Original Article

Long noncoding RNA *NEAT1* changes exosome secretion and microRNA expression carried by exosomes in hepatocellular carcinoma cells

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Background: This study aimed to investigate the roles and functions of nuclear-enriched abundant transcript 1 (*NEAT1*) in exosome secretion and exosomal microRNA (miRNA) changes in hepatocellular carcinoma (HCC) cells.

Methods: HepG2 and HuH-7 cells were divided into two groups: Lv-control (which were infected with lentivirus without *NEAT1* expression) and Lv-*NEAT1* (which were infected with lentivirus with *NEAT1* overexpression). Each group was used to study cell function (proliferation, invasion, and apoptosis) and exosome secretion by nanoparticle tracking analysis (NTA), electron microscopy, and nanoflow cytometry (nanoFCM). Different levels of messenger RNA (mRNA), miRNA, and exosomal miRNA were detected by RNA sequencing. Next, potential target RNAs were verified by reverse transcription polymerase chain reaction (RT-PCR). Changed exosomal miRNAs were found and miRNA mimics were used to study cell function in *NEAT1*-overexpression and *NEAT1*-knockdown HCC cells.

Results: The data showed that *NEAT1*-overexpression promoted exosome secretion. The overexpression of *NEAT1* altered global genes, including exosome-related genes. Compared with the control group, we observed that several miRNAs changed in the exosomes secreted by *NEAT1*-overexpressing cells. Our study found that these changed exosomal miRNAs played a suppressor role in HCC. Transfection of *miR-634*, *miR-638*, and *miR-3960* reversed the enhanced invasion and proliferation in HCC cells with a high level of *NEAT1* expression.

Conclusions: These results suggested that *NEAT1* regulates exosome-related genes, which might be associated with increasing exosome secretion by *NEAT1*-overexpressing cells. Furthermore, *NEAT1* promotes cell invasion and proliferation via downregulation of *miR-634*, *miR-638*, and *miR-3960* in exosomes. This study may provide potential targets for exosome-mediated miRNA transfer in HCCs with a high level of *NEAT1* expression therapy.

Keywords: Long noncoding RNA (lncRNA); nuclear-enriched abundant transcript 1 (*NEAT1*); exosome; microRNA (miRNA); hepatocellular carcinoma (HCC)

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Introduction

Hepatocellular carcinoma (HCC) has the third-highest cancer-related mortality rate (1), as well as a high risk of recurrence and poor prognosis. However, there are currently few biomarkers for the diagnosis of early HCC. Despite their limited benefits, resection and liver transplant are the primary options for the treatment of HCC (2). HCC progression is not only determined by the biological behavior of tumor cells but is also influenced by the tumor microenvironment (TME) (3,4). Increasing evidence has suggested that the TME plays a vital role in HCC progression (3,5).

Exosomes are double-layered vesicles (approximately 30–150 nm in diameter) that contain nucleic proteins, acids, and lipids, which regulate intercellular communication (6). Numerous studies have suggested that exosomes contribute to HCC progression by affecting chemoresistance, angiogenesis, metastasis, and the immune response (7,8). Knowledge about the contents of exosomes has contributed to the study of biomarkers in HCC, which has been useful for the prediction of disease progression and early diagnosis (9). These studies revealed the important effects of exosomes on the TME (10). Accumulating research has found that the TME is an important modulator in progression of HCC, which has enabled the increase/inhibition of exosomes as potential targets of therapeutic agents (11).

Numerous studies have investigated the role and application of microRNAs (miRNAs) in HCC. MiRNAs were found commonly in exosomes (12), and play vital roles in the pathogenesis of HCC (13). All cell types can release exosomes, which are nano-sized membrane-bound vesicles that are involved in intercellular communication (14). Exosomes can transfer biological messages between cells. These messages include miRNAs, proteins, long noncoding RNAs (lncRNAs), or DNA fragments (15). Recent studies have indicated that exosomal miRNAs might be more sensitive biomarkers compared with free miRNAs owing to their stability in exosomes (16).

LncRNA nuclear-enriched abundant transcript 1 (lncRNA-*NEAT1*) locates on chromosome 11 (17). Previous research has confirmed that the lncRNA *NEAT1* promotes invasion and proliferation by *hnRNP A2* regulation of HCC cells (18). Although an increasing number of studies have shown that *NEAT1* influences multiple biological functions that play an oncogene role in HCC (17), a relationship between *NEAT1* and exosome secretion or changes in exosome contents has not yet been identified. To determine whether *NEAT1* contributes to exosome secretion by HCC cells, we analyzed exosome secretion by nanoparticle tracking analysis (NTA) and nanoflow cytometry (nanoFCM). We demonstrated that *NEAT1* could promote exosome secretion by *NEAT1*-overexpressing HCC cells. Using a miRNA microarray system and reverse transcription polymerase chain reaction (RT-PCR) analysis, we found that several suppressor miRNAs changed in exosomes secreted by *NEAT1*-overexpressing cells. This study indicated oncogene function of *NEAT1* can continue to increase with increasing *NEAT1* expression levels. It will benefit for study in nude mice. This study also indicate *NEAT1* might be related with TME by changing exosomal miRNAs. Taken together, our results demonstrated that *NEAT1* could change exosome secretion and miRNA levels in the exosomes produced by HCC cells. We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/jgo-21-729).

Methods

Cell culture

HCC cell lines including HuH-7, HepG2, and 293T cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China (national collection of authenticated cell cultures of China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL).

Plasmid construction and transfection

According to the instructions of manufacturer, 293T cells were transfected using Lipofectamine 2000 (Invitrogen, USA). GV641 (Shanghai Genechem Co., Ltd., China) was selected as the expression plasmid vector; the component sequence of this vector is CMV-MCS-EF1-ZsGreen1-T2A-puromycin, and the cloning site is EcoRI/XbaI. As for the transfection of miRNA mimics, HCC cells were transfected using Lipofectamine 2000 for 6 h. The target gene (*NEAT1*) primers, small interfering RNAs for *NEAT1* (si-*NEAT1*), hsa-miRNAs (mimics), and cel-*miR-67* [negative control (NC)] were synthesized by Shanghai Genechem Co., Ltd. (China) as follows:

NEAT1 (47382-2D)-p1: GATCTATTTCCGGTGAAT

TCGGAGTTAGCGACAGGGAGGGATGCGCG;

NEAT1 (47382-2D)-p2: ATGGACTCGAGTCATCTAGTTCTAATGAGTTTAGAACTCAAACTTTATTTG;

si-*NEAT1*: target: GCCTCCGGTCATACTAGTT, forward: GCCUCCGGUCAUACUAGUUdTdT, reverse: dTdTCGGAGGCCAGUAUGAUCAA;

hsa-*miR-634* MIMAT0003304: forward: AACCAGCACCCCAACUUUGGAC, reverse: GUCCAAAGUUGGGGUGCUGGUU;

hsa-*miR-638* MIMAT0003308: forward: AGGGAUCGCGGGCGGGUGGCGGCCU, reverse: AGGCCGCCACCCGCCCGCGAUCCCU;

hsa-*miR-3960* MIMAT0019337: forward: GGCGGCGGCGGAGGCGGGGG, reverse: CCCCCGCCTCCGCCGCCGCC;

cel-*miR-67* (NC): forward: UCACAACCUCCUAGAAAGAGUAGA, reverse: UCUACUCUUUCUAGGAGGUUGUGA.

Lentiviral infection

Vectors with recombinant *NEAT1* or an empty control were cultured with 293T cells for 6 h at 37 ℃ and 5% CO_2 for transfection. The cellular supernatant was collected 48 h after transfection for lentivirus quality examination. Next, HCC cells were infected with the viral suspension for 72 h and observed under a fluorescent microscope (micropublisher 3.3RTV, Olympus, Japan).

Exosome isolation

HCC cells were cultured for 48 hours and the culture supernatant was used to prepare the exosomes. The supernatant was collected for centrifugation (2,000 ×g for 10 min and 1,000 ×g for 30 min). Subsequently, the supernatant was collected and centrifuged at 100,000 ×g for 70 min (4 ℃). The pellet was collected and resuspended in phosphate-buffered saline (PBS) as described previously (19).

