Research article

Distinct serum exosomal miRNA profiles detected in acute and asymptomatic dengue infections: A community-based study in Baiyun District, Guangzhou

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ABSTRACT

Background: In recent years, research on exosomal miRNAs has provided new insights into exploring the mechanism of viral infection and disease prevention. This study aimed to investigate the serum exosomal miRNA expression profile of dengue-infected individuals through a community survey of dengue virus (DENV) infection.
Methods: A seroprevalence study of 1253 healthy persons was first conducted to ascertain the DENV infection status in Baiyun District, Guangzhou. A total of 18 serum samples, including 6 healthy controls (HC), 6 asymptomatic DENV infections (AsymptDI), and 6 confirmed dengue fever patients (AcuteDI), were collected for exosome isolation and then sRNA sequencing. Through bioinformatics analysis, we discovered distinct serum exosomal miRNA profiles among the different groups and identified differentially expressed miRNAs (DEMs). These findings were further validated by qRT-PCR.
Results: The community survey of DENV infection indicated that the DENV IgG antibody positivity rate among the population was 11.97 % in the study area, with asymptomatic infected individuals accounting for 93.06 % of the anti-DENV IgG positives. The age and Guangzhou household registration were associated with DENV IgG antibody positivity by logistic regression analysis. Distinct miRNA profiles were observed between healthy individuals and DENV infections. A total of 1854 miRNAs were identified in 18 serum exosome samples from the initial analysis of the sequencing data. Comparative analysis revealed 23 DEMs comprising 5 upregulated and 18 downregulated miRNAs in the DENV-infected group (mergedDI). In comparison to AcuteDI, 18 upregulated miRNAs were identified in AsymptDI. Moreover, functional enrichment of the

predicted target genes of DEMs indicated that these miRNAs were involved in biological processes and pathways related to cell adhesion, focal adhesion, endocytosis, and ECM-receptor interaction. Eight DEMs were validated by qRT-PCR.

Conclusion: The Baiyun District of Guangzhou exhibits a notable proportion of asymptomatic DENV infections as suggested in other research, highlighting the need for enhanced monitoring and screening of asymptomatic persons and the elderly. Differential miRNA expression among healthy, symptomatic and asymptomatic DENV-infected individuals suggests their potential as biomarkers for distinguishing DENV infection and offers new avenues of investigating the mechanisms underlying DENV asymptomatic infections.

1. Introduction

Dengue is an acute arbovirus infectious disease caused by dengue virus (DENV) primarily transmitted by Aedes mosquitoes [1,2]. Prevalent in tropical and subtropical areas, more than half of the global population is at risk of DENV infection [3]. The manifestation of DENV infection ranges from asymptomatic, mild dengue fever (DF), even to severe dengue hemorrhagic fever (DHF) accompanied by life-threatening dengue shock syndrome (DSS) with a mortality rate of up to 5 % [4,5]. It is estimated about 25,000 human deaths out of 390 million dengue infections around the world each year, of which approximately 300 million are asymptomatic [6]. Though it does not appear to threaten the health condition of asymptomatic patients, the initial infection increases the risk of severe disease if the person contracts the virus again which may trigger the abnormal host immune response through antibody-dependent enhancement (ADE). Moreover, asymptomatic patients can spread the virus unknowingly, contributing to over 84 % of dengue transmission [7]. Since the first dengue outbreak in 1978 in Foshan City, Guangdong Province, China, cases have been reported every year in south-eastern coastal areas [8]. In 2014, Guangdong Province reported a record high of 45,230 dengue patients, with more than 80 % of the cases in Guangzhou City, the capital of the province, which has been considered the epicenter of DF in mainland China since then [9]. Baiyun District, situated within Guangzhou City, harbors the largest resident population and encompasses the most extensive area within the central city, posing a high risk for DENV infection.

As one primary type of extracellular vesicles (EVs), exosomes are cell-derived lipid bilayer-coated nanoparticles originating from the endosomal system and plasma membrane. Exosomes carry a variety of proteins, nucleic acids, and lipids, and their content reflects the state of the host cell to some extent [10]. Acting important roles for intercellular communication, exosomes transmit signals and substances from cell to cell [11]. Interestingly, microRNAs (miRNAs), small non-coding RNAs of approximately 19–22 nucleotides in length, can be secreted actively through exosomes that protect them from degradation by RNases [12]. Accumulating evidence concerning exosomal miRNAs has revealed their crucial functions in human health and potential application over the past few years. Clinically, it has been demonstrated that exosomal miRNAs have the potential to serve as innovative therapeutic targets and biomarkers in the treatment of human diseases, such as cancers, cardiovascular diseases, and metabolic diseases [13–16]. Moreover, exosomal miRNAs have been found associated with the regulation of multiple signaling pathways during the progression of many infectious diseases, including pneumonia, viral hepatitis, and dengue [17–19]. Collectively, research on exosomal miRNA provides new insight into viral pathogenesis including DENV, however, additional research is required to further enhance our understanding and seek alternative treatments for DENV. With increasing evidence suggesting the role that exosome-carried RNA molecules play during the viral infection, we hypothesized that the expression profile of plasma exosome microRNAs could impact the outcome of DENV infection.

In our study, we first investigated the epidemiology of DENV infection among citizen individuals in Baiyun District of Guangzhou to understand seroprevalence in the community. With the serum samples collected, we specifically investigated the role of exosome and its containing sRNA play during the infection. Aiming to identify specific exosomal miRNA biomarkers for DENV infection and to explore the protective mechanism in asymptomatic, we examined and compared the miRNA expression profiles carried by serum exosomes among acute and asymptomatic DENV-infected individuals and healthy controls. We believe our results shed light on new approaches for the prevention and treatment of DENV infection.

