ORIGINAL ARTICLE

Bali Medical Journal (*Bali MedJ*) 2025, Volume 14, Number 1: 115-121
P-ISSN.2089-1180, E-ISSN: 2302-2914

WNT/β-Catenin signaling pathway and clinicopathological factors in advanced stage non-small cell lung cancer: a multicenter study

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Received: 2024-05-07
Accepted: 2024-08-05
Published: 2024-09-05

ABSTRACT

Background: This study aimed to assess the association of GSK-3β, β-catenin, and CD44 expressions, which are the molecules involved in the WNT/β-catenin signaling pathway, with clinicopathological profiles and their correlation with the response to platinum-based chemotherapy using Response Evaluation Criteria in Solid Tumors (RECIST) criteria in advanced-stage Non-Small Cell Lung Cancer (NSCLC).

Methods: This research is a case-control study where we retrospectively collected data on every patient diagnosed with stage III-IV, adenocarcinoma EGFR-wild type and squamous cell carcinoma (SCC) who underwent three cycles of platinum-based chemotherapy and had their first RECIST evaluation. Immunohistochemical (IHC) staining for GSK-3β, β-catenin, and CD44 was performed on lung biopsy or surgical samples. RECIST statuses were classified as follows: complete response, partial response, and stable disease were considered favorable outcomes, while progressive disease was considered unfavorable.

Results: We analyzed 62 samples, including 37 patients with favorable outcomes and 25 patients with unfavorable outcomes. GSK-3β expression was higher in subjects who were male (p = 0,033), had stage III disease (p = 0,031), and had the SCC subtype (p = 0,015). While GSK-3β expression tended to be higher in subjects with unfavorable responses, while β-catenin and CD44 expression tended to be higher in subjects with favorable responses, these differences were not statistically significant. Correlation analysis among GSK-3β, β-catenin, and CD44 revealed no significant linear associations.

Conclusion: The WNT/β-catenin signaling pathway has a specific role in advanced-stage NSCLC. These findings emphasize the complex interplay between clinicopathological features and biological marker expression in NSCLC, warranting further investigation to elucidate underlying regulatory mechanisms and potential therapeutic targets.

Keywords: CD44, β-catenin, GSK-3β, NSCLC, platinum-based chemotherapy, RECIST.
Cite This Article: Kristiani, E., Syahruddin, E., Asmarinah., Rachmadi, L., Ham, M.F., Zaini, J., Kekalih, A., Heriyanto, D.S., Hidajat, H., Gultom, F.L. 2025. WNT/β-Catenin signaling pathway and clinicopathological factors in advanced stage non-small cell lung cancer: a multicenter study. *Bali Medical Journal* 14(1): 115-121. DOI: 10.15562/bmj.v14i1.5390

INTRODUCTION

According to recent studies, lung cancer ranks as the primary cause of cancer-related mortality globally, with a 5-year survival rate remaining below 15%.[1,2] Over 60% of lung cancer cases are diagnosed at advanced stages (stage III–IV), necessitating systemic therapy.[3] Current chemotherapy regimens for advanced lung cancer predominantly employ platinum-based alkylating agents.[4,5] The efficacy of chemotherapy treatment for lung tumors is evaluated using the RECIST.[6]

One identified resistance mechanism against platinum involves cancer stem cell (CSC) activity.[7] The Wnt/β-catenin pathway, known for its role in embryonic development and tissue homeostasis, is also implicated in CSC activity.[1,8] Nuclear β-catenin expression can be utilized as a CSC marker.[9] GSK-3β, a member of the GSK-3 kinase family, involved in the Wnt/β-catenin pathway. GSK-3β exhibits dual roles as both a tumor promoter and a tumor suppressor, depending on its ability to phosphorylate proteins, thereby inhibiting or stimulating cell growth.[10] CD44 functions as a CSC marker in the progression, migration, and drug resistance of colorectal, breast, and lung cancers.[11,12]

Most studies investigating the mentioned biomarkers are conducted through in vivo and in vitro studies, with limited data available from Indonesia. This study aimed to assess the association of these markers expression in advance stage NSCLC with clinicopathological profiles, and their correlation with the response to platinum-based chemotherapy using RECIST

criteria. Additionally, it seeks to determine their correlation with the response to platinum-based chemotherapy and their potential as targeted therapy for advanced stage NSCLC patients.

