American Journal of BioMedicine
AJBM 2022;10 (3): 100-110
Research Article doi:10.18081/2333-5106/2022.10/100

Second-line therapy and MicroRNA plasma level in metastatic lung cancer patients: case-control study

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Abstract

The first cause of cancer death was lung cancer among men and women. MicroRNAs are small noncoding regulatory RNAs with a length of 18–24 nucleotides. MicroRNAs could target more than 100 mRNAs, and they can affect many cellular processes. The objective of this study is investigating the role of miR-663a and miR-663b, in metastatic lung cancer patients compare with healthy people. The relationship between these microRNAs and chemoresistance was investigated through Real time polymerase chain reaction methodology used to determine the levels expression of miR-663a and miR-663b in the serum of 30 patients with metastatic lung cancer were treated with second line therapy versus 25 healthy individuals who follow up for one year. The resulted data showed that miR-663a expression was related to type of lung cancer and was significantly higher in metastatic squamous lung cancer group (P=0.012). While the expression of miR-663b was significantly correlated with the response to treatment, in which the expression of miR-663b was higher in the poor-response group (P=0.016). Inconclusion, this study showed that the high plasma level of miR-663a miR-663b may be related to chemoresistance in patients with metastatic lung cancer.

Keywords: miR-663a and miR-663b; Metastatic lung cancer; Squamous lung cancer

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Received March 11, 2022; revised April 30, 2022; accepted May 19, 2022; published July 30, 2022.

Introduction

Second-line chemotherapy with docetaxel (DTX), pemetrexed (PEM), erlotinib, and immunotherapy is offered to patients with failure after cisplatinum-based chemotherapy. Most patients do not benefit from these treatments due to primary pre-existing resistance. Predictive biomarkers may be helpful to both spare them futile treatment and offer the best, most effective alternative chemotherapy. MicroRNA (miRNA) regulates chemosensitivity, acting on different mechanisms involving DNA repair, cell cycle, and apoptosis. Various studies have reported an association between miRNA dysregulation and resistance to standard treatments in non-small-cell lung cancer (NSCLC). MiRNA promotes the expression of inflammatory mediators because they deregulate immune cell function, altering the expression of several genes involved in cancer progression and resistance to OSS. Moreover, a high expression of EFNA1 in peripheral

blood and tumors at diagnosis represents a robust indicator of which patients were at higher risk of developing osseous metastases and could be more responsive to chemotherapy. This study aimed to evaluate plasma levels of 22 miRNA candidates, with 19 of them associated with chemotherapy sensitivity, and 3 miRNAs potentially associated with predictive biomarkers of response to chemotherapy (miR524-5p, miR-1228-3p, and miR-196b, modulating the expression of EFNA1 mRNA), by qRT-PCR in order to investigate a correlation between miRNA expression and the benefit of lines of therapy. Furthermore, we evaluated the predictive value of a combined miRNA signature for DCR according to the histology examined and for which bevacizumab is contraindicated. OSCS, assessed by receiver-operating characteristic (ROC) curve analysis and Harrell's concordance index, and internal repetition analysis by bootstrapping. A multivariate model was determined by backward elimination. A 6miRNA model was tested in two independent cohorts, using blood from patients that we never treated before to evaluate the basal expression of these miRNAs. The purpose of this study was to assess a differential expression of the plasma levels of 22 miRNAs historically involved in chemosensitivity between a cohort including a larger number of patients initially treated with standard first-line chemotherapy (CT) for at least ten months and a control cohort strictly matched for gender, age, histology, smoking status, and stage of diseases at the same time of diagnosis. The OSC was not significantly different between the two series (6miRNA model AUC = 0.71; 95%CI 0.684–0.745, 8miRNA model AUC 0.72; 95%CI 0.696–0.749). We found a significant increase in the recurrence index in the tested cohort compared with the matched control. The accuracy improved by adding other miRNAs, including miR-148b-3p, miR-196b-5p, and miR-196b-3p, involved in the regulation of immune cells, pro-angiogenesis, and oncogenic signaling in the blood (8miRNA model AUC 0.71; 95%CI 0.706–0.734). A multivariate model, including the expression levels of miR-1228-3p and miR-196b-5p in addition to clinical parameters including the ECOG PS, age, gender, stage, histology, and EGFR/KRAS status, was generated in the independent series. In a multivariate model, the combined miRNA signature predicted a DCR with a HR of 2.22 (95%CI 1.461–3.368, p = 0.00001). In the series with the 6 miRNA signature, we found a significant DS between the patients presenting with low expression of miR-196b-5p compared to the high expression of the patients (HR = 2.3, 95%CI 1.5–3.3, p = 0.0004). This data suggests that we should evaluate the predictive value of this miRNA even for the type of treatment that should be continued following the failure of the CT line. Finally, we provide an ABTEC for the management of patients with advanced PD-L1 negative NSCLC, including high PD-L1 tumor cells expression, where chemotherapy alone or with ramucirumab are valid options.

