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# CEA and Cyfra 21-1 linked to serial miRNA expressions of advanced-stage non-small cell lung cancer in Indonesia

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## ABSTRACT

Globally, lung cancer is one of the cancers leading to dead, dominated by non-small cell lung cancer (NSCLC). In a previous study has shown those serial miRNA expressions (miR-148, miR-34, miR-222, and miR-155) had prognostic value in advanced-stage NSCLC patients. Meanwhile, CEA and Cyfra 21-1, pulmonary tumor markers, are sometimes considered in the Department of Pulmonology, Dharmais Cancer Hospital, Jakarta, although they are not used in routine clinics for prognostication. Both miRNA and CEA-Cyfra 21-1 are valuable biomarkers in NSCLC. This study aimed to evaluate their correlation between CEA and/or Cyfra 21-1 with miRNA expressions in NSCLC patients. It was a cohort retrospective study using data from the previous study. The correlation between variables was analyzed by Spearman-rho. A positive correlation was observed between CEA and Cyfra 21-1 with miR-148, miR-222, and miR-155 [(CEA: p=0.00369, r=0.522; p=0.00242, r=0.542; p 0.00106, r=0.576) (Cyfra: 21-1= p 0.01252, r=0.378; p=0.00035, r=0.519; p=0.01532, r=0.368)]. In conclusion, CEA and Cyfra 21-1 correlate with miR-148, miR-222, and miR-155 expressions in advanced-stage NSCLC.

## ABSTRAK

Secara global, kanker paru merupakan salah satu kanker penyebab kematian yang didominasi oleh *non-small cell lung cancer* (NSCLC). Pada penelitian sebelumnya menunjukkan bahwa ekspresi serial miRNA (miR-148, miR-34, miR-222, dan miR-155) memiliki nilai prognostik pada pasien NSCLC stadium lanjut. Sementara itu, CEA dan Cyfra 21-1, penanda tumor paru, terkadang dipertimbangkan di Departemen Pulmonologi, Rumah Sakit Kanker Dharmais, Jakarta, meskipun tidak digunakan secara rutin di klinik untuk prognosis. miRNA dan CEA- Cyfra 21-1 adalah biomarker yang berharga pada pasien NSCLC. Penelitian ini bertujuan untuk mengkaji hubungan antara CEA dan/atau Cyfra 21-1 dengan ekspresi miRNA pada pasien NSCLC. Penelitian ini merupakan penelitian kohort retrospektif yang menggunakan data dari penelitian sebelumnya. Hubungan antar variabel dianalisis dengan Spearman-rho. Hubungan positif diamati antara CEA dan Cyfra 21-1 dengan miR-148, miR-222, dan miR-155 [(CEA: p= 0,00369, r=0,522; p=0.00242, r=0,542; p=0,00106, r=0,576) (Cyfra 21 -1: p=0,01252, r=0,378; p=0,00035, r=0,519; p=0,01532, r=0,368)]. Simpulan, CEA dan Cyfra 21-1 berkorelasi dengan ekspresi miR-148, miR-222, dan miR-155 pada NSCLC stadium lanjut.

***Keywords***:
advanced stage;
NSCLC;
CEA;
Cyfra-21-1;
miRNA

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## INTRODUCTION

The burden of lung cancer is still a serious concern worldwide. Global data showed that lung cancer is the second highest of estimated new cases in 2020 for both sexes and all ages.[1] The American Cancer Society estimates there will be approximately 127,070 deaths in the United States due to lung cancer in 2023.[2] In Indonesia, the latest data showed that lung cancer placed the third highest among other cancers in men.[3] Thus, the evaluation is needed due to its burden is still an issue.

During past few decades, cancer management has been improved by genomic medicine transformation into health services. After a big project in human genome revealed, it is impressive to know that human genome is mostly constituted by non-coding regions instead of protein coding genes. These regions have key roles in gene regulation. The most explored molecule nowadays is micro RNA (miRNA), one of the small (~22 nucleotides) non-coding RNAs, mainly acts in translational repression and target gene degradation.[4] During development, miRNAs are essentially needed for cell growth and differentiation.[5] Meanwhile in cancer cell, their expressions are excessively affected to promote cancer proliferation and apoptotic resistance.[6] Several previous studies revealed that miRNAs can be found in the blood stream as well as other fluids, such as human saliva and breast milk.[6,7] This favorable condition is usually used in detecting miRNA as the biomarker besides its precision result.