METHODS

The research is designed as a case-control study in which individuals diagnosed with advanced stage (III-IV) Adenocarcinoma with EGFR-wild type and SCC from January 2018 and July 2023 were retrospectively 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. The current study employs consecutive sampling as its sampling technique, ensuring participants are systematically recruited in sequential order. The inclusion criteria were patients with complete medical records and appropriate and sufficient formalin-fixed paraffin-embedded (FFPE) tumor specimens. Exclusion criteria for this study encompass patients with malignancies other than lung cancer, aiming to maintain the homogeneity of the study population. Clinical data, including age, gender, clinical staging, chemotherapy drugs, and RECIST status, were obtained from their medical records. We categorized the complete response, partial response, and stable disease into a favorable RECIST outcome, whereas an unfavorable RECIST outcome was defined if the included sample was in the progressive disease group.

The histopathological confirmation and grading of the diagnosis were conducted by pathologists who reviewed the H&E slides. The immunohistochemistry staining procedure included multiple steps for the paraffin blocks containing tumor tissue, encompassing deparaffinization, rehydration, heat-induced antigen retrieval, and blocking. Subsequently, primary antibodies, such as 9315S (Cell Signaling™) for GSK-3ß, ACR406C (Biocare™) for β-catenin, and MA5-13890 (Thermo Scientific™) for CD44, were applied to the slides. Following this, secondary antibody incubation occurred with biotinylated (Novolink™), and the slides underwent counterstaining with Hematoxylin Mayer. The histopathological examination (HE) and immunohistochemistry (IHK) preparations undergo collaborative review by two pathologists, promoting agreement in diagnostic interpretation and bolstering the trustworthiness of the results through enhanced reliability. The positive control GSK-3β came from breast carcinoma tissue, β-catenin from colon carcinoma, and CD44 from tonsil tissue.

Five to ten well-stained photographs of tumor areas from each slide were taken at 400× magnification. Subsequently, a random selection process was run, assessing staining intensity in only five samples. Each chosen photo was partitioned into four quadrants (quadrants 1, 2, 3, and 4). Following this, another randomization was conducted, and only one quadrant was selected for staining intensity assessment, with a maximum of 100 cells evaluated per quadrant. Stained intensity was semi-quantitatively evaluated in each selected quadrant from the five photos, totaling 500 cells, using the Qu-path™ software.

The intensity of staining in tumor cells was assigned scores ranging from 0 to +3, corresponding to no staining, weak, moderate, and brown solid staining, respectively. Pathologists evaluated marker positivity without knowledge of the stage and RECIST status of the sample. Qu-path™ software was used for quantifying both the quantity and quality of expression. The analysis of marker expression utilized a histoscore (H-score) system using the following formula: H-score = [1 x (% cells +1) + 2 x (% cells +2) + 3 x (% cells +3)]. H-scores range from 0 to 300.

While the H-score was presented as numerical data, the clinicopathological data was categorized. Age, gender, stage, histopathological grading, RECIST status, and other categorical data were presented as frequencies and percentages. The entire research data was processed using Statistical Program for Social Science (SPSS) version 25. The correlation between clinical characteristics and histopathological features with the expression level of biological markers was analyzed using the Mann-Whitney test. Additionally, we used the Spearman correlation regression test to assess the association between GSK- 3β, β-catenin, and CD44. The analysis was accepted as significant if the p-value for each test was less than 0.05.

