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RESEARCH ARTICLE

Profile Expression and Validation of Plasma Exosomal miR-320a in Patients with Lung Adenocarcinoma as a Candidate Diagnostic Biomarker: A Study in the Indonesian Population

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Abstract

Lung cancer has the highest incidence rate in Indonesia, with a projected significant increase by 2040. Lung adenocarcinoma, the most common histological subtype, is often diagnosed at advanced stages due to the absence of early symptoms and reliance on invasive biopsy methods. Exosomal microRNAs (exomiRs), owing to their stability and ability to reflect tumor-specific changes, offer promise as non-invasive diagnostic biomarkers. This study aims to map the expression profile and validate exomiRs in Indonesian patients with lung adenocarcinoma so that it can be developed as a diagnostic biomarker. Plasma-derived exosomes were isolated from patients with lung adenocarcinoma and healthy controls. ExomiR profiles were first screened using NanoString analysis, followed by validation of candidate exomiRs with qRT-PCR. Differential expression was analyzed, and diagnostic performance was assessed using ROC curve analysis. The results indicate that in patients with locally advanced lung adenocarcinoma, there is upregulation of exosomal miR-320a, miR-604, miR-1261, and miR-648, as well as downregulation of exosomal miR-301b-3p, miR-605-5p, miR-924, miR-769-5p, miR-373-3p, miR-520h, miR-193a-5p, miR-3150b-3p, miR-3615, miR-4431, miR-6720-3p, miR-1909-3p, miR-574-3p, miR-382-5p, miR-593-3p, miR-572, miR-27a-3p, and miR-411-5p compared to healthy controls. In silico analysis showed that these regulated exomiRs play a role in chromatin structure formation and tumor immune cell infiltration pathways. Validation of exomiR-320a expression showed a 1.9-fold upregulation ($p = 0.00032$) in lung adenocarcinoma patients compared to healthy controls with an AUC value of 0.781, indicating that this miRNA has moderate potential as a diagnostic biomarker that requires further validation.

Keywords: ExomiRS, Exosome miRNAs, Lung adenocarcinoma, Biomarker lung cancer, miR-320a, Profiling exomiRs, Validation exomiR-320a

Introduction

Lung cancer is a global health challenge because it is the leading cause of cancer-related deaths worldwide. There were approximately 2,408,675 new cases of lung cancer globally and 1,817,469 deaths in 2022 [1]. Lung cancer is the most common type of cancer in Indonesia, accounting for 12.6% of all cancer deaths and 8.6% of all cancer cases in 2018. The number of lung cancer cases is projected to increase from 30,023 in 2018 to 54,983 in 2040 [2]. Lung cancer is broadly categorized into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), with NSCLC comprising about 80% of cases. Lung adenocarcinoma is the most common histopathological type found in non-small cell lung cancer [3,4]. Among NSCLC subtypes, lung adenocarcinoma is the most common histopathological type, making it a priority target for research on novel biomarkers and diagnostic approaches.

Lung cancer, particularly lung adenocarcinoma, often lacks specific early symptoms, leading many patients to be diagnosed only at advanced stages [5,6]. Advanced-stage lung cancer has a poor prognosis because it is more aggressive, resistant to chemotherapy, and prone to recurrence after tumor removal, with a short survival rate (12 - 20 months) [7,8]. Fine-needle aspiration is one of the methods used for tissue sampling to diagnose lung cancer in Indonesia [9]. This method is invasive and carries the risk of tissue necrosis in advanced-stage cancer, and it cannot be used for monitoring cancer progression. These limitations highlight the urgent need for non-invasive diagnostic strategies that can enable earlier detection and facilitate ongoing monitoring. In this context, liquid biopsy has emerged as a promising alternative, allowing the analysis of tumor-derived molecules from body fluids.

One type of cancer diagnostic biomarker from liquid biopsy that has been extensively studied is exosomal microRNAs (ExomiRs) [10,11]. Unlike free-circulating miRNAs, which can be degraded by RNases in the bloodstream, exomiRs are encapsulated within the lipid bilayer of exosomes, making them highly stable under various physiological conditions [12,13]. ExomiRs has good stability in the body and is suitable for non-invasive diagnostic approaches, readily detectable in circulation, and capable of reflecting tumor-specific molecular changes, making them attractive biomarkers for clinical application [14-16]. ExomiRs is miRNA found in exosomes and circulated throughout the body.

MiRNA is a non-coding RNA that plays a crucial role in regulating genes and is involved in pathophysiological conditions, particularly cancer [17,18]. MiRNAs also plays a role in promoting (oncomiRs) or inhibiting (tumor suppressor miRNAs) cancer progression. The real-time expression profile of specific miRNAs reflects the current state of various cellular conditions, both physiological and pathological, including during metastasis and healing [19]. Although several studies have investigated circulating and exomiRs diagnostic biomarkers for lung cancer in different populations [10,11,20,21], such findings may not be directly generalizable to the Indonesian setting. MiRNA expression profiles are known to be influenced by genetic background, lifestyle factors (such as diet and smoking prevalence) [22], environmental exposures [23,24], and population-specific characteristics [25]. These factors can lead to distinct biomarker signatures across populations, potentially altering diagnostic performance if markers validated elsewhere are applied without local verification. Therefore, identifying exomiRs signatures within the Indonesian population is scientifically significant, as it ensures that diagnostic tools are accurate, context-specific, and clinically relevant to this population. Based on this, exomiRs profiling have the potential to be developed as candidate biomarkers for the diagnosis of lung adenocarcinoma in Indonesia.

Despite extensive research on exomiRs expression profiles in lung adenocarcinoma, no mapping of such profiles has been conducted in the Indonesian population. This study aims to profile exomiRs expression and validate their diagnostic potential in Indonesian patients with lung adenocarcinoma, with the rationale that population-specific miRNA signatures may provide more accurate and clinically relevant biomarkers for early detection.

Materials and methods

Research sample collection

The research has obtained ethical clearance approval with number KE/FK/0177/EC dated January 30, 2024. The study was conducted at Dr. Sardjito

General Hospital with permit number DP.04.03/D.XI.2/7470/2024 and at Dr. Kariadi General Hospital with permit number DP.04.01/D.X.2/2376/2024. Patient samples with lung adenocarcinoma were collected from Dr. Sardjito General Hospital and Dr. Kariadi General Hospital. Healthy controls were recruited from individuals confirmed to be healthy based on normal for random blood glucose, cholesterol, and uric acid levels. They also reported no pain and had no family history of cancer.

This study used an exploratory observational design, involving eight subject including 4 for healthy control and 4 for locally advanced adenocarcinoma patients for exomiRs profiling. Respondents were recruited through purposive sampling using inclusion criteria such as newly diagnosed cases of primary lung adenocarcinoma and treatment-naïve at the time of diagnosis. Patients with a history of chemotherapy, radiotherapy, targeted therapy, immunotherapy, or surgical resection prior to sample collection were excluded to minimize treatment-related confounding effects on exomiRs expression.

After obtaining the exomiR profiles with the highest upregulation, validation was carried out by involving 75 lung adenocarcinoma subject, consisting of 5 with locally advanced and 70 with metastatic stage patients, dan control group consisting of 35 healthy subject that were collected from June to December 2025.

