

The Expression of miR-320b in Extracellular Vesicles as a Predictor of the Progression of Non-small Cell Lung Carcinoma and Its Association with Molecular and Clinical Therapy Response

Research Article

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Abstract: **Background:** Lung carcinoma remains a predominant contributor to cancer-related mortality globally. MicroRNAs (miRNAs) are pivotal regulators in carcinogenesis. Specifically, miR-320b is a tumor suppressor in the oncogenic process. This investigation probes the expression dynamics of miR-320b within extracellular vesicles (EVs) posttherapy in non-small cell lung cancer (NSCLC), correlating these patterns with differential therapeutic outcomes.

Patients and Methods: This analytical study employs a prospective cohort methodology including 16 advanced-stage NSCLC patients at Dharmas Cancer Hospital from November 2021 to August 2022. We obtained 12 ml of peripheral blood sequentially, pre- and post-therapy, encompassing chemotherapy, targeted, or radiotherapy. EVs were isolated utilizing the IZON qEV2 column, quantified via spectrophotometry, and further characterized using confocal microscopy. Statistical analyses were conducted with Statistical Package for the Social Sciences version 24.0.

Results: Participants ($N = 16$; mean age 53.44 ± 10.92 years; 68.8% male) predominantly received chemotherapy (81.2%). Post-therapeutic evaluation revealed nonsignificant elevation in miR-320b levels ($P > 0.05$). Distinctly, the partial response cohort exhibited a median 28% reduction in miR-320b, while the stable disease subset saw a 21% decrease and the progressive disease faction experienced a 29% augmentation. However, these changes bore no statistically significant correlation with treatment modality or response ($P > 0.05$).

Conclusions: Alterations in miR-320b expression within EVs posttherapy – decreases in the partial and stable disease cohorts and an increase in the progressive disease group – suggest a potential compensatory mechanism within the tumor milieu in reaction to therapeutic interventions. These statistically nonsignificant fluctuations underscore the complexity of miR-320b's role and its prospective utility in gauging NSCLC progression.

Keywords: miR-320b • NSCLC • tumor microenvironment • extracellular vesicles • therapeutic response

1. Introduction

Lung cancer is a malignant disease that originates from the epithelium of the respiratory tract and can spread to lymph nodes or other organs such as the brain. It is one of the leading causes of cancer and the

leading cause of cancer-related deaths in both men and women worldwide, with 2.2 million new cases and 1.8 million deaths. Lung cancer is divided into two types: non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC). Detection of lung cancer can be done using imaging techniques such as low-

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dose computed tomography (LDCT). However, LDCT has limitations when used as the sole screening tool, including a high false-positive rate of 96.4% and the risk of radiation exposure. The gold standard for diagnosing lung cancer is tissue biopsy, but this procedure also has limitations due to the difficult accessibility of tumor locations during biopsy. Another approach being developed as an alternative and minimally invasive method is liquid biopsy. Liquid biopsy has the potential to be used for screening, early diagnosis, treatment response monitoring, and prognostic evaluation in lung cancer. It involves examining tumor biomarkers in the patient's blood, including exosomes, circulating tumor DNA (ctDNA), tumor-associated antigens (TAAs), tumor-educated platelets (TEPs), tumor-associated autoantibodies (TAAbs), circulating tumor cells (CTCs), and microRNAs (miRNAs).^[1-3]

Extracellular vesicles (EVs) are nanosized vesicles released by various types of cells. They have various biological activities related to homeostasis and cancer development. miRNAs are short, single-stranded, noncoding RNA molecules. The role of miRNAs in various types of cancer is being investigated because they are associated with cancer cell development, such as proliferation, invasion, metastasis, apoptosis, and autophagy. Previous studies have shown a relationship between altered miRNA expression and the occurrence of various types of cancer. This is evidenced by the differential expression of miRNAs in tumor tissue compared to normal tissue. One specific miRNA, miR-320b, has been studied in various cancer tissues, including colorectal cancer, pancreatic cancer, glioblastoma, gastric cancer, and colorectal cancer. According to Cheng et al., increased miR-320b inhibits the proliferation, migration, and invasion of pancreatic cancer cells and induces apoptosis through the miR-320b/ARF1 axis. MiR-320b, in particular, is considered to play a role in the growth and development of lung cancer. A recent study stated that the expression of miR-320b can be used as a diagnostic and prognostic biomarker for lung cancer, especially NSCLC. This study aims to assess the potential role of miR-320b in evaluating therapy response and the molecular progression of NSCLC.^[4-9]

