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Circulating miR-30b and miR-30c predict erlotinib response in *EGFR*-mutated non-small cell lung cancer patients

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ABSTRACT

Objectives: MiR-30b, miR-30c, miR-221 and miR-222 are known to induce gefitinib resistance in lung cancer cell lines with activation of mutations in the *epidermal growth factor receptor* (*EGFR*). However, the role of these four microRNAs in tyrosine kinase inhibitor (TKI)-resistance in non-small cell lung cancer (NSCLC) patients is unknown. Thus, the aim of this study was to investigate the predictive value of miR-30b, miR-30c, miR-221 and miR-222 in plasma from *EGFR*-mutated lung cancer patients receiving erlotinib.

Materials and methods: The cohort consisted of 29 *EGFR*-mutated lung cancer patients receiving erlotinib. Plasma levels of miR-30b, miR-30c, miR-221 and miR-222 were analyzed by qPCR from blood samples collected before treatment start. Plasma concentration of each microRNA was correlated to clinical outcome.

Results: Plasma concentrations of miR-30b and miR-30c could be determined in all 29 patients. Low plasma concentrations of miR-30b and miR-30c showed significant correlation with superior progression-free survival (PFS) (miR-30b: HR = 0.303 [0.123–0.747], $p < 0.05$; miR-30c: HR = 0.264 [0.103–0.674], $p < 0.05$). Low plasma concentrations of miR-30c were also significantly correlated with superior overall survival (OS) (HR = 0.30 [0.094–0.954], $p < 0.041$).

Conclusion: High plasma concentrations of miR-30b and miR-30c predicted shorter PFS and OS. This implies that miR-30b and miR-30c could have clinical potential as biomarkers in *EGFR*-mutated lung cancer patients.

1. Background

Activating mutations in the tyrosine kinase domain of the epidermal growth factor receptor (*EGFR*) are found in approximately 15 percent of non-small cell lung cancer (NSCLC) patients with adenocarcinomas [1,2]. These mutations in *EGFR* predict response to tyrosine kinase inhibitors (TKIs) such as erlotinib and gefitinib, which currently are first-line treatment for *EGFR* mutation positive NSCLC [3,4]. Response rates are high with more than 70% of patients achieving a marked and durable response [5]. However, despite an activating *EGFR* mutation, around 20%–30% of the patients will not respond to *EGFR*-TKIs [6]. The mechanism behind this intrinsic resistance remains unclear and no marker of intrinsic resistance exists today. Therefore, it is crucial to identify new markers of resistance and potential targets for molecular-targeted treatments in order to overcome this resistance.

Among the numerous resistance mechanisms described, microRNAs (miRNAs) are being intensely studied as a contributing factor to resistance development [7,8]. MiRNAs are small non-coding RNAs that post-transcriptionally regulate gene expression by binding to messenger RNA (mRNA), thereby preventing protein formation [9,10]. MiRNAs are negative gene regulators with either oncogenic or tumor-suppressive capacity depending on the target gene [11]. MiRNAs are dysregulated in cancer patients [12] and are associated with cancer development [13], growth and metastasis [11].

A previous study by Garofalo et al. showed that high expression of miR-30b, miR-30c, miR-221 and miR-222 induces gefitinib resistance in gefitinib-sensitive *EGFR*-mutated lung cancer cell lines [14]. Also, inhibition of these four miRNAs restored sensitivity to gefitinib. Based on the previous findings in *EGFR*-mutated cells, miR-30b, miR-30c, miR-221 and miR-222 could play a role in intrinsic resistance to *EGFR*-TKIs.

Abbreviations: EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; NSCLC, non-small cell lung cancer; miRNA, microRNA; RT, reverse transcription; qPCR, quantitative polymerase chain reaction; CV, coefficient of variation; PFS, progression-free survival; RECIST, response evaluation criteria in solid tumors; OS, overall survival; CI, confidence interval

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However, their abilities to predict response to TKIs based on plasma concentration are unknown. Therefore, we hypothesized that high plasma levels of miR-30b, miR30c, miR-221 and miR-222 would be associated with intrinsic resistance to erlotinib. Thus, the aim of this study was to evaluate the predictive value of miR-30b, miR30c, miR-221 and miR-222 levels in plasma in a clinically relevant population with diverse activating *EGFR* mutations treated with first-line erlotinib.