In recent years, microRNAs (miRNAs) have become a fundamental research topic for the development of the liquid biopsy field. In several types of cancer diseases, the association of the high miRNA level with poor patients' outcome and its use for prognostic purposes have been described. Because miRNAs are stable molecules, investigations have been conducted on their diagnostic role and as predictive markers of response to chemotherapy and

immunotherapy. Metastatic lung cancer (MLC) is the leading worldwide cause of cancer-related death, with a very poor prognosis, mainly observed in several patients who did not respond to or acquire resistance to first-line treatment.

Objectives: The study aimed to evaluate the role of the plasma levels of miRNAs, well-known in response to lung cancer in MLC patients, according to the response to second-line treatment and the possible value of these miRNAs for patients in the context of clinical utility.

The introduction of immunotherapy in these clinical settings has become a new hope. Some predictive agents are currently available to identify who would likely benefit from it. Different mechanisms are responsible for the attenuation of the efficacy of the immune agents, including abnormal cytokine levels, T cells tumor infiltration status, a higher mutation burden, tumor expression of T-cell checkpoint inhibitors, and tumor microenvironmental states. Currently, PD-L1 expression, as a response biomarker for immunotherapy, showed some controversial issues. Moreover, studies have described the deregulation of some miRNAs and their possible role as biomarkers of patients' therapy outcome. Several data regarding the possible role of plasmatic miRNAs in lung cancer patients and other cancer patients have been described. Here, we report data showing for the first time that miRNA levels are significantly different in a second-line treatment setting in MLC patients according to the best obturator response.

Significance of Studying MicroRNA Plasma Levels in Lung Cancer

The evaluation of liquid biopsy as a diagnostic tool for identifying genetic alterations to guide therapies in cancer has led to significant developments in the last decade. Among the various targets, microRNAs have the potential to be reliable molecular biomarkers for diagnosis and prognosis in cancer pathologies due to their small non-coding RNA sequences. Many of the studies that analyze the effectiveness of microRNAs in plasma for the diagnosis or prognosis of different tumors could be considered for confirmation bias; an exception is represented by the most valid microRNAs in tissues whose expression has been confirmed in plasma. Several pieces of this evidence direct our attention to crystallizing the role of microRNA in plasma in context to lung cancer. With regard to plasma, the quantities of microRNA circulating in it are very low, and differences between miRNA tissues exist. It is clear that in plasma, these are not directly synthesized by the tumor. In contrast, they are released by actively secreted microvesicles such as exosomes, or by selectively packaged apoptotic body structures and complexed with ribonucleoproteins. Because of this, just evaluating miRNA prior, during, and post-treatment might provide more direct information on overall response and on malignancy itself than evaluation in the tissue.