Tumor markers, such as carcinoembryonic antigen (CEA) and cytokeratin 19 fragements 21-1 (Cyfra 21-1) are considerably being tested among lung cancer cases.[8-10] CEA is a glycoprotein formed by the fetus's intestinal epithelial cells used as a tumor biomarker, especially for adenocarcinoma types, such as colorectal, gastric, and lung cancer.[9,10] Cyfra 21-1 measures the cytokeratin fragment 19 found in epithelial cells and is associated with epithelial cell cancer.[11,12] Several studies have found that the increase 37.1% of CEA and 68.5% of Cyfra 21-1 have a diagnostic value and role as a negative predictor in non-small cell lung cancer (NSCLC).[13,14]

In the previously study reported that serial miRNA expressions, such as miR-34, miR-148, miR-222, and miR-155 have prognostic values in NSCLC.[15,16] The miR-34, and miR-148 repress tumor growth and induce tumor cell apoptosis through regulation of some signaling pathways, such as p53 targeting and mitogen-activated protein kinase/Jun N-terminal kinase (MAPK/JNK) signaling.[17,18] Meanwhile, as miR-222 and miR-155 promote tumor cell proliferation and invasion,[19,20] they implied in the correlation with clinicopathology indicators and survival rates from the previous study. More specifically, they both correlate with tumor metastatic and EGFR mutation, leading to poor prognosis.[15]

It is suggested that the tumor markers (CEA and Cyfra 21-1) and miRNA (miR-34, miR-148, miR-222, and miR-155) are related to each other and could be used as biomarkers in NSCLC prognostication. This study aimed to evaluate the correlation between the tumor markers with the miRNA expression.

## MATERIAL AND METHODS

### Study design and participant

It was retrospective study involving about 52 patients from the previous study. The patients have undertaken informed consent.[15,16] This study has been approved by the Dharmais Cancer Hospital Ethics Committee (No. 032/KEPK/V/2016). The study was performed following the ethical standards of the Helsinki Declaration.

### Inclusion and exclusion criteria

The inclusion criteria were the patients diagnosed with advanced-stage NSCLC based on histopathological examination done in-house or outside the Dharmais Cancer Hospital, Jakarta aged 18 yr or above and patients have not received any lung cancer treatment, yet. The exclusion criteria were the patients have a primary tumor besides lung cancer or a double primary tumor with lung cancer and the patients refused to be enrolled in the study.

### Data collection

The data of serial miRNA expressions (miR-148, miR-34, miR-222, and miR-155) were obtained from previous study.[15,16] Meanwhile, the other data were retrieved from patients' medical records, including patients' demographic data and the level of serum tumor marker (CEA and Cyfra 21-1) which have recorded on routine laboratory examination results following the miRNA test at the time patients enrolled.

### Sample collection

CEA and Cyfra 21-1 were obtained from different plasma from those for serial miRNA expressions but had the same sampling time.

### CEA and Cyfra 21-1 examination

The patient's whole blood was drawn 5-10 mL and centrifuged to obtain serum. All procedures for CEA and Cyfra 21-1 were undertaken at the Clinica Laboratory of Dharmais Cancer Hospital, Jakarta with the standard protocol applied for clinical routine.[15,16]

### miRNA examination

About 5-10 mL of whole blood was drawn from the patient. The sample was directly centrifuged at 1800 rpm for 10 min to obtain 200 uL of plasma. Plasma was collected into 1.5 uL cryotube and stored at -80°C until RNA extraction was conducted. The RNA extraction was conducted using the miRCURY RNA Isolation Kit (Exiqon) protocol. miRNA concentration was quantified by NanoDrop 2000 (Thermo Scientific). An A260/280 ratio between 1.9-2.1 was considered contaminated-free sample and continued to the next step.