2. Materials and methods

2.1. Patients

This study was a prospective observational study conducted at the Department of Oncology, Aarhus University Hospital, Denmark. Patients were enrolled from April 2014 to July 2017. All patients had NSCLC, adenocarcinoma and an activating mutation in the *EGFR* (patients with the ex20 in. mutation were excluded). All patients selected for this study had stage IV disease and received erlotinib as first-line treatment. All patients provided informed consent before inclusion. The study was approved by the Committees on Health Research Ethics of the Central Denmark Region (Record number: 1-10-72-83-14) and Danish Data Protection Agency (Record number: 1-16-02-431-14). The study was conducted according to The Helsinki Declaration.

2.2. Blood collection and miRNA extraction

A blood sample was collected from each patient before treatment start. Blood samples were collected in 10 ml EDTA tubes and plasma was isolated by centrifugation at 1850 *g* for 9 min. There was no sign of hemolysis in any of the blood samples. Plasma was subsequently frozen at −80 °C until analysis.

MiRNA was extracted from 200 µl plasma using miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) according to the manufacturer´s instruction. The variation of miRNA extraction efficiency was < 10% (Supplementary Table 1).

2.3. miRNA quantification

Reverse transcription (RT) and quantitative polymerase chain reaction (qPCR) miRNA analysis were performed using TaqMan® (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. A specific reverse transcription was conducted for each miRNA. A volume of 5 µl of the 50 µl miRNA eluate was added to each RT reaction. Afterwards, semi-quantitative miRNA concentrations were determined using TaqMan® probes. The analyses were conducted on a LightCycler® 480 (Roche Molecular Systems, Inc., Pleasanton, USA) with an analytical coefficient of variation (CV) < 11% (Supplementary Table 2). All samples were conducted in triplicate (mean SD = 0.06 [range 0.00–0.70]).

Concentrations were calculated by means of standard curves for each target. Dilution series of the four target miRNAs formed the basis for this calculation. The dilution series were conducted by diluting plasma 1:2 six times. Next, the concentration of each target miRNA was determined by TaqMan® in the six samples.

Table 1
Patient and tumor characteristics (N = 29).

Characteristics	
Age, median years (range)	66 (44-88)
	N (%)
Gender	
Female	20 (69)
Male	9 (31)
ECOG Performance status*	
0	11 (38)
1	15(52)
2	3 (10)
Smoking status	
Active	1 (3,4)
Former	16 (55,2)
Never	12 (41,4)
EGFR mutations	
Del19	15 (52)
L858R	10 (34)
L861Q	2 (7)
G719x	1 (3,5)
G719x/S768I	1 (3,5)
Progression 1. line erlotinib (at time of data analysis)	
Yes	22 (76)
No	7 (24)

* Oken M, Creech R, Tormey D, et al. Toxicity and response criteria of the Eastern Cooperative Oncology Group. *Am J Clin Oncol.* 1982;5:649-655.

2.4. Statistics

MiRNA plasma levels were stratified by the median plasma concentration for each target in a *high level* and a *low level* group. The primary endpoint was PFS, which was defined as time from treatment start until progression or death due to any cause. Progression was defined as either radiological progression judged by Response Evaluation Criteria in Solid Tumors (RESIST criteria) [15] or clinical progression. Responders were defined as *partial response*, *complete response* or *clinical benefit > 6 months* as described by Jackman et al. [16] Patients still treated with erlotinib at time of analysis (December 5, 2018) were excluded on that date. The secondary endpoint was OS, defined as time from treatment start until death due to any cause or until last follow up date (December 5, 2018). Kaplan-Meier curves were created, stratified by median values to determine differences in PFS and OS. P-values were calculated by a log-rank (Mantel-Cox) test and values below 0.05 were considered statistically significant. Hazard ratios (log-rank) between low and high concentrations were calculated.