The sample size was determined using the *comparison of 2 proportions* method [26], based on inclusion and exclusion criteria. The prevalence of lung cancer patients in the population was estimated at 35%. From this calculation, the minimum required sample size was 49 patients with lung adenocarcinoma, with a significance level (alpha) of 0.05 and a statistical power of 0.80 [26].

Blood samples were collected intravenously, 4 mL each, and placed in EDTA vacutainer tubes carried in a cool box (4 °C) for a maximum of 15 min before centrifugation. Blood was centrifuged at 1,500 rpm for 15 min to obtain plasma. Plasma was stored at −80 °C until further exosome and total RNA extraction.

Exosome extraction

Plasma sample preparation was performed in 3 centrifugation steps to eliminate red blood cells and cell debris. Plasma sample centrifuged at 300 g for 10 min. Centrifugation was then continued at 1,200 g for 20 min, and the final centrifugation step was performed at 10,000 g for 30 min. The plasma is separated from the pellet and transferred into a 1.5 mL tube. A total of 250 µL plasma samples underwent exosome extraction using a kit. Plasma exosome extraction is performed according to the procedure manual in the EXO-Prep kit for plasma isolation, catalog number: HBM-EXP-B5 [27].

Exosome particle concentration testing using Nanoparticle Tracking Analysis (NTA)

Exosome concentration was measured using the NTA ViewSizer 3000 machine from Horiba Scientific. Exosome particle concentration and size analysis was performed using ViewSizer software [28,29]. The exosome concentration from which total RNA was extracted was 2.9×10^{10} particles/ml with a size range of 30 - 150 nm.

Exosome RNA extraction

The exosome RNA extraction process refers to the manual procedure book from the HansaBioMed Life Sciences RNA Basic Kit [30]. Next, the eluted RNA is stored at −80 °C for use in the next downstream stage. The RNA concentration is measured using Nanodrop maestrogen, with a minimum concentration of 100 ng/3 µL required for exomiRs profiling analysis and an RNA purity ratio (260/280) of 1.9 - 2.0.

ExomiRs profiling using NanoString

Eight RNA samples, consisting of 4 locally advanced lung adenocarcinoma samples and 4 healthy control samples, were processed using the nCounter miRNA Expression Panel V3B. The panel uses nanostring technology to perform expression profiling through direct quantification of individual RNA molecules. The nCounter miRNA Expression Panel analyzes up to 827 human miRNAs. RNA samples underwent quality control for RNA concentration and purity, Field of View, Binding density, positive control linearity, fM detection threshold, and normalization factor [31,32].

The output file from the nCounter NanoString machine is in RCC (Reporter Code Count) format. RCC files are analyzed using the ROSALIND platform to obtain data in the form of volcano plots and heatmap of exomiRs expression that significantly increased or decreased in the locally advanced lung adenocarcinoma group compared to healthy controls. The Kyoto Encyclopedia of Genes and Genomes (KEGG) dot plot and gene ontology bar plot pathway enrichment analysis using R multimiR with references from various databases such as miRecords, MIRTarbase, miRDB, TargetScan, and miRanda. Pathway enrichment analysis using libraries from clusterprofiler, biomaRt, org.Hs.eg.db, and annotationDbi. Word cloud analysis using the R multimiR package based on miRNA-mRNA interaction data.

cDNA synthesis

cDNA synthesis was performed using the MiRCURY LNA RT Kit (cat no. 339340), following the procedure outlined in the kit insert manual. The RNA template used was 50 ng. Once the RT-PCR process was complete, the cDNA was immediately stored at 4 °C [33].

Validation of exomiRs expression using qRT-PCR

Quantification of exomiR-320a expression was performed using the miRCURY® LNA® miRNA PCR Assays and PCR Panels kit (cat. No. 339345). A low concentration of ROX dye is used for the Applied Biosystems® 7500. The ROX concentration must be diluted 20× from the 200× stock. Two times miRCURY SYBR Green Master Mix (5 µL) is added to the PCR tube panel and supplemented with 0.5 µL of ROX reference dye at low concentration, vortexed, and spin down. The solution is then mixed with 1 µL of PCR primer mix and 3 µl of cDNA (previously diluted 20-fold), followed by the addition of 1 µL of RNase-free water, bringing the total volume to 10 µL, vortex and spin down. The PCR tube plate is loaded into the ABI 7500 qRT-PCR machine using the following protocol. Initial heating activation step for 2 min at 95 °C (maximal/fast mode), followed by denaturation for 10 s at 95 °C (maximal/fast mode), combined annealing/extension step for 60 s at 56 °C (maximal/fast mode), total number of cycles is 40× with melting curve analysis at 60 - 95 °C [34,35]. The next step is data interpretation. The validated exomiRs using qRT-PCR are exomiR-320a and the reference gene is exomiR-103a-3p. MiRNA 103a-3p was selected as the reference gene in this study because it has been shown to be reliable for normalization in extracellular vesicles derived from cartilage, adipose tissue, and bone marrow cells [36]. In addition, miR-103a-3p was identified as a candidate reference gene based on qPCR-based miRNA expression profiling across 14 human tissues, including lung cancer [37]. Further evaluation of endogenous reference gene candidates using BestKeeper, geNorm, NormFinder, and the ΔCt comparative method confirmed that miR-103a-3p is a stable reference gene in FFPE lymph node samples [41]. Relative quantification of miR-320a expression was calculated using the $2^{-\Delta\Delta Ct}$ (Livak) method with miR-103a-3p as the reference gene [39].

Receiver operating characteristic (ROC) analysis of exomiRs expression

The cycle threshold (Ct) values for exomiR-320a and exomiR-103a-3p were analyzed for data homogeneity. Homogeneity of Ct values for exomiR-320a was assessed using Levene's test, while normality was evaluated using the Kolmogorov-Smirnov test. Differential expression of exomiR-320a Ct values among the 3 groups healthy controls, locally advanced lung adenocarcinoma, and metastatic lung adenocarcinoma was analyzed using the Kruskal-Wallis test, followed by the Wilcoxon test for pairwise comparisons. The expression of exomiR-320a was calculated using the Livak method to determine whether there was an increase or decrease in expression in adenocarcinoma compared to healthy controls. To evaluate the diagnostic performance of exomiR-320a, Receiver Operating Characteristic (ROC) curve analysis was performed by plotting sensitivity against 1-specificity using ΔCt values. The Area Under the Curve (AUC) was calculated along with its 95% confidence interval (CI). The AUC was calculated by entering the ΔCt value of miR-320a in the lung adenocarcinoma group compared to healthy controls.

Results and discussion

Overview of the study sample

Demographic data for subjects with lung adenocarcinoma included age, gender, stage, and *EGFR* mutation type, while healthy controls included age and gender (**Table 1**). However, subgroup stratification by these factors was not performed in the current analysis due to sample size limitations. Most of the healthy control samples were under 45 years of age because the inclusion criteria were normal based on health examinations, but it was quite difficult to obtain normal criteria in individuals over 45 years of age. Lung cancer patients in Indonesia are mostly detected at ages 56 - 70, and it is very difficult to find patients in the early stages of the disease; even locally advanced cases only account for 5 patients**.** This poses the greatest challenge for biomarker research for early diagnosis of lung cancer, as research subjects are extremely difficult to find in the early stages of the disease.

Table 1 Clinical and pathological characteristics of LUAD patients for ExomiR-320a expression analysis.