Reverse transcription was conducted with real-time polymerase chain reaction (qPCR) using miRCURY LNA Universal RT microRNA PCR, Polyadenylation, and cDNA synthesis Kit (Exiqon) protocol. cDNA was stored at 4°C before proceeded to qPCR machine. A 7500 Fast (Applied Biosystem) engine is used to amplify the target genes with specific primers. Firstly, denaturation is occurred at 95°C. Next, it continuously performed a melt-curve analysis. Then, cycle threshold (CT) produced and showed as the number of amplification cycles. Finally, miRNA expression level is appeared as a fold change (the level of absolute and relative expression). The complete technical steps are extracted and can be seen from previous study.[15]

### Statistical analysis

The data were statistically analyzed with Mann-Whitney U test (for two type variables) or Kruskal-Wallis test (more than two types). Spearman-rho analysis is considered to find correlation between variables with a 95% of confidence interval.

## RESULTS

The demographic characteristics of patients were presented in TABLE 1. Some data were collected from the previous study.[15,16] Patients were mostly male, with about one fourth proportions are female (28.8%). Most of the patients

were aged more than 40 yo, with a mean age of 55.87 ±11.47 yr. Both active and non-active smokers had tiny differences in proportion. End-stage lung cancer (stage IVb) was the most common stage (55.8%), with adenocarcinoma being the most contributor (82.7%). Meanwhile, the data on CEA and Cyfra 21-1 showed more than half of the total patients showed above-normal results on both CEA and Cyfra 21-1, although high Cyfra 21-1 was found in almost all patients (82.7%) (TABLE 1).

The correlation of lung tumor markers (CEA and Cyfra 21-1) with serial miRNA expressions (miR-34, miR-148, miR-222, and miR-155) are presented in FIGURE 1 and 2. Significant values were observed among them, except in correlation with miR-34 (TABLE 2).

TABLE 1. Demographic and clinical characteristic of patients[15]

| Variable | n (%) | p |
|---|---|---|
| Gender | | |
| • Male | 37 (71.2) | 0.002 |
| • Female | 15 (28.8) | |
| Age (mean ±SD yr) | 55.87 ± 11.47 | 0.928 |
| • Mean | 55.87 | |
| • Range | 23 – 88 | |
| Age group (yr) | | |
| • <40 | 4 (7.7) | |
| • 40-60 | 28 (53.8) | 0.000 |
| • >60 | 20 (38.5) | |
| Smoking | | |
| • Yes | 24 (46.2) | 0.579 |
| • No | 28 (53.8) | |
| TNM Stage | | |
| • IVb | 29 (55.8) | |
| • IVa | 20 (38.5) | 0.000 |
| • IIIb | 3 (5.8) | |
| Type of cancer cell | | |
| • Adenocarcinoma | 43 (82.7) | 0.000 |
| • Squamous cell carcinoma | 9 (17.3) | |
| Tumor markers | | |
| CEA | | |
| • >Normal | 29 (55.8) | 0.782 |
| • Normal | 23 (44.2) | |
| Cyfra 21-1 | | |
| • > Normal | 43 (82.7) | 0.000 |
| • Normal | 9 (17.3) | |