2. Methods

This study is an analytical study with a prospective cohort design to examine the role of miR-320b levels in predicting progression in NSCLC patients. The study was conducted at Dharmais Cancer Hospital from November 2021 to August 2022. The target population of this

study was NSCLC patients at tertiary referral hospitals in Indonesia from 2021 to 2022, while the accessible population of this study was lung cancer patients with pathological examination results indicating NSCLC who sought treatment at Dharmais Cancer Hospital during 2021–2022. Exclusion criteria were patients with primary tumors in locations other than the lungs and patients lost to follow-up. The samples that met the inclusion and exclusion criteria using consecutive sampling method were blood samples between two periods; before and after therapy (third-cycle chemotherapy, targeted therapy for 3 months, or have been finishing radiotherapy). After the blood samples were collected and centrifuged in a vacutainer containing ethylenediaminetetraacetic acid (EDTA), blood plasma was immediately used for extraction and isolation using EVs with utilizing IZON qEV2 column. Furthermore, the protein concentration was obtained by using a known equation through a standard curve quantified via spectrophotometry and was further characterized using confocal microscopy. This study was approved by the ethical committee of Dharmais National Cancer Center Hospital, Indonesia (No. 041/KEPK/IV/2021).

2.1. Statistical Analysis

All data collected was analyzed using International Business Machine Statistical Package for the Social Sciences statistics version 24 for Windows and presented in the form of tables and charts. Demographic and clinical characteristics were processed using univariate or descriptive analysis and presented as frequencies and percentages. Chi-square test was used to test the hypotheses for categorical data to examine the relationship between independent and dependent variables. If the Chi-square test did not meet the requirements, Fischer's exact test was used. A *P*-value of <0.05 was considered statistically significant.

3. Results

In this study, there were 16 research subjects with a mean age of 53.44 ± 10.92 years. The pathological examination results indicating NSCLC consisted of adenocarcinoma (14 samples, 87.5%) and squamous cell carcinoma (SCC; two samples, 12.5%). The research subjects consisted of 11 males (68.8%) and five females (31.3%). The average body mass index was 21.72 ± 3.70 kg/m². All research subjects were in stage IV and had no previous history of malignancy. Some subjects had comorbidities such as hypertension, diabetes mellitus, heart disease, and

cataracts. The majority of subjects (81.2%) did not have a history of coronavirus disease 2019 (COVID-19). Of the subjects, 81.2% underwent chemotherapy, while 18.8% underwent treatment with tyrosine kinase inhibitors (TKIs) (Table 1). The type of molecular mutations that received treatment with TKIs was epidermal growth factor receptor (EGFR) positive with mutation in exon 19. The symptoms found in patients before treatment included shortness of breath, back pain, lumps, chest pain, cough, coughing up blood, decreased appetite, nausea, vomiting, weight loss,

and hemiparesis. Based on the symptom parameters, the most common symptom before treatment was shortness of breath. Shortness of breath significantly decreased after treatment ($P < 0.05$) (Table 2). Other symptoms such as cough, hemoptysis, chest pain, and metastasis also decreased after treatment, but the decrease was not significant. Among the laboratory parameters, a significant decrease in leukocyte count was found after therapy compared to before therapy ($P < 0.05$) (Table 2). Other parameters, such as hemoglobin and platelets, also showed a decrease, but it was not significant (Table 2).

Table 1: Characteristic of Study Subjects.

Clinical Characteristics of Study Subjects (n = 16)	Mean $\pm$ SD	Frequency n (%)
Age (years)	53.44 $\pm$ 10.92	
Gender		
Male		11 (68.8)
Female		5 (31.3)
Weight (kg)	57.66 $\pm$ 10.54	
Height (m)	1.63 $\pm$ 0.09	
BMI	21.72 $\pm$ 3.70	
Duration (months)	9.44 $\pm$ 2.63	
Treatment option		
Chemotherapy		13 (81.3)
Tyrosine kinase inhibitor		3 (18.8)
COVID-19 history		
Present		3 (18.8)
None		13 (81.2)
Cancer history		
None		16 (100)
Stage		
Stage IV		16 (100)
Comorbidities		
Hypertension		1 (6.3)
Diabetes mellitus		2 (12.5)
Cardiac disease (CAD)		1 (6.3)
Cataract		1 (6.3)
None		11 (68.8)

3.1. Comparison of miR-320b Before and After Therapy

Posttherapy, miR-320b showed an increase, although it was not significant ($P > 0.05$) (Table 3). The relationship between therapy response and miR-320b level after therapy response ($P > 0.05$) is shown in Table 4.