RESULTS

Patients with newly diagnosed NSCLC who had received platinum-based chemotherapy for 3 cycles had undergone their first RECIST evaluation, and sufficient FFPE specimens were included in the study, with a total of 62 patients. With a median age of 59 years (range: 31-80), the study comprised 53.2% individuals below 60 years and 46.8% aged 60 years or older, with males constituting 79% and females 21%. The majority of disease staging leaned towards advanced stages, with 75.9% diagnosed at stage IV and 24.1% at stage III. Adenocarcinoma emerged as the predominant cancer subtype (80.6%), whereas SCC accounted for 19.4%. Most patients (82.2%) received carboplatin-based therapy, while 17.8% received cisplatin. Evaluation of treatment response by RECIST criteria showed no complete responses, 8% partial responses, 51.6% stable disease, and 40.3% progressive disease, ultimately categorizing treatment response as favorable in 59.7% and unfavorable in 40.3% of patients. The immunohistochemistry stains were assessed using Qu-Path software. GSK-3β expression was identified as positive in the cytoplasm, β-catenin expression in the nucleus, and CD44 expression in the cell membrane of the tumor cells. (Figure 1, Figure 2, and Figure 3)

When considering the age, there was no significant difference observed in the expression levels of GSK-3β ($p = 0.832$), β-catenin ($p = 0.631$), or CD44 ($p = 0.677$) between patients younger than 60 years and those aged 60 years or older. However, a notable gender-based difference was identified, with males demonstrating significantly higher levels of GSK-3β expression compared to females ($p = 0.033$). No significant differences were found for β-catenin ($p = 0.802$) or CD44 ($p = 0.938$) based on gender. Additionally, the levels of GSK-3β expression exhibited a notable contrast between Stage III and Stage IV tumors, displaying elevated levels in the former ($p = 0.031$). Conversely,

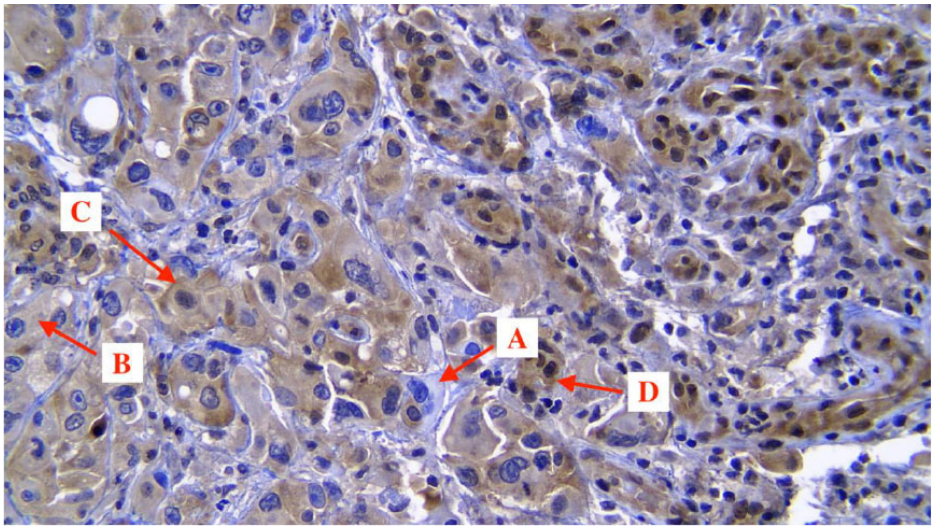

Figure 1. Immunohistochemical expression of GSK-3β in the cytoplasm of tumor cells (IHC 400x); A, B, C, and D are negative, weak, moderate, and strong expression, respectively.

