# Hsa-circ-0064636 regulation of the target gene VDAC1/UBE4A by hsa-miR-326/hsa-miR-503-5 in osteosarcoma

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# Abstract

Background: Circular RNAs (circRNAs) are a subclass of non-coding RNAs that play a critical role in regulating gene expression in eukaryotic organisms. Recent studies have revealed the critical role of circRNAs in cancer progression. However, there are few studies on the function of hsa-circ-0064636 in osteosarcoma (OS).

Methods: The differential expression of hsa-circ-0064636 in OS cell lines was detected by [1] quantitative real-time PCR (qRT-PCR). Differentially expressed mRNAs and miRNAs were screened in OS mRNA and miRNA expression datasets. MiRNAs that interacted with hsa-circ-0064636 were predicted by RNAhybrid, TargetScan and miRanda, and were further detected using RNAhybrid, TargetScan and miRanda. MiRWalk, miRMap, and miRNAMap were used to perform target gene prediction on the intersected miRNAs to construct a circ-miRNA-mRNA interactor network. The target genes were then subjected to survival analysis using PROGgeneV2, and then a circ-miRNA-mRNA interaction subnetwork was formed, and the prognostic impact of target genes was revealed.[2]

Results: The qRT-PCR experiments showed that hsa-circ-0064636 was significantly overexpressed in the OS cell lines. Hsa-mir-326(miR-326) and hsa-mir-503-5p(miR-503-5p) are targeting miRNAs of hsa-circ-0064636 in the target genes obtained from the miR-326 and miR-503-5p screens. UBE4A and VDAC1 have a significant effect on prognosis. UBE4A is a target gene of miR-326, while VDAC1 is a target gene of miR-503-5p.

Conclusion: Hsa-circ-0064636 may be involved in OS development by inhibiting the expression of miR-326 and miR-503-5p, thus regulating that of VDAC1 and UBE4A.


# 1. Introduction

Osteosarcoma (OS) is one of the most frequent primary malignant bone tumors, with high aggressiveness and metastatic potential, rapid progression, and chemoresistance. It is most common seen in children and adolescents[1]. In the past few decades, with extensive resection surgery and multidrug adjuvant chemotherapy, the 5-year survival rates have improved to 70–80%[2], while that of drug-resistant patients have significantly reduced to less than 20% due to poor sensitivity to chemotherapy. Additional therapies may be more effective for OS patients, such as small molecule targeted agents, but significant progress is failed to make in the clinical trials of these therapies[3,4]. Therefore, it is urgent to find new OS therapies, especially complex gene regulatory networks.

In recent decades, studies have successively identified new classes of non-coding RNAs (ncRNAs), for instance, circular RNAs (circRNAs), microRNAs (miRNAs), and long ncRNAs (lncRNAs). With different regulatory functions, they can effectively regulate protein effectors necessary for cellular function and play an important role in the development of OS[5,6]. CircRNA is a highly stable and conserved special RNA with covalent closed-loop properties, which is not easily degraded by endonucleases and widely exists in

tissues and organs[7]. Increasing research shows that circRNAs are widely involved in disease development in many ways and have important roles in cancer[8,9]. However, the potential molecular mechanisms of OS and the regulatory relationships between circRNAs expression and prognosis in OS remain unclear.

This study mainly focused on the differential expression of hsa-circ-0064636 in OS and studied its regulatory mechanism in the perspectives of bioinformatics to clarify its expression and prognostic significance and to provide a reference for future OS molecular marker studies.

## 2. Materials And Methods

## 2.1. Materials and equipment

Human normal osteoblasts (hFOB1.19) and OS cell lines (HOS, SJSA-1 and MG63) were purchased from Shenzhen Haodi Huatuo Biotechnology Co., Ltd. (Shenzhen, China). The TRIzol kit was purchased from Invitrogen (California, USA). The cDNA reverse transcription kit and SYBR green PCR mix were purchased from TaKaRa Biotechnology (Tokyo, Japan). The real-time quantitative PCR instrument (LightCycler® 96) was purchased from Roche (Basel, Switzerland).

## 2.2. Cell culture

The hFOB1.19 cells were cultured with DMEM/F12 medium containing 10% fetal bovine serum with G418 (0.3 mg/mL), penicillin (100 U/mL) and streptomycin (100 U/mL). OS cell lines were all cultured with DMEM/F12 medium containing 10% fetal bovine serum with streptomycin (100 U/mL) and penicillin (100 U/mL). All cells were incubated in 5% $CO_2$ at 37°C in a constant temperature incubator. Fresh complete medium was replaced every 2 or 3 days for further incubation, and when the cell density reached 90% or more, cells were then passaged to the next generation at a ratio of 1:3.

## 2.3. RNA Extraction and qRT-qPCR

Total cellular RNA was extracted by TRIzol reagent, and cDNA was synthesized using a reverse transcription kit. The qRT-qPCR was performed using SYBR green PCR mix and divergent primers from hsa-circ-0064636 under the following reaction conditions: pre-denaturation at 95°C for 10 mins, denaturation at 95°C for 15 s, annealing at 60°C for 30 s, with a total of 45 cycles performed. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 expression were used as a control. The relative expression of the final screened genes was calculated using a comparative method $2^{-\Delta Ct}$. The primers used in this study are shown in Table 1.

