hsa-miR-124-3p: A New Potential Prognostic Target For Multiple Myeloma

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Abstract

The relationship of Exosomal RNAs released within the bone marrow microenvironment and Prognosis of patients with multiple myeloma has not been thoroughly studied. This study aims to evaluate which exosomal RNAs can prompt the prognosis of patients with multiple myeloma. Exosomes were isolated from the bone marrow fluid of patients with good treatment effect or not. Then they were characterized by dynamic light scattering, transmission electron microscopy, and Western blot analysis. The isolated exosomes stimulate PRMI8226 cells, observe the migration ability and proliferation ability of PRMI8226 cells. Then we performed exosome miRNA sequencing, performed bioinformatics analysis of differential miRNAs, and evaluated several miRNAs that may affect the treatment effect. To determine the actual value of these miRNAs in clinical applications, we collected a collection of clinical samples for verification. We found that bone marrow-derived exosomes from patients with different therapeutic effects have different effects on PRMI8226 cells. Exosomes with a poor prognosis can promote the proliferation and migration of PRMI8226 cells, while exosomes with a good prognosis have no impact on PRMI8226 cells. The miRNA sequencing results showed differentially expressed miRNAs in exosomes from different sources. These differential miRNAs can be enriched in pathways related to cancer development. Validation in clinical specimens found that exosomal mir-124-3p are highly expressed in patients with poor therapeutic effects. This study suggested exosomal miRNA plays a vital role in the development and treatment of multiple myeloma. Some miRNAs that can promote tumor progression are highly expressed in bone marrow exosomes of patients with poor therapeutic effects, such as hsa-miR-124-3p, hsa-miR-451b, hsa-miR-509-3p. Among them, hsa-miR-124-3 is promising as a predictor of therapeutic effect.

Introduction

Multiple myeloma (MM) is a hematological malignancy characterized by the clonal proliferation of plasma cells in the bone marrow microenvironment. [1, 2]The clinical and biological differences of this malignant tumor lead to different responses to treatment and prognosis. With the massive increase in the treatment of MM and the improvement of the results, it becomes more and more critical to analyze the impact of this difference on the treatment because it may lead to tailor-made therapies for different patient groups.[3] It is currently known that the progression of MM mainly depends on the microenvironment of the bone marrow (BM), which includes different factors, including extracellular vesicles. These factors can affect not only tumor progression but also affect the treatment effect of patients. [4, 5]

Exosomes are nano-sized vesicles (30–200 nm) secreted by various cells, whether normal or tumor cells. They are wrapped in lipid bilayers and carry multiple biological molecules, including proteins, glycans, lipids, metabolites, RNA, and DNA. [6]The exosome will affect the recipient cell,if they are taken up. It is considered to be a key mediator of cell-to-cell communication. Tumor-derived exosomes (TDE) are involved in forming and developing different cancer processes, including tumor microenvironment

(TME) angiogenesis, invasion, metastasis, and drug resistance. Exosomes transmit heterogeneous cargo to initiate or inhibit various signal transduction pathways in recipient cells. [7, 8]

MicroRNA is a type of 17–24 nt small non-coding RNA, which mediates post-transcriptional gene silencing by binding to the 3'-untranslated region (UTR) or open reading frame (ORF) of the target mRNA. The participation of miRNA in many biological activities has been fully demonstrated, including cell proliferation, cell differentiation, cell migration, disease initiation, and disease progression. More and more evidence shows that miRNA can be stably present in body fluids, including saliva, urine, breast milk, and blood. [9, 10] More and more evidence has shown that exosomes secreted by cells also contain a large number of miRNAs in recent years. Given the transportability of vesicles, the role of miRNA in exosomes has attracted more and more attention. [11] The search for biomarkers is of great significance to clinical patients. By detecting these markers, doctors can quickly and accurately determine the patient's condition and provide targeted treatment. Because of exosomes' protective effect and the readily available characteristics, exosomes' contents have broad prospects as biomarkers. The majority of scientific researchers have conducted in-depth research on it in various fields. Exosome's contents have been proven to indicate the occurrence and progress of tumors and patients' drug resistance by The existing research projects. As biomarkers, the RNA and protein in exosomes, especially miRNA, have always been a research hotspot because small RNA fragments are small, not easily degraded, and the expression abundance of long-chain RNA is relatively high. [12, 13]

In this study, we isolated exosomes from the bone marrow fluid of patients with different prognoses, evaluated their effects on PRMI8226 cells, and then sequenced the exosomal miRNA, performed pathway analysis and clinical sample verification. The purpose of this study is to explore potential biomarkers of exosomal miRNAs that predict the prognosis of patients.

