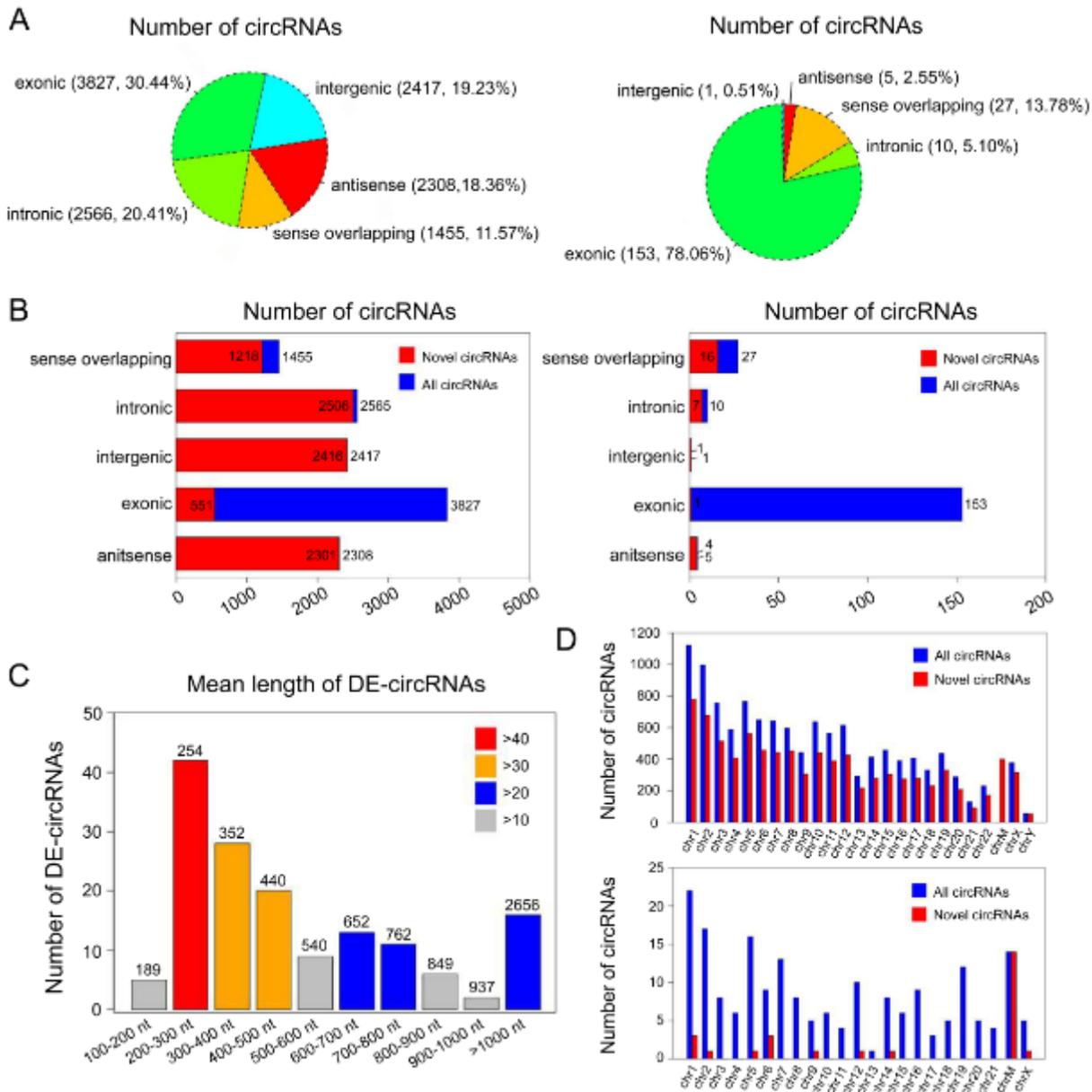


Figure 2

Expression patterns of circular RNAs. (A) The proportion of 5 isoforms of all predicted and differentially expressed (DE) circRNAs. (B) The further distribution of novel circRNAs among all predicted and DE-circRNAs. (C) The mean length of the exonic circRNAs among the known DE-circRNAs. (D) The chromosomal distribution of novel circRNAs among all predicted and DE-circRNAs.