2. Methods

2.1. Enrolled subjects

Baiyun District has the largest population and the highest number of historical dengue infections in 2019 among the eleven urban districts in Guangzhou, China [20]. To assess the prevalence of DENV antibodies in the healthy population, a cross-sectional serosurvey was conducted in Baiyun District in 2019 with the assistance of the local CDC using a stratified random sampling method (Fig. S1). Ethical approval for this study was granted by the Ethics Review Committee for Medical Research of the School of Public Health, Sun Yat-Sen University (SYSU). The study participants were provided with information and signed written informed consent documents. To obtain an appropriate and representative number, the study sample size was calculated based on the average reported incidence of DF from 2013 to 2017, the incidence of DF in 2018, and the results of the 2015 survey on the status of DENV antibodies in the Guangzhou population [21]. Recruited subjects require at least one year of residence in Baiyun District. The demographic information and informed consent were obtained by the in-person survey conducted by two CDC staff members. Data entry was conducted utilizing

EpiData 3.1 (Available from http://epidata.dk/download.php) and crosschecked by two researchers. Descriptive and inferential statistical analyses were carried out using SPSS software (Version 20.0. Armonk, NY: IBM Corp.). Spatial analysis of antibody positivity was performed using ESRI ArcMap software (Version 10.8.1. ESRI, Redlands, California, U.S.A.), and graphing was conducted using GraphPad Prism software (Version 8.0.0, San Diego, California USA, www.graphpad.com). In the influence factor analysis stage, univariate and multivariate analyses were conducted using chi-square analysis and binary logistic regression, respectively. The antibody results were the dependent variable, and sex, age, type and years of residence, and mosquito control behavior were independent variables. The Odds Ratio (*OR*) and its 95 % confidence interval (95 % *CI*) were calculated. All subject data were de-identified and analyzed anonymously.

2.2. Seroprevalence test to determine the groups

The serum specimens collected from healthy individuals were collected in 3–5 mL whole blood (ordinary dry non-anticoagulated tubes), clotted at room temperature for 30 min, and then centrifuged at 23000 rpm for 5 min in the community health service center according to the guidelines issued by the Chinese Center for Disease Control and Prevention. Serums were divided into centrifuge tubes and stored frozen at $-20\,^{\circ}$C in the refrigerator. Specimens were sent to the laboratory located in SYSU within a week and moved to $-80\,^{\circ}$C freezers for storage. The serum specimens of acute DENV infection were collected from diagnosed acute dengue patients in the Third Affiliation Hospital of SYSU. After exclusion, the qualified serum samples (n = 1253) were first subjected to the indirect commercial DENV IgG enzyme-linked immunosorbent assay (ELISA) kit (Abcam, USA) to detect the DENV-specific IgG antibody according to the manufacturer's instruction. In addition, a subset of serums (n = 340), proportionally distributed among different streets to the total number, were subjected to the commercial DENV IgM ELISA kit (Abcam, USA). Screened IgG or IgM-positive samples were further tested with ELISA again to ensure reproducibility. The participants were informed of the testing results by telephone and written report. For those individuals with positive results, additional questions were asked about the history of dengue infection or probable symptoms in the past six months.

Six asymptomatic infections (AsymptDI) and six healthy controls (HC) were selected based on seroprevalence results from both IgG and IgM tests. The inclusion criteria for the AsymptDI group were positive for dengue IgM antibodies by ELISA and no history of DF or dengue-related symptoms, such as fever, rash, and muscle aches, within the past six months. Accurate detection of asymptomatic infections requires a synthesis of laboratory diagnostics and clinical presentations, while the term "AsymptDI" here was to denote participants with "most likely" asymptomatic infections, and it is probably the best approach we could have in the current study. The inclusion criteria for the HC group were no history of DF, negative for DENV by ELISA, and no relevant symptoms within the past six months. In addition, serum samples from six hospitalized patients with acute DENV infection (AcuteDI) were collected for comparison. The inclusion criteria for the AcuteDI group were patients with clinically relevant symptoms who had been diagnosed with DF by medical institutions. Individuals with various types of tumors, neurological diseases, immune-related diseases, diabetes, hypertension, psychiatric diseases, infectious diseases, and other diseases that have been shown to affect or may affect exosomal miRNA expression were excluded from the selection. The age and sex of the samples were matched among the three groups (Table S1).

2.3. Exosome isolation and characterization

The obtained serum samples underwent exosome isolation using the exoEasy Maxi kit (Qiagen, cat. No. 76064) following the manufacturer's instructions. Subsequently, validation and identification experiments were executed to ensure assay reliability and reproducibility. The morphology of isolated exosomes was determined by transmission electron microscopy (TEM, HT7700, 100 kV, Hitachi, Tokyo, Japan) and exosome size distribution was identified using nanoparticle tracking analysis (NTA, N30E, NanoFCM Inc., Xiamen, China) according to the manufacturer's software manual. The exosome pellet was dissolved in protein lysis buffer, and exosomal surface marker protein expression of TSG101 and CD9 was detected using western blotting. The specific protein bands were visualized using the chemiluminescence gel imaging system (ChemiScope 3000mini, Clinx Science Instrument Co., Ltd., Shanghai, China) and imaged by autoradiography.

2.4. sRNA library preparation

Following the manufacturer's protocol, miRNA-enriched total RNA was extracted from the exosomal product obtained in the preceding step, utilizing the miRNeasy Serum/Plasma Kit (Qiagen, cat. No. 217184). The resultant RNA was dissolved in 20 μl of RNase-free water. To prepare the small RNA (sRNA) library, the quality and quantity of total RNA were assessed by the Agilent Bioanalyzer 2100 System (Agilent Technologies, CA, USA). Subsequently, approximately 1 μg of total RNA (with a qualified RIN number >7.0) processed to generate a small RNA library employing the TruSeq Small RNA Sample Prep Kits (Illumina, San Diego, CA, USA) by the manufacturer's guidelines. Purified cDNA library products with a size of 50 base pairs (bp) were subjected to single-end sequencing utilizing the Illumina Hiseq2500.