Table 1
qRT-qPCR primer sequences.

| Gene | Sequence(5’ to 3’) |
|---|---|
| has_circ_0064636- LEFT | GCTTCCCCTGTCTCCACATA |
| has_circ_0064636- RIGHT | ATGTCCAAAGGGTTTCAGCA |
| GAPDH - LEFT | CCACTCCTCCACCTTTGAC |
| GAPDH – RIGHT | ACCCTGTTGCTGTAGCCA |
| UBE4A – LEFT | TCCAGAGAACCTGCTACCCT |
| UBE4A –RIGHT | AGTTACATCTTCAAAATGGGCTCC |
| VDAC1– LEFT | GGAAGGCAGAAGATGGCTGT |
| VDAC1–RIGHT | GTCCACGTGCAAGCTGATCT |

# 2.4. CircRNA-miRNA-mRNA network construction

The miRNAs interacting with hsa-circ-0064636 were predicted via the circRNA target miRNA prediction tools, RNAhybrid[10] (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/), TargetScan[11] (http://www.targetscan.org/) and miRanda[12] (http://www.microrna.org/microrna/home.do) to identify intersected miRNAs that were simultaneously targeted by the four tools. For miRNA targeting, the gene prediction tools RNAhybrid, TargetScan, miRanda, miRWalk[13]（http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/index.html）, miRMap[14] (http://mirnamap.mbc.nctu.edu.tw/), and miRNAMap[15]（http://mirnamap.mbc.nctu.edu.tw/） were used to predict target genes on the intersected miRNAs. They were compared with the screened miRNAs to identify 7 intersections of differentially expressed miRNAs. The final selected circRNA-miRNA and miRNA-mRNA were used to construct a visual regulatory network using Cytoscape 3.8.0.

# 2.5 Wayne diagramming

Wayne diagrams are plotted using the R package ‘Venn’.

# 2.6 Differential expression analysis

The miRNA expression dataset GSE65071 (9 normal samples, 14 OS samples) and the mRNA expression dataset GSE1608816 (9 normal samples, 14 OS samples) were downloaded from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/, GEO) database and the Bioconductor affy package was used. The Limma package was adopted to identify differentially expressed genes between normal and tumor samples. Log2|foldchange (FC) | > 1 and adj.P-values < 0.05 were considered to be statistically significantly different, and the resulting differentially expressed genes were used for volcano mapping.

# 2.7. Survival analysis

The PROGgeneV2[16] (http://genomics.jefferson.edu/proggene) tool can be used along with public data to investigate the prognostic significance of genes. The previously obtained data of common target genes were entered into a database, and the dataset for OS was selected. The overall survival map was constructed based on the median expression of a given gene that could be classified into high and low expression groups (Kaplan-Meier, KM plots). PROGeneV2 and the R survival package were used for hypothesis testing. The data were analyzed using the log-rank test. Statistical analysis was performed using the log-rank test, and the threshold for a meaningful survival prognosis was $p < 0.05$. Based on the survival analysis, a new circRNA-miRNA-mRNA visual regulatory subnetwork was obtained.

## 2.8 Statistical analysis

GraphPad Prism 8 was used to statistically analyze the experimental data, which showed the statistical test data were normally distributed. $P < 0.05$ was considered to be statistically significant, and a t-test was used for comparisons between the two groups.

# 3. Results

## 3.1 Analysis of expression variance

According to the miRNA expression profile data, 114 miRNAs were upregulated and 117 miRNAs were downregulated in OS, and the differential expression volcanoes of miRNAs were plotted (Fig. 1A). The differential analysis of mRNA expression spectrum data yielded 716 mRNAs for downregulated genes and 1924 mRNAs for downregulated genes, and the differential expression volcanoes of mRNAs were mapped (Fig. 1B).

## 3.2 Hsa-circ-0064636 targeting miRNA prediction

To explore the regulatory mechanism of has-circ-006463 in OS species, has-circ-006463 targeting miRNAs were predicted using four databases. TargetScan, miRanda and RNAhybrid were adopted to predict 498, 965 and 226 targeting miRNAs, respectively, while differential analysis yielded 88 targeting miRNAs. The four intersections were used to obtain common targeting miRNAs of hsa-mir-326 (miR-326) and hsa-mir-503-5p (miR-503-5p) (Fig. 2).

## 3.3 Prediction and analysis of miRNA target genes

The regulatory mechanism of miR-326 and miR-503-5p in OS was studied, and the possible binding of target genes was further predicted. MiRWalk, miRanda, miRMap, miRNAMap, RNAhybrid, and TargetScan databases were adopted to predict miR-326 target genes, respectively, with obtained 5071, 3364, 6616, 6716, 16373, and 4830 target genes. The intersection with the 1924 upregulated differential genes obtained from the analysis yielded 31 target genes, which were plotted on a Wayne diagram and labeled with the names of these 31 target genes (Fig. 3A). MiR-503-5p target genes were analyzed using miRWalk, miRanda, miRMap, miRNAMap, RNAhybrid and TargetScan databases, which yielded 2034,

844, 6692, 1496, 12416 and 2196 target genes, respectively. These genes were intersected with the 1924 upregulated differential genes in the previous analysis to obtain 31 target genes, which were plotted in a Wayne diagram, and the six target gene names were labeled (Fig. 3B).

