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The Role of MiR-544a in the Wnt/β-Catenin Pathway and its Association with Platinum-Based Chemotherapy Resistance in Advanced Non-Small Cell Lung Cancer

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Abstract

Introduction

MicroRNAs (miR)-544a has been identified as a potential regulator in the Wnt/β-Catenin pathway, but its specific role in non-small cell lung cancer (NSCLC) and its relationship with platinum-based chemotherapy resistance, remains unclear. Thus, we aim to determine the relationship between the expression of miR-544a and GSK-3β, β-catenin, and CD44 with platinum-based chemotherapy resistance in advanced stage NSCLC patients.

Methods

The research is designed as a case control study in which individuals diagnosed with advanced stage (III-IV) NSCLC from January 2018 and July 2023 from 6 different hospitals in Indonesia.

The analysis of miR-544a levels was done using the real-time QuantiTect SYBR Green PCR Master Kit. The expression of GSK-3β, β-catenin, and CD44 expression using immunohistochemistry (IHC) staining was performed from the formalin-fixed paraffin embedded (FFPE). The evaluation of IHC intensity was divided into four expression categories: negative or unstained, weakly positive, moderately positive, and strongly positive. From 500 cells, we used the semi-quantitative H-score formula.

Results

This study of 62 advance NSCLC patients undergoing platinum-based chemotherapy and found miR-544a were higher in poor responders, with a significant p-value of 0.009. The predictive model for MiR-544a demonstrated a AUC value of 0.6957. A miR-544a cutoff value of 2.08 yielded sensitivity of 64% and specificity of 67.57%. MiR-544a levels above 2.08 were significantly associated with poorer treatment response (OR 2.159, 95% CI 1.132 - 4.117, p = 0.016).

Conclusions

The study demonstrates that miR-544a levels are a significant biomarker for predicting chemotherapy response in patients with advance NSCLC.

Keywords

β-catenin, CD44, GSK-3β, miR-544a, platinum-based chemotherapy.

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Introduction

MiRNAs (miR) are pivotal in tumor initiation, progression, and metastasis, with miR-544a playing a significant role in platinum-based chemotherapy resistance in advanced non small cell lung cancer (NSCLC).[1] The challenges in treating advanced NSCLC arise from multiple mechanisms of resistance to platinum-based chemotherapy, encompassing

increased drug efflux, decreased drug uptake, altered drug metabolism, impaired deoxyribonucleic acid (DNA) repair, disruptions in cell cycle arrest and apoptosis, the tumor microenvironment's influence, phenotypic changes via epithelial-mesenchymal transition (EMT), cancer stem cells (CSCs), and epigenetic modifications such as deregulated miRs.[2,3]

MiR-544a has emerged as a pivotal factor in driving platinum-based chemotherapy resistance in advanced NSCLC, displaying heightened expression in NSCLC cell lines with augmented invasive capabilities and lung CSCs.[1] Its upregulation correlates with the downregulation of glycogen synthase kinase (GSK)-3β expression, thereby activating the Wnt signaling pathway and facilitating the translocation of β-catenin into the nucleus.[4] The Wnt/β-catenin pathway is integral to various cellular processes, including stem cell regeneration and organogenesis. Moreover, miR-1246 has been found to promote metastasis and invasion in lung cancer cells through its targeting of GSK-3β-mediated Wnt/β-catenin signaling. Additionally, miR-544a has been implicated in promoting cancer metastasis by targeting cadherin 1 (CDH1) and activating the Wnt signaling pathway across various cancer types, including lung cancer.[4,5] Furthermore, miR-544a regulates the migration and proliferation of vascular smooth muscle cells by targeting the miR-544a/phosphatase and tensin homolog (PTEN) axis, while its downregulation of GSK-3β helps sustain the self-renewal capacity of lung cancer stem cells.[6–8]