2.5. Bioinformatics analysis

The raw Fastq files were demultiplexed and subsequently subjected to adapter trimming using Cutadapt. Quality reports were generated post-trimming using FastQC. Further refinement involved the elimination of low-quality reads by discarding sequences smaller than 15 nucleotides or longer than 35 nucleotides using Prinseq. These filtered reads underwent alignment with databases

including Silva, GtRNAdb, Rfam, and Repbase successively, employing the Bowtie2 aligner. This sequential alignment was conducted to exclude ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), other non-coding RNA (ncRNA), and repeats. The resulting reads were utilized for the identification of known miRNAs and the prediction of novel miRNAs by comparison with the human genome and miRNAs from miRBase. Secondary structure prediction for novel miRNAs was performed using the Randfold tool. Estimation of miRNA expression levels involved aligning sRNAs back onto precursor sequences and obtaining read counts for each miRNA from the alignment results. Raw read counts were normalized to transcripts per million (TPM) across all samples and employed for pairwise differential expression analysis through EdgeR. Significantly differentially expressed miRNAs (DEMs) were identified based on log2 |foldchange| >1 with a false discovery rate (FDR) or P-value <0.05. To elucidate the potential functions of exosomal miRNAs, TargetScan was employed for gene target prediction with the DEMs. Furthermore, Gene Ontology (GO; www.geneontology.org) enrichment analysis of DEMs was performed using the GOseq R packages based on Wallenius non-central hyper-geometric distribution. The Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome. jp/kegg) analysis was applied to delineate potentially altered molecular pathways. GO terms and KEGG pathways were considered significant at $P < 0.05$.

2.6. Validation of real-time quantitative PCR

To evaluate the identified candidate miRNAs from the differential expression analysis, quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the EzOmicsTM One-Step qPCR Kit (Biomics, China) on the ABI ViiATM7Dx Real-Time PCR System (Life Technologies, USA). Following the manufacturer's instructions, each reaction was conducted in a 25 μl reaction volume containing 0.1–100 ng of RNA, 0.5 μl of each primer, 12.5 μl of SYBR Green OneStep Mix (2 ×), and 0.5 μl of RT/Taq Mix (50 ×). RNase-free water was added to achieve a total RNA volume of 25 μl. The PCR protocol commenced with reverse transcription at 40 °C for 60 min, followed by an initial denaturation step at 95 °C for 10 min. Subsequently, it involved 40 cycles, each consisting of denaturation at 95 °C for 20 s, followed by annealing at 62 °C and extension at 72 °C for 30 s. The U6 snRNA expression levels were employed as an endogenous control for normalization purposes. All assays were conducted in triplicate. miRNAs with cycle threshold values exceeding 35 were excluded from subsequent statistical analyses.

3. Result

3.1. Dengue seroprevalence and potentially associated factors

The investigation into dengue infection seroprevalence was conducted among healthy individuals in Baiyun District, Guangzhou City from March to May in 2019. To ensure a comprehensive representation, fifty individuals from each of the district's sub-divisions (Jiedao, n = 22) were recruited across different sex and age groups. Following the exclusion of low-quality samples and those lacking demographic information, a total of 1253 serum samples were subjected to this study. Among these, 150 individuals (11.97 %) tested positive for DENV IgG antibody and were informed by follow-up calls regarding the test results. Of the 144 IgG serum-positive individuals who could be reached during the follow-up, 10 (6.94 %) either had been diagnosed with dengue infection or reported fever-related symptoms within the past six months, while the remainder were unaware of recent infections. Additionally, Dengue IgM
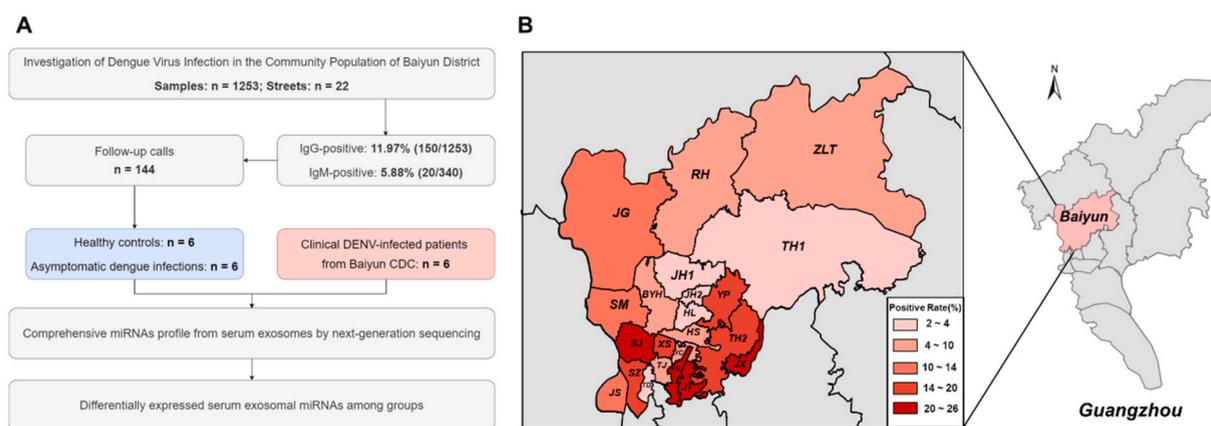

Fig. 1. Recruitment of study population and distribution of DENV IgG antibody positivity rate. (A) Outline of the investigation of DENV infection. (B) Spatial distribution of DENV IgG antibody positivity rate in Baiyun District, Guangzhou City. Different colors represent variations in the antibody positivity rate, with the highest rate observed in Shijing Street, reaching 26 %. Abbreviations: JG, Jianggao Town; RH, Renhe Town; ZLT, Zhongluotan Town; TH1, Taihe Town; SM, Shimen Street; BYH, Baiyunhu Street; JH1, Junhe Street; JH2, Jiahe Street; HL, Helong Street; YP, Yongping Street; SJ, Shijing Street; XS, Xinshi Street; HS, Huangshi Street; YC, Yuncheng Street; TH2, Tonghe Street; JX, Jingxi Street; JS, Jinsha Street; SZ, Songzhou Street; TD, Tongde Street; TJ, Tangjing Street; SYL, Sanyuanli Street; JT, Jingtai Street. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