TNM: cancer staging's notation system using alphanumeric codes

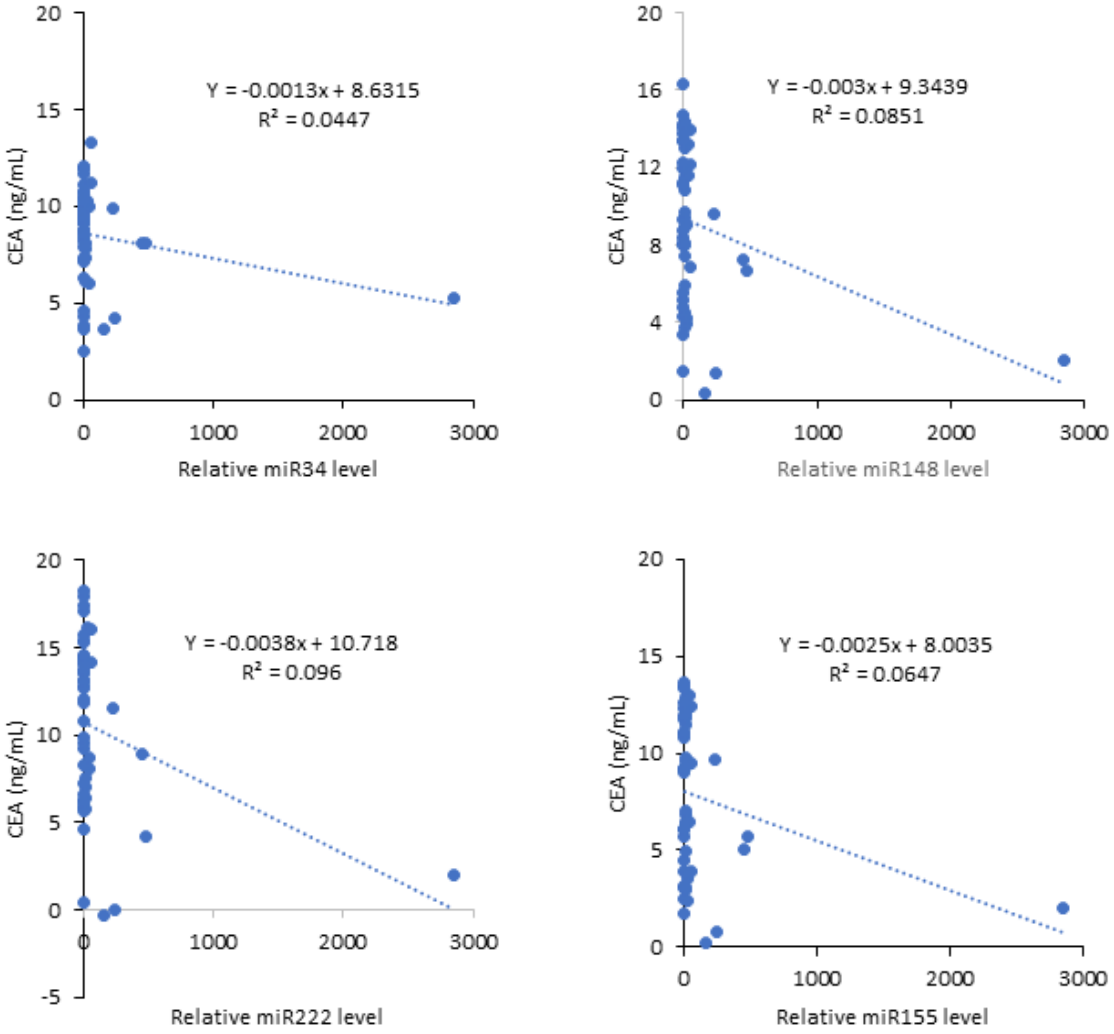


FIGURE 1. The correlation between the plasma CEA level and serial miRNA expression (miR-34, mir-148, miR-222, and miR-155)

TABLE 2. Correlation of lung tumor markers with serial miRNA expressions

| Tumor markers | miRNA | p (r) |
|---|---|---|
| CEA | • miR-34 | 0.270 (0.156) |
| • miR-148 | | 0.004 (0.522) |
| | • miR-222 | 0.002 (0.542) |
| | • miR-155 | 0.001 (0.576) |
| | • miR-34 | 0.253 (0.161) |
| Cyfra 21-1 | • miR-148 | 0.013 (0.378) |
| | • miR-222 | 0.000 (0.519) |
| | • miR-155 | 0.015 (0.368) |