# 3.4 CircRNA-miRNA-mRNA network construction

Six databases were used to predict binding targets miR-326 and miR-503-5p and 7 intersections of differential genes to obtain 31 and 9 target gene relationships, respectively, and construct the relationship networks (Fig. 4). Hsa-circ-0064636 may targets and binds to miR-326 and miR-503-5p. The target genes that miR-326 targets and binds to were TNC, HNRNPA2B1, ATXN1, DPYSL2, RGS3, UBE4A, MRC2, PSMC1, FOXO3, SRRM1, SAMD4A, MYO1D, ADAM17, PDIA4, VGLL4, FYN, NFATC3, VCL, SPAG9, MED13L, RAI14, MYOF, NDEL1, TUBB, ALPL, USP11, CEP350, LSM1, GOLPH3, C9orf3 and CUX1 (31 target genes). The target genes that miR-503-5p targets and binds to were PSMD7, INSR, PTPN12, TCF7L2, VDAC1 and TPM1 (6 target genes).

# 3.5 Survival analysis

Overall survival curves of the 31 target genes were obtained, and details are shown in Table 2. HNRNPA2B1, FOXO3, MYOF, C9orf3, and CUX1 could not be retrieved in the database. The survival analysis of 31 target genes showed only UBE4A and VDAC1 genes had statistical differences ($p < 0.05$), and their survival curves were shown in Fig. 5. UBE4A is a target gene of miR-326, while VDAC1 is a target gene of miR-503-5p.

Table 2
Target gene survival analysis results

| gene | HAZARD RATIO | LCI (95%) | UCI(95%) | P VALUE |
|---|---|---|---|---|
| TNC | 0.8 | 0.59 | 1.09 | 0.1608707 |
| ATXN1 | 1.06 | 0.45 | 2.51 | 0.896135 |
| DPYSL2 | 0.72 | 0.31 | 1.66 | 0.4461738 |
| RGS3 | 0.59 | 0.21 | 1.61 | 0.3011995 |
| UBE4A | 1.6 | 1 | 2.57 | 0.0489371 |
| MRC2 | 0.81 | 0.38 | 1.73 | 0.5945315 |
| PSMC1 | 0.38 | 0.1 | 1.4 | 0.1471227 |
| SRRM1 | 1.11 | 0.35 | 3.51 | 0.8532273 |
| SAMD4A | 0.75 | 0.25 | 2.28 | 0.610971 |
| MYO1D | 0.76 | 0.4 | 1.45 | 0.4085244 |
| ADAM17 | 0.4 | 0.06 | 2.86 | 0.3620074 |
| PDIA4 | 0.81 | 0.38 | 1.74 | 0.5882791 |
| VGLL4 | 1.03 | 0.46 | 2.27 | 0.9508824 |
| FYN | 1.66 | 0.7 | 3.95 | 0.251584 |
| NFATC3 | 4.94 | 0.76 | 32.03 | 0.0938036364009382 |
| VCL | 0.6 | 0.29 | 1.24 | 0.1688802 |
| SPAG9 | 0.45 | 0.18 | 1.17 | 0.1008696 |
| RAI14 | 0.33 | 0.16 | 0.68 | 0.0026146 |
| NDEL1 | 0.81 | 0.35 | 1.9 | 0.6275625 |
| TUBB | 1.59 | 0.92 | 2.78 | 0.0992659 |
| ALPL | 1.07 | 0.83 | 1.39 | 0.5919638 |
| USP11 | 0.5 | 0.18 | 1.38 | 0.1832391 |
| CEP350 | 0.88 | 0.35 | 2.21 | 0.7837919 |
| LSM1 | 1.58 | 0.78 | 3.22 | 0.2074225 |
| GOLPH3 | 1.54 | 0.94 | 2.5 | 0.0836026 |
| PSMD7 | 0.7 | 0.32 | 1.54 | 0.3788017 |
| INSR | 0.13 | 0.01 | 1.79 | 0.1255452 |

| gene | HAZARD RATIO | LCI (95%) | UCI(95%) | P VALUE |
|---|---|---|---|---|
| PTPN12 | 1.62 | 0.89 | 2.94 | 0.1136242 |
| TCF7L2 | 3.99 | 0.79 | 20.11 | 0.0939648 |
| VDAC1 | 4.09 | 1.26 | 13.29 | 0.0191903 |
| TPM1 | 0.73 | 0.47 | 1.16 | 0.1857696 |

## 3.6 CircRNA-miRNA-mRNA network construction with prognostic impact and significance

Based on the original circRNA-miRNA-mRNA ceRNA interaction axes, only the two target genes (UBE4A and VDAC1) with prognostic impact on survival of mRNA were retained (circRNA and miRNA were also retained), and a new subnetwork with prognostic implications were constructed, which is shown in Fig. 6A.

## 3.7 Validation of differential expression of hsa-circ-0064636in OS cell lines

First, qRT-PCR was used to detect the differential expression of hsa-circ-0064636 in human OS cell lines (HOS, SJSA-1, MG63, U2OS) and osteoblast cell lines (hFOB1.19). The results showed that the expression of hsa-circ-0064636 was significantly upregulated in the human OS cell line compared to osteoblasts (Fig. 6B). The expression of has-miR-326 (Fig. 6C) and has-miR-503 (Fig. 6D) were significantly decreased in OS cell lines. UBE4A (Fig. 6E) and VDAC1(Fig. 6F) were significantly overexpressed in OS cell lines relative to human osteoblast cell lines.

# 4. Discussion

OS is one of the common malignant bone tumors. However, breakthroughs are failed to realize in the OS treatment. This disease is mainly treated with surgery and chemotherapy, but the prognosis of OS patients is still unsatisfactory. Previous studies have shown that many genes are associated with the development and progression of OS. However, the emphasis has been on protein-coding genes or miRNAs[17]. The molecular mechanisms of OS are still unknown, and recent studies show that some non-coding RNAs play an important role in the development of OS[18,19]. For example, lncRNA(TMPO-AS1) acts as a ceRNA that promotes OS tumorigenesis by regulating the miR-199a-5p to WNT7B axis [20]. MiRNA-1236-3p is significantly overexpressed in OS, inhibiting cell proliferation and inducing apoptosis by targeting KLF8.[21].