	Lung adenocarcinoma patients (n = 75)				**Healthy subject (n = 35)**			
	Male (n = 44)		**Female (n = 31)**		**Male (n = 14)**		**Female (n = 21)**	
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**
Ages								
< 25	0	0.00	0	0.00	1	2.86	0	0.00
26 - 40	1	1.33	0	0.00	8	22.86	12	34.29
41 - 55	6	8.00	8	10.67	5	14.29	8	22.86
56 - 70	27	36.00	19	25.33	0	0.00	1	2.86
> 70	10	13.33	4	5.33	0	0.00	0	0.00
Stage								
1	0	0.00	0	0.00	-	-	-	-
2	0	0.00	0	0.00	-	-	-	-
3 (locally advanced)	3	4.00	2	2.67	-	-	-	-
4 (metastasis)	41	54.67	29	38.67	-	-	-	-
Type of *EGFR* mutation								
EGFR exon 18 G719X	1	1.33	0	0.00	-	-	-	-
EGFR exon Ins19	1	1.33	0	0.00	-	-	-	-
EGFR exon Del19	4	5.33	5	6.67	-	-	-	-
EGFR exon Ins20	0	0.00	1	1.33	-	-	-	-
EGFR exon 20 T790M	0	0.00	2	2.67	-	-	-	-
EGFR exon 20 S7681	1	1.33	1	1.33	-	-	-	-
EGFR exon 21 L861Q	1	1.33	2	2.67	-	-	-	-
EGFR exon 21 L858R	8	5.33	9	5.33	-	-	-	-
EGFR wildtype	28	37.33	11	14.67	-	-	-	-

Note: N = number of subject; stript mark (-) = the data is not available

The most common type of EGFR mutation in lung cancer in Indonesia is the exon 21 L858R mutation [40,41]. The exon 21 L858R mutation is common in Asian populations and accounts for approximately 40% of all *EGFR* mutations in NSCLC [42]. Patients with the L858R mutation are commonly found in advanced-stage lung cancer and among non-smokers [43] and exhibit a more aggressive disease course [42]. Patients with the L585R mutation generally exhibit lower sensitivity to *EGFR* tyrosine kinase inhibitors (TKIs) compared to patients with the del19 mutation [42]. Based on previous studies, 67.7% of patients with the L858R mutation responded to EGFR-TKI therapy; however, PDL-1-positive patients had a higher likelihood of non-response and progression to interstitial lung disease [44].

Signature microRNA exosomes in locally advanced lung adenocarcinoma and healthy controls

Based on the differential expression analysis of exomiRs, the upregulated and downregulated exomiRs were visualized in the form of a volcano plot (**Figure 1**). Differential expression analysis applied a filter for upregulated exomiRs with a fold change value of 1.5, while downregulated exomiRs had a fold change value of −1.5, and a p-value of ≤ 0.05.

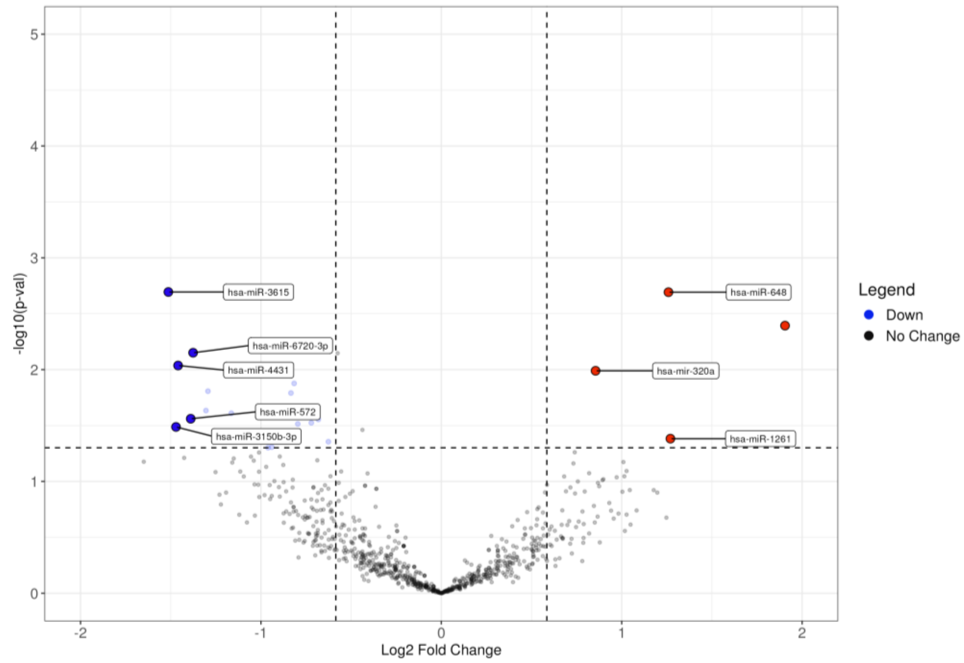

Figure 1 Screening differentially expressed exomiRs using a NanoString. Candidate differentially expressed in lung adenocarcinoma (LUAD) and healthy control are shown in a volcano plot. The log ratio of the fold change is on the X axis, and the negative log of p-value is on the Y axis. Each dot represents a exomiR within the comparison performed. The coloring on the dots reflects the clustering information for each exomiR, and those in black are miRNA that do not pass the parameters of the filter selected. ExomiR with the same coloring are grouping together based on the similarity of their expression pattern.

ExomiRs expression in patients with locally advanced lung adenocarcinoma compared to healthy controls showed significant upregulation of 4 exomiRs (miR-320a, miR-604, miR-1261, miR-648) and significant downregulation of eighteen exomiRs (miR-301b-3p, miR-605-5p, miR-924, miR-769-5p, miR-373-3p, miR-520h, miR-193a-5p, miR-3150b-3p, miR-3615, miR-4431, miR-6720-3p, miR-1909-3p, miR-574-3p, miR-382-5p, miR-593-3p, miR-572, miR-27a-3p, miR-411-5p) (**Figure 2**). Potential exomiR candidates for development as diagnostic biomarkers in lung adenocarcinoma are those with significantly increased expression levels to ensure their expression values remain measurable during patient validation. ExomiR candidates with decreased expression can be used as mimic-miR therapy candidates in lung adenocarcinoma patients [45].

The clustering by disease status shows that patients with locally advanced lung adenocarcinoma and healthy controls form distinct groups. This indicates that exomiRs expression within each group is relatively uniform but differs significantly between groups. In locally advanced lung adenocarcinoma, the number of oncogenic miRNAs (oncomiRs) is lower compared to tumor-suppressor miRNAs.