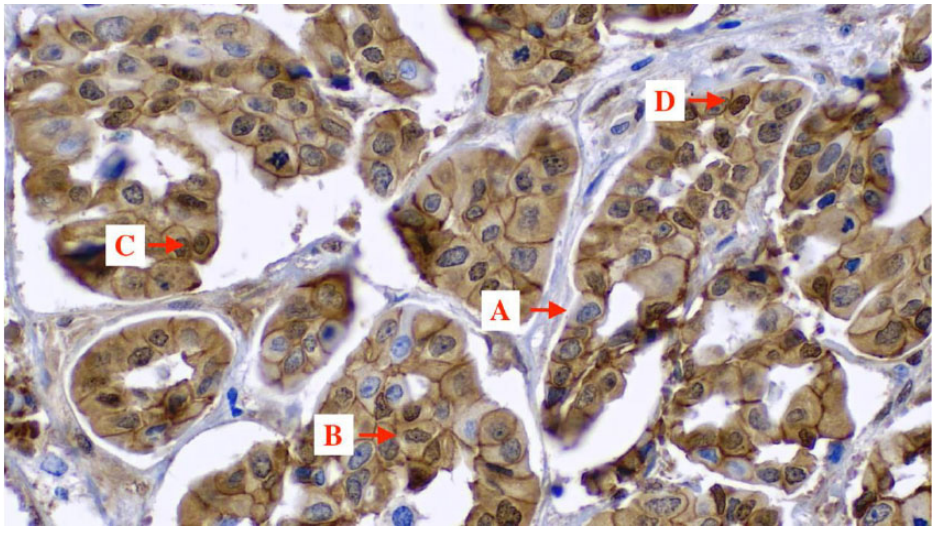

Figure 2. Immunohistochemical expression of β-catenin on the nucleus of tumor cells (IHC 400x): A, B, C, and D are negative, weak, moderate, and high expression, respectively.

no statistically significant variances were observed in the expression levels of β-Catenin and CD44 between these two stages (p = 0.537 and p = 0.588, respectively). No significant differences were observed for β-catenin (p = 0.537) or CD44 (p = 0.588) across different stages. In terms of cancer subtype, individuals with SCC demonstrated significantly higher levels of GSK-3β expression compared to those with Adenocarcinoma (p = 0.015), whereas no significant differences were noted for β-catenin (p = 0.387) or CD44 (p = 0.109) (Table 1).

Treatment response analysis indicated no significant differences in the expression levels of GSK-3β or β-catenin between patients with favorable and unfavorable treatment responses, while CD44 expression levels showed a trend towards significance (p = 0.126), being lower in patients with unfavorable treatment responses. (Table 2) In the favorable response group, β-catenin and CD44 exhibit higher expression levels compared to the unfavorable response group. Specifically, the median expression of β-catenin within the favorable response subset is 6 (range: 0 – 229.2), contrasting with 5.4 (range: 0.6 – 33.6) within the unfavorable response subset. Similarly, CD44 manifests increased expression, displaying a median of 81.2 (range: 2.1 – 167.8) in the favorable response subset, as opposed to 46.2 (range: 2.4 – 223.3) in the unfavorable response subset. Conversely, GSK-3β expression is attenuated within the favorable response subset, characterized by a median expression of 101.6 (range: 2.2 – 129), compared to 104.6 (range: 56.6 – 145.5) within the unfavorable response subset (Table 2).

The correlation analysis between GSK-3β, β-catenin, and CD44 was conducted to investigate the potential associations among these biological markers (Table 3). The results revealed no statistically significant correlations between GSK-3β and β-catenin (p = 0.673, r =0.065), β-catenin and CD44 (p = 0.618, r = 0.065), or GSK-3β and CD44 (p = 0.605, r = 0.067).

DISCUSSION

The expression levels of GSK-3β, β-catenin, and CD44 have been extensively studied in relation to various clinical parameters in different types of cancer. CD44, a cell surface glycoprotein, has been associated with tumor recurrence and poor prognosis in various cancers such as colorectal carcinoma, hepatocellular carcinoma, renal cell carcinoma, ovarian cancer, endometrial cancer, pancreatic carcinoma, breast cancer, gastric cancer, and others, linking its expression to clinical outcomes, disease-free survival, and overall survival rates.[13–21] Conversely, GSK-3β, a serine/threonine protein kinase, has been implicated in the pathogenesis of chronic allograft dysfunction in renal transplantation, with significantly higher expression levels observed in colorectal carcinoma tissue compared to normal tissue, and its dysregulation associated with various diseases including cancer.[22] Additionally, β-catenin, a key component of the WNT signaling pathway, interacts with GSK-3β and has been studied in relation to CD44 expression, though no significant association was observed between the expression levels of β-catenin, CD44, and other biomarkers, suggesting a complex interplay in cancer progression.[23,24] Our study found no significant correlations between age and

the expression levels of GSK-3β, β-catenin, or CD44 while showing significantly higher levels of GSK-3β expression in males than females. Notably, no significant differences were found for β-catenin or CD44 based on gender.