In an in vitro study conducted by Mo et al. it was shown that miR-544a is highly expressed in NSCLC cell lines with significant invasive potential. Additionally, another investigation by Mo et al. on lung cancer cells revealed that the overexpression of miR-544a leads to a decrease in GSK-3β expression and the activation of the Wnt signaling pathway. This activation results in the translocation of β-catenin to the nucleus, which in turn induces the expression of various cancer stem cell markers. As miR-544a has been identified as a potential regulator in cancer biology, but its specific role in NSCLC and its relationship with platinum-based chemotherapy resistance, remains unclear. Thus, we aim to determine the relationship between the expression of miR-544a and GSK-3β, β-catenin, and cluster of differentiation (CD44) with platinum-based chemotherapy resistance in advanced stage NSCLC patients.

Materials and Methods

The research is designed as a case control study in which individuals diagnosed with advanced stage (III-IV) adenocarcinoma with epidermal growth factor receptor (EGFR)-wild type and squamous cell carcinoma (SCC) from January 2018 and July 2023 were retrospectively and randomly gathered from six hospitals in Indonesia—specifically, Persahabatan National Respiratory Referral Hospital, Mochtar Riady Comprehensive Cancer Center, Medistra Hospital, Gatot Soebroto Army Hospital, Dharmais Hospital, and Dr. M. Goenawan Partowidigdo Respiratory Hospital. Patients with complete medical records, appropriate and sufficient formalin-fixed paraffin-embedded (FFPE) tumor specimens available were included. It is important to note that the expression levels of this miR were derived directly from the tissue. Clinical data, including age, sex, clinical

staging, chemotherapy drugs, and response evaluation criteria in solid tumors (RECIST) status, were obtained from their medical records. We categorized the complete response, partial response, and stable disease into a good RECIST outcome, whereas poor RECIST outcome was defined if the included sample was in the progressive disease group. The study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia (Approval No. KET-1030/UN2.F1/ETIK/PPM.00.02/2022), and permission was obtained from each participating institution.

Examination for miR-544a:

- Ribonucleic acid (RNA) Extraction from FFPE: Tissue isolation was performed using the Qiagen RNeasy FFPE kit. Paraffin tissue sections were cut, treated with xylene and ethanol, then incubated with PKD buffer. RNA was extracted and checked for quality.
- Circle DNA (cDNA) Synthesis: RNA templates were reverse transcribed into cDNA using Quantiscript Reverse Transcriptase. The synthesized cDNA was either used immediately or stored at -20°C.
- Real-time Quantitative polymerase Chain Reaction (PCR) (qPCR): miRNA-544a levels were quantified using QuantiTect SYBR Green PCR Master Kit. PCR was conducted, and expression levels were analyzed relative to miR-103a-3p as a reference gene and non-cancerous lung tissue as the control using the Livak formula.

The change in expression level (fold change) was calculated using the Livak formula, which was $2^{-\Delta\Delta CT}$ and expressed unitless, specifically for the fold change value of miRNA expression in poor chemotherapy response compared to good chemotherapy response.

Immunohistochemistry Staining Procedure (Manual Staining)

- Tissue sections from FFPE were cut to a thickness of 3–4 μm and placed on poly-L-lysine-coated slides. Each slide was labeled with the sample number and the type of antibody to be applied. Slides were warmed on a warmer for 30–60 minutes at a temperature of 56.5–60°C. Deparaffinization was performed using three changes of xylene (xylene I, II, and III) for 5 minutes each. Subsequent rehydration was done with decreasing concentrations of alcohol (absolute ethanol, 96% alcohol, and 70% alcohol), each for 5 minutes, followed by a 5-minute rinse with running water.
- Heat-induced antigen retrieval was carried out using 0.1 M NaOH citrate buffer (pH 7.0) in an autoclave at 121°C for 15 minutes, followed by rinsing in PBS (phosphate-buffered saline, pH 7.4) for 5 minutes. Endogenous peroxidase was blocked using 3% v/v hydrogen peroxide in methanol for 30 minutes at room temperature. Subsequently, slides were rinsed in running water for 5 minutes, followed by blocking of nonspecific protein using Background Sniper Universal for 15 minutes.
- Incubation with primary antibodies was performed as follows:
  - GSK-3β (9315S, Cell Signaling) overnight at room temperature at a dilution of 1:100.
  - β-catenin (ACR406C, Biocare) overnight at room temperature at a dilution of 1:200.
  - CD44 (MA5-13890, Thermo Scientific) for 1 hour at room temperature at a dilution of 1:200.

