

ORIGINAL ARTICLE

Plasma Exosomal Mir-375-3p As A Potential Diagnostic Biomarker For Nasopharyngeal Cancer

Tan Yadan^{1,2}, Rabiatul Basria S.M.N. Mydin¹, Adam Azlan^{1,3}, Chandrarohini Saravanan¹, Nur Hakimah Abu Mansor¹, Nigel J. Gooderham⁴, Muhammad Yusri Musa^{5,6,7}

¹ Department of Biomedical Sciences, Advanced Medical and Dental Institute, Universiti Sains Malaysia, 13200, Kepala Batas, Pulau Pinang, Malaysia.

² Medical Department, Yunnan College of Business Management, Wuhua District, 650032, Kunming City, Yunnan Province, People's Republic of China

³ Asian Institute of Medicine, Science and Technology (AIMST) University, 08100, Bedong, Kedah, Malaysia.

⁴ Department of Metabolism, Digestion, Reproduction, Imperial College London, Sir Alexander Fleming Building, London, SW7 2AZ, United Kingdom

⁵ Pusat Perubatan USM Bertam (PPUSMB), Universiti Sains Malaysia, 13200, Kepala Batas, Pulau Pinang

⁶ Department of Clinical Medicine, Advanced Medical and Dental Institute, Universiti Sains Malaysia, 13200 Bertam, Kepala Batas, Pulau Pinang Malaysia

⁷ Islamic Medical Association of Malaysia's (IMAM), 68100 Batu Caves, Selangor, Malaysia

ABSTRACT

Introduction: Nasopharyngeal cancer (NPC) is a malignant tumor with a poor prognosis, primarily due to the lack of specific symptoms and effective diagnostic markers. Plasma-derived exosomal miRNAs have emerged as potential cancer biomarkers. This exploratory study aimed to assess the potential of plasma exosomal miR-375-3p as a biomarker for NPC by comparing its levels in NPC patients to those in healthy controls. **Methods:** Using relative quantitative qRT-PCR, we found that miR-375-3p levels were 2.3 times higher in NPC patients. **Results:** ROC analysis indicated that miR-375-3p has strong diagnostic potential, with 75% sensitivity, 75% specificity, and an AUC of 0.8. KEGG and GO pathway analyses revealed that miR-375-3p is involved in the Hippo signaling pathway and promotes tumor cell invasion through the YAP1 gene. The miRanda tool also predicted a high-confidence interaction between miR-375-3p and YAP1 in this pathway. **Conclusion:** These findings highlight the potential of plasma exosomal miR-375-3p as a promising biomarker for NPC, suggesting the need for further research to explore its diagnostic and therapeutic applications.

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Corresponding Author:

Assoc. Prof. Ts. Dr. Rabiatul Basria S. M. N. Mydin,
PhD

Email: rabiatulbasria@usm.my

Tel :+604-5622351

INTRODUCTION

Nasopharyngeal cancer (NPC) is a prevalent malignancy and one of the leading causes of cancer-related deaths in Southeast Asia. NPC mainly associated with risk factors such as dietary, lifestyle, and Epstein-Barr virus (EBV) infection (1). Early symptoms, such as nasal congestion,

tinnitus, and hearing loss, are often misattributed to respiratory infections, leading to late-stage diagnoses (2). Timely, accurate and less invasive diagnosis is crucial for improving survival rates. While methods like nasopharyngeal endoscopy and tissue biopsy provide diagnostic evidence, their invasiveness and discomfort limit their widespread use (3). Therefore, there is a critical need for less-invasive, highly sensitive, and specific diagnostic methods. Current approaches using EBV DNA and EBV-related miRNAs have limitations in sensitivity, specificity, and early-stage detection, and are primarily focused on EBV-associated NPC (4, 5). On the other hand, exosomes from plasma have become a current research focus because they can carry stable

onco-miRNAs in the blood, potentially providing less-invasive biomarkers for early cancer detection (6, 7). This study aims to explore plasma-derived exosomal miRNAs as potential biomarkers for NPC, comparing profiles between healthy individuals and NPC patients to identify reliable diagnostic markers.