NTA

Exosomes were tracked using the NanoSight NS 300 system (PARTICLE METRIX, ZetaVIEW S/N 17-310, Germany) to detect the number and size. Samples in each group were diluted 1,000–2,000 times with Dulbecco's PBS (DPBS). Finally, the samples reached a concentration that was less than 20×10^8 particles/mL. Each sample was distributed to three tubes that were measured using a camera (Hitachi, HT-7700, China). According to the data, the distribution of the number and size were measured using the Stokes-Einstein equation (20).

Transmission electron microscopy

Samples were fixed using glutaraldehyde in phosphate buffer at 4 ℃, and the fixed samples were sliced at a thickness of approximately 70 nm. Exosomes in the suspension droplets were placed on the copper mesh of the electron microscope (Hitachi, HT-7700, China) and then subjected to negative staining with 20 μL 2% phosphotungstic acid for 10 min. We used a H-7700 electron microscope (Hitachi, China) to analyze samples at 100 kV.

RNA extraction and RT-PCR assay

Total RNAs were extracted using TRIzol reagent (Invitrogen) and detected by nanodrop for quality. Exosomal RNAs were extracted using TRIzol reagent. For messenger RNA (mRNA), we used glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal reference, and for miRNA we used U6 as an internal reference. A High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was used to transcribe RNA into cDNA. The reaction volume was 20 μL. RT-PCRs were analyzed in a reaction volume of 20 μL using the SYBR Green mix on an Applied Biosystems 7500. RNA expression in each group was normalized to GAPDH or U6. We used the $2^{-\Delta\Delta Ct}$ method to calculate fold change. The primers were as follows:

NEAT1: forward: CTCACTCCACCCCTTCT, reverse: TCCTCCACCATTACCAA;

GAPDH: forward: GAACGGGAAGCTCACTGG, reverse: GCCTGCTTCACCACCTTCT;

Alanine-tRNA ligase, cytoplasmic (*AARS*): forward: CGACCATACACTGGGAAA, reverse: AGGGACTGGACGACAACA;

Heat shock protein family A member 8 (*HSPA8*): forward: TACAAGGGAGAGACCAAAA, reverse: GTAGCCTGACGCTGAGAGT;

U6: forward: CTCGCTTCGGCAGCACA, reverse: AACGCTTCACGAATTTGCGT;

Solute carrier family 3 member 2 (*SLC3A2*): forward: CGAGAAGCAGCCGATGA, reverse: TGGACAGGCCCGTGAAC;

DnaJ heat shock protein family member A1 (*DNAJA1*): forward: CAAACCCAATGCTACTCA, reverse: CCACCT

GCTCCACCCTCT;

Heat shock protein (*HSP90AA1*): forward: AAGTT GAAAAGGTGGTTGTG, reverse: TGTTGAGT TGTCTCTTAGGG;

Methionyl-tRNA synthetase (*MARS*): forward: ACCCA ATGCCCAGTTTATC, reverse: TTCTTCCACCA TCTCTCCC;

Seryl-tRNA synthetase (*SARS*): forward: AAGCGA GCAGGGCATCAACTCC, reverse: GCCATCCTCAA AAAGCCGAAAA;

Solute carrier family 3 member 2 (*SLA7A5*): forward: TCTTCCTGATCGCCGTCTCC, reverse: CCACTTG GGCTTGTTTTTCC;

Transmembrane protein 109 (*TMEM109*): forward: CCAACAGAAGAGAGAAGC, reverse: TCCCAGACA GAGCAAAGA;

miR-6089: forward: ACACTCCAGCTGGGGGAGG CCGGGGTGGGGCG, reverse: TGGTGTCGT GGAGTCG;

miR-634: forward: ACACTCCAGCTGGGAACCAGC ACCCCAACT, reverse: TGGTGTCGTGGAGTCG;

miR-3960: forward: ACACTCCAGCTGGGGGCGG CGGCGGAGG, reverse: TGGTGTCGTGGAGTCG;

miR-6127: forward: ACACTCCAGCTGGGTGAGG GAGTGGGT, reverse: TGGTGTCGTGGAGTCG;

miR-638: forward: ACACTCCAGCTGGGAGGGAT CGCGGGCGGGTGG, reverse: TGGTGTCGTG GAGTCG.

Cell proliferation assay

Cells were seeded in 96-well plates with a density of 2,000 per well. We used a Cell Counting Kit-8 (CCK-8) assay to analyze cell viability at 0, 24, 48, and 72 h after seeding. An automatic microplate reader (Infinite M1000, TECAN, Switzerland) was used to detect absorbance at 450 nm.

Invasiveness analysis

The invasive function was assessed with a Transwell assay (Millipore, Billerica, MA, USA). A total of 1×10^4 HCC cells were suspended in DMEM without serum. Next, the cells were seeded in the upper chamber. Matrigel (BD Biosciences, USA) (200 mg/mL, 100 mL) was used to cover the membrane. To produce a chemoattractant environment, the lower chamber contained FBS (final concentration: 15%). After culturing for 48 h, we cleaned the cells in the upper chamber. Invasive cells were fixed with methanol and stained with 0.1% crystal violet. For calculation, cells in the lower chamber were detached by trypsin after cleaning cells in the upper chamber. The number of cells was counted using a hemocytometer (Z359629-1EA, Merck, China).

FCM analysis

For apoptosis analysis, we used propidium iodide (PI) and annexin V-FITC (BestBio, Shanghai, China) to incubate with HCC cells for 15 minutes at room temperature. FCM was performed using a FACSCalibur device (BD Biosciences, USA), and the data were analyzed using Cell Quest software (BD Biosciences, USA). For exosome FCM analysis, the exosomes were diluted and incubated with CD9-FITC, CD81-FITC and IgG-FITC (BD Biosciences) for 30 minutes at 37 ℃. FCM was performed using a NanoFCM (N30E).

RNA sequencing and data analysis

The integrity of RNA was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). A RNA integrity number of samples (RIN) ≥7 was used for analysis. Libraries were constructed using the stranded mRNA prep kit (Illumina, USA), and were sequenced on the Illumina sequencing platform (HiSeq™ 2500 or Illumina HiSeq X Ten). Next, 125 bp/150 bp paired-end reads were generated. Feature Extraction software (version 10.7.1.1, Agilent Technologies) was used for microarray analysis. Raw data were normalized using the quantile algorithm. Probes that had flags of "Detected" in any one of two conditions were selected for further data analysis. Changed miRNAs were then identified through the fold change and P value using *t*-tests. A fold change ≥2 and P value <0.05 were set for up- and down-regulated genes. Target miRNAs were predicted using two databases (miRDB, miRWalk). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were applied to determine the roles of target genes. Hierarchical clustering was performed to display the different miRNA expression patterns.

Statistical analysis

Analyses were performed using Prism 8.0 software (USA). Analyses included the Student's *t*-test and two-way analysis of variance (ANOVA) with Dunnett's multiple comparisons. The data graph was presented using Prism GraphPad 8.0, and significant differences between each group were marked

using *, P<0.05, **, P<0.01, ***, P<0.001, or ****, P<0.0001.

Results

NEAT1-overexpression promoted the invasion and proliferation of HCC cells

The efficacy of the *NEAT1* overexpression was measured in HCC cells (approximately 30 times for HepG2 cells and 20 times for HuH-7 cells; *Figure 1A*). Previous studies assessed functions of *NEAT1* knockdown cells. The aim of our research was to confirm whether the oncogene function could increase with increasing *NEAT1* expression levels, as *NEAT1* expression levels are naturally high in HCC cell lines and tissues. Proliferation was assessed by CCK-8 assays, and these experiments showed that overexpression of *NEAT1* significantly promoted the proliferation of HCC cells compared with the Lv-control and NC groups (P<0.01; *Figure 1B,1C*). We also analyzed the effect of Lv-*NEAT1* on invasiveness using Transwell assays. Compared with the Lv-control and NC groups, NEAT1 overexpression promoted invasiveness, which was observed using crystal violet staining (*Figure 1D*). Markedly more cells detached from the lower chamber filters in the Lv-*NEAT1* group compared with the Lv-control and NC groups (P<0.01; *Figure 1E*). Apoptosis was assessed by FCM, which indicated that overexpression of *NEAT1* considerably decreased the apoptosis of HCC cells compared with the Lv-control and NC groups (P<0.01; *Figure 1F,1G*).