Materials And Methods

Patients and Samples

During April 2019, bone marrow fluid was collected from 1 patient with multiple myeloma with good prognosis and 1 patient with poor prognosis of multiple myeloma, exosomes were isolated, and cell function experiments and sequencing were performed. During March 2020-2021, collect the bone marrow fluid of patients with good prognosis of multiple myeloma (n=10) and patients with good prognosis of multiple myeloma (n=10) to verify the selected potential biomarkers, from Each subject obtained written informed consent, and all aspects of the study were approved by the Ethics Committee of Shenzhen Luohu District People's Hospital. The research was conducted in accordance with relevant guidelines.

Isolation and Identification of Exosomes

The bone marrow's exosome is isolated by ultracentrifugation. The isolated process needs to be kept at four °C throughout the process. Western Blot (WB) analysis identified the isolated exosomes' surface

proteins, such as CD63 and TGS101. The size of exosomes is analyzed by nanoparticle tracking analysis. The exosomes' morphology is identified through transmission electron microscopy.

Migration Assays

Migration rate was measured by wound healing assay. The wound was made using a pipette tip, and pictures were taken immediately and 24h after wounding. The distance migrated by the cell was measured during the period.

Cell Proliferation

CCK8 is used to evaluate the effects of different sources of bone marrow exosomes on the proliferation of PRMI8226 cells. In short, PRMI8226 cells were seeded into a 96-well plate, the cell density was 5000 cells/well, and bone marrow exosomes containing different sources were added for stimulation. After 12, 24, 36, and 48 hours, each Add 10 μ L CCK8 solution to the wells and incubate the plate in an incubator for 1-4 hours. Then, use a microplate reader to measure the wells at 450 nm.

miRNA library sequencing and analysis

Total RNAs from exosomes was used for miRNA library preparation and sequencing. RNA-seq was conducted on an Illumina HiSeq 2500 platform. The quality of the sequencing data was analyzed by the bioinformatics pipeline. Briefly, read sequence quality checks were performed by FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Adapters from the 3' ends of reads were trimmed using Cutadapt [14] with a maximum allowed error rate of 0.1. Reads shorter than 17 nucleotides in length were excluded from further analysis. Reads were mapped to human hg19 using Bowtie version 1.1.1. [15] Then reads were annotated to human miRNAs from miRBase Release 21 [16] using miRDeep2 (version 2.007). [17] The number of reads in each miRNA was enumerated by miRDeep2, and differences in expression between groups were statistically assessed by DeSeq (version 1.16.0). [18] Differential expression miRNAs were determined based on log2 fold change (log2 fold change) and false discovery rate (FDR) with $|\log_2 \text{fold change}| \geq 1$ and $\text{FDR} \leq 0.05$. Pathway analysis were constructed in GO and KEGG.

qRT-PCR Analysis for Evolution and Validation of Candidate Serum Exosomal miRNA

The candidate miRNAs were further confirmed with quantitative real-time polymerase chain reaction (qRT-PCR). qRT-PCR was performed using the QuantiFast® SYBR® Green PCR Master Mix in an LightCycler® 96 qPCR system (Roche Life Science, USA). The relative gene expression values of the target miRNAs were normalized to that of cel-miR-39-3p (U6) and the differences were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method.²⁵

Results

The Characterization of Exosomes from the bone marrow

Identification of exosomes. Exosomes were isolated from the bone marrow of the patient and morphological characterization was observed by Transmission Electron Microscope, showed that exosomes were cup-like vesicles (Figure 1A). The surface protein markers (CD63-TSG101) were identified by Western blot analysis (Figure 1C). The isolated exosomes' size is between 128nm and 165nm. The poor prognosis' exosome is mainly concentrated in 165nm and 128nm, and the excellent prognosis' exosome is concentrated primarily in 165 nm (Figure 1B).

Different exosomes affect the biological function of PRMI8226 cells

We observed the effects of exosomes from different patients on PRMI8226 cells and found that the exosomes of patients with poor prognosis can significantly promote the proliferation and migration of PRMI8226 cells. The exosomes of patients with a good prognosis have no such effect. (Figure2.A-C)

miRNA expression profile

Exosomes have been demonstrated to contain both mRNAs and miRNAs, which can be delivered to other cells and affect cellular function. we compared the expression level of miRNAs in HXR-TDE and YRJ-TDF. Using a 2-fold change and $qvalue < 0.05$ as the threshold cutoff, we found 12 miRNAs were significantly up-regulated and 24 miRNAs were significantly down-regulated (Figure 3A). The differentiated expressed microRNAs were clustered (Figure 3B). To further investigate the internal mechanisms on exosomes miRNA, pathway analysis of target genes was performed. For the results, some up-regulated genes were most enriched in PI3K-Akt signaling pathway, Ras signaling pathway and Metabolic pathways (Figure 3C), while down-regulated genes enriched in MAPK signaling pathway, Transcriptional misregulation in cancer and so on (Figure 3D).