- After incubation with primary antibodies, slides were rinsed with PBS for 2 minutes. Subsequent incubation with secondary antibodies using biotinylated (Novolink) was performed for 30 minutes at room temperature. Slides were then washed with PBS for 5 minutes.
- Color reaction was developed using diaminobenzidine (DAB) substrate-chromogen for 1 minute, followed by rinsing with deionized water for 2 minutes and washing in running water for 10 minutes. Slides were then dipped in Hematoxylin Mayer counterstain for about 1−2 minutes and washed with running water for 3 minutes, followed by bluing with lithium carbonate for 5 seconds and another wash with running water. Dehydration was done with graded alcohol (70%, 96%, and absolute ethanol) for 3 minutes each, followed by clearing with xylene I, II, and III for 5 minutes each, mounting with mounting medium, and cover slipping. Negative and positive controls were included with each staining run. Positive controls for GSK-3β came from breast carcinoma tissue, for β-catenin from colon carcinoma, and for CD44 from tonsil tissue.

  The evaluation of staining intensity had been divided into four expression categories: negative or unstained (0), weakly positive (+1), moderately positive (+2), and strongly positive (+3), alongside the proportion of tumor cells stained. In assessing 500 cells, we used the H-score formula to determine each immunohistochemistry expression: ([% of weakly stained cells × 1] + [% of moderately stained cells × 2] + [% of strongly stained cells × 3]) divided by 130.[9] The assessment of β-catenin expression was conducted through two methods: initially employing the H-score to examine nuclear expression, followed by determining the percentage of staining expression with weak, moderate, and strong intensity across the membrane, cytoplasm, and nucleus in each sample. The evaluation of each sample staining was performed by two pathologists with agreement using QuPath Software™, blindly without knowing the RECIST status.

  Data analysis in this study was conducted using Statistical Packages for Social Sciences (SPSS) version 27. Normality assessment was performed using the Kolmogorov-Smirnov test for numerical data. MiR-544a, along with GSK-3β, β-Catenin, and CD44, was assessed for associations with age, sex, cancer stage, cancer subtype, and RECIST status using the Mann Whitney test. Additionally, the correlation between miR-544a and GSK-3β H-Score, β-Catenin H-score, CD44 H-score, CD44 strong expression, as well as the different localizations of β-Catenin (membrane, cytoplasm, nucleus), was examined through regression analysis. Discrimination and differentiation between surviving and deceased patients were assessed through Area Under the Curve (AUC) analysis. A higher AUC value indicates better predictive capacity of patient mortality outcomes by the model. Calibration, which measures the agreement between predicted and observed results, was assessed using the Hosmer-Lemeshow test. A p-value above 0.05 suggests satisfactory calibration of the scoring system. Sensitivity and specificity evaluations were conducted using optimal cut-off values derived from the Youden index.

Results

The study comprised 62 patients newly diagnosed with NSCLC who underwent three cycles of platinum-based chemotherapy. Regarding RECIST status, we observed that 5 patients exhibited a partial response, while 32 patients fell into the stable disease category. These groups were consolidated into a single category, referred to in this study as the favourable RECIST status response group (n = 37). Conversely, 25 patients were classified under the progressive disease status and were thus categorized into the unfavourable RECIST status response group. Age categories exhibited no significant differences in miR-544a (3.5 ± 5.9 vs. 5.3 ± 8), GSK-3β (94.8 ± 29 vs. 97.68 ± 28), β-Catenin (18.8 ± 43 vs. 7.7 ± 8.9), or CD44 (69.3 ± 58.6 vs. 72.4 ± 48). However, females showed significantly lower GSK-3β levels (83.1 ± 28.4) than males (100.4 ± 27.6, p=0.033). Stage III and IV patients differed significantly in GSK-3β levels (109.2 ± 23.5 vs. 92.8 ± 28.9, p=0.031) but not in other biomarkers. SCC had higher GSK-3β levels than adenocarcinoma (114.1 ± 15.8 vs. 92.6 ± 29.3, p=0.015); other biomarkers showed no subtype differences. Detailed demographic characteristics was presented in **table 1**.