antibodies were tested in 340 serum samples, where 20 (5.88 %) were found positive (Fig. 1A). As depicted in Fig. 1B, all 22 sub-districts in Baiyun District exhibited IgG-positive cases, with Shijing, Jingtai, Sanyuanli, and Jingxi streets recording the highest prevalence rates of 26.00 %, 24.00 %, 22.00 %, and 22.00 %, respectively. Conversely, Tongde, Jiahe, Junhe, Hailong streets, and Taihe town displayed lower prevalence rates of 4.00 %, 4.00 %, 4.00 %, 2.00 %, and 2.00 %, respectively. Regions with elevated IgG
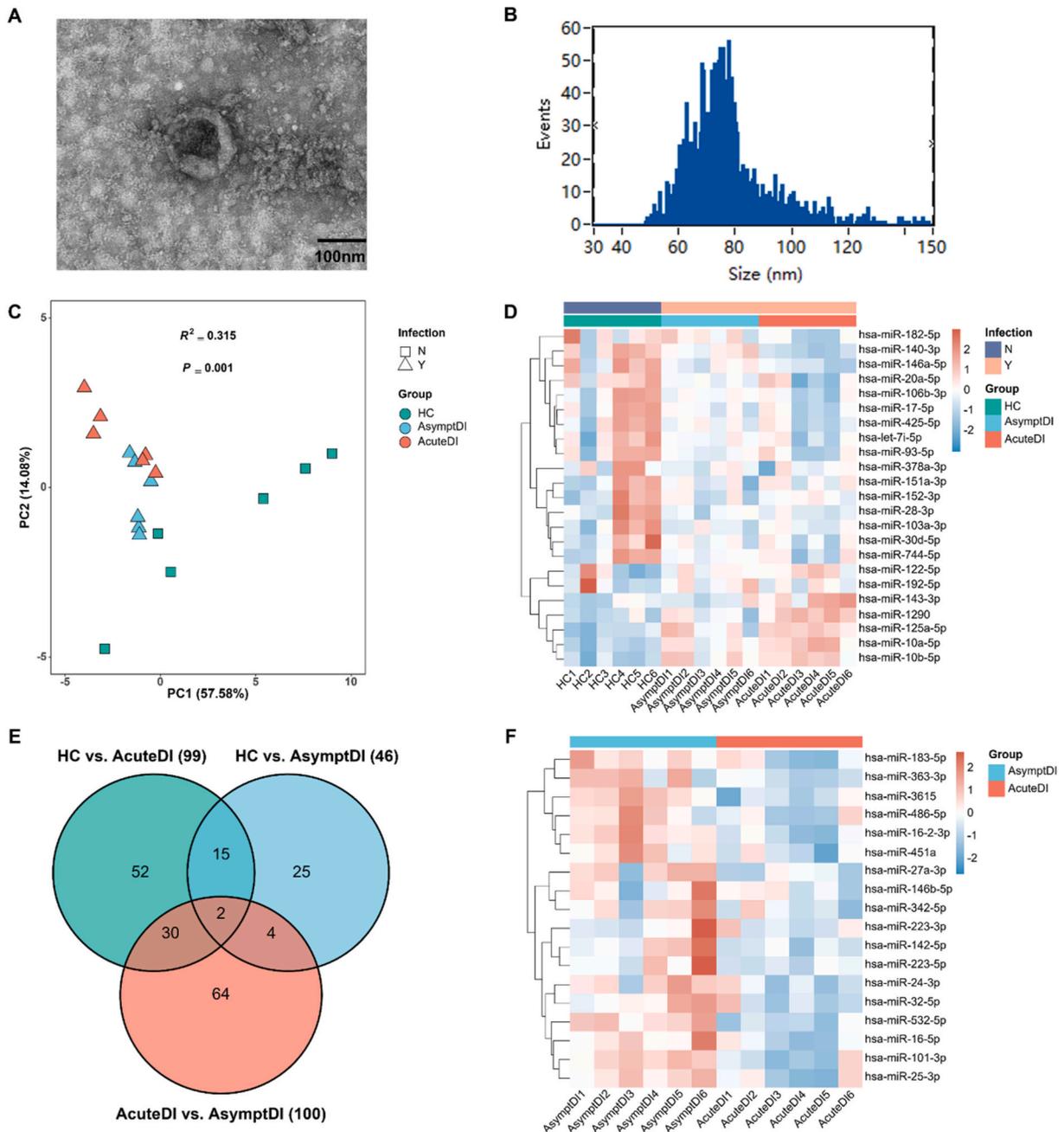

Fig. 2. miRNA profiling of DENV-infected patients and healthy controls. (A) Morphological characterization using transmission electron microscopy. (B) Physical characterization employing nanoparticle tracking analysis. (C) Principal Component Analysis (PCA). The triangle represents DENV infection, while the square represents healthy controls. Different colors signify the three distinct groups. The R-square and P-value were calculated using Adonis test. (D) Clustering of differentially expressed miRNAs (DEMs) among the three groups. The heatmap demonstrates the 23 miRNAs exhibiting differential expression in HC and mergedDI. (E) The Venn diagram showcases the amount of DEMs among HC, AcuteDI, and AsymptDI groups. Overlapping regions denote shared miRNA counts, while non-overlapping regions indicate uniquely expressed miRNA counts. (F) The heatmap illustrates the 18 miRNAs found to be differentially expressed in AcuteDI and AsymptDI. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

positivity rates were predominantly clustered in the southern area of Baiyun District.

Compared to a similar survey in 2015 (8.35 %, unpublished data by Baiyun CDC), the overall positive rate of IgG has increased by 43 %. Integrating the information collected from the questionnaire, we analyzed the odds of seroprevalence among different demographic factor groups, including age, gender, occupation, education level, residential area type, Guangzhou household registration status, mosquito prevention measures, etc. There was only a significant difference among different age groups in the distribution of both DENV IgG and IgM antibody positivity rates. Regression analysis showed that compared with the group age >60, the group 15–35 ($P = 0.005$) and 35–50 years old ($P = 0.066$) were less likely to be DENV IgG antibody-positive. Compared with those non-natives, Guangzhou natives were more likely to be detected as DENV IgG antibody-positive ($P = 0.042$). See details in Tables S2–5.

3.2. Exosomal sRNA sequencing and miRNA profiles

The completion of the DENV seroprevalence survey enabled us to differentiate healthy controls without dengue infection (IgG negative and IgM negative, HC) and inapparent individuals with asymptomatic DENV infection (IgG negative but IgM positive, AsymptDI). Along with another six serum samples collected from patients with acute DENV infection (AcuteDI), serum samples from six HC and AsymptDI individuals were subjected to exosome isolation in preparation for miRNA expression profile characterization. The morphology, size, concentration, and presence of envelope-enriched marker proteins of exosomes were examined to ensure the quality of the isolation procedure. TEM images confirmed that exosomes were transparent spherical particles with a diameter ranging from 45 to 150 nm (Fig. 2A). NTA results revealed that the mean particle size of the measured samples was 77.43 nm and a concentration of 2.5x10^9 particles/ml, which falls within the range of 10^7 to 10^11 particles/ml (Fig. 2B). Western blotting analysis showed that exosomal protein markers may lack distinct bands due to factors such as low protein concentration or antibody immunotyping (Fig. S2).