3.2. Relationship Between miR-320b and Mortality

No significant difference in miR-320b levels was found between subjects who survived until the end of the observation period and subjects who died (Table 5).

3.3. Relationship Between Treatment Type and miR-320b Levels

No significant difference was found in miR-320b levels between patients undergoing chemotherapy and those undergoing TKI therapy (Table 6).

Table 2: Symptoms and Laboratory Parameters of Research Subjects.

Parameters	Before therapy n (%)	After therapy n (%)	Before therapy (mean $\pm$ SD)	After therapy (mean $\pm$ SD)	P
Symptoms					
Cough	3 (18.8)	1 (6.3)			0.625*
Hemoptysis	4 (25.0)	0 (0)			0.125*
Shortness of breath	10 (62.5)	1 (6.3)			0.004*
Chest pain	2 (12.5)	0 (0)			0.500*
Metastasis symptoms	6 (37.5)	2 (12.5)			0.125*
Laboratory					
Hemoglobin			12.38 $\pm$ 2.02	11.52 $\pm$ 1.94	0.122**
Leucocytes			10.90 $\pm$ 5.82	8.52 $\pm$ 4.88	0.034**
Platelets			377.56 $\pm$ 151.36	359.50 $\pm$ 154.30	0.650***

*McNemar Test

**Paired T-Test

***Wilcoxon Signed-Rank Test

Table 3: miR-320b Parameter Before and After Therapy.

miR-320b Before Therapy (mean $\pm$ SD)	miR-320b After Therapy (mean $\pm$ SD)	P***
1.00 $\pm$ 0.0	1.72 $\pm$ 2.40	0.856

***Wilcoxon signed-rank test

Table 4: Relationship between Treatment Response and miR-320b Levels After Therapy.

miR-320b PR group (med (min - max))	miR-320b SD group (med (min - max))	miR-320b PD group (med (min - max))	P****
0.72 (0.38 - 4.55)	0.79 (0.14 - 9.70)	1.29 (0.52 - 1.66)	0.767

****Kruskal-Wallis Test

Table 5: Relationship between miR-320b and Mortality.

Mortality	miR-320b (med (min - max))	P*****
Yes	0.93 (0.14 - 2.76)	0.791
No	0.85 (0.34 - 9.7)	

*****Mann-Whitney U Test

Table 6: Relationship between Treatment Type and miR-320b Levels.

Treatment	miR-320b (med (min - max))	P*****
Chemotherapy	0.93 (0.14 - 9.70)	0.737
TKI	0.45 (0.38 - 4.55)	

***** Mann-Whitney U Test

3.4. Survival of Research Subjects

Overall, the mean survival of research subjects was 11.57 months with a median of 13.00 months. No significant difference in survival was found between subjects receiving chemotherapy and those receiving TKI therapy ($P < 0.249$) (Figures 1 and 2).

3.5. ROC Curve of the Relationship between miR-320b and Treatment Response

Based on the receiver operating characteristic (ROC) curve analysis, the area under the curve for miR-320b in predicting treatment response was 0.450 (Figure 3). The sensitivity and specificity of miR-320b levels for various threshold values are shown in Table 7.

4. Discussion

NSCLC accounts for 85% of all lung cancer cases. NSCLC comprises several histological types, including

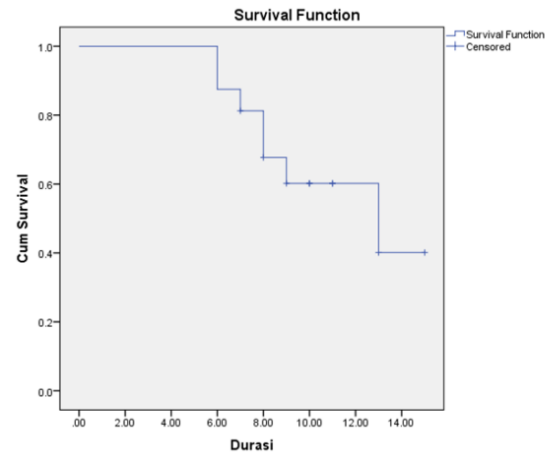


Figure 1: Overall Survival of Research Subject.