MiR-375-3p has emerged as a promising plasma biomarker for cancer diagnosis, with distinct expression patterns across various malignancies (8, 9). In prostate cancer, elevated plasma miR-375-3p levels differentiate malignant cases from benign hyperplasia (10). In contrast, colorectal cancer shows miR-375-3p downregulation in plasma, suggesting its potential for non-invasive liquid biopsy diagnostics (11). Elevated exosomal miR-375-3p is associated with metastasis in small cell lung cancer (SCLC) (12), while low levels in oral squamous cell carcinoma correlate with lymph node metastasis prediction (13). However, the cancer-specific expression of miR-375-3p necessitates context-dependent interpretation. Currently, limited information exists regarding miR-375-3p in NPC using blood plasma, highlighting the need for further research to evaluate its potential as a diagnostic and therapeutic target for NPC. The present exploratory research investigates plasma-derived exosomal miR-375-3p expression in NPC and examines its potential regulatory roles using bioinformatics tools. This study aims to provide insights into miR-375-3p's regulatory functions in NPC, exploring its potential as a biomarker for diagnosis and treatment strategies.

MATERIALS AND METHODS

Exosomal MIR-375-3p analysis

This exploratory study (14) involving plasma samples from 10 NPC patients and 10 healthy subjects were obtained from Pusat Perubatan USM Bertam (PPUSMB), Pulau Pinang, Malaysia, following the protocol approved by the Human Research Ethics Committee USM (HREC): 21110714. The inclusion criteria for healthy volunteers are the absence of chronic diseases such as cancer, chronic obstructive pulmonary disease, and ischemic heart disease, an age of 18 years or older, and a normal body mass index (BMI). Exclusion criteria include pregnancy or breastfeeding, as well as chronic infectious diseases like hepatitis B, hepatitis C, and HIV. For NPC subjects, inclusion criteria are being newly diagnosed with NPC, aged 18 years or older, and having a normal BMI. Exclusion criteria are a history of other malignant tumors, pregnancy or breastfeeding, chronic infectious diseases such as HIV, hepatitis B, and hepatitis C, and previous surgery, chemotherapy, or radiotherapy.

Exosomes were isolated from plasma using the protocol from Total Exosome Isolation Kit (Invitrogen, cat# 4484450), and small RNA was enriched with Qiazol extraction. Reverse transcription was performed using

the miRCURY LNA RT Kit (Qiagen) protocol. Expression levels of miR-375-3p (Qiagen, YP00204362) and miR-185-5p (Qiagen, YP00206037, endogenous control) were assessed with the miRCURY LNA SYBR® Green PCR Kit (QIAGEN Biotechnology) protocol and analyzed using the Bio-Rad CFX96 Real-Time PCR detection system. Statistical analyses were conducted with SPSS version 27, employing the independent sample t-test for group comparisons. ROC (Receiver Operating Characteristic) curves were used to evaluate the diagnostic performance of biomarkers, particularly for distinguishing between diseased and non-diseased individuals.

Bioinformatics analysis

For mRNA target genes of miRNAs prediction, use the PITA and EIMMO databases. Enrichment analysis was conducted with the clusterProfiler package in R (15), and miRNA-related mRNA functions were examined through Gene Ontology (GO) and KEGG to link them to cancer development. miRanda identified binding sites between miRNAs and target mRNAs via sequence alignment and free energy calculations, providing data on binding sites, free energy, and alignment scores to assess regulatory potential (16). Additionally, miRanda predicted interactions between target genes and mRNAs, focusing on cancer-related pathways to elucidate their roles in NPC.

RESULTS

The extracted exosomes ranged in size from 30 to 90 nanometers, with smooth surfaces, clear edges, and no visible refractive abnormalities or defects (Fig. 1). Despite some size variation, their uniform morphology distinguished them from other extracellular vesicles or debris. Under bright-field microscopy, they exhibited typical characteristics, including a consistent round or oval shape and smooth surfaces, confirming their identity as reported by previous studies (17). Exosomes were evenly dispersed, without aggregation or clustering, and maintained consistent contours and transparent internal structures typical of exosomes.

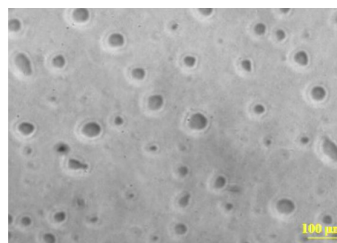


Figure 1: Visualization of plasma-derived exosomes. Exosomes, small vesicles secreted by cells ranging in diameter from approximately 30 to 90 nm, appear circular or elliptical under a bright-field microscope Magnification: 10x40. These extracellular vesicles are enclosed by phospholipid bilayers and exhibit a characteristic shallow transparency or light reflection when viewed under bright-field microscopy.