Overexpression of NEAT1 promoted exosome secretion by HCC cells

A recent study showed that lncRNAs can regulate abnormal exosome-related gene expression, which results in abnormal exosome secretion by HCC cells (20). We used transmission electron microscopy to detect the purity of exosomes in each group. As shown in *Figure 2A*, for each group, the exosomes were double membrane and 100–150 nm in diameter. NTA indicated that the sizes of the released exosomes were approximately 150 nm (*Figure 2B*). By combining the particle number and volume (*Figure 2B*), we found that exosomes were secreted in greater quantities by the overexpression of *NEAT1* HCC cells than by the Lv-control group (P<0.05) (*Figure 2C*). To explore surface molecular differences of exosomes, we detected the markers (CD81 and CD9) of the Lv-*NEAT1* group compared with those of the Lv-control group. The data indicated that *NEAT1* overexpression increased exosome secretion (*Figure 2D,2E*). Exosomes released by *NEAT1* overexpression cells contained CD81 and CD9 (*Figure 2D*). These results indicated that *NEAT1* could affect the secretion of exosomes in HCC cells.

Overexpression of NEAT1 caused global gene expression changes in HCC cells

The data showed that the expression levels of 1,158 genes were changed in the *NEAT1*-overexpressing cells (including 362 up-regulated and 796 down-regulated genes, fold change ≥2.0 and P value <0.05) (*Figure 3A*). GO analysis indicated that the changed genes were involved in biological and molecular functions, including extracellular matrix changes (*Figure 3B*). The top 20 variations after the overexpression of *NEAT1* were presented in *Figure 3B*, which were associated with biological and molecular functions. Also, KEGG pathway analysis identified variations in the MAPK signaling pathway, apoptosis, p53 signaling pathway, and the HIF-1 signaling pathway after overexpression of *NEAT1* (*Figure 3C*).

Based on the bioinformatics analysis data as well as the results showing that *NEAT1* overexpression promoted HCC cell invasion, proliferation, and exosome secretion, we selected genes that are involved in cellular components relevant to exosome synthesis and secretion. We found that these genes were associated with each other. We drew a protein-protein interaction network of these genes based on the bioinformatics analysis (*Figure 3D*). Using RT-PCR assays, we confirmed the upregulation of *HSPA8*, *DNAJA1*, *HSP90AA1*, and *TMEM109*, and the downregulation of *AARS*, *SARS*, *MARS*, *SLC3A2*, and *SLC7A5* in *NEAT1*-overexpressing HCC cells (*Figure 3E*). We found that the PCR results matched the protein-protein interaction network of these exosome-related genes.

Overexpression of NEAT1 altered global miRNA levels in HCC cells

MiRNAs play a vital role in the interaction between *NEAT1* and HCC pathogenesis. In addition, it is possible that *NEAT1*-related miRNAs participate in exosome synthesis, secretion regulation, and even exosome-containing changes. We used gene sequencing to analyze miRNA level changes in HCC cells 72 h after Lv-*NEAT1* infection. Overall, 169 miRNAs were differentially expressed in *NEAT1*-overexpressing cells (including 92 upregulated and 77 downregulated miRNAs, fold change >2.0 and P value

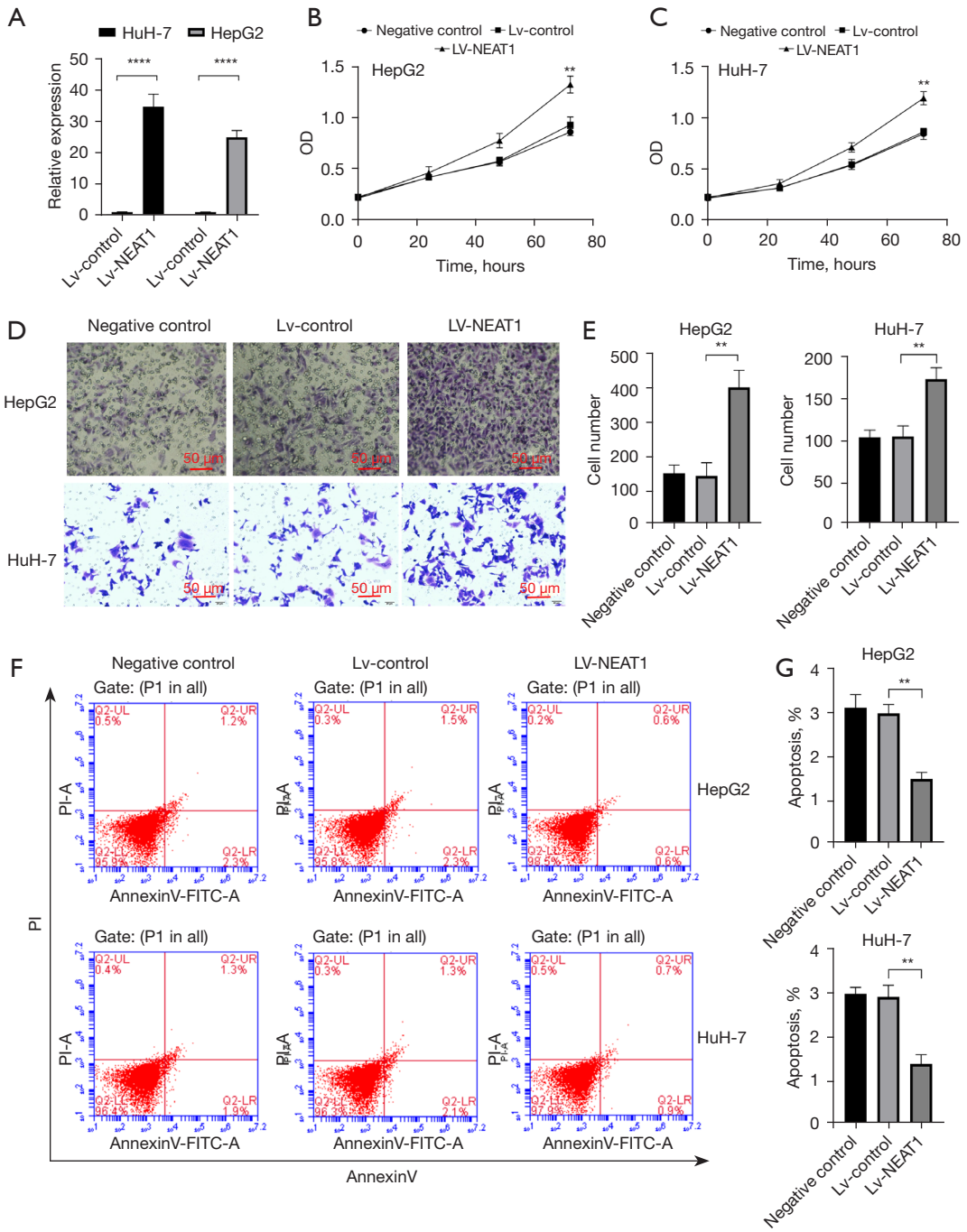

Figure 1 *NEAT1*-overexpression promoted the invasion and proliferation of HCC cells. (A) Expression levels of *NEAT1* in Lv-NEAT1 and Lv-control groups. (B,C) After Lv-NEAT1 or Lv-control treatment for 48 h, 2×10^3 HCC cells were used for CCK-8 assay. Ten μL of CCK-8 was added and incubated for 1 h at 37 ℃. An automatic microplate reader was used to detect cell viability on the OD450 value. (D) Cells infected with Lv-*NEAT1* or Lv-control were used for Transwell assays. After invasion for 48 h, invasive cells were stained with 0.1% crystal violet and photographed. The scale bar represents 50 μm. (E) The number of cells that penetrated the filters was counted and presented on the statistical graph. (F) HCC cells transfected with Lv-*NEAT1* or Lv-control were collected and cultured with PI and Annexin V for 30 minutes in 4 ℃. PI and Annexin V intensity were detected by FCM. (G) PI and Annexin V intensity data were presented on the statistical graph. Significant differences between each group were marked by **, P<0.01 or ****, P<0.0001. *NEAT1*, nuclear-enriched abundant transcript 1; HCC, hepatocellular carcinoma; CCK-8, Cell Counting Kit-8; OD450, optical density 450 nm; PI, propidium iodide; FCM, flow cytometry.