Exosomes isolated from these 18 serum samples were subsequentially prepared for RNA extraction and Illumina sRNA Sequencing. Each sample yielded 15.1 ± 2.7 (mean ± sd) million raw reads and 11.9 ± 1.8 million reads after quality filtration (Table S6). By comparing the paired sequences with the Homo sapiens reference genome and miRBase (v22) database, 1854 known miRNAs were identified. Length distribution analyses showed that the majority of small RNAs ranged from 21 to 24 nt in length (>85.3 %), and the
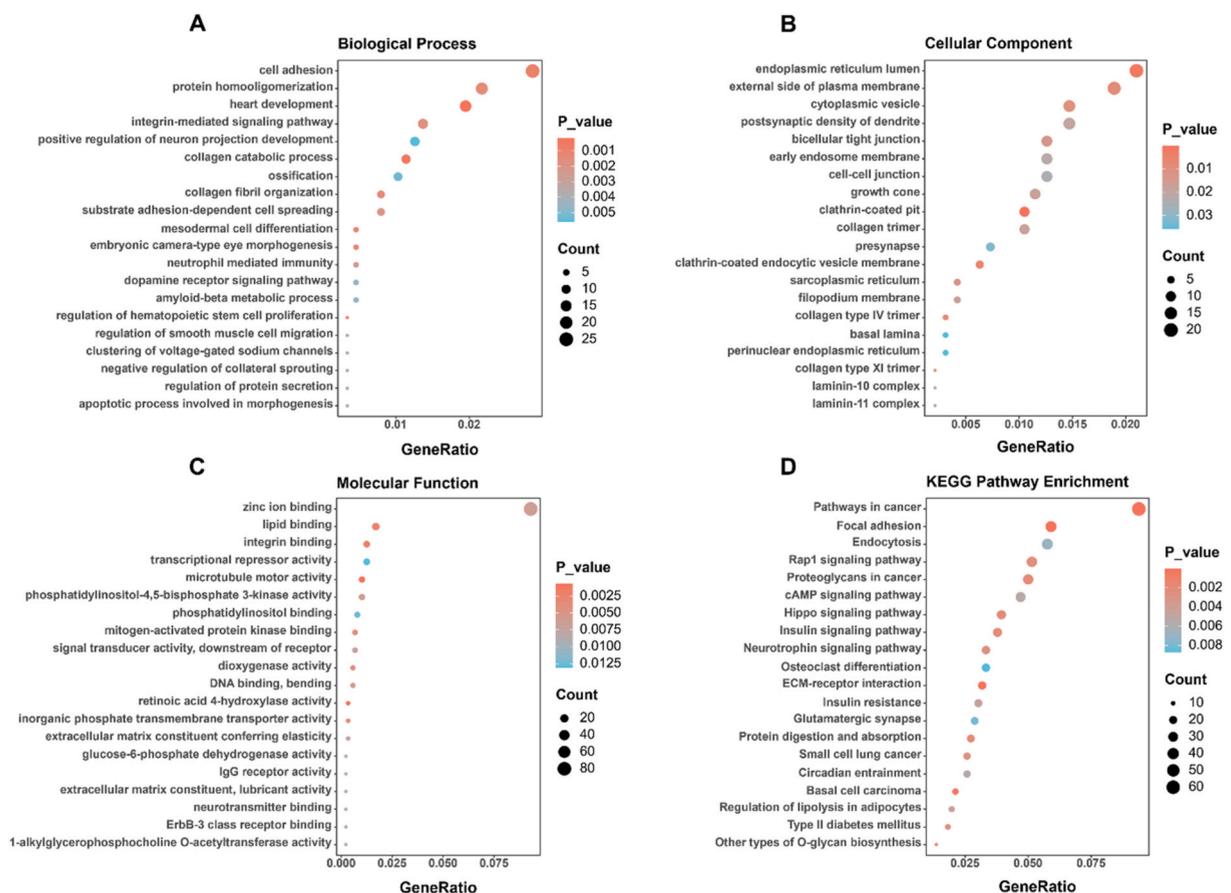

Fig. 3. The GO and KEGG enrichment analysis interpretation for the functions of the mergedDI and HC groups. (A–C) The GO terms interpretation for the functions of these 23 DEMs. (D) The KEGG terms interpretation for the functions of these 23 DEMs.

small RNAs with 22 nt were the most abundant (>47.6 %), indicating reliable miRNA data quality.

To examine the exosomal miRNA signature in the DENV infection, the AsymptDI and AcuteDI were combined into the DENV infection group (mergedDI) to present DENV patients. Among these differential miRNAs, only those with homogenized expression (TPM value) greater than or equal to 100 were selected as candidate miRNAs for subsequent analysis. The principal component analysis (PCA) plot revealed distinct segregation between HC and mergedDI groups based on the expression patterns of the screened miRNAs (Fig. 2C). As depicted by the hierarchical clustering, a total of 115 serum exosome-derived miRNAs were detected, of which 23 miRNAs fulfilled the screening criteria. Among the 23 DEMs, 5 were upregulated and 18 were downregulated in the mergedDI group compared with the HC group (Fig. 2D), with hsa-miR-122-5p and hsa-miR-1290 exhibiting the largest differential multiplicity, displaying log2 FC of −3.316 and 3.397, respectively (Table S7).

Further exploration of distinct exosomal miRNA signatures among varied groups holds considerable significance. As indicated in the Venn diagram (Fig. 2E) and supplementary tables (Tables S8–10), a pairwise analysis was performed among HC, AcuteDI, and AsymptDI. The analysis revealed 52 miRNAs with significantly different expression in HC vs. AcuteDI, 25 miRNAs differentially expressed in HC vs. AsymptDI, and 64 miRNAs differentially expressed in AcuteDI vs. AsymptDI. Following the exclusion of common differential miRNAs with TPM values lower than 100, we identified 2 up-regulated miRNAs and 8 down-regulated miRNAs in AcuteDI samples compared to HC. Notably, only hsa-miR-122-5p was identified as a down-regulated miRNA in AsymptDI samples compared to HC. Furthermore, all 18 identified miRNAs were found to be up-regulated in AsymptDI samples in contrast to AcuteDI (Fig. 2F).