Selection and Verification Serum Exosomal miRNA as the Diagnostic Biomarker

Three miRNAs (miR-124-3p, miR-451b, miR-503-3p) with the most significant increase in expression were selected as Multiple myeloma biomarkers for miRNA verification. Among patients with different prognosis types, no significant differences were observed between miR-451b and miR-503-3p, while significant differences were observed for miR-124-3p. ($P = 0.025$).

Discussion

In clinical trials, the drug resistance of patients with multiple myeloma is still a problem. Some patients use the most conventional drugs to achieve sound therapeutic effects, and some patients use the most advanced drugs to be still unable to control their condition effectively. Suppose a diagnostic biomarker that can effectively predict the effect of the patient's treatment can be found. In that case, it will be helpful for the personalized treatment of patients and greatly help develop patient resistance, which can help researchers develop new drugs to avoid the drug resistance and improve the therapeutic effect.

Exosomes are one of the essential tools for mutual "communication" between cells. Many studies have shown that exosomes can be related to the occurrence, development and drug resistance of tumors. Luan

W et al. found that melanoma transfers mir-106b-5p to melanocytes by secreting exosomes, promoting the EMT, migration, invasion, and adhesion of melanocytes. Senlin Zhao et al. found that exosomes secreted by colorectal cancer cells can induce M2 macrophage polarization to promote liver metastasis of colorectal cancer. The bone marrow microenvironment can promote the survival and drug resistance of myeloma cells. Therefore, we studied the BM exosomes of MM and found that different patients' BM exosomes have different effects on the proliferation and migration of PRMI8226 cells.

microRNA is closely related to cancer. Mikhail K Ivanov et al. estimated the levels of 25 microRNAs and 12 mRNAs involved in cervical carcinogenesis in 327 air-dried Pap-stained cervical smears from patients with cervical precancerous lesions, cancer, or without the disease by real-time PCR and found that mir-124-2 and the methylation level of MAL promoter is related to the degree of cervical cancer. Farah Hady El Kilany and others proposed that miR-744 promotes breast cancer progression through miR-744/eNOS/NO axis. We performed miRNA sequencing on the bone marrow fluid-derived exosomes of patients with different multiple myeloma, and we found the expression of miRNA in BM exosomes in patients with different therapeutic effects has a difference. Through the enrichment of KEGG pathways, we found that these differential miRNAs are concentrated in tumor development pathways, such as transcription disorders and tumor microenvironment-related proteoglycans.

After verification, we found that mir-138-5p and mir-142-3p are significantly low-expressed in patients with poor prognoses. Mir-138-5p has been proven to be a tumor suppressor in lung cancer, glioblastoma, bladder cancer, and breast cancer. It can inhibit cell proliferation and invasion and slow down tumor progression, while mir-142-3p can target NUCKS1 to inhibit the proliferation and invasion of pancreatic cancer cells. It has been proven to target the ZFX gene to inhibit tumor progression in non-small cell lung cancer. Therefore, we believe that the expression of mir-138-5p and mir-142-3p in BM exosomes is closely related to patients' treatment effects.

In short, we found in this study that the bone marrow exosomes of patients with multiple myeloma are heterogeneous, affecting the proliferation and migration of cancer cells. After sequencing and verification, we found that mir-138-5p and mir-142-3p can be used as a predictor of patient prognosis-the lower the expression, the worse the patient's treatment effect.

Declarations

Author Contributions Statement

Hui Gong designed the research; Hui Gong, Chunxiang Kang, Fang Du and Shengtang Zhou performed the research; Chunxiang Kang analyzed the data; Hui Gong wrote the paper.

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Declaration of Interest Statement

The authors declare there is no conflicts of interest regarding the publication of this paper.

Availability of data and materials

None.