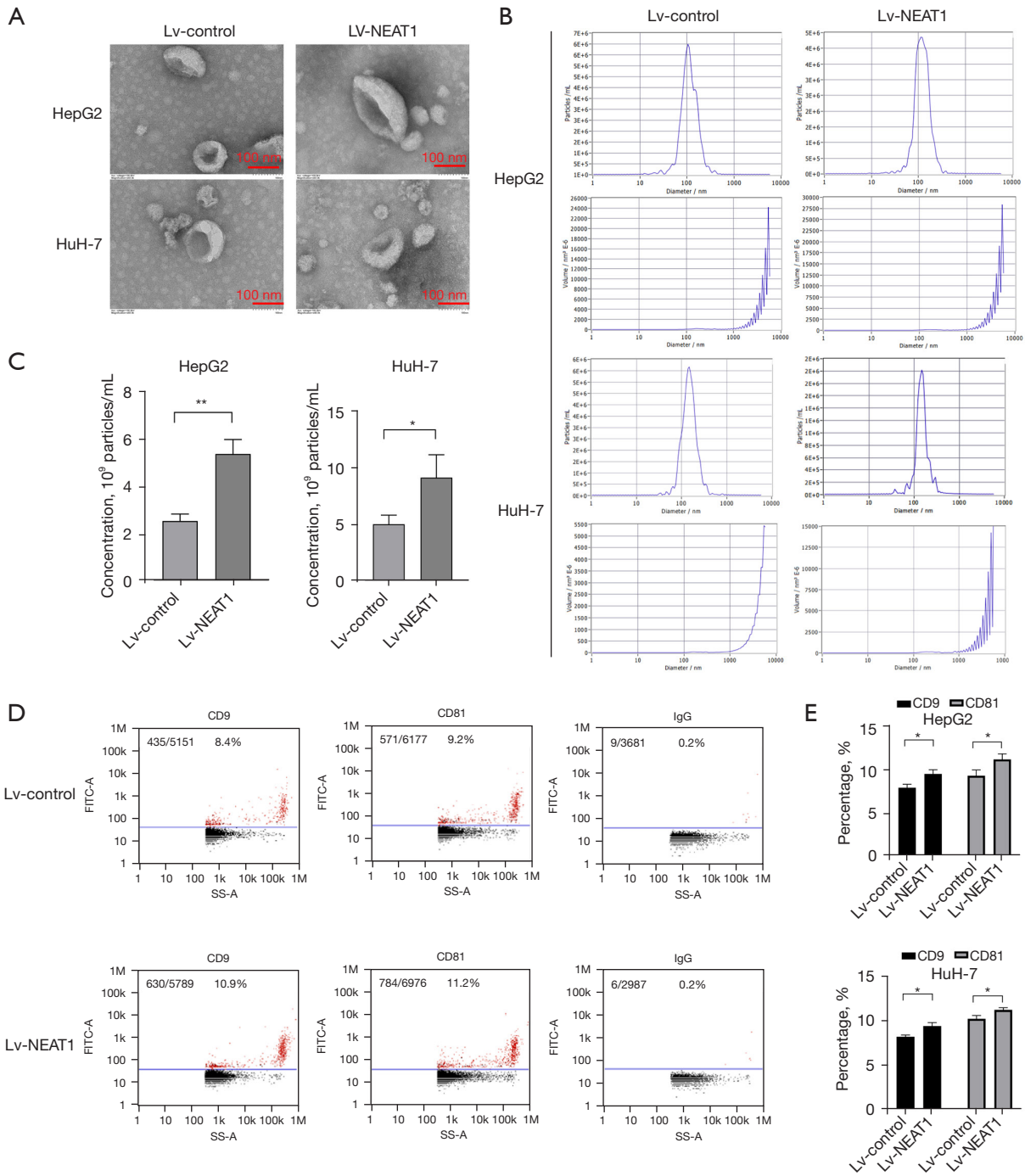

Figure 2 Overexpression of *NEAT1* promoted exosome secretion by HCC cells. (A) Fifty mL of cell culture medium were collected, and exosomes were isolated using ultracentrifugation in each group. Purified exosomes were analyzed using transmission electron microscopy to detect their purity and particle size. The scale bar represents 100 nm. (B) The concentration of exosomes purified from HCC cells (transfected with Lv-control of Lv-*NEAT1*) culture medium was analyzed using NTA. (C) The concentration of exosomes in each group was calculated according to the NTA data. (D) Exosomes were diluted and incubated with fluorescently-labeled antibodies (CD9, CD81, IgG) at 37 ℃ for 30 min. Samples were analyzed by nanoFCM to detect CD9 and CD81 in each group. (E) Flow data was presented on the statistical graph. Significant differences between each group were marked by *, P<0.05 or **, P<0.01. *NEAT1*, nuclear-enriched abundant transcript 1; HCC, hepatocellular carcinoma; NTA, nanoparticle tracking analysis; FCM, flow cytometry.