3.3. Functional analysis with GO and KEGG

GO and KEGG enrichment analyses were performed to explore the function of these miRNAs. In the comparison between mergedDI and HC, as seen in Fig. 3A, the biological processes (BP) indicated that miRNAs were mainly associated with cell adhesion, collagen catabolic processes, heart development, mesodermal cell differentiation, and the regulation of hematopoietic stem cell proliferation. As seen in Fig. 3B, the cellular components (CC) showed that these miRNAs were found in the clathrin-coated pit, endoplasmic reticulum lumen, clathrin-coated endocytic vesicle membrane, collagen type IV trimer, and external side of the plasma membrane. In addition, the molecular functions (MF) suggested that these miRNAs regulate lipid binding, zinc ion binding, integrin binding, microtubule motor activity, and inorganic phosphate transmembrane transporter activity (Fig. 3C). Moreover, the KEGG indicated that these miRNAs participate in the extracellular matrix (ECM)-receptor interaction, focal adhesion, endocytosis, neurotrophin signaling pathway, and pathways in cancer (Fig. 3D).

In the comparison between AcuteDI and AsymptDI, the BP indicated that miRNAs were mainly associated with synapse assembly, receptor clustering, positive regulation of GTPase activity, negative regulation of telomerase activity, and hematopoietic progenitor cell differentiation (Fig. S3A). The CC showed that these miRNAs were found in the axon, collagen trimer, dendrite, sarcomere, and cell junction (Fig. S3B). In addition, the MF suggested that these miRNAs regulate ATP binding, Rac GTPase binding, Rho guanyl-nucleotide exchange factor activity, protein binding and bridging, microtubule motor activity, and N-acetylglucosamine 6-O-sulfo-transferase activity (Fig. S3C). Moreover, the KEGG indicated that these miRNAs participate in the mitogen-activated protein kinase
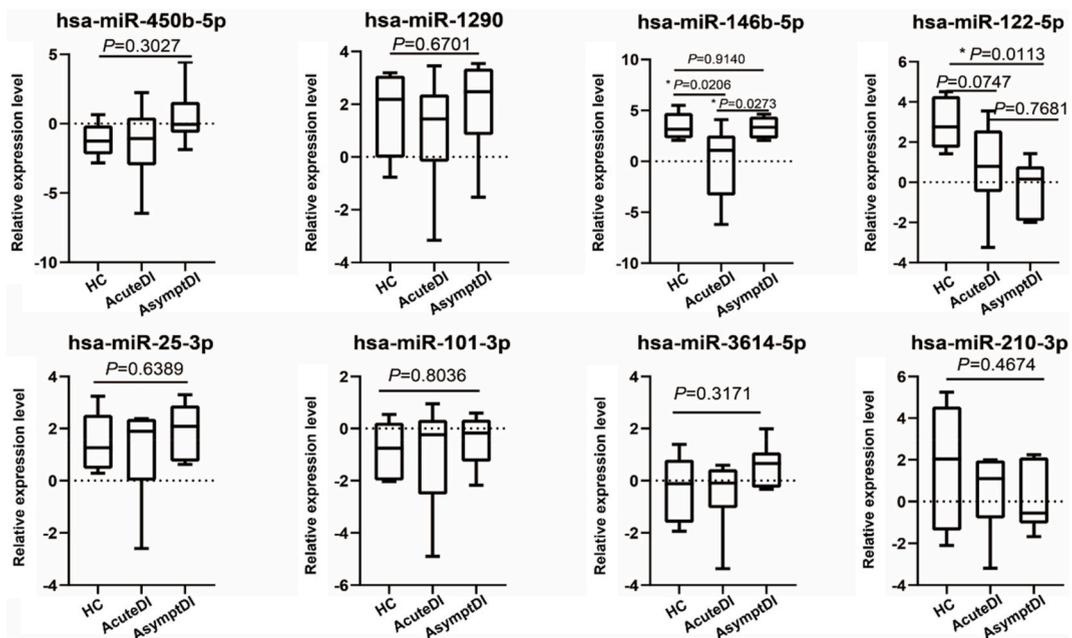

Fig. 4. Expression levels of DEMs in qRT-PCR assay. Only the hsa-miR-146b-5p and hsa-miR-122-5p expression levels were significantly different between AcuteDI or AsymptDI and HC.

(MAPK) signaling pathway, focal adhesion, axon guidance, ECM-receptor interaction, and the Notch signaling pathway (Fig. S3D)

3.4. qRT-PCR verification of miRNA expression

To validate the sRNA sequencing results, eight highly DEMs, hsa-miR-450b-5p, hsa-miR-1290, hsa-miR-146b-5p, hsa-miR-122-5p, hsa-miR-25-3p, hsa-miR-101-3p, hsa-miR-3614-5p, and hsa-miR-210-3p, were selected for qRT-PCR validation using the aforementioned 18 exosome samples of sequencing. All the tested miRNAs passed the quality control assessment. The findings demonstrated that the expression levels of hsa-miR-146b-5p and hsa-miR-122-5p were significantly different, aligning with the sequencing results between AcuteDI or AsymptDI and HC. Without showing statistical significance, qRT-PCR results of hsa-miR-450b-5p, hsa-miR-25-3p, hsa-miR-101-3p, hsa-miR-3614-5p, and hsa-miR-210-3p exhibited alterations in the same direction as observed in the sequencing analysis, whereas the expression level of hsa-miR-1290 contradicted the sequencing findings (Fig. 4).