Association between miRNA plasma concentrations and clinical characteristics was tested by Fischer´s exact test. Survival analyses were performed using SPSS Statistics Version 24.0 for Windows (IBM SPSS Statistics, Chicago, IL, USA). All graphic presentations were performed using GraphPad Prism Version 7.0b (GraphPad Software, Inc. San Diego, USA).

3. Results

3.1. Patient characteristics

Of 75 included patients with advanced NSCLC, 29 patients had available pre-treatment samples and were included for analysis in this study. Median age was 65 years (range: 44–88). Median PFS was 12.8 months (95% CI: 8.0–17.6) and median OS was 13.3 months (95% CI: 2.4–54.6). Seven patients were still treated with erlotinib and had stable disease at the time of analysis (December 5, 2018). Patient characteristics are presented in Table 1.

3.2. MiRNA concentration in plasma and clinical outcome

MiRNA plasma levels were stratified by the median plasma concentration for each target. Statistically significant association was observed between miR-30b and miR-30c plasma concentrations and PFS. PFS was significantly longer in the group with low plasma concentration of miR-30b compared to high plasma concentration (24.3 vs 7.0 months; $p < 0.05$; Fig. 1a; HR = 0.3 [95% CI: 0.1–0.8]; Table 2) and miR-30c (24.3 vs 7.5 months; $p < 0.05$; Fig. 1b; HR = 0.3 [95% CI: 0.1–0.7]; Table 2). MiR-221 and miR-222 showed a similar tendency, but no significant difference in PFS between the high- and low-concentration groups was demonstrated (Table 2).

Furthermore, OS was significantly longer in the groups with low plasma concentration of miR-30b ($p = 0.05$; Fig. 1c) and miR-30c

Table 2
Median PFS, OS and HR. Patients (n = 29) were stratified by the median plasma concentration for each target.

	PFS		OS	
	Median months (95% CI**)	Crude HR* (95% CI**)	Median months (95% CI**)	Crude HR* (95% CI**)
miR-30b				
Low	24.3 (10.4-38.1)	0.3 (0.1-0.8) p < 0.05	NR***	0.4 (0.1-1.1) p = 0.06
High	7.0 (0.0-17.3)	1.0	16.0 (6.9-25.1)	1.0
miR-30c				
Low	24.3 (11.1-37.4)	0.3 (0.1-0.7) p < 0.05	NR***	0.3 (0.1-1.0) p < 0.05
High	7.5 (0.4-14.5)	1.0	19.2 (11.4-27.0)	1.0
miR-221				
Low	19.3 (10.4-28.2)	0.5 (0.2-1.3) p = 0.13	NR***	0.6 (0.2-1.7) p = 0.33
High	11.2 (3.4-19)	1.0	19.2 (3.1-35.2)	1.0
miR-222				
Low	19.3 (10.4-28.2)	0.5 (0.3-1.3) p = 0.13	NR***	0.6 (0.2-1.7) p = 0.33
High	11.2 (3.4-19.0)	1.0	19.2 (3.1-35.2)	1.0

* HR ratios were calculated as low plasma concentration/high plasma concentration.
** 95% Confidence Interval.
*** Not reached.

(p < 0.05; Fig. 1d). With miR-30b the median OS was not reached in the low-concentration group, compared to 16.0 months in the high-level group (HR = 0.4 [95% CI: 0.1–1.1]) and 19.2 months for miR-30c (HR = 0.3 [95% CI: 0.1–0.9]). Median survivals are presented in Table 2.

The plasma concentration of miR-221 and miR-222 showed no significant difference in OS between the groups, but a trend toward association between low plasma concentration and longer OS was indicated (Table 2).

Plasma-miR-concentration for each patient and matching treatment response is listed in supplementary table 3.

We found no statistical differences in the clinical characteristics (listed in Table 1) between the two groups with high and low miRNA plasma concentration (data not shown).

4. Discussion

Erlotinib possesses high efficacy in the treatment of *EGFR*-mutated NSCLC patients. However, approximately 20%–30% will not respond to erlotinib despite an activating mutation [6]. Here, we investigated circulating miRNAs known to influence *EGFR*-TKI sensitivity in order to reveal a new biomarker of intrinsic resistance to erlotinib.