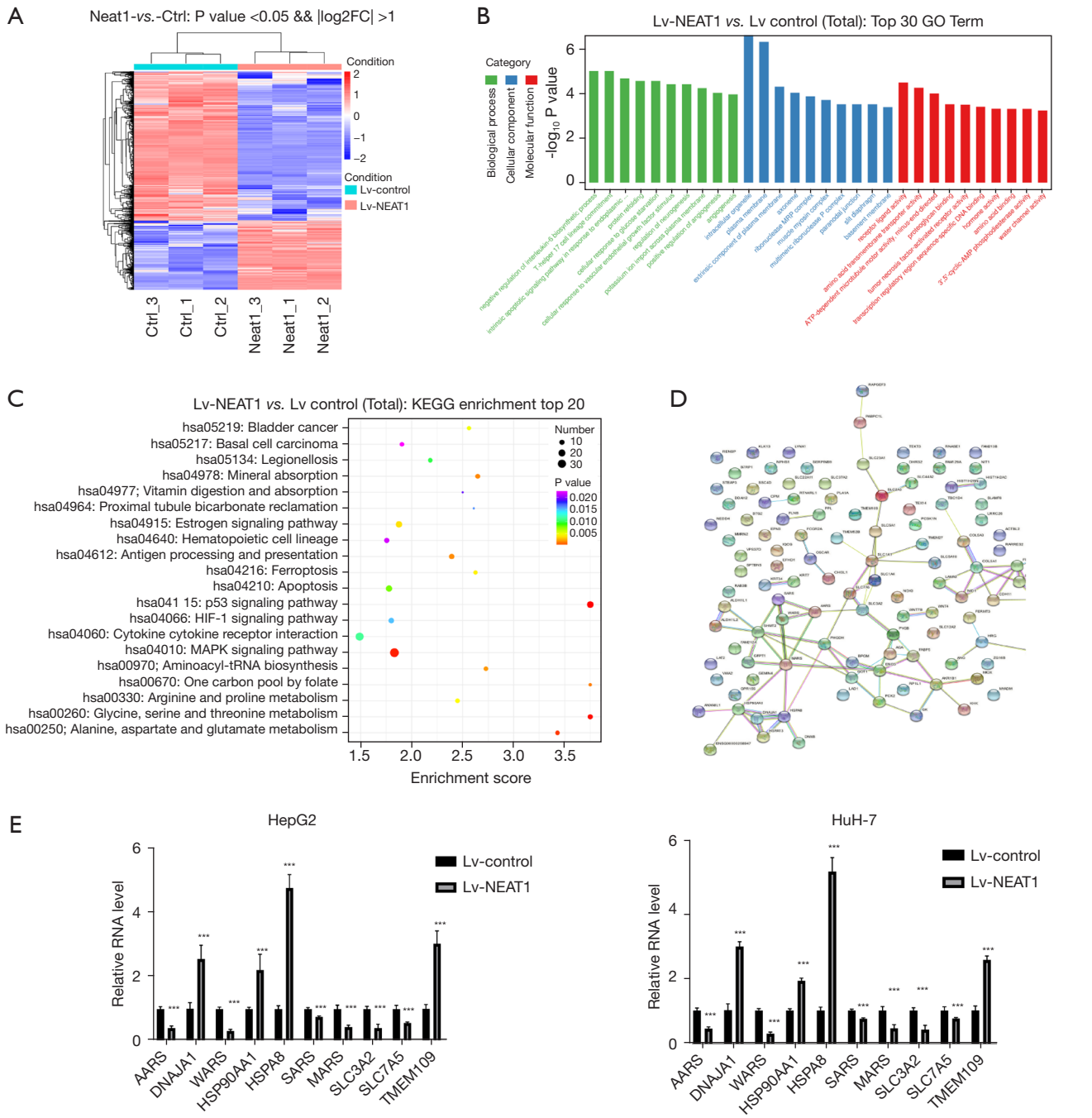

Figure 3 Overexpression of *NEAT1* caused global gene expression changes in HCC cells. (A) The heat map shows the transcriptional profile of HCC cells transfected with Lv-*NEAT1* and Lv-control independent replicates (n=3). The color key and histogram in the upper right represents the distribution of lowly-expressed genes (blue) and highly-expressed genes (red). (B) Based on the GO analysis, hits for changed genes and associated function are presented. (C) Based on the KEGG pathway, hits for changed genes and pathways are presented. (D) Protein-protein interaction network of genes that participate in exosome secretion-related pathways. (E) Genes for further validation were analyzed by RT-PCR. Significant difference between each group were marked by ***, P<0.001. *NEAT1*, nuclear-enriched abundant transcript 1; HCC, hepatocellular carcinoma; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; RT-PCR, reverse transcription polymerase chain reaction; FC, fold change.

<0.05) (*Figure 4A*). Combined with the miRNA-mRNA interaction analysis, GO analysis showed that the changed miRNA-related mRNAs were involved in biological and molecular functions, which were related to HCC pathogenesis, including exosome and extracellular matrix changes (*Figure 4B*). KEGG pathway analysis identified the classification and target mRNA number of variation miRNA levels after *NEAT1* overexpression (*Figure 4C*). The data show that miRNAs regulated by *NEAT1* reacted to a large number of mRNAs, which significantly contributed to cancer pathogenesis (*Figure 4C*).

Overexpression of NEAT1 changed miRNAs carried by exosomes in HCC cells

MiRNAs carried by exosomes (exosomal miRNAs) secreted by HCC cells play a vital role in cellular communication, which might contribute to tumor growth, metastasis, angiogenesis, and immunologic escape. *NEAT1* changes exosome secretion and induces global miRNA level changes in HCC cells, and it is possible that *NEAT1* also changes the miRNA content in exosomes. We used a miRNA microarray system to analyze the miRNA level changes in exosomes secreted by HCC cells 72 h after Lv-*NEAT1* infection, in order to identify the miRNA changes carried by the exosomes. The data showed that five miRNA levels were different in exosomes secreted by *NEAT1*-overexpressing cells. All altered miRNA levels were downregulated (including one miRNA, fold change >5.0 and P value <0.05 and four miRNAs, fold change >2.0 and P value <0.05) (*Figure 5A*).

Combined with the miRNA-mRNA interaction analysis, GO analysis showed that the changed miRNA-related mRNAs were involved in biological and molecular functions relevant to the pathogenesis of HCC (*Figure 5B*). KEGG pathway analysis identified variations in MAPK activity that are associated with *NEAT1* and HCC progression, and identified a close association between miRNA and mRNA changes caused by *NEAT1* in our bioinformatics analysis and previous work (*Figure 5C*). We confirmed the downregulation of *miR-6089*, *miR-634*, *miR-638*, *miR-6127*, and *miR-3960* in *NEAT1*-overexpressing HCC cells by RT-PCR assays (*Figure 5D*).

In addition to bioinformatics analysis data, we also found that most altered miRNAs in exosomes, such as *miR-6089*, *miR-634*, *miR-638* and *miR-3960*, played suppressor gene functions in HCC, which has been shown in other studies. It will be interesting to confirm whether *NEAT1* downregulated these suppressor miRNAs, which are transported between cells carried by exosomes in HCC. Using RT-PCR assays, we also confirmed that *miR-6089*, *miR-634*, *miR-638*, *miR-6127*, and *miR-3960* were downregulated in exosomes secreted by *NEAT1*-overexpressing HCC cells (*Figure 5D*).

MiR-634, miR-638, and miR-3960 reverse proliferation and invasion function in NEAT1-overexpressing HCC cells

We observed the alteration of several exosomal miRNAs in *NEAT1*-overexpressing HCC cells. We proposed the hypothesis that NEAT1 promotes cell progression through regulation of exosomal RNAs. We used miRNA mimics of downregulated exosomal RNAs to transfer *NEAT1*-overexpressing HCC cells and *NEAT1*-knockdown HCC cells. We found that transfection of *miR-634*, *miR-638*, and *miR-3960* reversed the enhanced proliferation function in *NEAT1*-overexpressing HCC cells (P<0.01, *Figure 6A*). However, the reversion was not observed in *NEAT1*-knockdown HCC cells because transfection of these miRNA mimics did not significantly reduce proliferation in *NEAT1*-knockdown HCC cells (*Figure 6A*).

Transfection of *miR-634*, *miR-638*, and *miR-3960* reduced invasion in *NEAT1*-overexpressing HCC cells and *NEAT1*-knockdown HCC cells (P<0.05; *Figure 6B,6C*). In *NEAT1*-knockdown HCC cells, the extent of inhibition might be relenting compared *NEAT1*-overexpressing HCC cells group (*Figure 6B,6C*). Transfection of *miR-634*, *miR-638*, and miR-3960 markedly increased the apoptosis rate in HCC cells of Lv-NEAT1 and si-NEAT1 groups (P<0.0001; *Figure 6D,6E*). As for the change of apoptosis, there was no difference in the extent of increase between *NEAT1*-overexpressing HCC cells and *NEAT1*-knockdown HCC cells (*Figure 6D,6E*).

Discussion

Our previous study determined that lncRNA *NEAT1* was an oncogene. When NEAT1 was downregulated, the invasion and proliferation of HCC cells were inhibited, as was tumor growth in xenograft models. These effects were correlated with *hnRNP A2*, which is regulated by *NEAT1* (18). A number of studies have confirmed the oncogene function of NEAT1 and its specific molecular mechanism, including its function through lncRNA-miRNA interactions in cells [*miR-124-3p* (21), *miR-204* (22), *miR-296-5p* (23), *miR-22-*