In contrast to other non-coding RNAs, lncRNAs, miRNAs, and circRNAs are highly conserved and stable in mammalian cells, making circRNAs potentially ideal biomarkers and therapeutic targets[22]. In recent

years, increasing research reveals the important role of circRNAs in different biological processes, especially in tumorigenesis, development, and metastasis, such as the development of gastric cancer[23], colorectal cancer[24], lung cancer[25], and cervical cancer[26]. A previous study showed that circTADA2A is differentially expressed between OS cell lines and corresponding noncancer cell lines[27]. However, the role of many circRNAs in the development of OS is still largely unknown. In this study, we focused on the significantly upregulated expression of hsa-circ-0064636 in OS cell lines and normal tissue cell lines and further predicted the regulatory network between its targeted regulatory miRNAs and corresponding target genes. MiR-326 and miR-503-5p were predicted to be the targeting miRNAs of hsa-circ-0064636 according to multiple databases of circRNA-miRNA interactions. To verify the expression of miR-326 and hsa-miR-503-5 in OS, they were also used in the GSE65071 dataset and the difference-in-differences analysis showed that they were under-expressed in the OS samples compared to normal samples. As mentioned in a previous study, hsa-circ-0064636 is significantly upregulated in OS, regulates down-regulation of miR-326 and promotes cervical cancer progression through up-regulation of ELK1. MiR-326 overexpression transfection experiments verified that it could inhibit proliferation and invasion of cervical cancer cells and induce their apoptosis and autophagy[28]. MiR-326 is also significantly under-expressed in prostate cancer[29] and correlated with prognosis. However, miR-503-5p has been reported to inhibit tumorigenesis, angiogenesis, and lymphangiogenesis of colon cancer through directly downregulating VEGF-A[30]. Hepatocellular carcinoma studies showed miR-503-5p could regulate epithelial to mesenchymal transformation, metastasis, and prognosis of hepatocellular carcinoma cells by inhibiting WEE1[31]. Thus, miR-326 and miR-503-5p play a key role in the suppression of many cancers, with low expressions in cancers. Therefore, hsa-circ-0064636 may lead to the development of OS by suppressing the expression of miR-326 and miR-503-5p in OS. However, the function of miR-326 and miR-503-5p in OS are rarely studied.

This study also used the miRNA prediction mRNA database to analyze the significantly differentially expressed genes in OS to screen the potential target genes of miR-326 and miR-503-5p and obtain a circRNA-miRNA-mRNA ceRNA Interaction Network. Based on that, we screened the mRNAs with prognostic impact to obtain a circRNA-miRNA-mRNA regulatory subnetwork. UBE4A was a potential direct target of miR-326, and VDAC1 was a potential target of miR-503-5p. We also found significant prognostic differences between UBE4A and VDAC1 in the survival analysis of OS patients. The results of survival analysis plots showed the prognosis of UBE4A and VDAC1 was significantly worse in the high expression group compared to the low expression group. UBE4A belongs to the U-box ubiquitin ligase class of enzymes. The encoded protein is involved in polyubiquitin chain assembly and plays a key role in chromosome condensation and polyubiquitination segregation via securin. Autoantibodies against the encoded protein may become a marker for scleroderma and Crohn's disease[32,33]. UBE4A is significantly upregulated in the comparison between ovarian plasmatic cystic carcinoma and adjacent normal tissues. The VDAC1 voltage-dependent anion channel protein is a major component of the outer mitochondrial membrane[34]. VDAC1 is expressed in all compartments, including mitochondria, the plasma membrane and other cells. It regulates major metabolic and energetic functions of cells, including $Ca^{2+}$ homeostasis,

oxidative stress, and mitochondria-mediated apoptosis. The overexpression of VDAC1 triggers cell death, which may be related to the destruction of nerve cells [35]. VDAC1 was identified as a tumor promoter in cervical cancer, and knock-down of VDAC1 in cell lines of cervical cancer increase the incidence of apoptosis, which could be partially reversed by overexpression of VDAC1[36].

The qRT-PCR experiments showed that hsa-circ-00063636, VDAC1 and UBE4A were highly expressed in OS cell lines, and miR-326 and miR-503-5p were lowly expressed in OS cell lines. These results were consistent with those of our bioinformatics analysis. However, the combination of the regulatory network needs to be verified in the subsequent experiments.

# 5. Conclusion

The target miRNAs that hsa-circ-0064636 binds to were miR-326 and miR-503-5p. A further prediction of the mRNAs to which the miRNAs might bind and the intersection results with differentially expressed mRNAs showed that miR-326 had 31 potential targets. MiR-503-5p had six potential binding coding genes, and a circRNA-miRNA-mRNA regulatory network was derived accordingly. Survival analysis results showed that UBE4A and VDAC1 had a significant impact and significance. A survival-significant sub-regulatory network of hsa-circ-0064636-miR-326 /miR-503-5p-UBE4A/VDAC1 was established. The qRT-PCR experiments showed that hsa-circ-0064636, UBE4A and VDAC1 were significantly differentially overexpressed in OS cell lines while miR-326 and miR-503-5p were under-expressed in OS cell lines. In conclusion, hsa-circ-0064636 may be involved in OS development by suppressing the expression of miR-326 and miR-503-5p to facilitate up-regulation of VDAC1 and UBE4A.