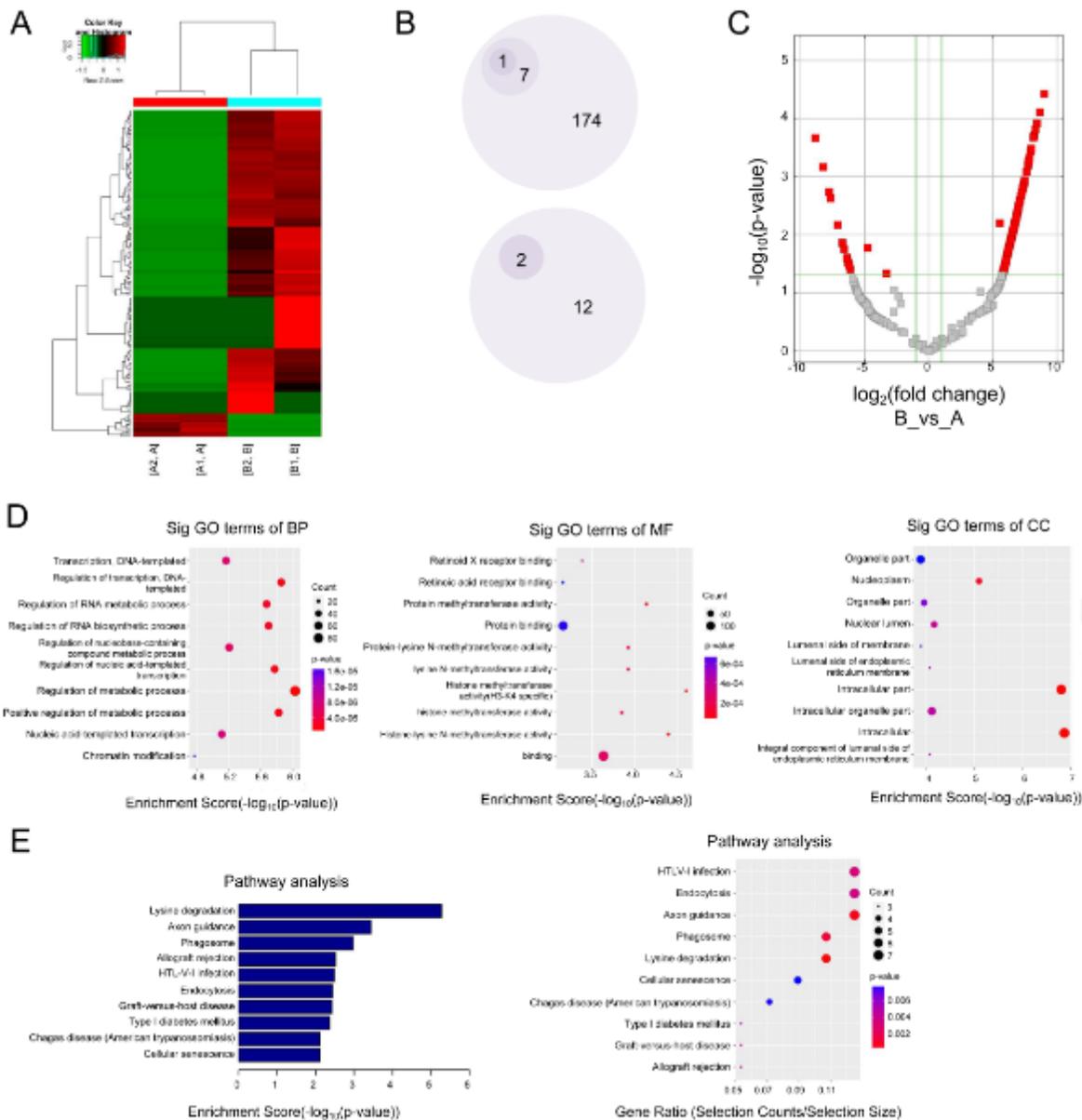


Figure 3

Annotation, visualization and functional clustering of DE-circRNAs. (A) Heatmap of the DE-circRNAs between group A and group B. (B) Bullet graph of the DE-circRNAs between two groups. (C) Volcano plot of the DE-circRNAs. The vertical lines correspond to fold-change, and the horizontal lines to p-value. Red rectangles represent DE-circRNAs ($|\text{fold change}| > 2.0$ and $p < 0.05$). Top 10 items of GO and KEGG enrichment of DE-circRNAs were represented in (D, E).