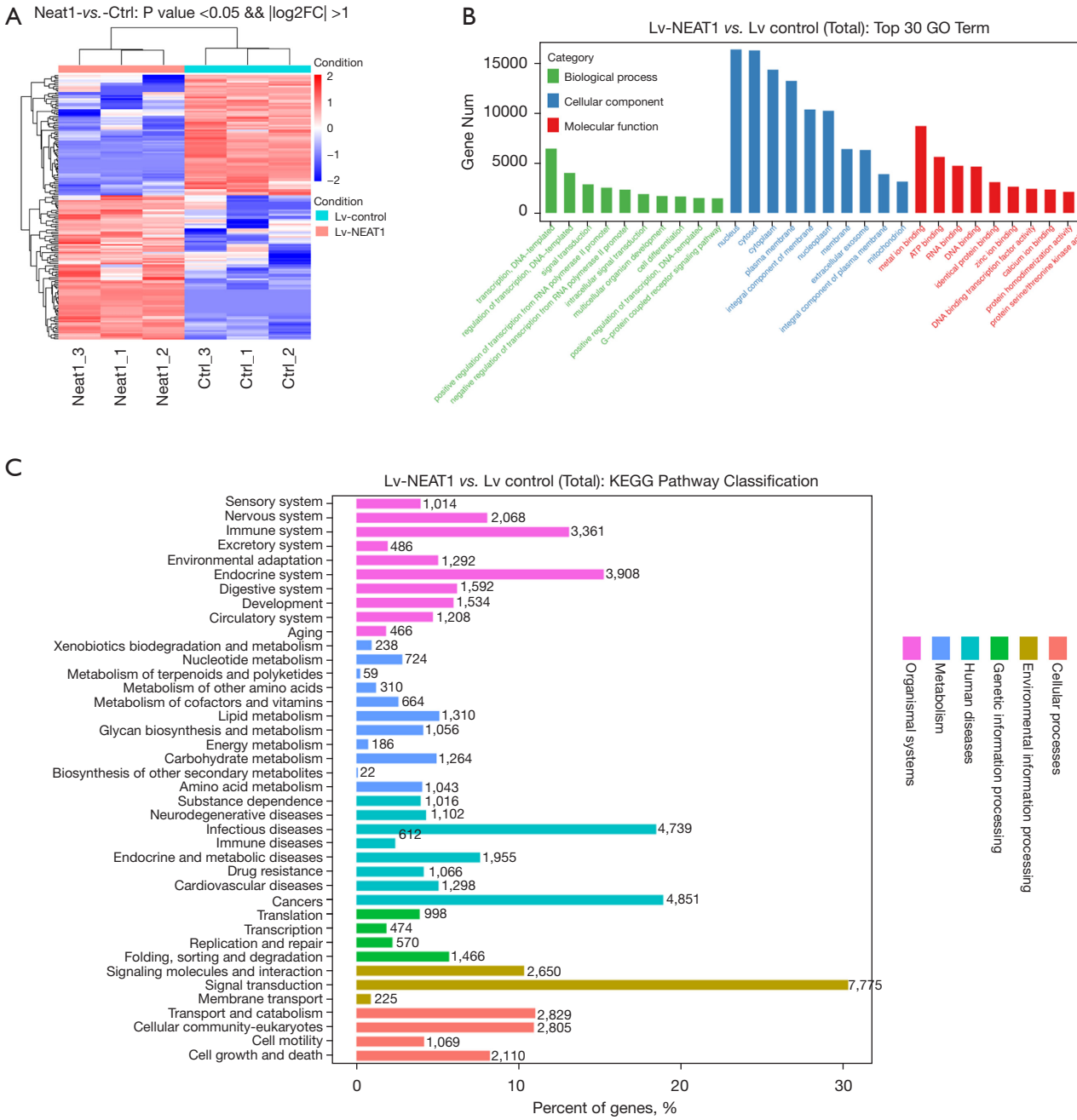

Figure 4 Overexpression of *NEAT1* altered the global miRNA levels in HCC cells. (A) The heat map shows the miRNA level profile of HCC cells transfected with Lv-*NEAT1* and Lv-control independent replicates (n=3). The color key and histogram in the upper right represent the distribution of lowly-expressed genes (blue) and highly-expressed genes (red). (B) Based on the GO analysis, hits for different miRNA target mRNAs and associated biological molecular functions. (C) Based on the KEGG analysis, hits for different miRNA target mRNAs and associated biological molecular functions. *NEAT1*, nuclear-enriched abundant transcript 1; miRNA, microRNA; HCC, hepatocellular carcinoma; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; mRNA, messenger RNA; FC, fold change.

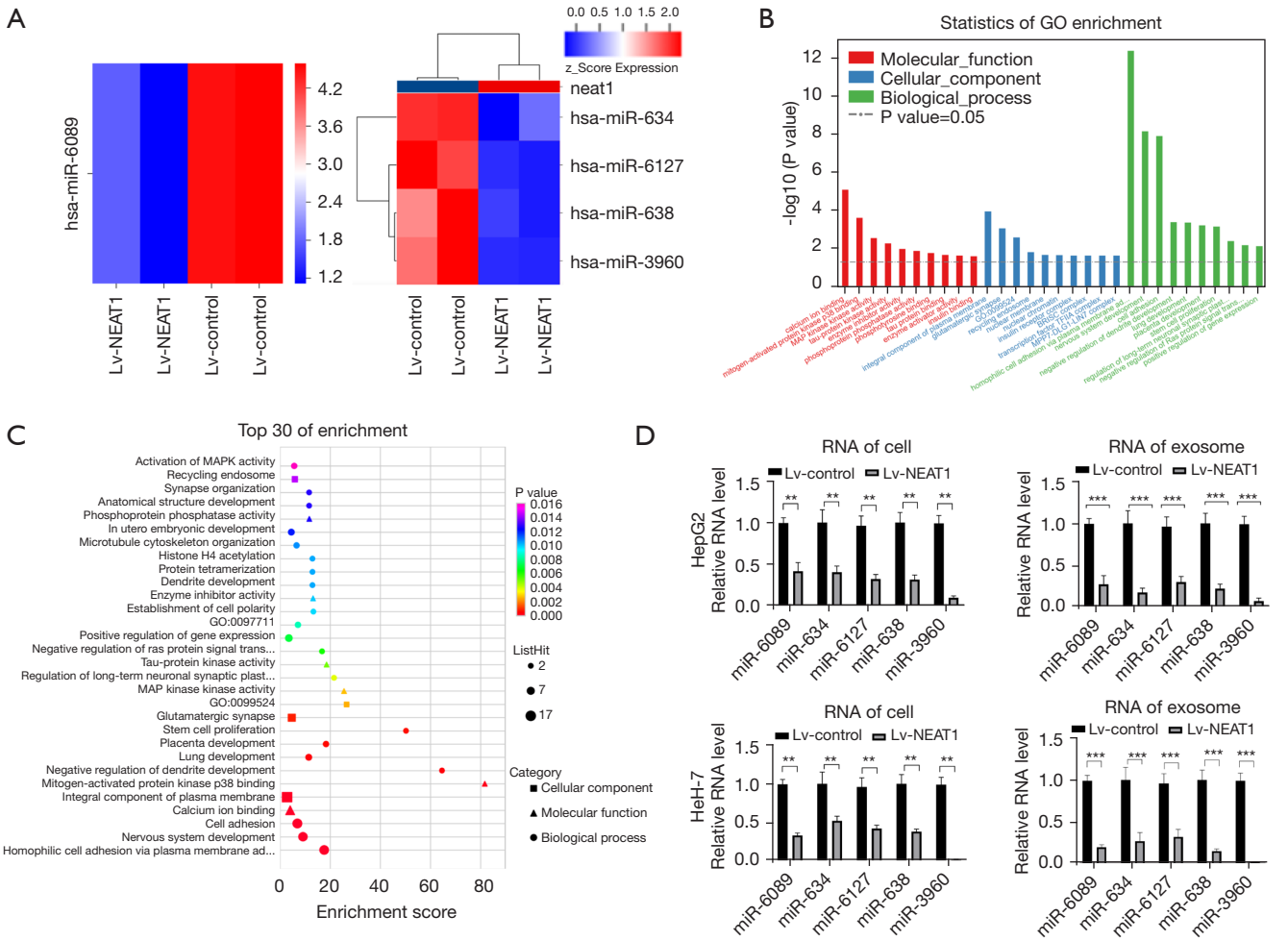

Figure 5 Overexpression of *NEAT1* changed the miRNAs carried by exosomes in HCC cells. (A) RNA of exosomes in each group was extracted for detection using the miRNA Microarray System with the miRNA Complete Labeling. Data from the microarray scanning were analyzed by feature extraction software to quantitatively analyze the change in miRNA carried by exosomes in each group. (B) Based on the GO analysis, hits for different miRNA target mRNAs and associated biological molecular functions. (C) Based on the KEGG analysis, hits for different miRNA target mRNAs and associated biological molecular functions. (D) The miRNA levels of the HCC cells and their exosomes were normalized by the level of U6 according to the $2^{-\Delta\Delta Ct}$ method. Significant difference between each group were marked by **, P<0.01 or ***, P<0.001. *NEAT1*, nuclear-enriched abundant transcript 1; miRNA, microRNA; HCC, hepatocellular carcinoma; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; mRNA, messenger RNA.