# Abbreviations

GEO
Gene Expression Omnibus
MiRNA
MicroRNA
OS
Osteosarcoma
CircRNAs
Circular RNAs
HR
Hazard ratio
lncRNA
long ncRNAs
qRT-PCR
quantitative real-time PCR

# Declarations

# Declarations

### Ethics approval and consent to participate

Ethics approval was obtained from the Ethics Committee of Guangxi Medical University (Nanning, China).

# Declarations

### Conflicts of Interest

The authors declare that no conflicts of interest exist in this study.

### Consent for publication

The manuscript has not been published or presented elsewhere, nor has it been considered by other journals.

# Authors' Contributions

Guohua Yan, Hanji Huang and Yongnian Qin are equal contributors to this research. Guohua Yan, Hanji Huang and Yongnian Qin designed the research program and wrote this paper. Kanglu Li, Mingjun Zheng, Lichuan Mo, Junjian Zheng, Rui Jiang, and Nanchang Huang conducted the research and analyzed the data. Jianwen Cheng and Renjie Wei revised the manuscript and sought financial support.

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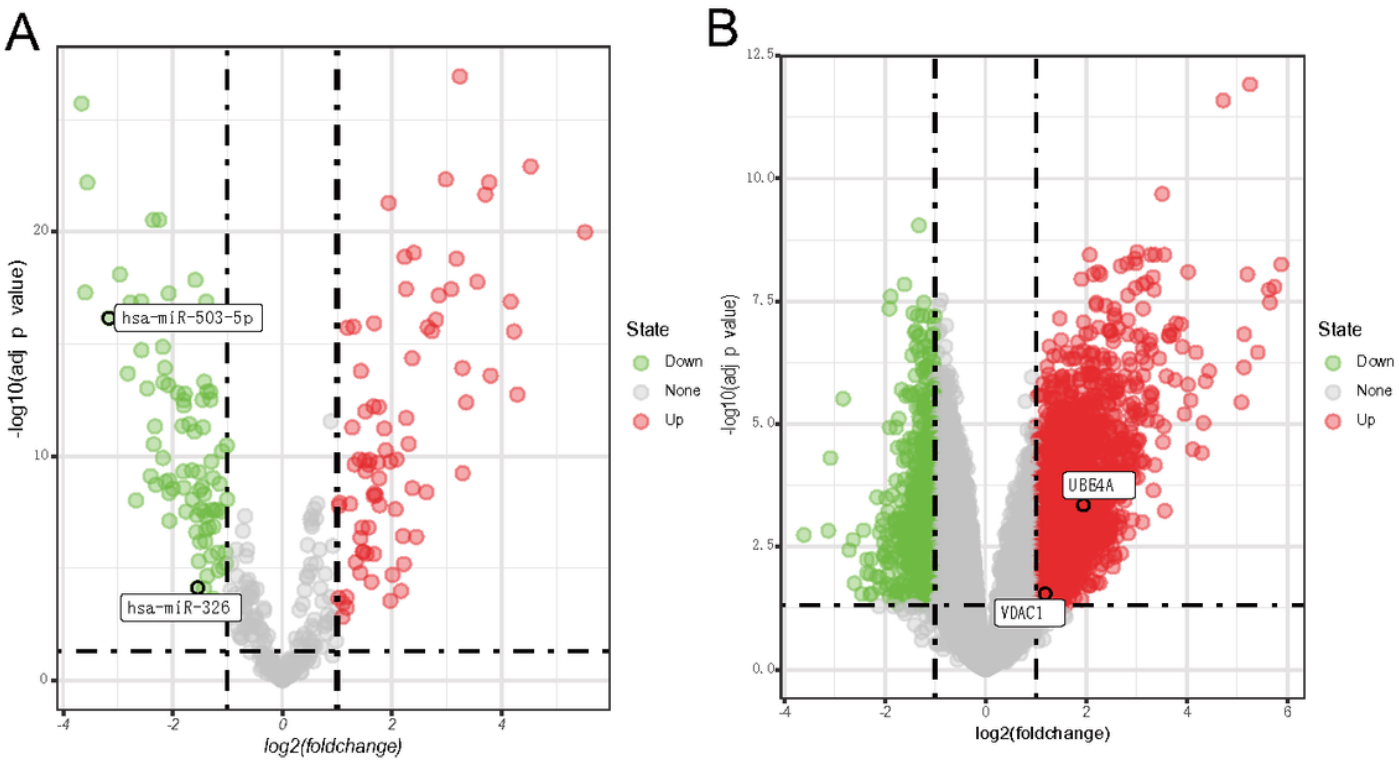
## References

Bibliography not available with this version

## Figures




## Figure 1

Differential gene expression volcano plot, with red representing upregulated genes and green representing down-regulated genes. (A) miRNA differential expression volcano plot, where the positions of miR-326 and miR-503-5p are labeled. (B) mRNA differential expression volcano plot with the positions of VDAC1 and U8E4A labeled.