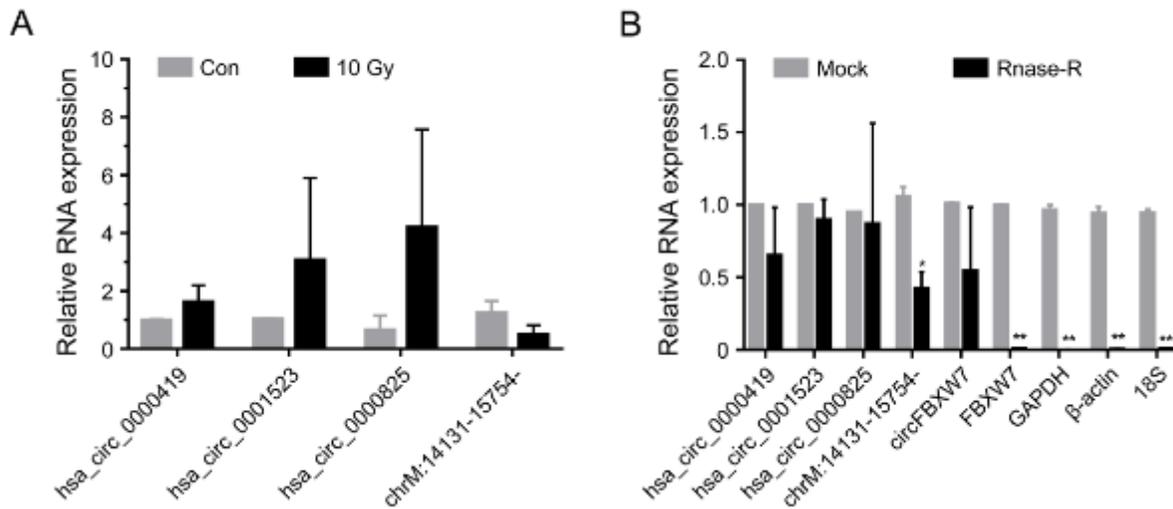


Figure 4

Validation of the circRNA-sequencing results. (A) Expression levels of hsa_circ_0000419, hsa_circ_0001523, hsa_circ_0000825, and chrM: 14131-15754 in the irradiation group (group B) with that in the non-irradiation group (group A). The results of quantitative real-time PCR (qRT-PCR) were evaluated by $2^{-\Delta\Delta CT}$ method. Results were represented as means \pm standard deviation (SD). *p-value <0.05. (B) CircRNAs were resistant to RNase R digestion, whereas the linear RNAs were sensitive to RNase R digestion. 4 circRNAs were examined, which were qualified in expression level. And FBXW7, GAPDH, β -actin, and 18S RNA were used as negative controls and known circRNA FBXW7 was used as a positive control. The results of quantitative Real-time PCR (qRT-PCR) were evaluated by $2^{-\Delta CT}$ method. Results were represented as means \pm standard deviation (SD) *p-value <0.05, ** p-value <0.001.