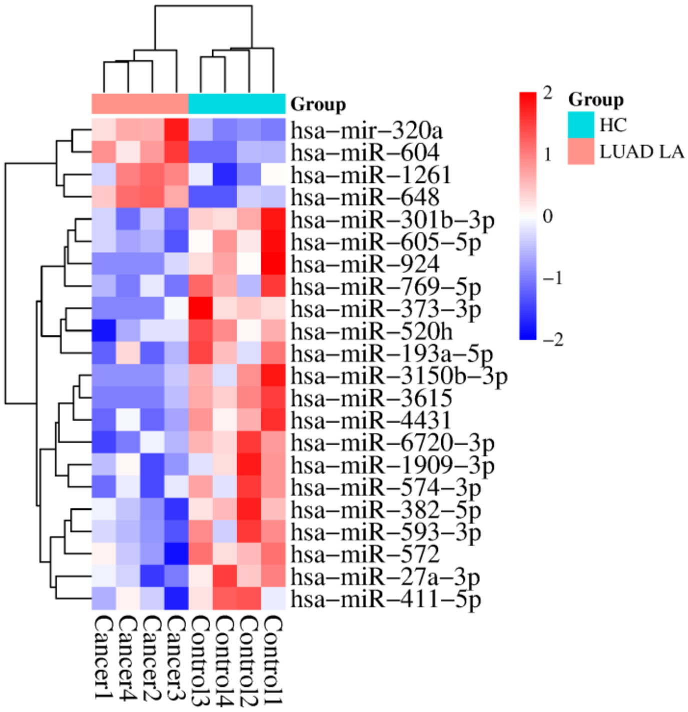

Figure 2 Screening differentially expressed exomiRs using a NanoString. Candidate differentially expressed in lung adenocarcinoma (LUAD) and healthy control are shown in a heat map.

This reflects a shift in the balance between oncogenic and tumor-suppressive functions of exomiRs. At the locally advanced stage, tumor-suppressor activity remains relatively strong in inhibiting cancer progression compared to the metastatic stage [46]. The lower abundance of exomiRs may reduce inhibition of tumor-suppressor genes, thereby limiting metastatic activity at this stage.

For validation, candidate single biomarkers were selected from the overexpressed exomiRs. ExomiR-320a was chosen as the diagnostic biomarker for validation because it was consistently overexpressed in the NanoString results. In addition, bioinformatics analysis showed that exomiR-320a had a greater number of validated and predicted target genes compared to the other 3 overexpressed exomiRs. Validation using lung adenocarcinoma patient samples in Indonesia has limitations, as there are very few patients in the early stages of the disease. Even at Dr. Sardjito General Hospital in Yogyakarta, Indonesia, no early-stage lung adenocarcinoma patients were found between June and December 2024.

Expression of exomiRs in locally advanced lung adenocarcinoma compared to healthy controls

Based on the fold change analysis of the exomiR profile, the highest expression of exomiRs in the locally advanced lung adenocarcinoma group and healthy controls was miR-604, which increased by 3.75 times (**Table 2**). Research on exosomes miR-604, miR-1261, and miR-648 in lung adenocarcinoma has not been widely conducted. According to the miRTarBase database (https://mirtarbase.cuhk.edu.cn/~miRTarBase/miRTarBase_2019/php/index.php), exosomal miR-604, miR-1261, and miR-648 have validated target genes using NGS, thus falling into the category of less strong evidence. In the miRBase database https://www.mirbase.org/, exosomal miR-604, miR-

1261, and miR-648 lack strong evidence regarding their expression and target genes. Additionally, no studies have reported the expression of exomiR-604 in lung adenocarcinoma, making this marker a candidate biomarker for validation due to its novelty. However, after validating exomiR-604 in lung adenocarcinoma patients, its expression was found to be unstable (data not yet published). Previous research indicates that miR-1261 is regulated by circular RNA. MiR-1261 is downregulated by Circ-0072088, where increased expression of the *PIK3CA* gene is associated with poor outcomes in lung adenocarcinoma patients [47].

Table 2 Differentially expressed exosome miRNA in locally advanced lung adenocarcinoma groups compared to healthy controls.

miRNA	Fold change	*p*-value
hsa-miR-604	3.74615	0.004042
hsa-miR-1261	2.41162	0.041439
hsa-miR-648	2.39358	0.002030
hsa-mir-320a	1.80893	0.010243
hsa-miR-3615	−2.85452	0.002022
hsa-miR-3150b-3p	−2.7717	0.032548
hsa-miR-4431	−2.74969	0.009189
hsa-miR-572	−2.6193	0.027500
hsa-miR-6720-3p	−2.59559	0.007065
hsa-miR-520h	−2.47049	0.023235
hsa-miR-27a-3p	−2.45146	0.015588
hsa-miR-593-3p	−2.24087	0.024531
hsa-miR-924	−2.16897	0.038621
hsa-miR-411-5p	−1.94614	0.049934
hsa-miR-193a-5p	−1.94432	0.036078
hsa-miR-1909-3p	−1.91838	0.049334
hsa-miR-382-5p	−1.7821	0.016190
hsa-miR-301b-3p	−1.7605	0.013285
hsa-miR-605-5p	−1.73578	0.030589
hsa-miR-373-3p	−1.64806	0.029913
hsa-miR-574-3p	−1.60399	0.027886
hsa-miR-769-5p	−1.54335	0.044143

The exomiRs with the lowest expression, miR-3615, was 2.85 times lower in locally advanced lung adenocarcinoma compared to healthy controls. Exosomes miR-3615, miR-3150b-3p, and miR-4431 have not been extensively studied in lung adenocarcinoma. In the miRtarBAse database, exosomal miR-3615, miR-3150b-3p, and miR-4431 have validated target genes using NGS, placing them in the “less strong evidence” category. Based on analysis in the miRBase database, exosomes miR-3615, miR-3150b-3p, and miR-4431 lack strong evidence regarding their expression and target genes. Previous studies have shown that extracellular vesicle miR-3651 is a miRNA signature that can be used for early detection of lung adenocarcinoma [48]. LncRNA RP11-116G8.5 promotes the progression of squamous cell lung carcinoma by sponging miR-3150b-3p/miR-6870-5p, thereby increasing the expression of *PHF12/FOXP4* [49].

Interactions between exomiRs, target genes, and signaling pathways involved in carcinogenesis

The analysis of exomiR interactions, target genes, and signaling was performed using R tools and David (https://davidbioinformatics.nih.gov), and mapping was performed using Cytoscape. Based on in silico analysis, it was found that exomiRs that were upregulated (exomiR-320a, exomiR-648, exomiR-1261) share common target genes. ExomiR-320a, and exomiR-1261 have common target genes involved in chromatin structure formation pathways and gene expression regulation. The genes targeted by exomiR-320a and exomiR-1261 are genes that express histone proteins, namely H3 clustered histone 15 (*H3C15*), H3 clustered histone 14 (*H3C14*), Histone acetyltransferase 1 (*HAT1*), H3 clustered histone 2 (*H3C2*), H2B clustered histone 4 (*H2BC4*), and H2B clustered histone 12 (*H2BC12*) (**Figure 3**). ExomiR-604 has target genes involved in cell cycle processes, endocytosis, cellular senescence, metabolic pathways, and ubiquitin-mediated proteolysis, and does not share any target genes with exomiR-320a, exomiR-648, or exomiR-1261. ExomiR-320a and exomiR-648 target genes involved in the tumor-infiltrating immune cell pathway. The pathways involved in tumor-infiltrating immune cells include the inflammatory response, targeted by the NF-κB subunit (*RELA*) gene; actin beta (*ACTB*); the toll-like receptor signaling pathway, targeted by the high mobility group box 1 (*HMGB1*) gene; membrane ruffling phagocytosis, targeted by the integrin subunit beta 3 (*ITGB3*) gene; Rac family small GTPase (*RAC1*), NADPH oxidase (Nox2-based) targeted by the *RAC1* gene, lytic NETosis targeted by the AKT serine/threonine kinase 3 (*AKT3*) gene, and the *PI3K-AKT* signaling pathway targeted by the phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) and *AKT* genes.