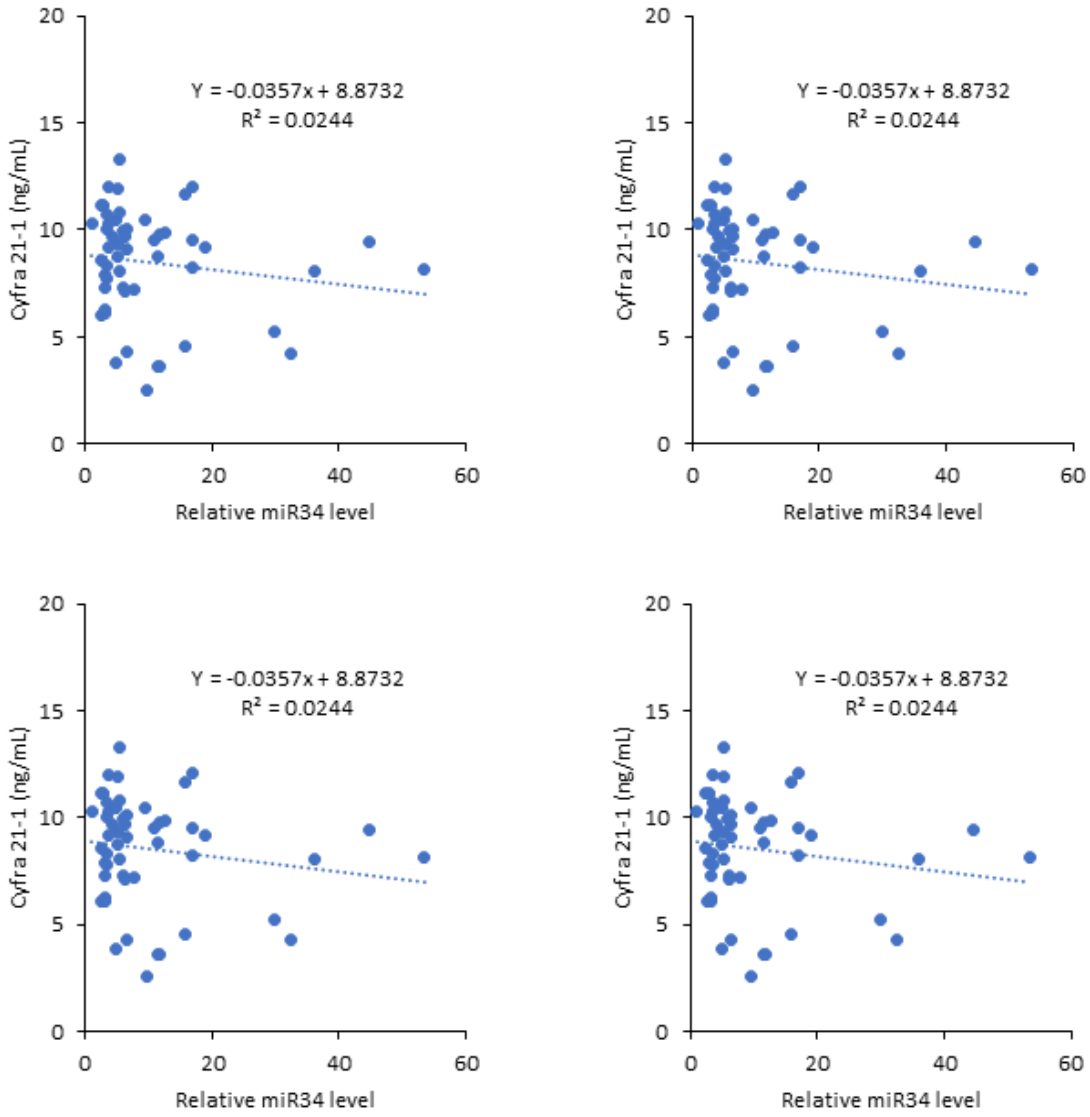


FIGURE 2. The correlation between the plasma Cyfra 21-1 level and serial miRNA expression (miR-34, mir-148, miR-222, and miR-155)

## DISCUSSION

Lung cancer is one of global health's biggest concerns since it is the top 2 cancer burden of all ages in recent years.[1] In Indonesia, the current data on cancer burden showed that lung cancer is the highest among all cancer types in men.[3] The data is in tune with this study, showing that lung cancer incidence is higher among men than women. Smoking as an independent risk factor for lung cancer[21] is considered in this study. Passively smoked with chronic exposure are proven as a high risk factor that contribute to lung cancer.[22] This study showed that patients with lung cancer demonstrate a little difference in numbers between actively and passively smoked, proving that both conditions may contribute to lung cancer causes. The age range among cancer patients is varied, but this study showed that the patients recruited are commonly adults and elderly, with a mean age of around 55 yr. Patients in this hospital are generally advanced-stage cancer patients since this hospital is the national reference

hospital for cancer in Indonesia. Patients included in this study were mostly diagnosed by end staged lung cancer and dominated by adenocarcinoma type.