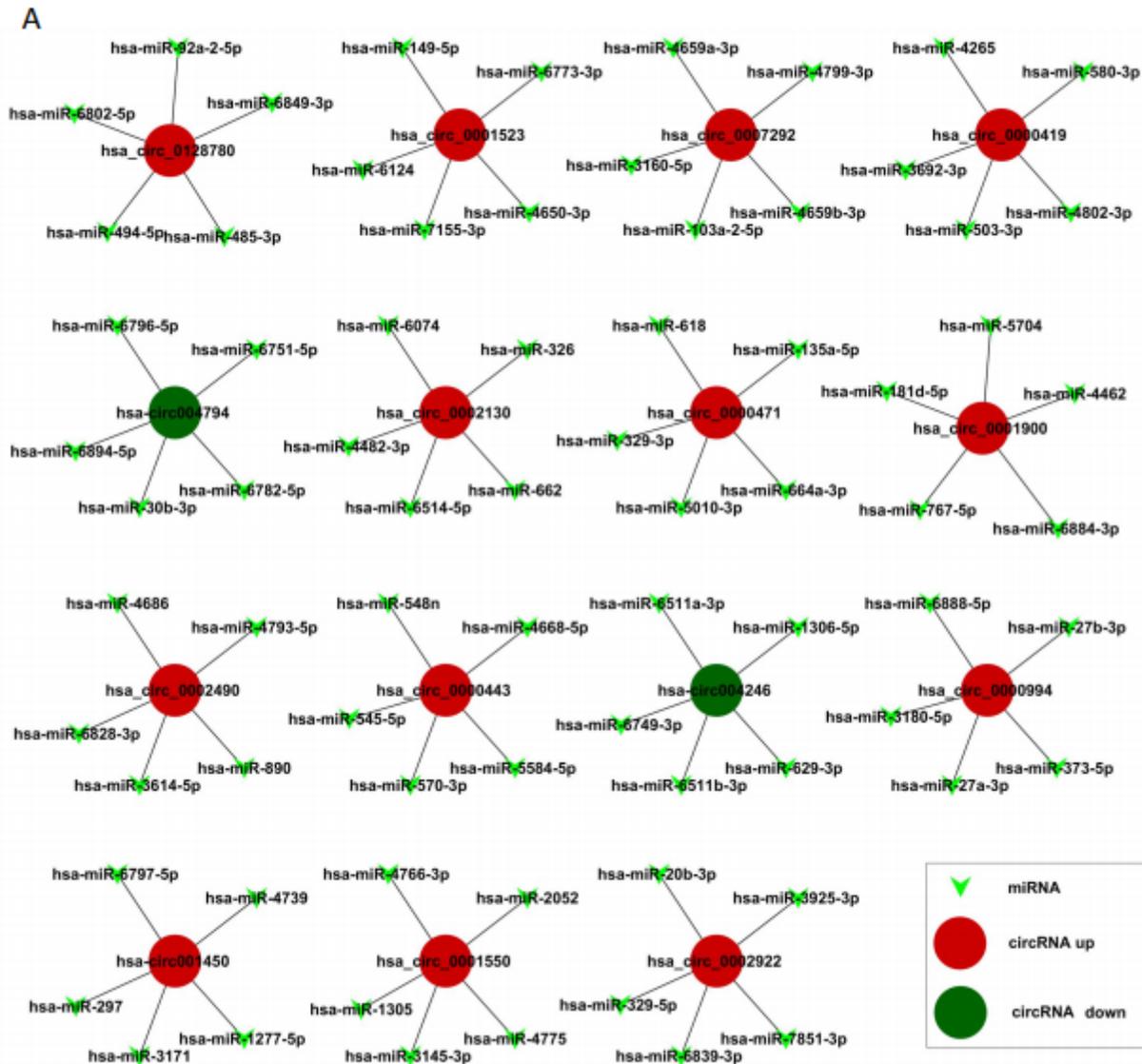


Figure 5

circRNA-miRNA network prediction. The top 15 significantly expressed circRNAs and top 5 predicted miRNAs were selected to generate a network map. The circRNA-miRNA network was built using bioinformatics online programs (TargetScan and miRBase). The red circle indicated the up-regulated circRNAs and the green ones indicated the down-regulated ones.