3p (24), *miR-613* (25), *miR-485* (26)], its function through the lncRNA-RBP complex [*NEAT1-U2AF65* (18), *NEAT1-PSPC1* (27)], and its function through gene regulation [*TUG1* (28), *ATGL* (21), *TGF-β1* (29), *STAT3* (26)]. Most of these studies confirmed the function and mechanism associated with intracellular regulation using *NEAT1* knockdown in HCC cell lines. Hence, there are two problems to be solved according to the current study of NEAT1. The first problem is whether the oncogene function of *NEAT1* can continue to increase with increasing *NEAT1* expression levels, and the second issue relates to the identification of the different mechanisms.

NEAT1 has a high expression level in HCC cell lines and tissues (30), which makes it possible that the basic expression level of *NEAT1* could perform an oncogene function in HCC and that overexpression of *NEAT1* might not increase the intensity of its oncogene function. In previous work on *NEAT1*, one study used *NEAT1*-overexpressing

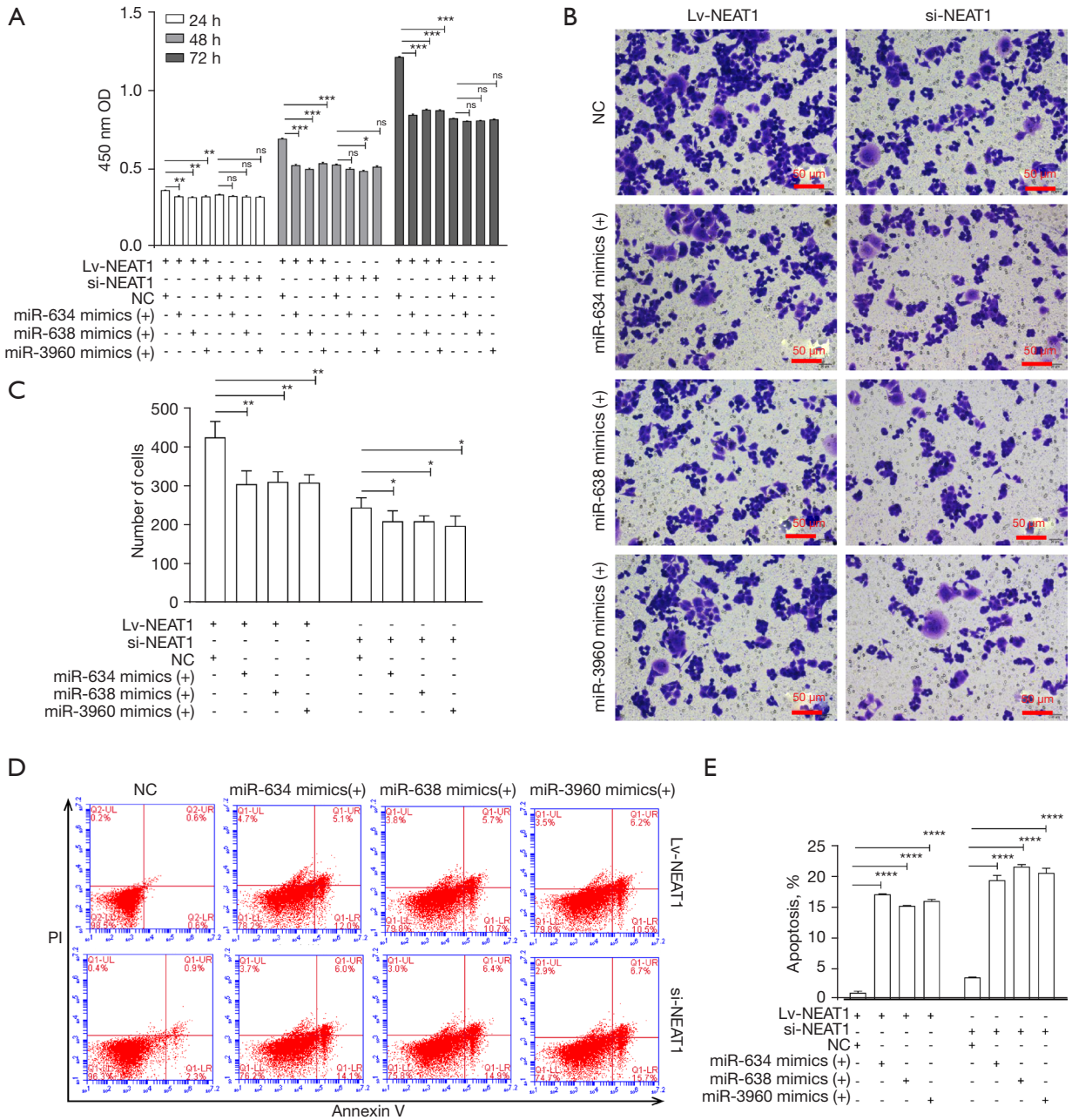

Figure 6 *MiR-634*, *miR-638*, and *miR-3960* reversed the proliferation and invasion function in *NEAT1*-overexpressing HCC cells. (A) After Lv-*NEAT1*, si-*NEAT1*, cel-miR-67 (NC), *hsa-miR-634*, *hsa-miR-638*, or *hsa-miR-3960* (mimics) treatment for 48 h, 2×10^3 HepG2 cells were seeded for the CCK-8 assay. Ten μL of CCK-8 were added and incubated for 1 h at 37 ℃. An automatic microplate reader was used to detect the cell viability based on the OD450 value. (B) After invasion for 48 h, invasive cells were stained with 0.1% crystal violet and photographed. The scale bar represents 50 μm. (C) The number of cells that penetrated the filters was counted and was presented on the statistical graph. (D) HepG2 cells of each group transfected with Lv-*NEAT1* or Lv-control were collected and cultured with PI and Annexin V for 30 minutes in 4 ℃. The intensities of PI and Annexin V were detected by FCM to measure cell apoptosis. (E) Data of PI and Annexin V intensity were presented on the statistical graph. Significant difference between each group were marked by ns: P>0.05, *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. *NEAT1*, nuclear-enriched abundant transcript 1; HCC, hepatocellular carcinoma; NC, negative control; CCK-8, Cell Counting Kit-8; OD, optical density; PI, propidium iodide; FCM, flow cytometry.

cells instead of *NEAT1*-knockdown cells to explore the metastatic function and mechanism of HCC *in vitro* and *in vivo* under hypoxia, as decreasing the growth and metastatic function of *NEAT1*-knockdown cells did not demonstrate positive findings in a metastasis model (31). Compared with the *NEAT1* knockdown HCC cell model, the *NEAT1* overexpression cell model has advantages in tumor growth and metastasis studies *in vivo*. *NEAT1*-overexpressing cells also made it possible to explore extracellular matrix changes that might be associated with *NEAT1* overexpression. Our study confirmed that lentivirus transfection was a reliable method for *NEAT1* overexpression in HCC cell lines and that the oncogene function of *NEAT1* was not limited to its basic level of expression in HCC cell lines (*Figure 1*). Our study also revealed alterations in global gene expression patterns and miRNA levels in HCC cells, which might be associated with cancer pathogenesis in *NEAT1*-overexpressing HCC cells (*Figures 3*,*4*). These data might support further *NEAT1* overexpression studies on metastasis, drug resistance, hypoxia, angiogenesis, and extracellular matrix changes *in vivo*, considering the various functions of lncRNAs (12).