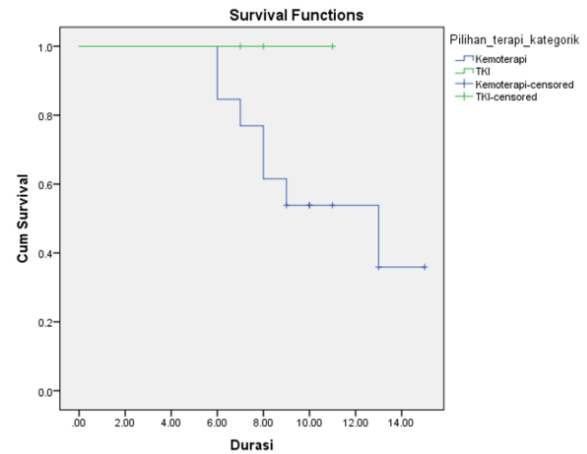


Figure 2: Survival of Research Subjects undergoing Chemotherapy and Tyrosine Kinase Inhibitor Therapy.

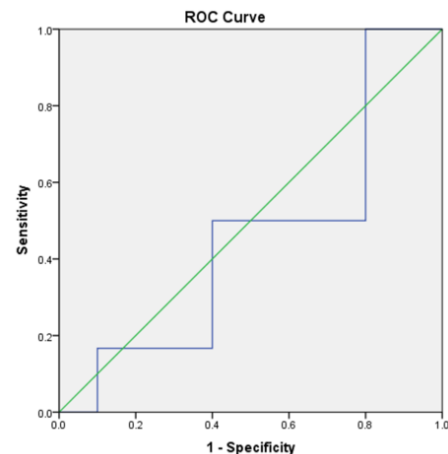


Figure 3: ROC Curve of the Relationship between miR-320b and Treatment Response.

Table 7: Sensitivity and Specificity of miR-320b for Predicting Treatment Response.

Positive for CR/PR if miR-320B is greater than or equal to	Sensitivity	1 - Specificity
0.240	1.000	0.900
0.360	1.000	0.800
0.400	0.833	0.800
0.435	0.667	0.800
0.485	0.500	0.800
0.620	0.500	0.700
0.785	0.500	0.600
0.890	0.500	0.500
0.960	0.500	0.400
1.195	0.333	0.400
1.525	0.167	0.400
1.655	0.167	0.300
2.210	0.167	0.200
3.655	0.167	0.100

SCC, large cell carcinoma, and adenocarcinoma, which together make up 87% of all lung cancer cases. NSCLC is a heterogeneous disease caused by complex molecular aberrations. According to the National Lung Screening Trial, LDCT can be used for the detection of NSCLC. However, LDCT has shown a high rate of false positives and low specificity for early detection of lung cancer. Therefore, biomarker testing in body fluids becomes important for early diagnosis of lung cancer. Molecular changes in epithelial cells in the bronchi have the potential to be developed as biomarkers for the detection of NSCLC. Sputum samples can be used to obtain bronchial epithelial cells. Examination of bronchial epithelial cells in the sputum can identify molecular changes associated with tumors to diagnose NSCLC, such as the miRNAs miR-31-5p and 210-3p. Biomarker testing can also be performed using blood samples, such as the miRNA miR-21-5p, which has a sensitivity of 85.5% and specificity of 91.7% for diagnosing NSCLC.^[7-9]

This study involved 16 research subjects with an average age of 53.44 ± 10.92 years. Of the research subjects, 68.8% were male, with an average body mass index of 21.72 ± 3.70 kg/m². The most common clinical symptoms found were shortness of breath (62.5%), followed by coughing up blood (25%) and cough (18.75%). This is consistent with a previous study by Iyer et al., which stated that shortness of breath was found in 95% of patients with NSCLC, followed by cough (93%), pain (92%), and coughing up blood (63%). Each patient received therapy with chemotherapy or TKIs^[10]. After therapy, the patients experienced significant improvement in shortness of breath symptoms ($P < 0.05$). Other symptoms such as cough, hemoptysis,

chest pain, and metastasis symptoms also showed improvement, although the difference was not statistically significant. In terms of laboratory parameters, the patients showed a significant decrease in white blood cell count after therapy ($P < 0.05$). Hemoglobin and platelet concentrations decreased, but not significantly.^[9,10]