The research findings from Alves M et al. and Ren J et al. indicate a notable association between GSK-3β expression levels and disease stage in NSCLC, with heightened expression observed in advanced stages (III and IV) compared to early stages (I and II).[25,26] While Alves M et al. established a significant correlation between GSK-3β expression and disease stage (p = 0.007), Ren J et al. found a prevalence of positive GSK-3β expression in stage III relative to stage I-II, although statistically insignificant.[26] Kaplan-Meier analysis highlighted a more favorable prognosis among patients exhibiting negative GSK-3β expression. Notably, this study encompassed individuals diagnosed with advanced stages, categorized into stage III and stage IV, revealing a significant association between GSK-3β expression and disease stage (p = 0.031).

Despite the elevated median expression of GSK-3β in stage III compared to stage IV, concurrent reductions in nuclear β-catenin expression and CD44 expression were not observed. Within the canonical WNT pathway, GSK-3β operates alongside APC and Axin proteins, forming a destruction complex that phosphorylates cytoplasmic β-catenin and facilitates its nuclear translocation, activating TCF/LEF transcription factors responsible for WNT target gene activation such as CD44. Additionally, studies by Cao X et al. and Zeng J et al. reported heightened GSK-3β expression within tumor tissues compared to normal lung tissue, with increased expression correlating with a poorer prognosis.[27,28] These findings collectively suggest a significant role of GSK-3β in NSCLC progression, impacting disease prognosis and potentially influencing therapeutic response.

The current study observed that the average expression level of GSK-3β was notably higher in the SCC subtype compared to the Adenocarcinoma subtype, and this disparity held statistical significance (p = 0.015). Both Ren J et al. and Alves M et al. studies reported a greater prevalence of positive GSK-3β expression in the SCC subtype; however, neither study obtained statistically significant findings.[25,26] Furthermore, Zeng J et al.'s study revealed that patients exhibiting positive GSK-3β expression in

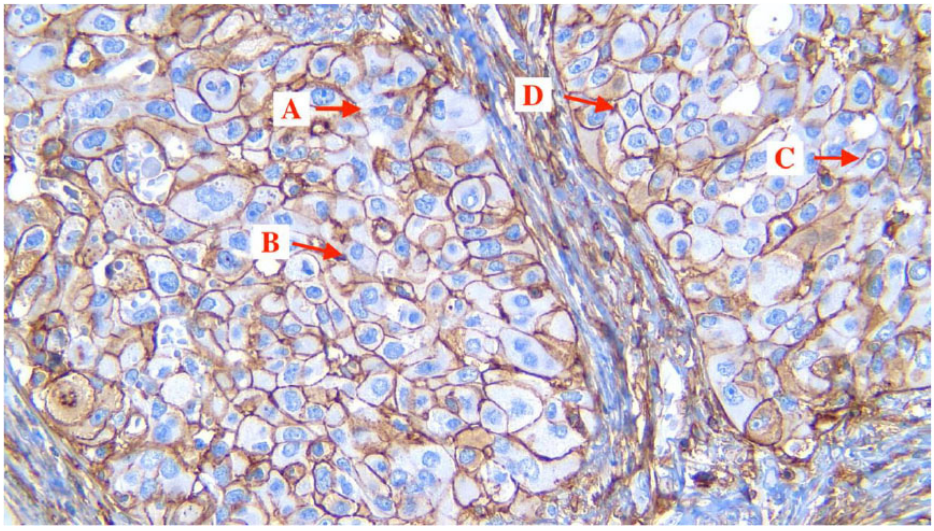

Figure 3. Immunohistochemical expression of CD44 on the membrane of tumor cells (IHC 400x); A, B, C, and D are negative, weak, moderate, and high expression, respectively.