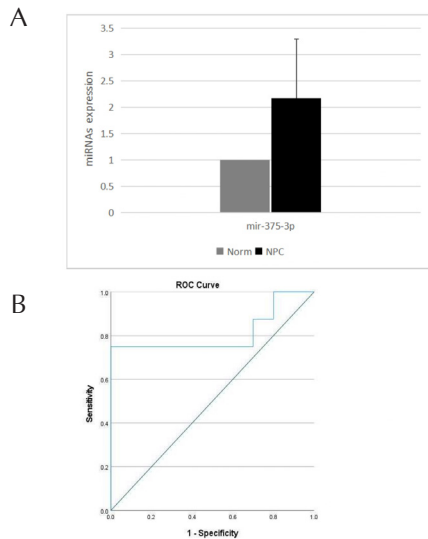


Figure 2: Plasma-derived exosomal miR-375-3p profile in NPC. (A) The expression of miR-375-3p in NPC than in healthy individuals. **(B)** Receiver operating characteristic (ROC) curves for miR-375-3p showed strong diagnostic potential with an AUC of 0.8.. Data points represent the mean \pm SEM of triplicate observations from a representative experiment. * $p < 0.05$ indicates a statistically significant result.

MiRNA extracted from exosomes showed that the expression level of miR-375-3p was more than 2-fold higher in NPC compared to healthy subjects, with a statistically significant difference ($P = 0.02$, $P < 0.05$) (Fig. 2 A). The ROC curve analysis was performed to understand the diagnostic potential of miR-375-3p (Fig. 2 B) showed an AUC (area under the curve) of 0.8 ($P = 0.033$), with sensitivity and specificity exceeding 75%. The high AUC indicates strong diagnostic accuracy in distinguishing NPC from healthy subjects, highlighting miR-375-3p's potential biomarker for NPC. Further bioinformatic analysis (Fig. 3) identified 252 and 919 potential mRNA target genes for miR-375-3p using the PITA and ELMMo databases (Fig. 3 A), respectively. By intersecting these lists, 116 target genes were selected. Functional analysis of the top 20 genes using the KEGG platform (Fig. 3B) and the GO enrichment platform (Fig. 3C) highlighted the Hippo signaling pathway, which is notably linked to cancer, including NPC. Additionally, the miRanda tool was used to predict the interaction between miRNA-375 and YAP1 mRNA (Fig. 3D). The analysis provided a prediction score of 130 and a binding energy of -81.199997 kCal/mol, suggesting a strong and stable interaction. This indicates that miRNA-375 likely regulates YAP1 by directly targeting its mRNA.

DISCUSSION

The increased levels of plasma-derived exosomal miR-375-3p in NPC suggest its potential role in cancer development. In SCLC cancer, elevated miR-375-3p levels are linked to metastasis and promote cancer spread by disrupting vascular barriers (18). Similarly, in prostate cancer, higher miR-375 levels are associated with greater disease severity and contribute to enhanced cancer cell proliferation, migration, and invasion (19).

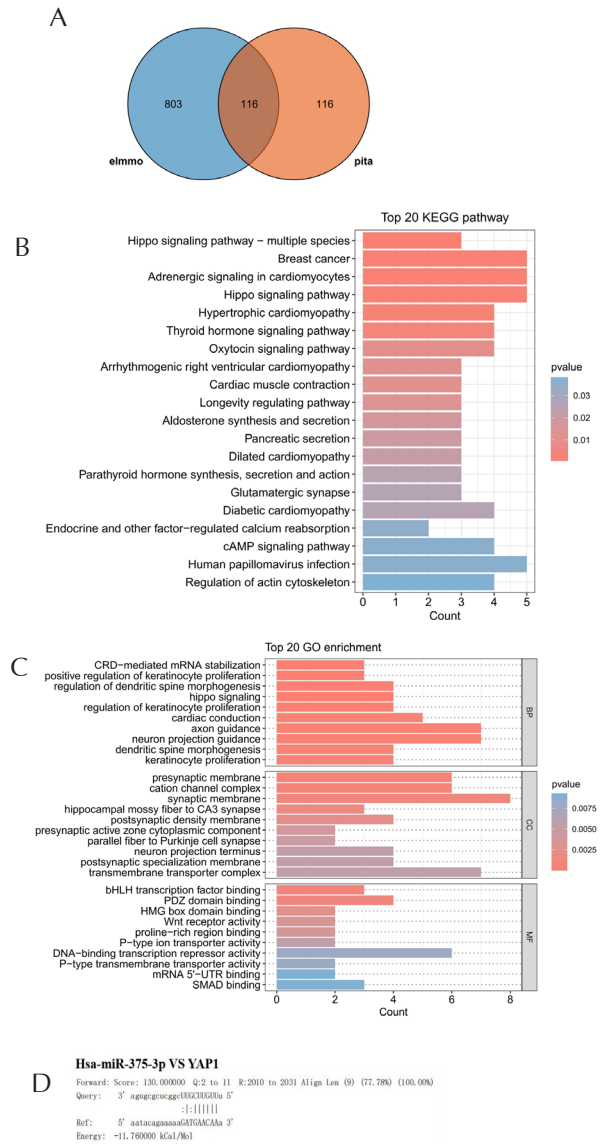


Figure 3: Target Gene Prediction and Functional Analysis of miR-375-3p. (A) Enrichment of target mRNAs in ELMMo and PITA databases. **(B)** KEGG analysis of miR-375-3p. **(C)** GO analysis of miR-375-3p targeting mRNAs. **(D)** miRanda prediction of interaction between miR-375-3p and YAP1 gene.