4. Discussion

4.1. Overview of dengue infection in Baiyun District, Guangzhou City

Baiyun District is the most densely populated district situated in the north-central region of Guangzhou. The extensive urban village areas and proximity to the International Baiyun Airport heighten the risk of imported cases of dengue fever within the district. This study is the first time to conduct a survey on the current situation of DENV infection based on all towns and streets in Baiyun District to understand the current situation of asymptomatic infection, which is highly representative. The survey results show an overall DENV IgG antibody positivity rate of 11.97 % in Baiyun District, Guangzhou City. Although the dengue infection rate is relatively lower compared to countries with high IgG antibody positivity rates (>50 %) such as India, Sri Lanka, Indonesia, and Singapore, these tropical countries have previously experienced severe local outbreaks [22–24]. Notably, in 2019, a total of 1655 confirmed cases of dengue fever were reported in Guangzhou, with an incidence rate of 11.10 per 100,000 people. The district with the highest number of confirmed cases was Baiyun, accounting for 356 cases, which represents 21.51 % of the total confirmed cases [20]. As Guangzhou is the third-largest city in China, frequent international trade activities and connections with Southeast Asian countries increase the chances of DENV infection among the residents. Therefore, with the further expansion of Guangzhou's opening to the outside world, it is important to prevent the local transmission of DF. In this study, 93.06 % of individuals with antibody positivity are considered to have asymptomatic DENV infection, accounting for 11.06 % of the total survey population. For many pathogens, asymptomatic infections may represent a significant proportion of the infection reservoir and largely contribute to pathogen transmission [25–27]. Considering the high infection rate of asymptomatic DENV carriers in the population, enhancing surveillance is crucial as an important DF intervention measure to reduce the chances of silent transmission.

Furthermore, the analysis of factors influencing DF infections unveils noteworthy insights. Comparatively, Guangzhou household registration residents exhibit a 1.514-fold higher risk of infection in contrast to non-Guangzhou residents. Moreover, the 15–35 and 35–60 age groups display reduced risks of infection by 0.444 and 0.597 times, respectively, when compared to the age group above 60. This suggests that aging and Guangzhou household registration are potential risk factors for DENV infection. This is because the areas with high rates of antibody positivity are located in the southern part of Baiyun District, where there are relatively more old residential areas with high population density and poor living conditions, serving as a breeding ground for mosquitoes. Additionally, most residents in these areas are local elderly people who are accustomed to growing flowers or aquatic plants in pots or courtyards, also providing suitable breeding conditions for mosquitoes [28]. In addition, in the season of vigorous mosquito breeding, elderly individuals spend more time outdoors, particularly in parks and markets during mornings and evenings, which coincides with the peak activity of Aedes albopictus [29]. Coupled with reduced immunity in the elderly compared to younger individuals, these conditions elevate the risk of viral infection from mosquito exposure. However, no significant difference in gender and other factors was observed between populations, which is consistent with previous studies [30,31]. Therefore, it is necessary to provide DF education for the elderly population, especially local elderly residents in Guangzhou, and to carry out mosquito control and monitoring in public places.

4.2. Analysis of differential miRNAs between infected and uninfected individuals

Currently, the hotspots at home and abroad mainly focus on severe dengue fever, while this study starts from asymptomatic infections and provides the first serum exosome miRNA expression profile of DENV clinically infected and asymptomatic infected patients based on serum exosome miRNA study, which tries to explore the biomarkers to identify DENV infection (including asymptomatic infection) and provides a reference for identifying DENV infected patients from the population. Through high-throughput sequencing analysis, this study has identified specific and highly expressed serum exosome differential miRNAs in DENV-infected individuals compared to healthy controls, including hsa-miR-122-5p, hsa-miR-1290, hsa-miR-146a-5p, hsa-miR-152-3p, hsa-miR-378a-3p, hsa-miR-106b-3p, hsa-miR-10a-5p, and hsa-miR-10b-5p, which are related to dengue infection.

DENV infection can affect multiple organs, with the liver being the most commonly affected. miR-122, a liver-specific homeostatic regulator, constitutes 72 % of total miRNAs in the human liver [32]. In a study by Oliveira et al. [33], a comparative examination of miRNA expression profiles in the liver tissues of DHF fatalities and healthy controls revealed differential expression of miR-122-5p, which is associated with DHF liver pathogenesis. Concurrently, research by Othumpangat et al. [34] also indicated the down-regulation of serum exosome miR-122-5p expression in influenza A patients. In this study, has-miR-122-5p is downregulated in DENV-infected individuals, which is consistent with the research by Juan Teng and colleagues [35]. This evidence suggests that

hsa-miR-122-5p may be a potential biomarker for DENV infection.

Furthermore, in this study, there was an observed upregulation of hsa-miR-1290, hsa-miR-10a-5p, and hsa-miR-10b-5p, as well as a downregulation of hsa-miR-146a-5p, hsa-miR-378a-3p, and hsa-miR-106b-3p, consistent with previous studies [35–39]. However, the expression of hsa-miR-152-3p contradicted the findings of the study [38], possibly due to variations in the range of DENV-infected individuals, warranting further investigation. Although there is no specific biological function attributed to these miRNAs in the pathological process of DENV infection, this study provides clues for the selection of biomarkers. Further research is needed to determine whether these biomarkers can be used as predictive indicators for dengue infection and to gain a deeper understanding of the role of these miRNAs in the pathogenesis of DENV infection.

Although the aforementioned studies focused on miRNAs directly present in serum, while this study focuses on serum exosome miRNA, their functions are consistent. On the one hand, exosomal miRNAs, due to their membrane encapsulation, can escape digestion by nucleases in the blood and possess high stability. On the other hand, because some miRNAs are enriched in exosomes, the detection rate of low-abundance miRNAs is higher, making exosomal miRNA a more advantageous biomarker [40]. In addition, the aforementioned studies mainly focused on clinical and severe dengue-infected individuals, while this study includes asymptomatic infected individuals. Currently, some research has also focused on asymptomatic infection or latent infection issues. Lyu et al. explored the serum exosomal miRNA that can distinguish latent and active pulmonary tuberculosis patients from the population [41]. Chen et al. also demonstrated that miR-122 may be a novel biomarker for occult HBV infection [42]. This indicates that the application scope of diagnostic biomarkers can not only be used for clinical diagnosis but also for case screening in the field of public health to detect hidden sources of infection early on. Moreover, to date, no direct research evidence has been established to associate DENV infection with the onset of cancer. The appearance of cancer-related pathways in our KEGG analysis may be attributed to the extensive role of miRNAs in the regulation of gene expression and cellular functions within the cell. During the pathogenesis of dengue fever, certain miRNAs may be upregulated or downregulated, resulting in the modulation of pathways that are also implicated in cancer.