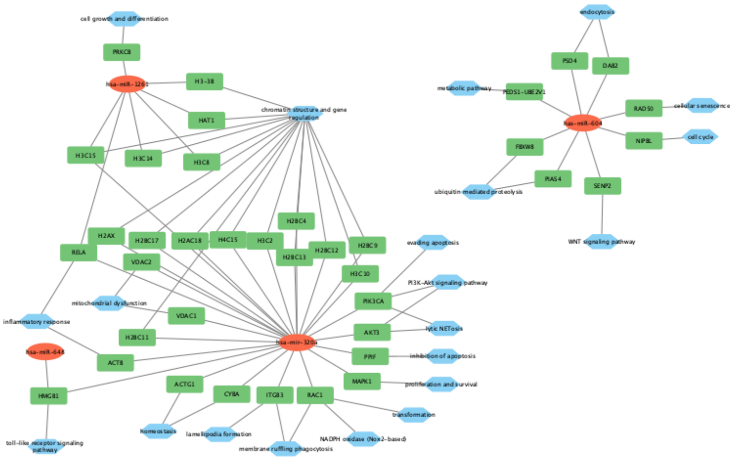

Figure 3 Mapping of genes and biological pathways of cancer targeted by exomiRs in locally advanced lung adenocarcinoma compared to healthy controls. Four exomiRs showing significant increases (miR-604, miR-320a, miR-648, miR-1261) were analyzed using R tools to identify validated target genes with significant expression (figure created using Cytoscape). Most genes targeted by miR-320a are involved in tumor-infiltrating immune cell signaling, including the RELA proto-oncogene, NF-κB subunit (*RELA*), actin beta (*ACTB*), high mobility group box 1 (*HMGB1*), integrin subunit beta 3 (*ITGB3*), Rac family small GTPase (*RAC1*), AKT serine/threonine kinase 3 (*AKT3*), and phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit alpha (*PIK3CA*).

ExomiR-320a was found to target histone cluster genes (*H3C15*, *H3C14*, *H3C2*, *H2BC4* and *H2BC12*) and chromatin regulators such as *HAT1*, linking its expression to epigenetic regulation in lung adenocarcinoma. Dysregulation of histone genes and acetylation balance is strongly associated with genomic instability and tumor progression [50,51]. In addition, exomiR-320a regulates genes involved in immune-related signaling, including *RELA (NF-κB), HMGB1, ITGB3, RAC1, AKT3*, and *PIK3CA*, which are critical for inflammation [52-54], *PI3K-AKT* signaling, and tumor-immune interactions. These findings suggest that exomiR-320a plays a dual role in both chromatin remodeling and modulation of the tumor microenvironment. Clinically, this highlights its potential as a non-invasive diagnostic biomarker, while also suggesting prognostic relevance in predicting disease aggressiveness and therapeutic resistance.

Mapping of genes targeted by exomiR-320a based on previous research

Based on validated studies, exomiR-320a has been shown to target genes involved in several key oncogenic pathways. These include immune system regulation, ranging from cancer growth inhibition, to maintaining equilibrium, and ultimately driving immune escape, as well as cell cycle control that promotes proliferation, and signaling pathways that facilitate migration and invasion. Collectively, these molecular effects contribute to cancer progression (**Figure 4**).

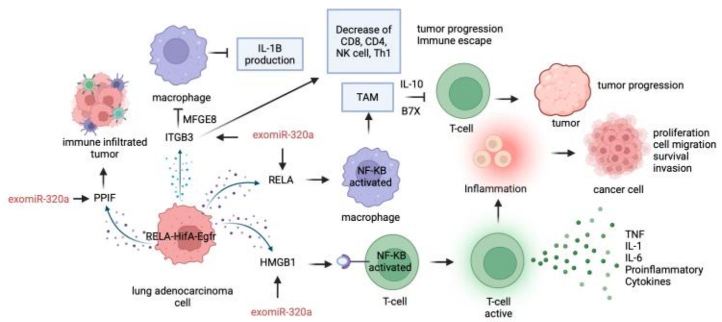

Figure 4 Schematic representation of the genes and signaling pathways targeted by exomiR-320a. ExomiR-320a targets genes involved in immune processes that support cancer progression, leading to immune escape. Immune signaling pathways create a tumor microenvironment that can support tumor development. Chronic inflammation caused by the genes *HMGB1, RELA*, and *ITGB3* induces proliferation, migration, and invasion of tumor cells.

MiR-320 has been shown in previous studies to act as a tumor suppressor, thereby targeting oncogenes. However, in this study, the expression of miR-320 will be validated in Indonesian patients with lung adenocarcinoma, thereby confirming the profiling results as an oncomiR. The development of cancer begins with the initiation, promotion, progression, and metastasis stages, which occur at different times depending on the type of cancer, as well as internal and external factors that influence it [55]. ExomiRs expression in locally advanced lung adenocarcinoma compared to healthy tissue represents the promotion and progression stages.

The initiation stage of lung adenocarcinoma is very difficult to study in terms of exomiRs expression, target genes, and carcinogenesis pathways because most lung cancer patients in Indonesia are already in advanced stages. The initiation stage is characterized by irreversible genetic changes, causing cells to proliferate continuously and form tumor cell clones [56]. The initiation stage marks the beginning of the disruption of the balance between the immune system attacking cancer cells and the immune system undergoing evasion (tolerance toward cancer cells) [57].

The tumor microenvironment drives tumor cell development. Components of the tumor microenvironment, including cytokine accumulation, chemokine secretion, reactive oxygen species (ROS) secretion, growth factors, and pro-angiogenic mediators, accelerate the initiation stage into tumor progression [57]. This aligns with the genes and biological pathways targeted by exomiRs in lung adenocarcinoma, particularly miR-320a, which is involved in cell proliferation and the tumor microenvironment through the secretion of pro-inflammatory cytokines and suppression of the immune system, ultimately leading to immune escape and cancer progression.

During the promotion phase, genetic changes cause cells to proliferate, forming neoplasms. The progression phase involves additional genetic and epigenetic changes that drive the cancer to an advanced stage. During this phase, cancer cells undergo clonal selection, characterized by enhanced survival, invasion, and metastasis capabilities. The advanced stage is characterized by cells undergoing proliferation and invasion, angiogenesis, and metastasis [55]. The processes of proliferation and differentiation are marked by the activation of signal transduction from the *Wnt/β-catenin* and *MAPK/ERK* signaling pathways. The *PI3K/AKT/mTOR* signaling pathway causes increased cell growth, survival, and metabolism. TGF-β functions in cell growth and differentiation. The *JAK/STAT* signaling pathway is frequently activated by cytokines and growth factors, playing a role in inflammatory events. The *NOTCH* signaling pathway influences cell proliferation, repair, and apoptosis [55].