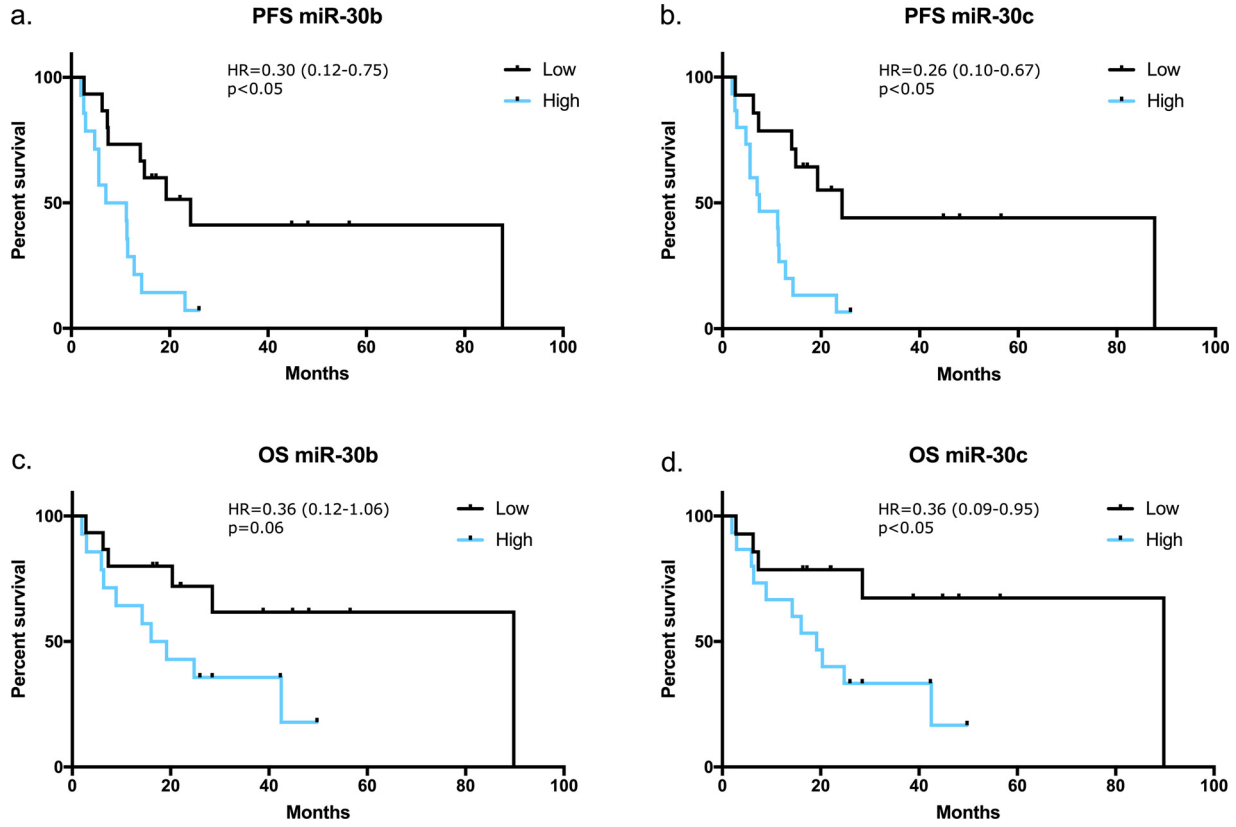

Fig. 1. Kaplan-Meier curves illustrating PFS and OS from erlotinib start, stratified by median miR plasma concentration for a) miR-30b (median PFS: 24.3 vs 7.0 months; p < 0.05); b) miR-30c (median PFS: 24.3 vs 7.5 months; p < 0.05) c) miR-30b (median OS: Not Reached (NR) vs 19.3 months; p = 0.05) and d) miR-30c (median OS: NR vs 19.3 months; p < 0.05). N = 29.