These findings indicate that targeting miR-375 with antisense oligonucleotides could offer therapeutic benefits. Additionally, studies in breast cancer (20), Merkel cell carcinoma (21), and esophageal squamous cell carcinoma (22) also have highlighted the important role of exosomal miR-375 in cell communication and tumor progression, suggesting its potential as a non-invasive biomarker for early cancer detection and monitoring.

Previous studies have shown that miR-375-3p could regulate cancer invasion by targeting PDK1, a key factor in cell survival and proliferation (23). miR-375 binds directly to the 3' untranslated region of PDK1 mRNA, downregulating its expression and inhibiting cancer cell proliferation, migration, and invasion (23). Overexpression of miR-375 reduces cell viability and colony formation, while PDK1 overexpression reverses

these effects, suggesting that miR-375's downregulation of PDK1 plays a crucial role in modulating carcinogenesis (23). The PDK1-targeting effects of miR-375 also have been observed in pancreatic carcinoma (24), where its downregulation correlates with increased tumor aggressiveness. Additionally, the interaction between miR-375 and the PI3K/AKT signaling pathway, a key driver of tumor progression and drug resistance (25), further highlights its potential as a therapeutic target.

In present study, further bioinformatic analysis predicted 252 and 919 mRNA target genes of miR-375-3p using the PITA and EIMMo databases, respectively. Intersection analysis of these datasets identified 116 target genes, which were then subjected to functional analysis using KEGG and GO pathways. GO analysis revealed that miR-375-3p target genes are enriched in biological processes such as 'CRD-mediated mRNA stabilization' ($P = 2.71E-05$), 'positive regulation of keratinocyte proliferation' ($P = 7.34E-05$), 'regulation of dendritic spine morphogenesis' ($P = 9.60E-05$), and 'Hippo signaling' ($P = 0.000105119$). Notably, the Hippo signaling pathway is critically involved in cancer development, including NPC. Dysregulation of key components such as MST1/2 and LATS1/2 in the Hippo pathway often leads to the hyperactivation of YAP/TAZ, promoting tumor growth and progression. KEGG pathway analysis also highlighted the 'Hippo signaling pathway - multiple species' ($P = 0.000539719$) as a crucial pathway associated with miR-375-3p targets, emphasizing its role in NPC. YAP1, a central effector of the Hippo signaling pathway, drives carcinogenesis through multiple interconnected mechanisms across various cancers (26-28).

The miRanda tool was used to predict the interaction between miR-375 and YAP1 gene mRNA, revealing a strong interaction with a score of 189, indicating a high likelihood of binding. The predicted binding region spans from the 2nd to the 57th position of miR-375 and from the 329th to the 392nd position of YAP1 mRNA, with a matching rate of 58.93% and a similarity of 69.64%. The calculated binding energy of -81.2 kCal/Mol suggests a stable interaction, implying that miR-375 likely regulates YAP1 expression by directly targeting its mRNA. This interaction highlights miR-375's significant role in gene regulation and its potential involvement in carcinogenesis pathways related to YAP1. The role of plasma-derived exosomal miR-375-3p in cancer progression underscores the complex relationship between miRNAs, exosomes, and cancer development, opening new avenues for research and therapeutic strategies. However, further studies are needed to validate these findings. Overall, plasma-derived exosomal biomarkers could provide non-invasive diagnostic and prognostic capabilities, serving as a promising alternative to traditional biopsy methods.

CONCLUSION

This study found that miR-375-3p was significantly upregulated in plasma-derived exosomes from NPC patients ($p < 0.05$), with ROC analysis showing high diagnostic potential ($AUC = 0.8$), suggesting it as an effective biomarker for NPC. KEGG and GO analyses revealed that YAP-1, a target gene of the Hippo pathway influenced by miR-375-3p, plays a role in tumor development. These results emphasize miR-375-3p's key involvement in NPC pathogenesis and its potential as both a diagnostic marker and therapeutic target. However, further validation in larger cohorts with different stages and functional studies are needed for broader clinical applicability.

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Ethics approval

Human ethical approval was obtained from JEPeM USM (JEPeM Code:USM/JEPeM/21110714).

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