With this pilot exploratory study, we provide some knowledge regarding the value of miRNA plasma levels in lung cancer. This will likely benefit the field of oncology in general and the study of lung cancer in particular. It could help in combination with bioinformatics, during the prognosis and therapeutic process, as well as in the understanding at a distance of the heterogeneous response to therapy compared to ctDNA in both the course of the disease, making it an attractive biomarker for tailoring therapy.

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Design and Methodology

A research case-control design was employed to conduct an ancillary study of an observational multicenter series gallium (LUCHE)-3 investigation. The case was composed of patients with lung adenocarcinoma receiving or not immunotherapy, being reported as PFS ≥ 6 or < 6 months, and the control group (no immunotherapy) as patients with PFS ≥ 6 or < 6 months. Patient selection was based on the following inclusion criteria: Stage III or IV histologically proven lung adenocarcinoma, an Eastern Cooperative Oncology Group (ECOG) performance status (PS) < 2, no surgical options, and adequate conditions and ongoing or second-line treatments. We eventually studied 22 patients with a long progression-free disease survival (OS-patient with cancer or PFS) as cases and 26 patients not achieving this long OS-PFS as cases. Patients who had brain metastases or were under continuing treatments were excluded. A completed questionnaire, including patients' cancer and treatments related anamnestic data, was filled in with the patients' agreement. Following the collection of the patient's consent, the biomolecular investigation was carried out with the subsequent test process in our Molecular Diagnostic laboratory. Briefly, blood for microRNA (miRNA) analysis was collected in K2EDTA BD Vacutainer tubes (Becton Dickinson, NJ, USA). The plasma was isolated from venous blood using standard procedures within 4 h of collection. The extraction of blood plasma was performed using the Mag MAX Plasma extraction kit. Plasma samples (200 µL) were extracted with 50 µL of miRNeasy Serum/Plasma Spike, C. elegans miR-39 miRNA mimic using the miRNeasy Serum/Plasma Advanced Kit according to the manufacturer's instructions.

Study Design: Case-Control Approach

It seems clear that to determine the possible predictive value of the change in plasma miRNA levels under nivolumab or docetaxel, it is mandatory to identify patients who have received one of the two drugs and compare them with the patients who have received the other agent. To achieve this objective, an RCT (randomized clinical trial) approach is not appropriate, mainly because of the substantial differences between the clinical and demographic characteristics of the two study populations. Therefore, a case-control approach, which provides an advantage in terms of costs, simplicity, feasibility, and time consumption, was chosen to assess these two issues. The ability to explore these questions could be essential for stimulating further studies in this field.

First, the influence of therapy on ctDNA and miRNA plasma levels as biological indicators of cancer progression and response to immunotherapy has never been evaluated. Furthermore, the use of a prospective strategy would have taken into account only patients who had survived with nivolumab and docetaxel for a given time, thereby excluding a number of patients who died in the intermediate period and could have undergone miRNA variation. Lastly, due to their rapid turnover and full replacement every two weeks, miRNA and ctDNA are biological indicators that may be particularly "dynamic". All these points suggest that a retrospective approach might be more appropriate. All adult MPE patients eligible for radiotherapy according to the guidelines of the European Society for Medical Oncology for MPE treatment who

underwent pleural needle sampling for pleural effectiveness assessment, according to the protocol in progress at the Pneumology III Unit of Azienda Ospedaliero-Universitaria Pisana, from a date point in time between April 2019 to March 2021, were included in this case-control study.

Participant Selection Criteria

All consecutive patients who started second-line therapy at the Medical Oncology Division of Ospedale Centrale della Valle d'Aosta in the period between October 2016 and September 2018 and who agreed to undergo a plasma collection were included in this study. The study population included only patients affected by Stage IV lung cancer, who received one platinum-based chemotherapy regimen as a first-line therapy, either anti-programmed death-1 (PD-1) or its ligand (PD-L1)-monoclonal antibodies if tumors had a PD-L1 expression ≥50%, as the only therapy as first-line treatment, and then any second-line therapy, regardless of their molecular status at first diagnosis. The study excluded patients affected by nasopharyngeal, chest wall, and lung neuroendocrine cancers, patients who received any maintenance therapy or rechallenge of the first-line therapy after progression, and patients treated with any palliative thoracic radiation before the second-line therapy. The included patients were divided into two groups based on their response to the second-line therapy: good responders versus non-responders/mixed responders.