Kyoto encyclopedia of genes and genomes pathways, gene ontology, and genes targeted by exomiRs are upregulated in locally advanced lung adenocarcinoma

Based on pathway enrichment analysis using KEGG, a total of 42 pathways were identified and classified into 5 categories: Environmental information processing (6 pathways), human diseases (22 pathways), organismal systems (9 pathways), genetic information processing (1 pathway), and cellular processes (4 pathways). From these, 10 pathways were selected according to the highest gene ratios, with neutrophil extracellular trap formation emerging as the most significantly affected pathway in locally advanced lung adenocarcinoma compared to healthy controls (**Figure 5**).

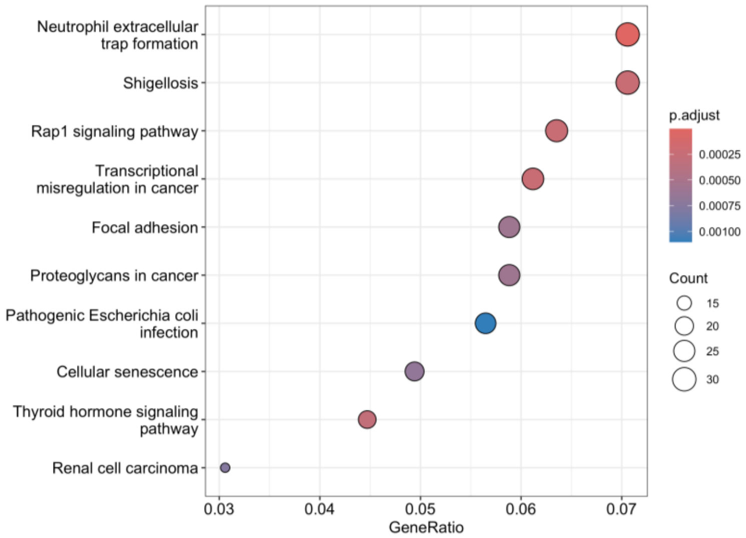

Figure 5 Top ten validated biological activities based on gene ratio targeted by upregulated exomiRs in locally advanced lung adenocarcinoma compared to healthy controls.

Neutrophils are abundant immune cells that are the first to arrive at the site of infection. Neutrophil activation leads to the secretion of pro-inflammatory cytokines, causing inflammation in the tumor microenvironment (TME), triggering tumor cell proliferation, survival, immune evasion, and migration. Neutrophils are activated by microbial extracellular components, PMA, LPS, IL-8, and calcium. Neutrophil activation leads to the activation of the protein kinase C (PKC) signaling pathway to *MEK/Erk*, resulting in *NOX2* phosphorylation [58], *JKN, PI3K* to Akt signaling also causes *NOX2* to undergo phosphorylation, which in turn leads to the formation of ROS, causing neutrophil elastase (NE) and myeloperoxidase (MPO) granules to translocate to the nucleus and cause chromatin de-condensation (chromatin unfolding) and damage to the nuclear membrane, with the help of calcium-dependent protein arginine deiminase type 4 (*PAD4*), which citrullinates histones. The unfolded chromatin mixes with granule proteins, is first released into the cytoplasm, and then exits the cell membrane, forming Neutrophil Extracellular Traps (NETs) [59]. Affected pathway based on KEGG related to immune regulation aligns with the previous study finding of the interaction of exomiR-320a interactions with target genes involved in tumor-infiltrating immune cells, thereby influencing the tumor microenvironment and causing tumor cell proliferation and invasion [60,61], as illustrated in **Figure 4**.

Based on gene ontology analysis, a total of ten biological processes with significant adjusted p-values were identified out of 214 processes targeted by upregulated exomiRs. Among the biological processes, cytoplasmic translation exhibited the lowest adjusted p-value; however, the process with the largest number of targeted genes was positive regulation of phosphorylation (**Figure 6(A)**). Furthermore, a total of 30 targeted genes, 4 genes were most frequently regulated by upregulated exomiRs across various biological processes, including *H3C15*, followed by *H3C14*, *HAT1*, and *VDAC1* **(Figure 6(B)**).

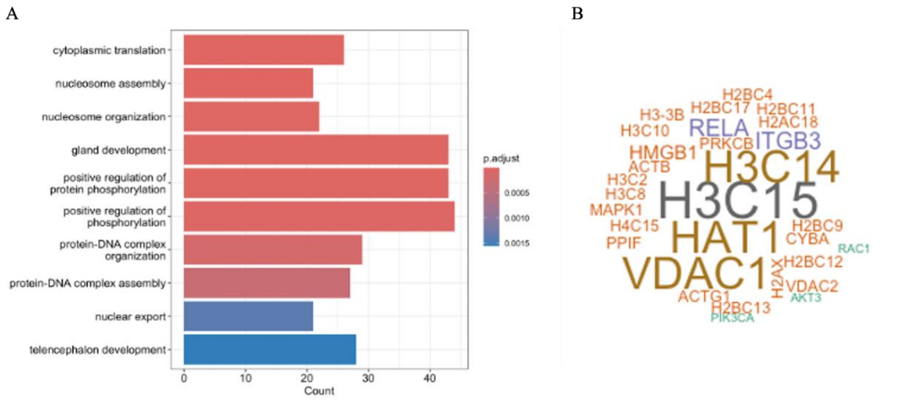

Figure 6 Top ten biological process based on p-adjust that targeted upregulated exomiRs in locally advanced lung adenocarcinoma available in bar plots (A) and targeted genes available in word clouds (B).

This finding is consistent with the role of miRNAs as post-transcriptional silencers of mRNA, thereby preventing translation. The translation of mRNA into protein represents a critical step in gene expression. Under cancerous conditions, numerous oncogenes are translated into proteins that produce factors promoting tumor development and progression. Cancer promoting factors regulate cyclin proteins, anti-apoptotic factors, pro-angiogenic factors, cellular metabolism, pro-metastatic factors, immune modulators, and proteins involved in DNA repair. Changes in the expression of initiation factors during translation were first identified in cancer cells, showing overexpression of the initiation factor eukaryotic translation initiation factor 4E (*eIF4E*), which transforms NIH 3T3 cells *in vitro*. Eukaryotic translation initiation factor 4E (*eIF4E*) acts as an oncogene [62].

The gene targeting results by exomiR-320a, exomiR-648, exomiR-1261, and exomiR-604, which target *H3C15*, illustrated with the results of target gene interaction analysis (**Figure 3**). *H3C15* is a gene involved in histone protein translation. Mutations in histone-modifying enzymes and histone-encoding genes occur in cancer cells, leading to changes in chromatin methylation patterns, which contribute to tumor development and metastasis [50]. Abnormal histone modifications and the enzymes involved in them, when their expression is altered, are responsible for tumor cell proliferation, invasion, apoptosis, and stemness [63].

This study has limitations because it did not perform profiling and validation of target genes in Indonesian patients with lung adenocarcinoma, so the genes and signaling pathways targeted are based on *in silico* analysis. However, the expression of exomiRs from profiling Indonesian patients with lung adenocarcinoma has novelty and uniqueness. ExomiR-320a has been reported in many published studies as a tumor suppressor miRNA, whereas in this study, its expression increased. Further research is needed to develop a profile of genes and proteins that show increased or decreased expression in Indonesian patients with lung adenocarcinoma.