miRNAs are endogenous noncoding, single-stranded RNA molecules that can regulate gene expression. miRNAs play an important role in various biological and pathological processes, including inflammation and cancer. miR-320 functions as a tumor suppressor in tumorigenesis and progression of various types of cancer. The expression of miR-320 has been studied in several types of cancer, such as breast cancer, SCC of the oral cavity, colon cancer, leukemia, glioma, and osteosarcoma. According to Lei et al., miR-320 expression in NSCLC cells is decreased compared to adjacent normal tissue. Statistical analysis also shows that miR-320 expression is significantly associated with TNM classification and metastasis. miR-320 inhibits cell growth, migration, and invasion of NSCLC cells. miR-320 targets the enzyme involved in fatty acid synthesis, which is called fatty acid synthase (FAS).^[7] FAS is an enzyme complex with various functions. Endogenous FAS expression is found to be low in normal human tissue. However, FAS expression is increased in various types of tumors. FAS is also associated with various clinicopathological characteristics of cancer. Excessive FAS expression in NSCLC is significantly associated with bone metastasis. Therefore, therapies targeting FAS have the potential to be used in patients with NSCLC.^[11-13]

After therapy, the levels of miR-320B show an increase. Based on the therapy response, miR-320b levels do not show significant differences between the partial response (PR), stable disease (SD), and progressive disease (PD) groups. However, the median miR-320 in the PR group is lower than in the SD group, and the median miR-320 in the SD group is lower than in the PD group. Research by Peng et al. showed findings similar to those of this study. Peng et al. reported that the expression of hsa-miR-320b is higher in the PD group compared to the PR group in patients with NSCLC who received PD-1/PD-L1 inhibitors.^[14,15]

Overall, the average survival and median survival of the research subjects are 11.57 and 13.00 months, respectively. The survival of the research subjects is longer compared to that reported in a previous study by Lou et al., which stated that the median survival of patients with stage IV NSCLC is 5.9 months.^[16]

In this study, it was found that the median level of miR-320B in patients who survived at the end of the

observation period was not significantly different from that of patients who died at the end of the observation period. This is different from a previous study by Ma et al., which stated that decreased miR-320b is associated with worse patient survival. According to Ma et al., excessive miR-320b expression not only reduces cancer cell invasion, but also decreases tumor volume and angiogenesis. miR-320b inhibits HNF4G, reduces the expression of IGF2BP2, and TKI.^[6]

In this study, no significant association was found between the type of therapy and miR-320b levels. However, miR-320b levels are lower in patients treated with TKIs compared to the levels in patients treated with chemotherapy. No mortality was observed until the end of the observation period in patients receiving TKI therapy. In the group of patients receiving chemotherapy, seven deaths (53.85%) were recorded at the end of the observation period. A previous study by Tfayli et al. indicated that therapy with EGFR TKIs shows better outcomes compared to chemotherapy in the population of patients with EGFR gene mutations.^[17]

To summarize, the research findings indicate that miR-320 plays a significant role in NSCLC. Decreased expression of miR-320 is observed in NSCLC cells compared to normal tissue, and its expression is associated with TNM classification and metastasis. miR-320 acts as a tumor suppressor by inhibiting cell growth, migration, and invasion in NSCLC, possibly by targeting FAS. The study also suggests that miR-320 levels may be used as a potential biomarker for therapeutic response and prognosis in NSCLC patients.

The study has limitations that need consideration. Firstly, the small sample size of 16 NSCLC patients raises concerns about generalizability and compromises statistical power. The study lacks clarity on controlling confounding variables such as age, gender, smoking status, cancer stage, and prior treatments. The duration of patient observation may be insufficient to fully understand the impact of miR-320b on prognosis or therapeutic response. In addition, while an association between miR-320b and NSCLC is identified, the study lacks detailed exploration of the specific biological mechanisms underlying this relationship.

It is important to note that these findings are based on the specific research cited and may not represent the entirety of scientific knowledge on the subject. Further studies and clinical trials are needed to validate and expand upon these findings to develop effective therapeutic strategies for NSCLC.

List of Abbreviations

MED: median
MIN: minimum
MAX: maximum
miRNAs: microRNAs
NSCLC: non-small cell lung cancer
SCLC: small cell lung carcinoma
EVs: extracellular vesicles
TKI: tyrosine kinase inhibitor
LDCT: low-dose computerized tomography
TAAs: tumor-associated antigens
TEP: tumor-educated platelets
TAAbs: tumor-associated autoantibodies
CTCs: circulating tumor cells
EGFR: epidermal growth factor receptor
FAS: fatty acid synthase
PR: partial response
SD: stable disease
PD: progressive disease
CR: complete response

Author's Contributions

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Conflict of Interest

All authors declare no conflict of interest.

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