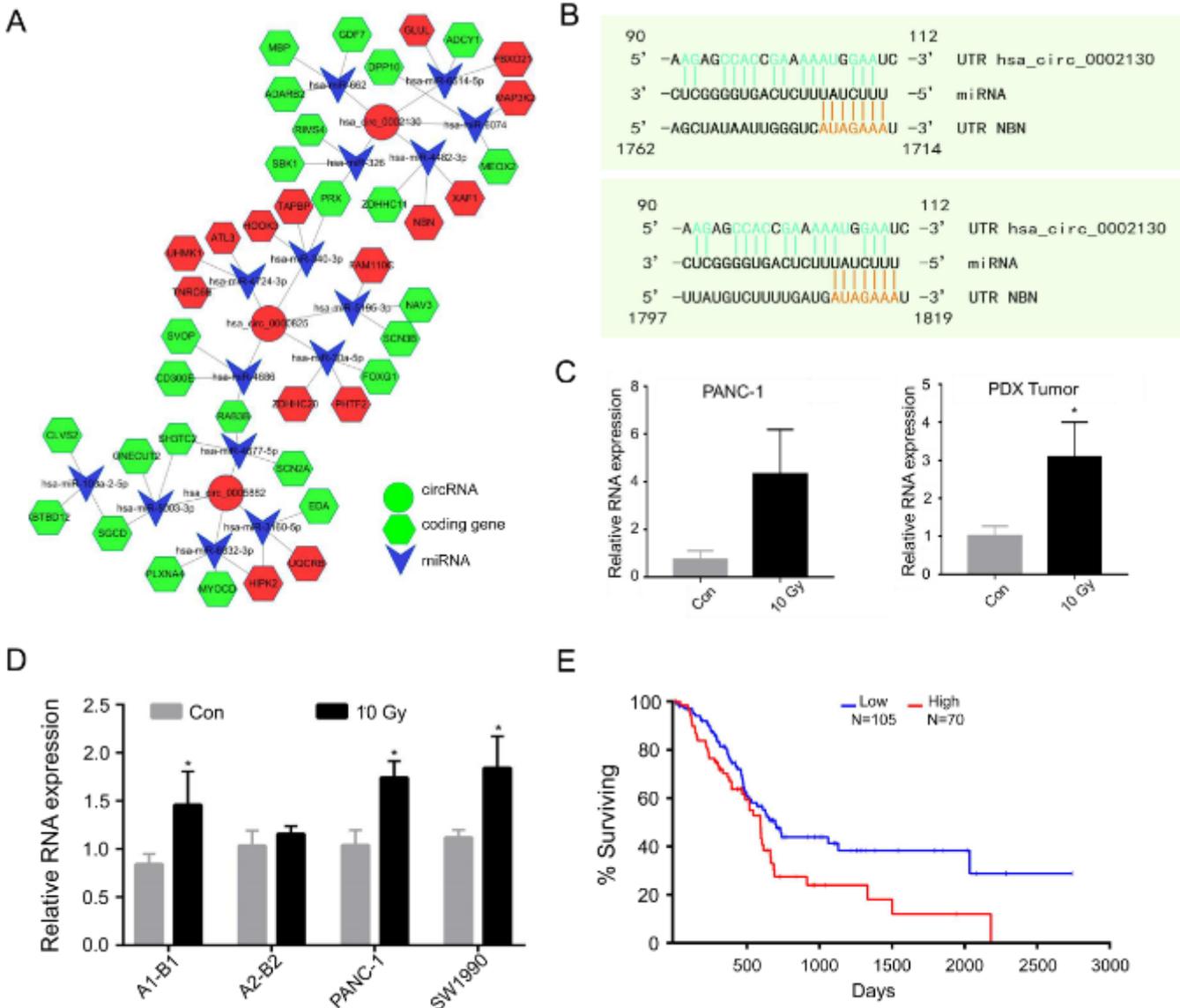


Figure 6

Hsa_circ_0002130-hsa_miR_4482-3p-NBN interaction axis. (A) The circRNA-miRNA-mRNA interaction network was predicted using online bioinformatics programs (Circinteractome, Circbank, TargetScan, and miRBase). The red circle indicates the up-regulated circRNAs, the green indicates the down-regulated ones, the arrow and the hexagon indicate miRNAs and target genes respectively. (B) The predicted binding sites between hsa_circ_0002130 and hsa_miR_4482-3p were exhibited. (C) The expression of hsa_circ_0002130 in PANC-1 cells (Left) and PDX tumors (Right) were examined respectively by qRT-PCR. (D) The expression of NBN was determined by qRT-PCR in samples of sequencing, PANC-1 and SW1990. *p-value <0.05 and data indicated the mean \pm SD. (E) The survival data from TCGA (Log rank p-value=0.0234).

Supplementary Files

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