Table 1. The correlation between clinical characteristics and histopathological features with the expression level of biological markers

Variable	GSK-3β	β-Catenin	CD44
	Median (range)	Median (range)	Median (range)
Age			
< 60 (n = 33)	101.8 (2.2 – 131.8)	5.4 (0.6 – 229.2)	58.6 (2.1 – 223.3)
≥ 60 (n = 29)	100.4 (52.8 – 145.4)	5.4 (0 – 34.2)	87.2 (10.4 – 167.8)
p-value	0.832	0.631	0.677
Sex			
Male (n = 49)	102.4 (2.2 – 145.4)	5.4 (0 – 229.2)	63.2 (2.1 – 223.3)
Female (n = 13)	82.2 (32.2 – 126.4)	5.4 (0.6 – 33.6)	62.2 (14 – 142.6)
p-value	0.033*	0.802	0.938
Cancer stage			
III (n = 15)	109.4 (53.4 – 145.4)	4.2 (0 – 100.8)	87.2 (2.4 – 223.3)
IV (n = 47)	100.6 (2.2 – 131.8)	6 (0 – 229.2)	62.2 (2.1 – 167.4)
p-value	0.031*	0.537	0.588
Cancer subtype			
Adenocarcinoma (n = 50)	100.2 (2.2 – 131.8)	5.7 (0 – 229.2)	60.5 (2.1 – 167.8)
SCC (n = 12)	109.8 (94 – 145)	3.9 (0.6 – 28.2)	111 (14.1 – 223.3)
p-value	0.015*	0.387	0.109

*Mann-Whitney test: statistically significant if p-value less than 0.05

Table 2. Comparison of GSK-3β, β-catenin, and CD44 expression between patients with a favorable and unfavorable response to platinum-based chemotherapy in advanced NSCLC

Variables	Frequency (n)		P
	Favorable Response	Unfavorable Response	
Biomarkers			
GSK-3β	101.6 (2.2 – 129)	104.6 (56.6 – 145.5)	0.590
β-catenin	6 (0 – 229.2)	5.4 (0.6 – 33.6)	0.304
CD44	81.2 (2.1 – 167.8)	46.2 (2.4 – 223.3)	0.126

*Mann-Whitney test: statistically significant if p-value less than 0.05

Table 3. Correlation between GSK-3β, β-catenin, and CD44

Variable	p	r
GSK-3β		
β-catenin	0.673	0.065
CD44	0.618	0.065
GSK-3β	0.605	0.067

*Spearman test: statistically significant if p-value less than 0.05

the SCC subtype experienced a poorer prognosis.[28]