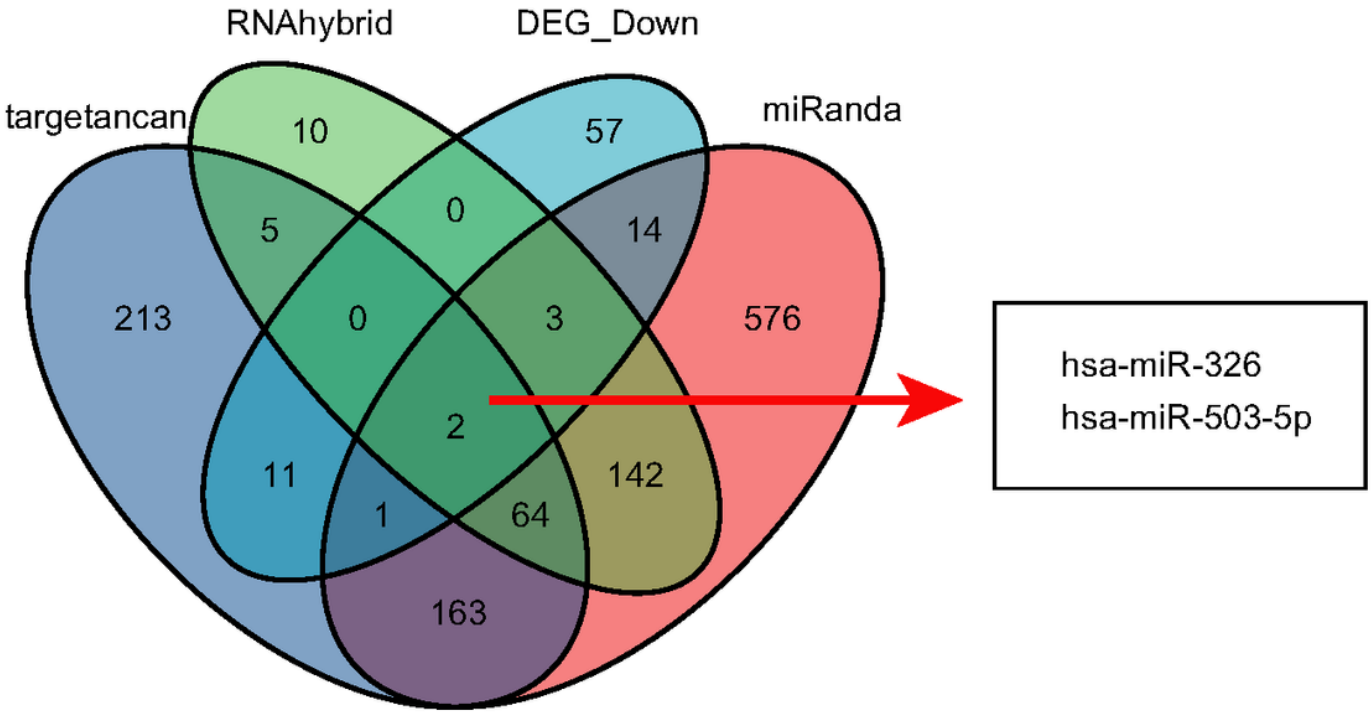
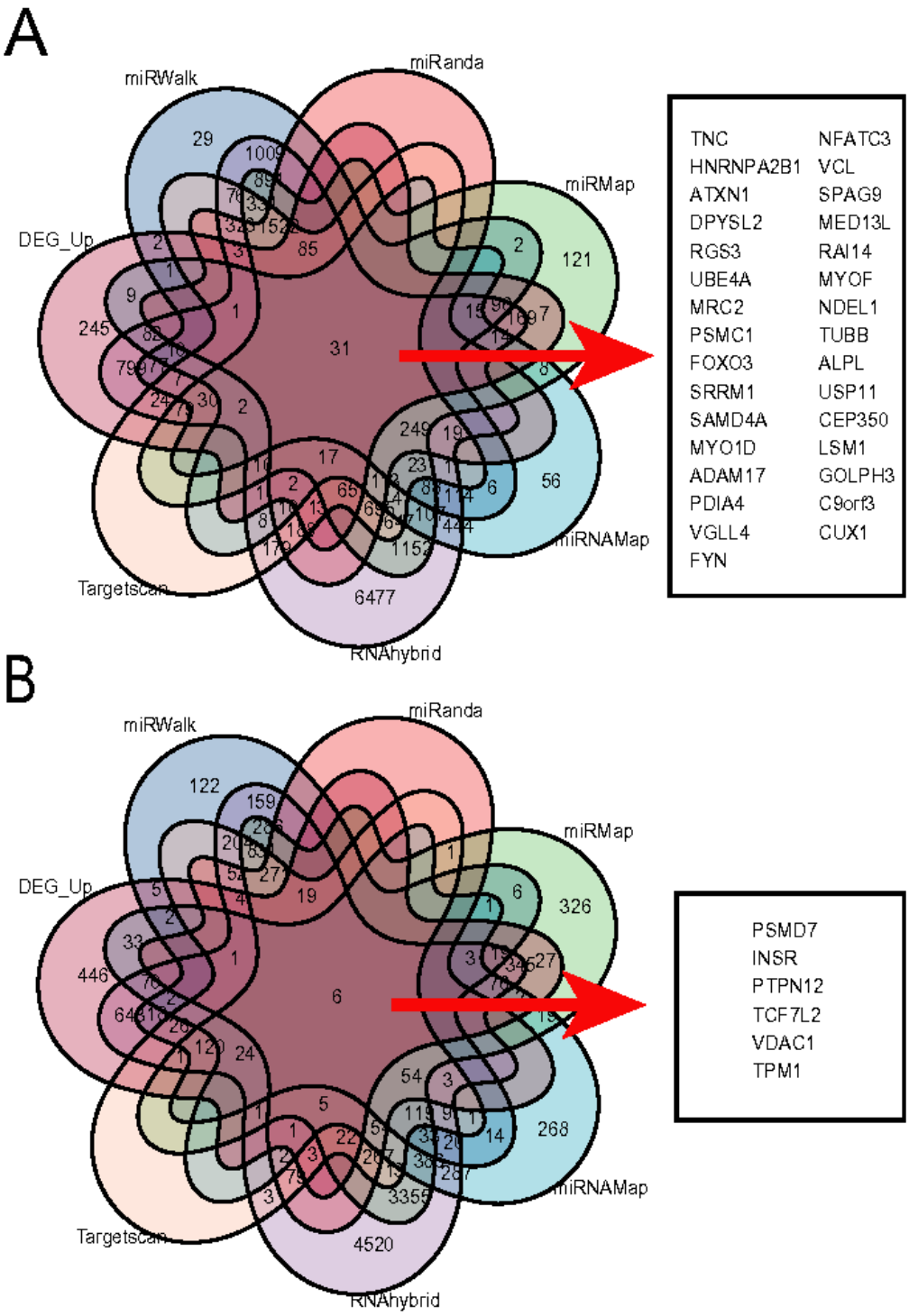