GSK-3β was detected positively in the cytoplasm, β-catenin was observed in the nucleus, and CD44 was present in the cell membrane of the tumor cells. (**Figure 1, Figure 2, and Figure 3)**

Median values for miR-544a were 2.88 in poor responders and 1.38 in good responders, with a significant p-value of 0.009. GSK-3β levels showed no significant difference between groups, with medians of 104.6 and 101.6 respectively (p=0.59). Similarly, β-Catenin and CD44 also did not significantly differ between poor and good responders, with p-values of 0.304 and 0.126 respectively (**table 2**).

Correlation analysis revealed miR-544a associations with various biomarkers (**table 3**). miR-544a moderately correlated negatively with β-Catenin in the cytoplasm (r = -0.383, p = 0.002), but not with other biomarkers.

The AUC value of the miR-544a model in relation to therapy response was determined to be 0.6957 (**figure 4**). This indicates that the predictive model for miR-544a can account for 69.57% of the variability in therapy response outcomes, with a 95% Confidence Interval ranging from 0.569 to 0.828. The remaining 30.6% of variability is attributed to other variables.

Following AUC analysis, the optimal cutoff value of 2.08 was identified for miR-544a, achieving a sensitivity of 64% and specificity of 67.57% (LR+ = 1.9733 and LR- = 0.5328). MiR-544a levels below 2.08 were significantly linked to a higher likelihood of poor treatment response (OR 2.159, 95% CI 1.132 - 4.117, p = 0.016), with 9 patients categorized as poor responders compared to 25 as good responders (**table 4**). Conversely, miR-544a levels at or above 2.08 indicated 16 patients classified as poor responders and 12 as good responders.

Discussion

MiR-544a regulates gene expression by binding to the 3' untranslated regions (3' UTRs) of target mRNAs, leading to their degradation or inhibition of translation, and is crucial

in various biological processes including cell proliferation, apoptosis, and differentiation.[10] In cancer biology, miR-544a relationship with GSK-3β (glycogen synthase kinase-3 beta), β-catenin, and CD44 is significant.[11] GSK-3β, a serine/threonine kinase, plays a key role in the Wnt signaling pathway by phosphorylating β-catenin, leading to its degradation and keeping β-catenin levels low in the cytoplasm in the absence of Wnt signaling.[12] Dysregulation of the canonical WNT/β-catenin signaling pathway contributes to the carcinogenesis of solid tumors and hematologic malignancies, playing a role in EMT and self-renewal, proliferation, and differentiation (SPK) activities.[13]

The study by Yanaka et al. employed an in vitro cell-based reporter system to identify miR-544a as a miRNA inducing EMT by directly targeting CDH1 and AXIN2, and indirectly regulating APC2, leading to β-catenin translocation into the cytoplasm and nucleus.[14] β-catenin plays dual roles in maintaining cell-cell adhesion via the cadherin-catenin complex and as a pivotal regulator of WNT signaling.[15] Within the nucleus, β-catenin acts as a transcriptional activator that triggers EMT.[16] Elevated miR-544a expression in their research reduced E-cadherin, AXIN2, and APC2 expression without affecting GSK-3β, facilitating β-catenin translocation and activating TCF/LEF transcription factors, marked by increased Vimentin expression, thereby promoting gastric carcinoma cell motility and invasion through the EMT pathway.[14] Our own investigation revealed significant correlations between miR-544a levels and reduced cytoplasmic β-catenin expression, suggesting a regulatory role in this cellular compartment. However, miR-544a showed no significant links with GSK-3β and CD44 levels, and weaker associations with β-catenin expression in the membrane and nucleus, indicating potential selective interactions within specific pathways relevant to non-small cell lung cancer progression.