The expression of exomiR-320a was validated in patients with lung adenocarcinoma compared to healthy individuals

The selection of exomiR-320a was based on the consideration that this exomiR showed a 1.8-fold increase in expression compared to healthy controls after quantification using Nanostring. Additionally, bioinformatics analysis using R showed that hsa-miR-320a has numerous target genes and is involved in carcinogenesis signaling compared to 3 other exomiRs (hsa-miR-648, hsa-miR-604, hsa-miR-1261). Analysis using Wilcoxon test showed significant differences in exomiR-320a expression among healthy controls, locally advanced lung adenocarcinoma, and metastatic lung adenocarcinoma.

Analysis of the difference in exomiR-320a expression in lung adenocarcinoma compared to healthy controls using Wilcoxon test showed that there was a significant difference (p-value < 0.05) in exomiR-320a expression in metastatic lung adenocarcinoma compared to healthy controls (**Figure 7**). However, there was no significant difference (p-value > 0.05) in the expression of exomiR-320a in locally advanced lung adenocarcinoma compared to healthy controls or between metastatic and locally advanced cases. Based on these findings, it can be concluded that the expression of exomiR-320a can distinguish metastatic conditions in lung adenocarcinoma patients compared to healthy controls, but cannot yet be used to distinguish pre-metastatic conditions. ExomiR-320a have the potential to be developed as a progression biomarker to assess metastatic conditions in lung adenocarcinoma.

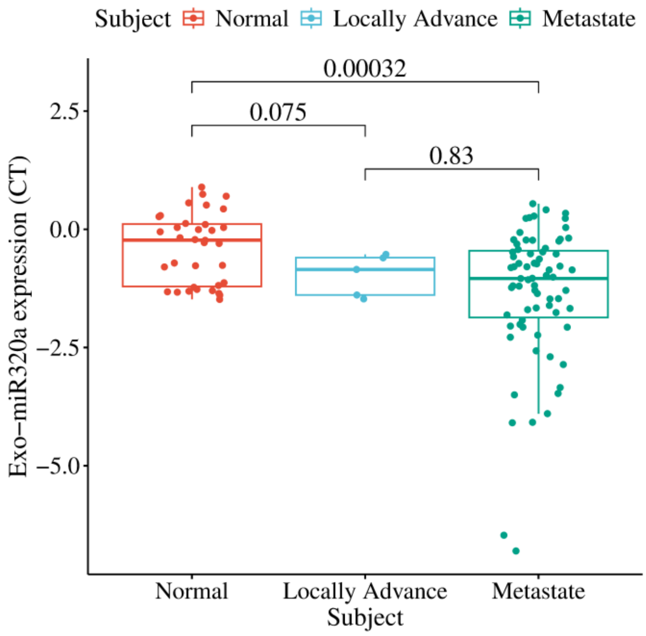

Figure 7 Expression of exomiR-320a validated using qRT-PCR in locally advanced and metastatic lung adenocarcinoma compared to healthy controls. The Y-axis represents the ΔCt values of exomiR-320a expression, while the X-axis represents the sample groups: healthy controls (normal), locally advanced lung adenocarcinoma, and metastatic lung adenocarcinoma.

The mean ΔCt value of exomiR-320a was lower in the metastatic group (–1.361) compared with healthy controls (–0.399), indicating higher expression. Similarly, the mean ΔCt value in the locally advanced group (–0.840) was also lower than in healthy controls (–0.399). Since lower ΔCt values reflect higher expression, these results indicate that exomiR-320a expression was elevated in both locally advanced and metastatic lung adenocarcinoma compared with healthy individuals.

ExomiR-320a may serve as a potential diagnostic biomarker for lung adenocarcinoma and could also be developed as a marker to predict disease severity based on clinical stage. This is supported by the observed increase in expression from locally advanced to metastatic disease, suggesting that higher exomiR-320a levels are associated with more advanced disease stages. However, validation in early-stage lung adenocarcinoma could not be performed due to the unavailability of patient samples at this stage. Fold-change analysis further confirmed these findings. Validation of exomiR-320a expression in lung adenocarcinoma showed an increase in expression up to 1.902 times compared to healthy controls (Livak method). Based on this, there is consistency in the increased expression of exomiR-320a in lung adenocarcinoma patients compared to healthy controls. ExomiR-320a expression increased 1.358-fold in locally advanced adenocarcinoma compared with healthy controls, 1.948-fold in metastatic adenocarcinoma compared with healthy controls, and 1.435-fold in metastatic compared with locally advanced adenocarcinoma. These results indicate that exomiR-320a has potential not only as a diagnostic biomarker but also as a contributor to the development of non-invasive approaches for early detection and disease monitoring in lung adenocarcinoma.

The expression of exomiR-320a is unique in the Indonesian population, showing an increase in lung adenocarcinoma conditions, thereby indicating its role as an oncomiR that inhibits tumor suppressor genes. However, most studies conducted outside Indonesia indicate that exomiR-320a expression decreases in cancer conditions, with most validated targets being oncogenes [64-69]. The progressive increase in exomiR-320a expression observed from locally advanced disease to metastasis suggests its role in reflecting tumor aggressiveness and disease progression. Circulating exomiR, in addition to its diagnostic role, also has the potential to predict therapeutic response in cancer. Although our study focused on newly diagnosed patients who had not yet received treatment and therefore did not evaluate treatment response, several studies have demonstrated the role of circulating and tumor-specific exomiR as predictors of sensitivity or resistance to chemotherapy. Recent evidence highlights that certain specific miRNAs can modulate drug response pathways and influence therapeutic outcomes in cancer patients [70-73]. The exomiR-320a identified in our study also has the potential to serve as a predictive biomarker of therapeutic response, either independently or in combination with other miRNAs, although this requires further investigation in prospective cohorts. Discussing the role of exomiR-320a in monitoring therapy effectiveness could broaden the clinical implications of our findings.

Studies using miRNAs as biomarkers to investigate their involvement in chemoresistance have shown promising results. In triple-negative breast cancer (TNBC), a total of 12 miRNAs were found to be either upregulated or downregulated and were correlated with chemoresistance in tumor tissues compared with chemosensitive tissues [72]. Previous research also demonstrated that miR-320a induces resistance to 5-fluorouracil (5-FU) in pancreatic cancer cells. Overexpression of miR-320a contributes to the pathogenesis of pancreatic cancer by promoting proliferation, invasion, metastasis, and chemoresistant characteristics [74]. These findings further highlight the potential role of exomiRs as therapeutic targets. Although the present study did not analyze the prognostic potential of exomiR-320a, several reports suggest that it may serve this purpose. Low circulating miR-320a expression in non-small cell lung cancer (NSCLC) patients has been associated with poorer prognosis and reduced survival rates compared to patients with higher expression [75]. Furthermore, exomiRs or miRNAs circulated can be explored as prognostic biomarkers by integrating survival analysis in lung cancer patients. Several genes involved in lung cancer survival, such as *ALDOC*, *SNRPG*, and *FN1* [76], are regulated by miRNAs, further supporting their potential utility as prognostic biomarkers.