Treatment response was evaluated using RECIST criteria, wherein complete, partial, and stable responses were categorized as favorable outcomes (control group), while progressive disease was classified as an unfavorable outcome (case group). The findings of the study revealed a higher proportion of participants in the favorable response category compared to the unfavorable response category. These results are consistent with previous research conducted in France, which reported a higher incidence of favorable responses (n = 391) than unfavorable responses (n = 73) among 464 patients with NSCLC.[29] Similarly, the outcomes of this study align with those reported by Kaira K et al., who identified a greater number of patients exhibiting favorable responses (n = 35) compared to unfavorable responses (n = 19) in NSCLC patients.[30] Importantly, no significant disparities were observed between the levels of biological marker expression and response to chemotherapy in this investigation. GSK-3β expression was found to be higher in cases associated with an unfavorable response, whereas β-catenin and CD44 expression levels were notably lower. These observations emphasize a distinct regulatory paradigm, wherein cytoplasmic GSK phosphorylation of β-catenin facilitates its translocation into the nucleus, consequently activating transcription factors such as the CD44. It is important to note that this study used a retrospective approach, thereby limiting the availability of clinical-pathological data. The assessment of therapy response in NSCLC patients encompassed subjective indicators such as patient-reported symptoms, including cough, dyspnea, or chest pain, semi-subjective measures such as appearance and weight, and objective evaluations based on tumor size utilizing RECIST criteria.[31] The relationship among GSK-3β, β-catenin, and CD44 in NSCLC involves complex molecular interactions, with GSK-3β influencing β-catenin expression and activity in various cancer types, indicating a cell type-specific correlation.[32] Correlations between nuclear GSK-3β and β- catenin in oral carcinogenesis and their involvement in myofibroblast transition in lumbar spinal stenosis patients underscore their intricate relationship.[32–34] In metastatic NSCLC, PLK1-mediated β-catenin phosphorylation affects extracellular matrix-related factors, including CD44, during epithelial-mesenchymal transition. Additionally, miR-27a's role in breast cancer progression through GSK-3β activity suggests a link to WNT/β-catenin signaling.[34] Dysregulated pathways highlight a core relationship between β-catenin and GSK-3β while inhibiting GSK-3β improves angiogenesis during VEGF-driven pathological processes. Prostaglandin E2 regulates stem cell differentiation via the GSK-3β/β-catenin pathway, and GSK-3β influences SNAIL protein expression and stability in NSCLC. The PI3K/AKT/GSK-3β/β-catenin pathway contributes to proliferation, migration, invasion, and epithelial-mesenchymal transition in gastric cancer cells, emphasizing their role in cancer progression.[35] However, this study found no statistically significant correlations between GSK-3β and β-catenin, β-catenin and CD44, or GSK-3β and CD44 in NSCLC, suggesting limited direct linear relationships between these markers' expression levels. Further investigation is necessary to elucidate their interactions fully.

The study has several notable limitations. Firstly, the retrospective design of the study, relying on historical data, potentially affects the accuracy and completeness of the collected clinical data. Furthermore, the implementation of exclusion criteria, such as excluding individuals with insufficient samples or inappropriate slides, may raise the potential for selection bias, thus compromising the study population's representativeness. While the study analyzes biomarker expressions across cancer stages, it does not delve into potential variations within each stage, suggesting a need for a finer-grained analysis to enhance the study's clinical relevance. The detection of further impactful molecules lies outside the objective of our investigation, with a notable aspect being that signaling pathways typically incorporate multiple molecular interactions.

Our study demonstrated the significance of the WNT signaling pathway, which is supported by statistically significant findings in the GSK-3β subgroup. Higher GSK-3β expression was observed in males compared to females, in stage III compared to stage IV NSCLC, and in SCC compared to adenocarcinoma. Our analysis of treatment responses revealed discrepancies in the expression levels of GSK-3β, β-catenin, and CD44; specifically, within the favorable response group, both β-catenin and CD44 exhibited higher expression levels compared to the unfavorable response group. This is related to the nature of CSC. However, we must acknowledge a limitation in our study about identifying other influential molecules. It is essential to highlight that signaling pathways

typically comprise myriad molecular interactions that remained unexplored in our analysis. This emphasizes the complex interdependence among clinical attributes, histopathological attributes, and the manifestation of biological markers in NSCLC and highlights the necessity of further investigation to clarify the underlying regulatory mechanisms and prospective therapeutic targets. It is suggested in the future that subsequent investigations will need to adopt a prospective research design, marking a paradigmatic shift towards anticipatory data collection and analysis. By prospectively following cohorts of subjects, researchers can longitudinally monitor the development of specific events or conditions, capturing dynamic changes and temporal relationships with greater precision.

CONCLUSION

The WNT/β-catenin signaling pathway has a specific role in advanced-stage NSCLC. These findings emphasize the complex interplay between clinicopathological features and biological marker expression in NSCLC, warranting further investigation to elucidate underlying regulatory mechanisms and potential therapeutic targets.

CONFLICT OF INTEREST

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

ETHICAL CLEARANCE

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.

FUNDING

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

AUTHOR CONTRIBUTIONS

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by 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.

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