MiR-544a has emerged as a significant regulator of chemotherapy response across various cancer types among the many miRNAs studied. Studies indicate that miR-544a plays a role in altering cancer cell sensitivity to chemotherapy agents.[17] For instance, a study by He et al. in 2018 showed that upregulated miR-544a has been associated with promoting migration and invasion in colorectal cancer cells.[18] Lu et al. showed that miR-544a promotes cancer metastasis by targeting cadherin 1 in lung cancer and inducing epithelial–mesenchymal transition via activating the WNT signal in gastric cancer.[19] Inhibition of miR-544a leads to decreased expression of MMP-2 and MMP-9 and increased levels of PARP and caspase 3. Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) inhibits the expression of various types of MMP at the post-transcriptional level, suppressing tumor angiogenesis and thereby inhibiting tumor invasion and metastasis. Zeng et al. study also demonstrated that inhibiting miR-544a results in increased expression of RECK, thereby suppressing the progression of endometrial carcinoma. This suggests that miR-544a could be developed as a targeted therapy for endometrial carcinoma.[20] In our study, we observed significantly higher levels of miR-544a in poor responders compared to good responders (median 2.88 vs. 1.38, p=0.009). This finding supports the hypothesis that increased expression of

oncogenic miRNAs, such as miR-21-5p, in response to chemotherapy is associated with cancer cell adaptation to treatment. One proposed mechanism is that MiR-544a inhibits caspase 3, a mechanism that leads to the suppression of apoptosis. Platinum-based chemotherapy agents act on cancer cells by causing DNA damage, nuclear and mitochondrial damage, disrupting DNA replication, and transcription in the nucleus, which results in ROS production, cell cycle arrest, and ultimately induces apoptosis. The elevated expression of miR-544a affects the response of cancer cells to platinum-based chemotherapy agents. Furthermore, previous research has indicated that chemotherapy-induced exosomal miR-378a-3p and miR-378d promote cancer stemness and resistance to chemotherapy.[21] Therefore, our study suggests that miR-544a may be linked to therapy response, but not directly through the WNT/β-catenin pathway.

Changes in miRNA expression profiles represent promising indicators of chemotherapy response in advanced NSCLC. For example, circulating exosomal miR-425-3p has emerged as a significant prognostic factor for the response of NSCLC to platinum-based chemotherapy.[22] Additionally, miR-21 has been identified as a suppressor of NSCLC sensitivity to chemotherapy and radiotherapy, underscoring its potential utility as a predictive marker for platinum chemotherapy in NSCLC patients.[23] However, previous literature primarily involved animal models, with a limited number of unpublished clinical trials utilizing miRNA to monitor chemotherapy response. In our study, we investigated human subjects and conducted AUC analysis, revealing a sensitivity of 64% and specificity of 67.57% for a miR-544a cutoff value of 2.08. Saito et al. identified a miRNA signature (miR-1290, miR-196b, and miR-135a) capable of accurately predicting chemotherapeutic response in lung adenocarcinoma patients, achieving sensitivities of 82.5% and 77.8% in test and validation cohorts, respectively.[24] Similarly, Yuwen et al. examined 203 patients and highlighted miR-425-3p role in upregulating autophagy levels by targeting AKT1, thereby reducing therapeutic response.[25] In contrast, our findings on miR-544a suggest its influence on drug efflux transporters, apoptosis-related proteins, and signaling pathways, potentially contributing to chemotherapy resistance and decreasing cancer cell susceptibility to chemotherapy-induced cell death.