Receiver operating characteristic (ROC) curve analysis of ΔCt data from patients with lung adenocarcinoma compared to healthy controls yielded an AUC value of 0.718 (**Figure 8**). This value is interpreted as moderate [77], so exomiR-320a can be considered as a diagnostic biomarker but must be combined with other exomiR biomarkers. ExomiR-320a have moderate discriminatory power to distinguish lung adenocarcinoma patients from healthy controls. While this suggests potential clinical utility, it is unlikely that exomiR-320a alone would be sufficient as a stand-alone diagnostic marker in routine practice. Instead, its translational relevance may lie in being part of a biomarker panel that integrates multiple circulating miRNAs or combines exomiRs signatures with established diagnostic tools such as imaging and protein-based assays. Such multi marker approaches have been reported to substantially improve both sensitivity and specificity, thereby increasing clinical applicability. ExomiRs obtained from liquid biopsies are important to develop because they are minimally invasive and compatible with qRT-PCR platforms that are already widely available in clinical laboratories, supporting the feasibility of future clinical implementation. Further large-scale validation studies are warranted to confirm these findings and optimize the diagnostic model before translation into practice.

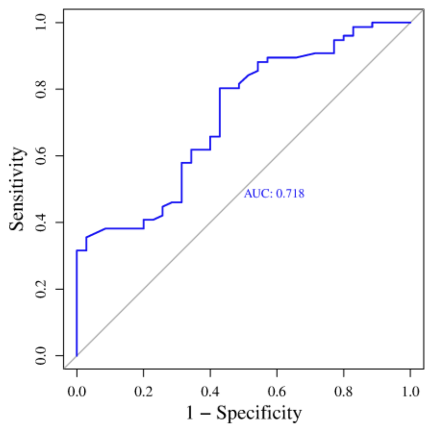

Figure 8 ROC analysis of exomiR-320a expression validated in locally advanced and metastatic lung adenocarcinoma compared to healthy controls.

The hsa-miR-320a encoding gene is located on chromosome 8, short arm region 2, band 1, sub-band 3. The role of miR-320a as an oncogene is evident from several studies on cancer. Hsa-miR-320a has been identified as the optimal upstream miRNA regulator of *CISD2*. MiR-320a inhibits *CISD2* expression by directly binding to the 3'UTR region, resulting in a negative correlation between the 2. MiR-320a, as a negative regulator of CDGSH iron-sulfur domain 2 (*CISD2*), participates in bone metastasis of lung adenocarcinoma via MYC activation and is associated with tumor infiltration into the immune system [78]. Hsa-miR-320 overexpression is associated with invasion and metastasis in ovarian cancer. miR-320a promotes epithelial ovarian cancer (EOC) cell proliferation and invasion by targeting RASSF8. The expression level of miR-320a in EOC specimens was found to be associated with ovarian cancer progression and infiltration. MiR-320a significantly promotes proliferation, migration, and invasion in EOC cells [79].

MiR-320a, a member of the miR-320 family, also plays a role as a tumor suppressor, so its dual role depends on the type of cancer, histopathology, and the target genes involved in the carcinogenesis process [80]. The role of miR-320a as a tumor suppressor miRNA is evident from previous studies, which showed that a decrease in miR-320a expression triggers proliferation and invasion in non-small cell lung carcinoma cells through increased *VDAC1* expression. Inhibiting *VDAC1* expression can suppress cell proliferation and invasion in NSCLC by reducing cellular energy and metabolism [80]. Research on miR-320a in LUAD demonstrates its role as a tumor suppressor. MiRNA-320 can regulate EMT by binding to the 3'UTR of EMT-related transcription factors (EMT-TFs). Additionally, miR-320a regulates EMT through several important signaling pathways such as *Wnt, PI3K/Akt*, and *TGF-β/Smad* signaling pathways. MiRNA-320 can act as a negative regulator of *TGF-β/Smad* signaling by regulating the expression of target genes [81].

In cancer types other than lung adenocarcinoma, serum exomiR-320a levels were significantly reduced in HCC patients compared to patients with chronic liver disease and healthy controls. The ROC curve indicates that serum exomiR-320a has good diagnostic value for distinguishing between HCC subjects and normal controls [82]. Downregulated miR-320a is associated with imatinib resistance in gastrointestinal stromal tumors [83]. MiRNA-320a also plays a role in the treatment process, either as a regulator causing resistance or sensitizing chemotherapy use. MiR-320a inhibits cell proliferation and induces apoptosis in A549 cells. Additionally, miR-320a inhibits the expression of both *STAT3* and *p-STAT3* [64]. Overexpression of miR-320 with pemetrexed effectively inhibits proliferation and invasion in human lung cancer cells (Calu-6) [68]. However, miR-320a also shows increased expression during the drug resistance process. miR-320a expression is induced by the demethylation agent 5-Aza-dc. These results suggest a role for miR-320a as a regulator of DDP resistance [84].

This study shows that there is a novel and unique exomiRs profile signature in Indonesian patients with lung adenocarcinoma compared to previous findings in various countries. ExomiRs with increased expression in locally advanced adenocarcinoma compared to healthy control target genes involved in chromatin structure formation and tumor-infiltrating immune cells that can influence the tumor microenvironment, thereby supporting tumor development. However, these genes, based on in silico analysis results, have not yet been validated. Further research that can be developed includes validating the genes targeted in silico to generate findings that can lead to biological processes for the development of therapeutic biomarkers. ExomiR-320a shows increased expression in lung adenocarcinoma, both in locally advanced and metastatic conditions, but the increase is significantly different in metastatic conditions compared to healthy controls. Based on AUC analysis, miR-320a has the potential to be developed as a diagnostic biomarker for lung adenocarcinoma, but it must be combined with other biomarkers such as miR-34 and miR-155, which have been validated in lung cancer in the Indonesian population [85]. Our findings suggest that exomiR-320a holds promise not only as a minimally invasive diagnostic biomarker for lung adenocarcinoma but also as a potential candidate for future studies exploring its role in predicting treatment response.

Conclusions

ExomiRs profiling in patients with locally advanced lung adenocarcinoma showed increased expression in exosomes miR-320a, miR-604, miR-1261, and miR-648 and decreased expression in exosomes miR-301b-3p, miR-605-5p, miR-924, and miR-769-5p. miR-373-3p, miR-520h, miR-193a-5p, miR-3150b-3p, miR-3615, miR-4431, miR-6720-3p, miR-1909-3p, miR-574-3p, miR-382-5p, miR-593-3p, miR-572, miR-27a-3p, miR-411-5p compared to healthy controls. ExomiRs upregulation in locally advanced lung adenocarcinoma, as shown by in silico analysis, targets signaling pathways involved in chromatin structure formation and tumor-infiltrating immune cells. Validation of miR-320a showed a 1.9-fold increase in expression in lung adenocarcinoma compared to healthy controls. ExomiR-320a is ready for clinical application, while the AUC of 0.781 ($p = 0.00032$) is considered only moderately potent. ExomiR-320a a promising marker that requires further validation as a diagnostic biomarker in lung adenocarcinoma.

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Declaration of generative AI in scientific writing

This manuscript only uses AI (deepL) for language translation, not for manuscript development.

CRediT author statement

Aprilia I Kartika: Conceptualization; Investigation; Methodology; Software; Writing - Original Draft. **Muchamad Dafip**: Data curation; Formal analysis**.** **Nastiti Wijayanti**: Methodology; Supervision. **Didik S. Heriyanto**: Resources; Supervision. **Kartika W. Taroeno-Hariadi**: Resources; Supervision. **Monica D Hartanti**: Project administration; Funding acquisition. **Sunarno Sunarno**: Project administration; Funding acquisition. **Sofia M. Haryana**: Conceptualization; Supervision; Reviewing and Editing.

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