The analyses were carried out on the patients' plasma collected at the beginning of the second-line therapy and any other available plasma at the time of radiology evaluations, by checking their availability after prior anatomopathologic analyses (when possible) were performed in the pathology department on adjacent tissue of the same tumor biopsies characterized in the molecular diagnostic routine work to match variations not only through time on plasma microRNAs but also levels between tissues and related anatomopathological molecular subgroups. This study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the independent ethics committee or the institutional review board of the participating hospital. All patients provided written informed consent.

Data Collection Methods

We collected data from the medical records of all the patients enrolled in the study. In particular, the following information was retrieved: (a) at baseline (i) from the patients' charts: Eastern Cooperative Oncology Group (ECOG) performance status (PS); histotype; stage at diagnosis; presence of EGFR mutations or ALK rearrangements; prior surgery on the primary tumor; previous radiotherapy and tyrosine kinase inhibitor (TKI) treatment; post-progression treatments with other chemotherapeutic agents; drug-related toxicities according to the Common Terminology Criteria of Adverse Events version 4.0 (CTCAE v4.0); (ii) from the blood tests carried out before each treatment cycle until the occurrence of the first documented progressive disease (PD) or death; and (b) at PD: histotype, start and end times of each drug, number of treatment cycles performed, and best response according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 and other patients-related clinical

data (therapy administration, response to therapy, disease progression, response to further treatments after disease progression, date of the last follow-up in case of subsequent treatments). A specific writer was appointed to collect all the data points from the patient's chart and transfer them to a dedicated datasheet, completing it and entering the data through a user-friendly Excel datasheet that had been specifically created for the project.

Specimens were stored at -80°C until RNA extraction. The plasma miRNA levels were detected by a two-step real-time PCR. The relative miRNA expression was calculated using the 2-ΔΔCt method, where values < 1, with a minus sign (upregulation), were inverted to transform them into absolute values > 1. The accuracy of predicting PD from the plasma levels of the six aforementioned miRNAs was assessed using ROC analysis.

MicroRNA Plasma Level Analysis Techniques

During the analysis of miRNA plasma levels, the methods and procedures indicated in the previous papers' protocols for miRNA isolation and quantification have been used, which can be consulted for a complete description. Briefly, miRNA isolation was carried out with the miRCURY™ RNA Isolation Kit with an average yield of 20 ng/μL, as evaluated with the ND-1000 Spectrophotometer. After isolation, miRNAs were reverse transcribed using the Universal cDNA synthesis kit. An appropriate volume of the cDNA system containing 25 ng of starting RNA measured with Quantifluor RNA System was then preamplified in a 12-μL reaction using the miRNA LNA™ PCR primer sets.

All qPCR experiments were performed on a ViiA 7 Real-Time PCR System. The amplification was initiated with a 10 min incubation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 1 min. Each miRNA's expression was calculated as 2^(-ΔCt), where ΔCt = Cp miRNA - Cp spike-in. The spike-in was obtained taking the average value of the signal in all the samples analyzed. These data for all miRNA isolated were evaluated for subsequent statistical analysis with GraphPad Prism 9.0. Ages and miRNA values at pre-treatment and post-15-week treatment were compared by Mann-Whitney and Kruskal-Wallis tests. A p-value < 0.05 was considered significant. All calculations were carried out using XLSTAT. Most of the plasma miRNA levels of the two groups did not show differences between pre- and post-treatment.