Another point to highlight is that we found miR-544a was moderately correlated negatively with β-Catenin in the cytoplasm ($r = -0.383$, $p = 0.002$), but not with other markers. One possible explaination is that miR-544a may directly regulate β-Catenin levels in the cytoplasm but might not affect upstream regulators (like GSK-3β) or downstream processes (like nuclear translocation). Moreover, the cellular distribution and function of β-Catenin are complex, involving multiple regulatory layers and interactions that might not be directly influenced by miR-544a alone. Tang et al. demonstrated that β-Catenin/TCF/LEF-1 can bind to the promoter of the miR-183-96-182 cluster gene, activating its transcription.[26] Additionally, Potenza et al. found that miR-544a can decrease CDH1 and AXIN2 expression while inducing the nuclear import and stabilization of β-catenin in the nucleus through the Wnt signaling pathway.[27] Xie et al. showed that exosomal miR-1246 directly targets GSK-3β, leading to enhanced β-catenin protein expression and inhibition of apoptosis and inflammation.[28]

Furthermore, Mo et al. demonstrated that miR-544a can inhibit the expression of GSK-3β, leading to increased levels of β-catenin.[6]

However, we encountered several study limitations. Firstly, the study comprised only 62 patients, which may limit the generalizability of the findings, and lacked long-term follow-up, potentially missing insights into the long-term effects and survival outcomes post-chemotherapy. Additionally, the study analyzed only a few biomarkers (miR-544a, GSK-3β, β-Catenin, CD44), not considering other potentially relevant biomarkers such as the APC and AXIN molecules. Despite these limitations, the study has important clinical applicability. MiR-544a can be used as a potential predictive biomarker to identify patients less likely to respond well to platinum-based chemotherapy. Using miR-544a levels as part of a risk stratification model could improve clinical decision-making and patient counseling regarding expected treatment outcomes. The findings could serve as a basis for larger, multicenter trials to validate the role of miR-544a and other biomarkers in predicting chemotherapy response in NSCLC and support the development of diagnostic tests or kits based on miR-544a levels to guide clinical practice in NSCLC management.

Conclusions

The study demonstrates that miR-544a levels are a significant biomarker for predicting chemotherapy response in patients with advance NSCLC, with higher levels associated with poor treatment outcomes. While other biomarkers such as GSK-3β, β-Catenin, and CD44 did not show significant differences in response prediction, miR-544a predictive value highlights its potential utility in clinical decision-making. Furthermore, sex and cancer stage were identified as influencing factors for GSK-3β levels, and histological subtype impacted GSK-3β levels as well, indicating the complexity of biomarker interactions in NSCLC. These findings suggest that personalized treatment strategies considering miR-544a levels could enhance therapeutic efficacy in NSCLC patients.

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Figure Legends

Figure 1. Immunohistochemical expression of GSK-3β in the cytoplasm of tumor cells (IHC 400x); A, B, C, and D are negative, weak, moderate, and strong expression, respectively.

Figure 2. Immunohistochemical expression of β-catenin on the nucleus of tumor cells (IHC 400x); A, B, C, and D are membrane, cytoplasm, and nucleus, respectively.

Figure 3. Immunohistochemical expression of CD44 on the membrane of tumor cells (IHC 400x); A, B, C, and D are negative, weak, moderate, and high expression, respectively.

Figure 4. MiR-544a has the potential to assist in predicting the likelihood of therapy response by approximately 69.57%.

Table 1. Relationship Between Clinical and Histopathological Characteristics and the Level of Biological Marker Expression (n = 62).

Table 2. Variations in the Expression Levels of Biological Markers.

Table 3. Relationship Between miRNA Expression and Expression of GSK-3β, β-Catenin, and CD44.

Table 4. The threshold of MiR-544a expression levels and its correlation with therapy response based on RECIST criteria.

Statements & Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed Erna Kristiani. The first draft of the manuscript was written by Erna Kristiani and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval

KET-1030/UN2.F1/ETIK/PPM.00.02/2022.

Consent to participate

We affirm that all subjects within our cohort study have granted informed consent, comprehending the utilization of their data for publication purposes, with strict adherence to confidentiality protocols.

Consent to publish

Cohort participants have explicitly consented to the publication of their data, with assurances of confidentiality upheld as per the terms outlined during the informed consent process.