**Figure 2**

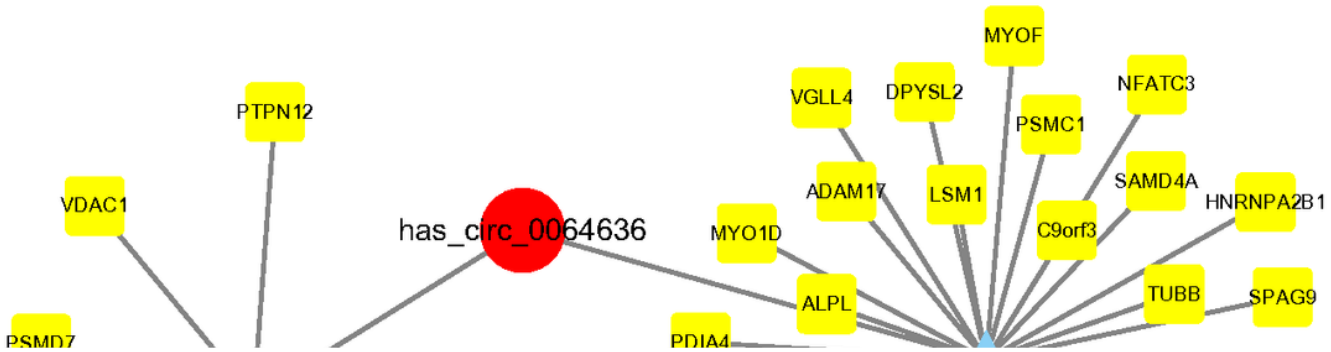
Schematic representation of targeting miRNAs of hsa-circ-0064636. TargetScan, miRanda, and RNAhybrid databases used to predict targeting miRNAs of hsa-circ-0064636 and miRNA Wayne plots were derived according to the differential analysis, with the two common miRNAs labeled on the right side of the figure.




## Figure 3

MiRNA target gene predictions and differential gene Wayne plots. Figure 3(A) Wayne plot of the intersection of miR-326 using six databases to predict binding target genes and differentially expressed genes. The names of 31 target genes of miR-326[1] are shown on the right side of Figure 3 (A). Figure 3 (B) Six databases were used to predict the intersection of binding target genes of miR-503-5p, and

differentially expressed genes are shown in a Wayne diagram. The names of the six miR-503-5p target mRNAs are shown on the right side of Figure 3 (B).




## Figure 4

CircRNA-miRNA-mRNA ceRNA regulatory network starting with hsa-circ-0064636. The red circle represents circRNA, the blue triangle represents miRNA, and the yellow square represents mRNA.