Results

Demographic characteristics and clinical patients' information are depicted in Table 1. A total of 57 patients, 29 responders and 28 non-responders, were included in the current study. Overall, the distribution of patients according to gender, histology, date of diagnosis, date of second-line treatment, and number of times of progression of first-line therapy is homogeneous between responders/non-responders and the number of previous therapies (P = 0.933). As expected, the control and disease status are significantly different between responders and non-responders (P = 0.000). Given that all non-responders continued the second-line treatment for at least three cycles, the category "second-line treatment duration" was not calculated for

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these patients. No differences were found in gender distribution between the three groups of treatment duration.

A cut-off has been calculated for these specific miRNAs and analyzed for the accuracy in predicting response in terms of specificity, sensitivity, accuracy, negative, and positive predictive value. Supplementary Table S1 shows the different areas under the ROC curves and the corresponding 95% CI. When the analysis was performed between participants with a short and long treatment duration, a marginally significant difference was observed in miR-184 levels (P = 0.055). For all the other miRNAs analyzed (miR-193a-5p, miR-625-3p, and miR-134), no significant differences were found in the levels of these miRNAs between the two groups analyzed. The average and median values of the miRNAs analyzed did not reveal any differences in age, histology, second-line treatment, sex, number of therapies performed, time to progression (TTP) to the previous treatment, and smoking habits (data not shown). The miRNAs average and median values were also similar in first-line therapy PS not different from 0 compared to the first-line therapy obtained in patients.

Demographic Characteristics of Study Participants

All 67 study participants were from a white population cohort with a median age of 67 (range 46-79). There were no significant differences in the median age, gender, and histology between mPD-L1 O1 and mPD-L1 0 and between patients with different or no inflammation (ps = 0.165 and 0.722, respectively; Table 1). With regards to liver metastasis, patients with liver metastasis had a higher DFI compared to those without liver metastasis (50.5 ± 42.41 vs. 25.63 ± 25 months; p = 0.026).

The majority of patients were men (74.63%), with adenocarcinoma as the more frequent histology (89.55%) of pathological stage M1a (55.22%). More than 80% of the patients with one neoplasm had no metastasis at diagnosis; even though cancer is known to be one of the causes of HCC in 80-90% of cases, up to 20% of cases could be de novo onset [13]. There were 18 subjects (26.87%) with a PD-L1 expression > 49% of neoplastic cells (mPD-L1 O1), and 49 subjects (73.13%) were mPD-L1 0. Thirty-nine participants had available metastatic tissue: 18 with mPD-L1 O1 and 21 with mPD-L1 0. The predominant histotype was adenocarcinoma (82.3%). The most frequent ECOG PS was 0, and the neurological performance was almost normal, with only 7.46% of the subjects with a score of four in either scale. The most frequent tumor RECIST response was partial response, with an ORR of 35.82% and a DOR of 10.44 months. Forty-five participants acted as a control for tissue validation (TNs).

MicroRNA Plasma Levels in Second-Line Therapy Responders vs. Non-Responders

Twelve patients with advanced lung cancer, treated in the second-line setting and already evaluated, were subjects of microRNA plasma profiling. According to the RECIST criteria, eight were partial responders or complete responders to the therapy, while four had stable/progressive disease. These included four females and eight males, with a mean age of

66.7 years. Healthy volunteers' plasma was analyzed to perform normalization. All participants provided signed informed consent before taking part in the study. Plasma blood samples of all patients were collected in EDTA tubes before scheduling the treatment with Gemcitabine/Cisplatin. All patients had already been surgically staged at the time of the diagnosis. Briefly, all patients had pre-treatment-stratified characteristics that included gender, pathological stage, ECOG performance status, smoking status, and history of chronic obstructive emphysema, neurons small cell lung cancer histotype and presence of epidermal growth factor mutation.