References

1. Moser-Katz T, Joseph NS, Dhodapkar MV, et al. Game of Bones: How Myeloma Manipulates Its Microenvironment. *Front Oncol.* 2020;10:625199.
2. van de Donk N, Pawlyn C, Yong KL. Multiple myeloma. *Lancet.* 2021;397:410–27.
3. Hideshima T, Anderson KC. Signaling Pathway Mediating Myeloma Cell Growth and Survival. *Cancers (Basel).* 2021; 13.
4. Chen T, Moscvin M, Bianchi G. Exosomes in the Pathogenesis and Treatment of Multiple Myeloma in the Context of the Bone Marrow Microenvironment. *Front Oncol.* 2020;10:608815.
5. Lopes R, Caetano J, Ferreira B, et al. The Immune Microenvironment in Multiple Myeloma: Friend or Foe? *Cancers (Basel).* 2021; 13.
6. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. *Science.* 2020; 367.
7. Bernardi S, Farina M. Exosomes and Extracellular Vesicles in Myeloid Neoplasia: The Multiple and Complex Roles Played by These "Magic Bullets". *Biology (Basel).* 2021; 10.
8. Srivastava A, Rathore S, Munshi A, et al. Extracellular Vesicles in Oncology: from Immune Suppression to Immunotherapy. *AAPS J.* 2021;23:30.
9. Rocchi A, Chiti E, Maiese A, et al. MicroRNAs: An Update of Applications in Forensic Science. *Diagnostics (Basel).* 2020; 11.
10. Shi Y, Ding D, Qu R, et al. Non-Coding RNAs in Diffuse Large B-Cell Lymphoma. *OncoTargets therapy.* 2020;13:12097–112.
11. Wu Y, Zhang Y, Zhang X, et al. Research advances for exosomal miRNAs detection in biosensing: From the massive study to the individual study. *Biosens Bioelectron.* 2021;177:112962.
12. Jimenez-Avalos JA, Fernandez-Macias JC, Gonzalez-Palomo AK. Circulating exosomal MicroRNAs: New non-invasive biomarkers of non-communicable disease. *Mol Biol Rep.* 2021;48:961–7.
13. Moloudizargari M, Hekmatirad S, Mofarahe ZS, et al. Exosomal microRNA panels as biomarkers for hematological malignancies. *Curr Probl Cancer.* 2021: 100726.
14. Kechin A, Boyarskikh U, Kel A, et al. cutPrimers: A New Tool for Accurate Cutting of Primers from Reads of Targeted Next Generation Sequencing. *J Comput Biol.* 2017;24:1138–43.

15. Langmead B, Trapnell C, Pop M, et al. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 2009;10:R25.
16. Griffiths-Jones S, Saini HK, van Dongen S, et al. miRBase: tools for microRNA genomics. *Nucleic acids research.* 2008;36:D154-8.
17. Friedlander MR, Mackowiak SD, Li N, et al. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucleic acids research.* 2012;40:37–52.
18. Anders S, Huber W. Differential expression analysis for sequence count data. *Genome Biol.* 2010;11:R106.

Figures

Figure 1

Characterization of bone marrow fluid exosomes in patients with different prognosis (A) Exosomes were isolated from the bone marrow of the patient and morphological characterization was observed by Transmission Electron Microscope. (B) The isolated exosomes' size is between 128nm and 165nm. (C) The surface protein markers of exosomes were identified by Western blot.

Figure 2

Effects of exosomes from bone marrow fluid in patients with different prognosis on the biological function of PRMI8226 cells. (A) Cell proliferation for PRMI8226 cells after the exosome stimulation. (B-C) Migration of PRMI8226 cells after the exosome stimulation evaluated by wound healing assay.

Figure 3

Bioinformatics analysis of differential miRNAs and their target genes. (A) Differentially expressed miRNA volcano map, 24 miRNAs including miR-142-3p, miR-138-5p, miR-215-5p are significantly down-regulated, including miR-124-3p, miR-451b, miR-509-3p of 12 miRNAs were significantly up-regulated. (B) Differentially expressed miRNA heat map. (C) Differentially highly expressed miRNA target gene KEGG pathway analysis shows that the highly expressed miRNA target genes of exosomes in patients with poor

prognosis are concentrated in PI3K-Akt signaling pathway, Ras signaling pathway, and Metabolic pathways. (D) Differentially under-expressed miRNA target genes KEGG pathway analysis shows that the low-expressed miRNA target genes in exosomes of patients with poor prognosis are concentrated in the MAPK signaling pathway, Transcriptional misregulation in cancer.

Figure 4

The expression of miRNA in clinical samples. (A) significant differences were observed for miR-124-3p among patients with different prognosis types. (B) no significant differences were observed between miR-451b and miR-503-3p.