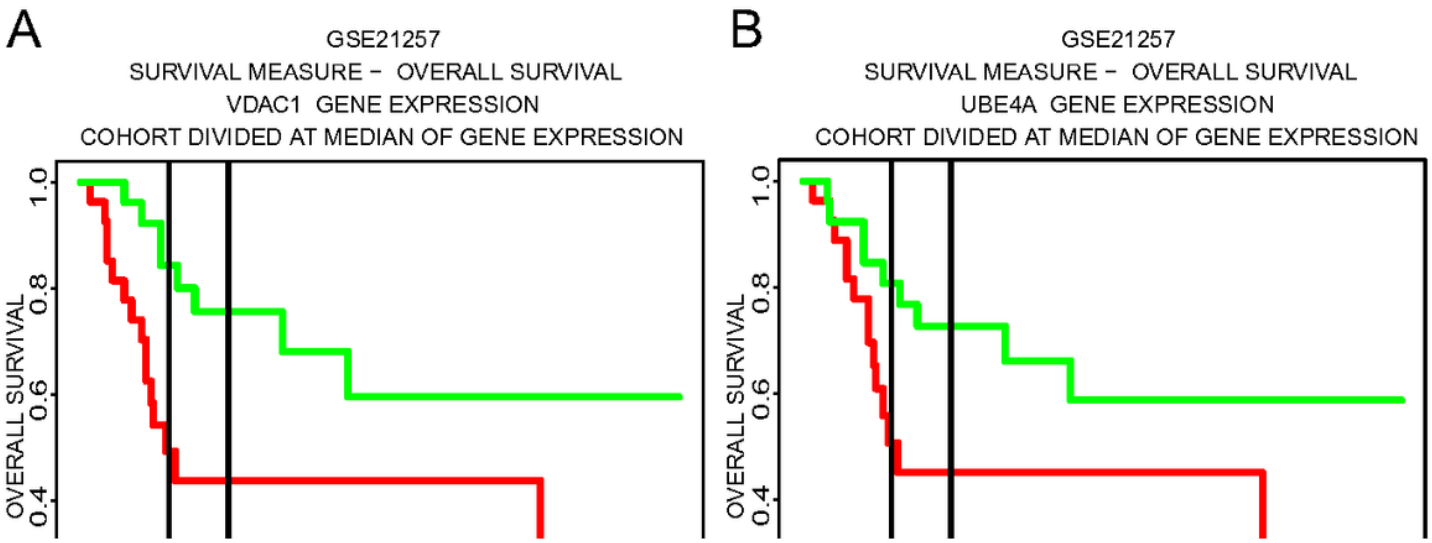
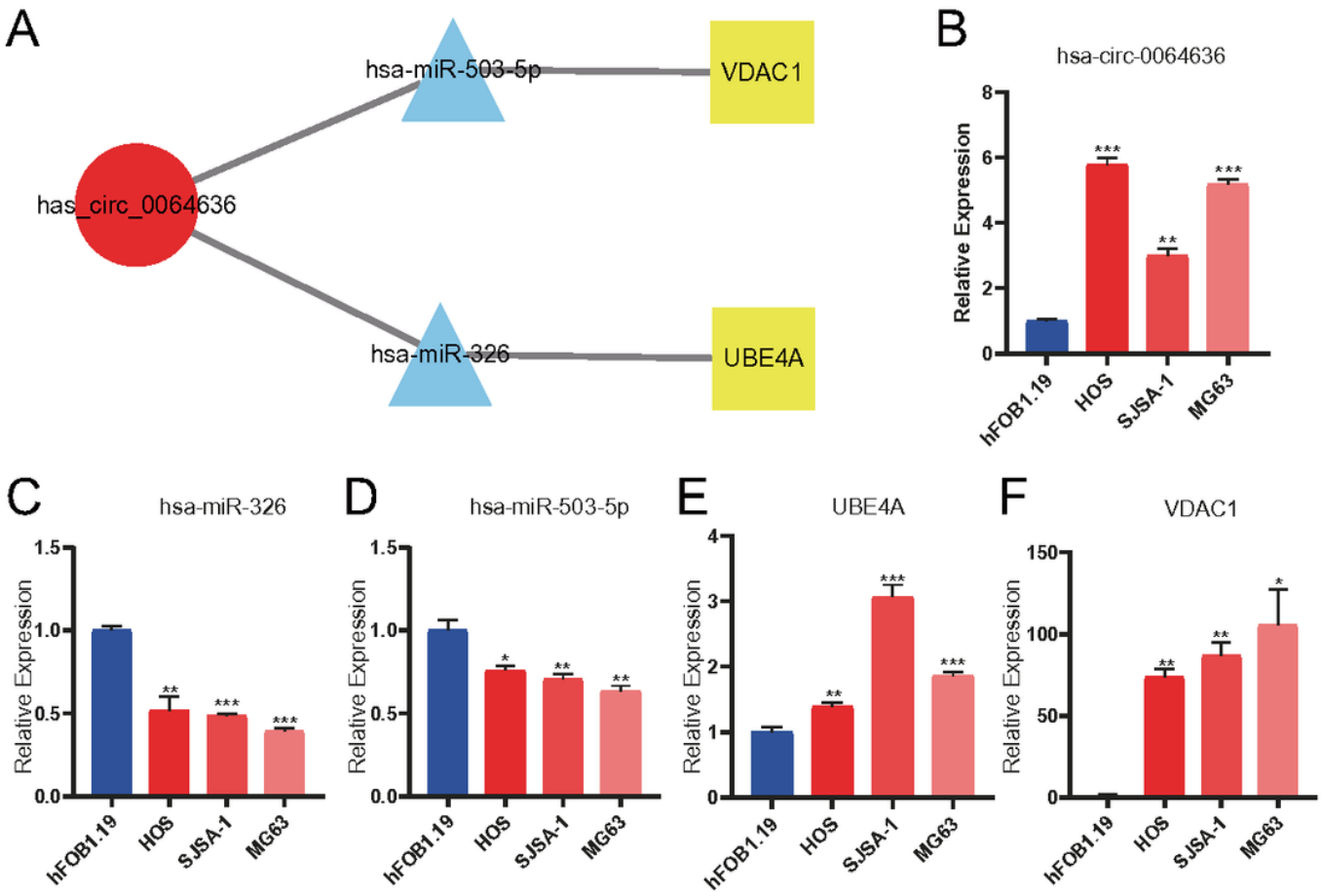



**Figure 5**

Survival curves of target genes were plotted using Kaplan-Meier curves on the PROGeneV2 online platform, with red lines representing a high expression of the genes and green lines representing a low expression of the genes. Figure 5 (A) Survival curve analysis of VDAC1. Figure 5 (B) Survival curve analysis of UBE4A.




## Figure 6

CircRNA-miRNA-mRNA ceRNA regulatory network construction according to target genes with prognostic impact and significance. Figure 6 (A) Circles in red represent circRNAs, triangles in blue represent miRNAs, and squares in yellow represent mRNAs. Figure 6 (B-F) Expression of Hsa_circ_006463/miR-326/ miR-503-5p/ UBE4A and VDAC1 in OS cell lines. *: p<0.05.