This study revealed that a half percents of patients perform high level of both CEA and Cyfra 21-1, those are commonly evaluated tumor markers among lung cancer.[8,9] Although a small amount of CEA and Cyfra 21-1 are also produced by normal cells, both marker levels are high in cancer due to normal cell response and tumor cell production.[11-13] Several studies showed that CEA and Cyfra 21-1 have diagnostic and prognostic value in lung cancer.[23-25] A study discovered a role of CEA that is functionally associated with anoikis-mediated cell death induction to promote liver metastasis.[26] Another study also demonstrated that the cytokeratin 19 (CK19) fragment is closely related to the expression of mRNA for CK19.[27] However, the exact molecular mechanism remains unclear.

Micro RNA or miRNA has two roles as an oncogene (oncomiR) and tumor suppressor miRs.[4,5] Several of them have been discovered in the previous study showing prognostic values among advanced stage NSCLC patients.[15] In this study, miR-148, miR-222, and miR-155 expression show significant correlation with CEA and Cyfra 21-1, suggesting that such miRNAs might role as regulator on several targeted proteins, including protein in the tumor markers formation.

As a tumor suppressor, miR-148 represses cancer growth through mitogen-activated protein kinase/ Jun N-terminal kinase (MAPK/JNK) signaling.[18] A study has found that CEA mRNA expression inversely correlated with miR-148b expression in NSCLC tissue. Furthermore, the overexpression of CEA was an inhibitory effect on miR-148, resulting in the proliferation and migration of NSCLC cells.[28] The previous finding showed that high CEA and Cyfra 21-1 levels are significantly correlated with miR-148 expression.[15] Unfortunately, it did not find any studies discussing Cyfra 21-1 with its molecular mechanism related to miR-148. miR-148 and miR-34 also acts as a tumor suppressor in NSCLC. Its target is the p53 gene which further will repress tumor growth and cancer cell invasion.[18] The correlation of CEA and Cyfra 21-1 to miR-34 expression in this study also does not show significant results. It is hypothesized that the sample size is small and perhaps the miR-34 expression has a small contribution to tumor marker formation. A previous study only showed that a combination of miR-34 and CEA increases diagnostic efficiency.[29] However, there is no prior study that describes the relation of miR-34 expressions to CEA or Cyfra 21-1. Further investigation is needed to evaluate this section.

This study revealed that CEA and Cyfra 21-1 levels were correlated with miR-222 and miR-155 expressions. A recent study found that a high level of miR-222 expression would induce cell proliferation and regulate apoptosis through several pathways, mainly in the cell cycle, such as the Akt pathway.[19] miR-155 expression also has a similar mechanism, which inhibits tumor cell apoptosis and DNA damage.[20] Another study investigated the relationship between tumor markers and several inflammatory cytokines concluded that they are involved in tumorigenesis through the tumor microenvironment.[30] Although, this study could not find any related research specifically describing the mechanisms by which these two oncomiRs are linked to CEA and Cyfra 21-1, it is suggested that oncomiRs expression may promote the production of CEA and Cyfra 21-1 in a certain way. Moreover, the expression of oncomiRs may trigger an increase in the production of CEA and Cyfra 21-1.

In this study, the sample size was small. Previously, a consecutive sampling method was conducted.[15] Extending the

timeframe to include the sample might be beneficial to gain more participants. Since this study is retrospective on the same subject as previous study, only the same sample size was gained. Besides, this study only shows tumor markers' correlation with miRNAs, and the exact mechanism underlying that correlation has not been discovered. However, this findings may be novel and have value for further investigation.

## CONCLUSION

There is a correlation between CEA and Cyfra 21-1 with miR148, miR222, and miR155 expressions in advanced-stage NSCLC patients in Indonesia. This result is valuable for further evaluation to describe the exact mechanism.

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