A recent study showed that lncRNAs regulate a large number of mRNAs and miRNAs in HCC, which suggests that lncRNAs affect exosome secretion (32,33). Recently, several studies have suggested that lncRNAs can regulate exosome secretion, such as lncRNA *HULC/miR-372-3p*, which plays a significant role in exosome secretion. LncRNA HOTAIR was found to promote exosome secretion by inducing the phosphorylation of *SNAP23*, which could be a mammalian target of rapamycin (mTOR) signaling (20,34). LncRNA *NEAT1* has various significant biological functions associated with RNA-binding proteins (RBPs), which form functional ribonucleoprotein (RNP) complexes. RBP has encouraged most *NEAT1*-related studies to focus on intracellular gene regulation or even paraspeckles (17), and few studies have focused on the association between *NEAT1* and exosomal miRNA.

In this study, we confirmed that NEAT1 overexpression promoted exosome secretion by NTA and nanoFCM (*Figure 3*). Combining the bioinformatics analyses of *NEAT1*-overexpressing cells, we found that a large number of altered genes were involved in exosome secretion-related cellular components, including the plasma membrane, cellular exosomes, and PKM2-related pathways (*Figures 3*,*4*). Using RT-PCR assays, we confirmed the presence of *HSPA8*, a traditional exosome marker (35) with high expression in *NEAT1*-overexpressing cells (*Figure 3D*). *HSPA8* also affects the expression of CD38, which is expressed on the surface of secreted exosomes (36). Based on the protein-protein interaction analysis, the expression of *MARS*, *WARS*, *AARS*, and *SLC* proteins might change due to *HSPA8* alterations (*Figure 3D*). These proteins are also associated with secretion and are contained in exosomes (37-39). Our RT-PCR data confirmed that the expression of exosome- or *HSPA8*-related genes, including *DNAJA1*, *HSP90AA1*, *TMEM109*, *AARS*, *SARS*, *MARS*, *SLC3A2*, and *SLC7A5*, was altered in *NEAT1*-overexpressing cells. Up- or down-regulated regulation of these genes was consistent with the bioinformatics analysis and the protein-protein interaction network, which indicates that *NEAT1* plays a vital role in exosome-related gene expression.

The phenomenon of increased exosome secretion in HCC is common; however, the molecular mechanisms have not been elucidated (20). Exosomes transfer effector messages between cells, including miRNAs, proteins, or DNA fragments (15,40). Exosomes have been studied for many years, but the biological roles of exosomal miRNAs are only just beginning to be understood in HCC (16). Exosomal miRNAs contribute to HCC progression and metastasis signaling networks, including cell metastasis, proliferation, immune escape, and interactions with the TME (16,32,41). Recent studies have investigated several types of exosomes and confirmed their biological function in HCC, such as exosomes containing *miR-122* secreted by Huh7 cells that inhibit growth and proliferation by targeting *CAT1* (42), exosomes containing *miR-142* secreted by macrophages that inhibit proliferation by targeting *STMN1* (43), exosomes containing *miR-320a* secreted by *CAFs* that inhibit migration and progression by targeting the MAPK pathway (44), and exosomes containing *miR-1247-3p* secreted by LM3 cells that promote the conversion of normal fibroblasts into CAFs and accelerate the lung metastasis of HCC by targeting *B4GALT3* (32).

Exosomal miRNAs might be more sensitive biomarkers compared with free miRNAs owing to their stability in exosomes (16). This study focused on biological roles and function of exosomal miRNAs regulated by *NEAT1* in HCC. The mechanism of NEAT1-exosomal miRNAs-target mRNAs could provide potential target for HCC diagnosis and therapy. Exosomes can play role of carriers for gene target therapy. Thus, advance study can try to use exosomal miRNAs as nucleic acid and exosomes as carrier for HCC target therapy study. Considering the exosome secretion-related function of *NEAT1*, the global alteration of miRNA levels in *NEAT1*-overexpressing cells, and the fact that the

target mRNAs of altered miRNAs are involved in HCC pathogenesis (*Figure 4*), we analyzed exosomal miRNAs in the *NEAT1*-overexpressing and control groups using a miRNA microarray system, and confirmed an alteration of exosomal miRNAs by RT-PCR. Interestingly, we found that in *NEAT1*-overexpressing HCC cells, most of the exosomal miRNAs were downregulated (*Figure 5A*), and they were previously shown to have tumor suppressor functions in HCC, such as *miR-6089* (45), *miR-634* (46), *miR-638* (47-49), and *miR-3960* (50). Our data also indicated that exosomal miRNAs were also downregulated in *NEAT1*-overexpressing HCC cells (*Figure 5D*). These data imply that increasing *NEAT1* expression downregulates several suppressor miRNAs both in cells and also in exosomes secreted by HCC cells. Therefore, confirming the suppressor function of these exosomal miRNAs will be meaningful for exosome-mediated miRNA transfer for HCC therapy, because compared with free miRNAs, exosomal miRNAs have much greater stability and possess important biological properties that ensure long-term persistence (16,51).

To determine whether *NEAT1* promotes HCC progression by regulation of exosomal miRNA, we used these changed exosomal miRNA mimics to transfer *NEAT1*-overexpressing HCC cells and *NEAT1*-knockdown HCC cells and detect cell function. Transfection of *miR-634*, *miR-638*, and *miR-3960* reversed the enhancement of proliferation and invasion by *NEAT1* in *NEAT1*-overexpressing HCC cells (*Figure 6*). However, this reversion phenomenon was not observed or relenting in *NEAT1*-knockdown HCC cells (*Figure 6*). Based on the fact that *NEAT1* significantly down-regulated *miR-634*, *miR-638*, and *miR-3960* in exosomes, these data suggest that the down-regulation or transmission reduction of *miR-634*, *miR-638*, and *miR-3960* in exosomes might contribute to cell invasion and proliferation in HCC cells with a high level of *NEAT1* expression. It also indicated that the suppressor functions of *miR-634*, *miR-638*, and *miR-3960* are reliant on *NEAT1* expression. However, increased apoptosis by *miR-634*, *miR-638*, and *miR-3960* mimics might have no association with *NEAT1* expression, as transfection of these miRNAs the increased apoptosis rate and there is no difference in the extent of increase between *NEAT1*-overexpressing and *NEAT1*-knockdown HCC cells (*Figure 6*). Bioinformatics data will not be used in clinic as clinicopathological and prognostic biomarker directly. We need to screen out potential data by cell lines and nude mice verification. In this study, we focused on *NEAT1*-miRNAs function. Our Bioinformatics data shown more than 100 miRNAs and target mRNAs change. However, not all miRNAs and target mRNAs can be biomarker. Thus, stability, function and relationship with *NEAT1* need to be verification. This study using exosomal miRNA to screen out potential miRNAs which might be stable. We also use miRNA mimics to verified function of exosomal miRNA to make sure these miRNAs as tumor suppressor gene. To explore if these miRNAs' functions are related with *NEAT1*, we used these changed exosomal miRNA mimics to transfer *NEAT1*-overexpressing HCC cells and *NEAT1*-knockdown HCC cells and found proliferation and invasion reversion phenomenon was not observed or relenting in *NEAT1*-knockdown HCC cells. It indicated function of *NEAT1* might be related with exosomal miRNA change. The same method will be used in target mRNA *in vivo*, vitro and clinical samples. Finally, clinicopathological and prognostic biomarker might be found combined these data. Taken together, the results of our study demonstrated that *NEAT1* promotes cell invasion and proliferation through downregulation of *miR-634*, *miR-638*, and *miR-3960* in exosomes.

Conclusions

This study indicates that lncRNA *NEAT1*-overexpression promotes proliferation and invasion, and inhibits apoptosis in HCC cells. Overexpression of *NEAT1* also promotes exosome secretion by HCC cells. This effect might be correlated with altered gene expression related to exosome synthesis and secretion, which is also regulated by *NEAT1*. Increasing *NEAT1* expression downregulated several suppressor miRNAs both in cells and exosomes secreted by HCC cells. Furthermore, *NEAT1* promoted cell proliferation and invasion via downregulation of *miR-634*, *miR-638*, and *miR-3960* in exosomes. The suppressor function of *miR-634*, *miR-638*, and *miR-3960* were found to be reliant on *NEAT1* expression.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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