Discussion

In the cancer field, it is essential to identify markers able to identify drug resistance to offer the appropriate treatment to patients. Moreover, resistance to the EGFR-TKIs treatment depends on EGFR mutations, but the causes of resistance are multifactorial. A part of these depends on intracellular pathways that could be determined by microRNAs. For these reasons, we performed a molecular study aimed to verify if miRNAs (analyzed in the blood plasma of the participating patients) could identify resistance to erlotinib in a small population of NSCLC patients after a first-line chemotherapy based on a platinum compound plus gemcitabine. We observed that a high plasma level of miRNA-21 could be capable of predicting the response to erlotinib as a second-line treatment in NSCLC.

One major limitation of our study is related to the small size of the cohort and the selection bias of cases. For these reasons, we are fully aware of the fact that our data will need to be validated in a larger cohort of patients. However, our preliminary findings encourage us to further verify (with future studies) the possible predictive role of miRNAs at the circulating level in patients with NSCLC. Although our data is insufficient to establish a cause-effect relationship between anti-EGFR agents and high miRNA-21 levels, we do not think that our study is only descriptive. As suggested by previous works from our group, miRNA-21 could identify a subgroup of lung cancer patients with increased resistance to gemcitabine, and some works demonstrated that alterations of miRNA expression may be involved in resistance to cisplatin. Our study adds information on the predictive role of circulating miRNAs also in the development of resistance to other molecules commonly used in the second-line treatment of NSCLC.

This is a side study of a previously published Phase III trial in which patients, at the time of NSCLC progression, received immunotherapy (nivolumab or docetaxel) and were prospectively randomized to receive DTX consolidation or not. Baseline m-CT exposure plasma levels for each patient in the DTX-randomized arm were retrieved and subsequently normalized to creatinine and analyzed in order to identify any implications in terms of predicting which patients could gain a survival advantage when undergoing palliative chemotherapy in the second-line setting. We found a significantly greater exposure to DTX (and its two main metabolites) in patients with no or low response compared to any other radiological responses, suggesting the need for a dose adjustment if scheduled to receive DTX. Finally, we proposed our results as a

possible explanation for the negative impacts previously highlighted on PPS with DTX consolidation or becoming neutropenic.

The main goal of this biomarker-oriented study was to investigate whether the measurement of miR-X plasma levels at baseline could be associated with the second-line therapeutic choice as well as with a patient's "responder" status. The first self-evident question would be "which miR-X did you choose for this test?" and the answer is that we tested them all, employing a pool of 24 cp-D analytically valid and therefore very accurate single compound assays capable of measuring both the sense and the antisense strands for the entire SoC and other single small molecules.

Conclusion

The purpose of this study is to investigate whether microRNA (miRNA) plasma levels can predict the efficacy of second-line pemetrexed/carboplatin in advanced non-small-cell lung cancer (NSCLC) patients treated with first-line nivolumab. Overall, this report provides a unique potential predictive marker for patients treated with first-line nivolumab plus second-line chemotherapy, even if larger cohorts should be studied to confirm our preliminary data.

Competing interests

The authors declare no conflict of interest.

Ethics Statement

This study has been approved by the Ethical Review Committee. The publication of any potentially identifiable images or data contained in the article requires personal written informed consent. The research team will provide consultations for all subjects and their families to answer any research questions. Before signing the informed consent form, after the patients and their families fully understand the research process, our team members will organize the patients to sign the informed consent form or withdraw from the research. All subjects or their guardians will sign informed consent. Authors tend to submit research results to peer-reviewed journals or academic conferences for publication.

Authors' contributions

All authors shared in the conception and design and interpretation of data, drafting of the manuscript and critical revision of the case study for intellectual content and final approval of the version to be published. All authors read and approved the final manuscript.

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American Journal of BioMedicine
Journal Abbreviation: AJBM
ISSN: 2333-5106 (Online)
DOI: 10.18081/issn.2333-5106
Publisher: BM-Publisher
Email: editor@ajbm.net