4.3. Potential protection mechanism of asymptomatic DENV infection based on DEMs in AcuteDI vs. AsymptDI

This study investigates the protective mechanisms of asymptomatic infections by exploring DEMs between AcuteDI and AsymptDI, providing new insights for DF prevention and treatment. Hsa-miR-101, a well-known miRNA, is involved in various cellular processes such as cell proliferation, tumor initiation and progression, megakaryocyte differentiation, neurodegenerative disorders, and auto-immune diseases [43–45]. Expression of miR-101 has been reported to downregulate VE-cadherin, which has a positive effect on the establishment and maintenance of endothelial barrier integrity, and its downregulation leads to increased endothelial permeability [46]. Vascular endothelial hyperpermeability is a recognized pathogenic feature of DF, leading to plasma leakage and severe outcomes in certain cases [47]. Contrary to expectations, our study found an upregulation of hsa-miR-101-3p in asymptomatic infections, warranting further investigation. Research has shown that hsa-miR-101 exhibits antiviral effects against Herpes Simplex Virus (HSV) and Influenza A Virus (IAV) infection [48–50]. Moreover, it has been reported that miR-101-3p inhibits the production of inflammatory cytokines in Treponema pallidum (TP) infection, which is relevant to DF severity [51]. These findings suggest that miR-101-3p may have implications for antiviral and anti-inflammatory effects in asymptomatic DENV-infected individuals, eliciting adequate inflammatory responses to clear the virus without clinical symptoms.

MiR-146b-5p has been shown to play a vital role in regulating the NF-κB signaling pathway during viral infections, which is crucial for innate and adaptive immunity and inflammation [52]. Wu et al. have identified the role of miR-223 in regulating DENV-2 replication by negatively regulating the microtubule-destabilizing protein STMN1 gene [53]. Additionally, Pham et al. have proposed that miR-142, one of the most abundant specific miRNAs in hematopoietic stem cells, inhibits the systemic spread of the virus by restricting DENV-2 replication to hematopoietic cells [54]. These findings are consistent with our study results, where hsa-miR-146b-3p, has-miR-223-3p, has-miR-223-5p, and has-miR-142-5p were found to be upregulated in asymptomatic infections compared to clinical patients, indicating a potentially stronger viral inhibitory effect in asymptomatic individuals.

Studies have demonstrated that miR-183-5p promotes the stability and integrity of the vascular endothelial barrier during DENV infection by interacting with ERG-associated lncRNA (ERGAL) [55]. MiR-27 targets and regulates the expression of various tight junction proteins, leading to vascular dysfunction and plasma leakage [19]. In addition, hsa-miR-25-3p and hsa-miR-142-5p respectively target KLF2/KLF4 and RAB11/FIP2 to regulate endothelial function [56,57]. These findings illustrate the multifaceted role of endothelial regulation factors in balancing vascular endothelial permeability. In our study, the upregulation of these miRNAs in asymptomatic individuals may contribute to protecting the stability of the vascular endothelium. Furthermore, GO term and KEGG pathway annotations further support the potential functions of miRNAs, reinforcing the reliability of our study results compared to other dengue-related research [58–61].

Nevertheless, the present study has some limitations. Firstly, the serological investigation used the ELISA method, which could lead to cross-reactivity of DENV antibodies with other flaviviruses like Japanese encephalitis, yellow fever, chikungunya fever, and Zika. However, the study considered positive serum antibodies as a more accurate reflection of DENV prevalence in the community, given the rarity of other flavivirus cases and minimal imported cases in the region, along with the low vaccination rates against yellow fever or DENV. Secondly, in asymptomatic infection samples used for high-throughput sequencing, only IgM antibodies were detected, and viral RNA was not identified. This could be attributed to the extended storage of serum samples, making it challenging to determine the infection stage. It's important to note that some clinical patients also test negative for nucleic acid, and diagnosis relies on a combination of factors. Research has demonstrated that the presence or absence of clinical symptoms does not strongly correlate with viral load [59], and others have used subjects with detectable IgM antibodies and no clinical manifestations as samples in gene expression profiling studies of asymptomatic infections [62]. Thirdly, obtaining enough symptomatic DENV infection samples from hospitals is

not straightforward, and successfully isolating exosomal miRNAs is inherently challenging, leading to a constrained sample size for the serum exosomal miRNA expression profile study and the samples available for qRT-PCR validation. Furthermore, the symptomatic cases in our study did not progress to severe dengue, limiting our ability to investigate the impact of infection severity on serum exosomal miRNA expression. Finally, a discrepancy was observed in the expression level of one miRNA between sequencing and qRT-PCR results. Such inconsistencies are normal as the two techniques can yield divergent outcomes [63].

5. Conclusion

Based on the DENV seroprevalence survey in the Baiyun District of Guangzhou City, our study presents the first depiction of serum exosomal miRNA profiles in acute and asymptomatic DENV infections. This notable proportion of asymptomatic DENV infections highlighted the need for enhanced monitoring and screening of asymptomatic persons and the elderly. There are differences in the expression of miRNAs in serum exosomes between healthy individuals and DENV-infected individuals (symptomatic and asymptomatic), and these differentially expressed miRNAs are involved in biological pathways and functions that can impact the body's response to infection. This study provides a reference for screening potential biomarkers for dengue fever infection and offers a new perspective for exploring the mechanism of asymptomatic infections.

Ethical approval

The study protocol was approved by the Ethical Review Committee for Biomedical Research, School of Public Health, Sun Yat-sen University (Approval number: SYSU-PHME-2019078) and all study participants gave their written informed consent to participate in this research.

Data availability

Serum exosome miRNA sequencing raw data were deposited in the NCBI BioProject (https://www.ncbi.nlm.nih.gov/bioproject) with BioProject accession no. PRJNA974807.

CRediT authorship contribution statement

Xiaokang Li: Writing – review & editing, Writing – original draft, Visualization, Software, Formal analysis, Data curation. **Conghui Liao:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Jiani Wu:** Writing – review & editing, Validation, Formal analysis, Data curation. **Boyang Yi:** Writing – review & editing, Visualization, Data curation. **Renyun Zha:** Writing – review & editing, Data curation. **Qiang Deng:** Validation, Formal analysis. **Jianhua Xu:** Writing – review & editing, Supervision, Resources, Investigation, Data curation. **Cheng Guo:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Jiahai Lu:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31546.

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