We successfully demonstrated that high plasma levels of miR-30b and miR-30c are associated with inferior effect of erlotinib in *EGFR*-mutated NSCLC patients. Our results suggest that miR-30b and miR-30c may have potential as predictive markers for erlotinib response in *EGFR*-mutated NSCLC patients.

MiRNAs have been studied intensely as a new group of biomarkers. Several studies have tried to screen innumerable miRNAs using large panels in order to construct a signature consisting of multiple miRNAs. Results have varied and the reproducibility is usually low [17]. The low reproducibility rate may be due to miRNAs' broad specificity and ability to bind to different targets [18]. Hence, miRNAs' utility as biomarkers may be dependent on the biological function in the disease concerned. In this study we solely focused on four miRNAs proven to adversely affect sensitivity to gefitinib. These four miRNAs are therefore more than just biomarkers, as they are biologically involved in reducing *EGFR*-TKI sensitivity. Our results indicate that their presence in plasma mirrors this fact, since PFS is more than three times longer in the low-level group.

Wang et al. demonstrated a signature of three miRNAs with the ability to identify intrinsic resistance to *EGFR*-TKIs in NSCLC patients with *EGFR* exon 19 deletions. They profiled 764 miRNAs and identified twelve deregulated miRNAs. They did not identify any of the *EGFR*-regulated miRNAs that were investigated in our study. However, their approach, methods and patient cohort were very different from ours. Nonetheless, if only patients with del19 mutations are included in our analysis, miR-30b and miR-30c are still very strong predictors of erlotinib efficacy.

Previously, high expression of miR-30b and miR-30c in paraffin-embedded tissue samples has been associated with superior response to erlotinib in a Chinese study [19]. These data are in contrast to our findings. Though, in this previous Chinese study the cohort consisted of both *EGFR*-mutated and *EGFR*-wild type patients receiving erlotinib in first-line treatment. This heterogenic cohort makes interpretation of their results difficult. Our cohort consists exclusively of patients with activating *EGFR* mutations, making the data clinically relevant and the conclusion more trustworthy.

Besides the potential as biomarkers, miRNAs have been suggested as possible targets for treatment. Previous results from cell lines have confirmed that by knocking out these specific miRNAs, sensitivity to gefitinib can be restored [14]. Our results support the hypothesis that miR-30b and miR-30c are contributing factors in decreasing sensitivity to erlotinib in lung cancer patients. This insensitivity could potentially be reduced by inhibiting miR-30b and miR-30c in mutated lung cancer patients with high plasma levels of miR-30b and miR-30c. However, due to the small sample size presented in this study, our results must be confirmed in a larger cohort before drawing a definitive conclusion.

A great hurdle when analyzing miRNAs in plasma is the lack of a gold-standard normalization method [20]. Normalizing the target miRNA to the total amount of miRNA in the sample has been suggested, but this approach is only feasible when numerous miRNAs are analyzed. Normalization to the synthetic cel-miR-39 is also widely used. However, it is uncertain if cel-miR-39 is incorporated in microvesicles, hence the ability to estimate efficacy of the purification is unclear [20]. Our method was highly robust with very low analytical CV (Supplemental Tables 1 and 2) and extremely low SD on the triplicates. Due to the quality of our analysis, normalization to amount of plasma added to the reaction was considered sufficient. By this approach we avoided introducing unnecessary bias into our data.

Our results could have major clinical implications. The identification of *EGFR*-mutated subgroups with reduced sensitivity to erlotinib offers possibilities for studies of the underlying molecular mechanisms of resistance and potential for further improvements in the management of *EGFR*-mutated lung cancer patients.

5. Conclusion

In the present study, we suggested that high plasma levels of miR-30b and miR-30c were predictive of inferior response to erlotinib treatment in *EGFR*-mutated lung cancer patients. Our results support the theory that miR-30b and miR-30c are involved in intrinsic *EGFR*-TKI resistance in NSCLC patients with an activating *EGFR* mutation. This indicates that patients with high plasma concentration of miR-30b and miR-30c could benefit from closer monitoring, as they are more likely to experience reduced effect of erlotinib despite an activating *EGFR* mutation.

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Declaration of Competing Interest

The Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2019.07.005.

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