Glycogen Synthase Kinase-3 Beta Expression Correlates With Worse Overall Survival in Non-Small Cell Lung Cancer—A Clinicopathological Series

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Background: Glycogen Synthase Kinase-3 beta (GSK-3β) regulates diverse cell functions including metabolic activity, signaling and structural proteins. GSK-3β phosphorlylates target pro-oncogenes and regulates programmed cell death-ligand 1 (PD-L1). This study investigated the correlation between GSK-3β expression and clinically relevant molecular features of lung adenocarcinoma (PD1 score, PTEN expression and driver mutations).

Methods: We evaluated 95 lung cancer specimens from biopsies and surgical resections. Immunohistochemistry was performed to analyze the expression of GSK-3β, PTEN, and PDL1. Epidemiological data, molecular characteristics and staging were evaluated from medical records. The histologic classification was performed by an experienced pulmonary pathologist.

Results: Most patients were female (52.6%) and the majority had a positive smoking history. The median age was 68.3 years, with individuals over 60 years accounting for 82.1%. The predominant histological subtype was adenocarcinoma (69.5%), followed by squamous cell carcinoma (20.0%). GSK-3β expression in tumors was cytoplasmic with a dotted pattern and perinuclear concentration, with associated membranous staining. Seven (7.3%) tumors had associated nuclear expression localization. Seventy-seven patients (81.1%) had advanced clinical-stage tumors. GSK-3β was positive in 75 tumors (78%) and GSK3-positive tumors tended to be diagnosed at advanced stages. Among stage III/IV tumors, 84% showed GSK3 positivity (p= 0.007). We identified a statistically significant association between GSK-3β and PTEN in the qualitative analysis (p 0.021); and when comparing PTEN to GSK-3β intensity 2+ (p 0.001) or 3+ expression (> 50%) – p 0.013. GSK-3β positive tumors with a high histological score had a worse overall survival.
INTRODUCTION

Lung cancer represents a serious public health problem. In addition to its high incidence, this malignancy has the highest mortality rate worldwide (1). Glycogen synthase kinase-3 (GSK3) is a serine/threonine kinase, initially described as an ATP-Mg-dependent protein phosphatase (2), subdivided into two isoforms: GSK3α and β (3). GSK3 was initially found to be related to various inflammatory processes, psychiatric disorders, neurodegenerative diseases, diabetes, cardiac dysfunction, autoimmune disorders, and more recently, it has been associated with cancer development (4, 5). It has been shown that GSK3 phosphorylates various components (TSC2, RICTOR, PTEN, and AKT) of the PI3K-AKT signaling network, an essential pathway for cell proliferation. Growth factors, cytokines, and chemokines are some of the signals that stimulate this process (6).

GSK3 is a central regulator of programmed cell death protein-1 (PD-1) expression, and GSK3 inhibition may downregulate PD-1 and enhance CD8+ cytolytic T cell (CTL) function (7). PD-1 and its ligand (PD-L1) are involved in the immune checkpoint pathway mechanism, of which activation promotes negative regulation of anti-tumor actions (8–11). In the same context, immunotherapy has become the new frontier to be explored in the cancer therapeutic arsenal, especially in lung cancer (12) (13). The level of PD-L1 protein phosphatase (2), subdivided into two isoforms: GSK3α and β (3). GSK3 was initially found to be related to various inflammatory processes, psychiatric disorders, neurodegenerative diseases, diabetes, cardiac dysfunction, autoimmune disorders, and more recently, it has been associated with cancer development (4, 5). It has been shown that GSK3 phosphorylates various components (TSC2, RICTOR, PTEN, and AKT) of the PI3K-AKT signaling network, an essential pathway for cell proliferation. Growth factors, cytokines, and chemokines are some of the signals that stimulate this process (6).

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PTEN negatively regulates the PI3K/AKT pathway, playing an important role in intracellular growth, leading to decreased phosphorylation of AKT substrates, including BAD (BCL-2-associated agonist of cell death) and GSK-3 (17, 18). PTEN functions as a tumor suppressor gene that antagonizes PI3K activity (19). Several mechanisms can identify PTEN loss, such as mutations, deletions, absence of protein expression, and promoter methylation. In lung tumors, the absence of immunohistochemical expression is seen in 30%–50% of cases (20). (Figure 1).

Considering these interactions with components of tumor growth pathways, GSK-3β has become a critical molecule to be used in the fight against cancer and the development of new drugs (30). In the search for the improvement and innovation in biomarkers, recognizing its potential to influence carcinogenesis, and considering its role in other inflammatory pathologies, our study analyzed the GSK-3β expression in lung cancer and its correlation with PDL1, the best predictor of response to immunotherapy used in clinical practice.

MATERIALS AND METHODS

Patient and Tissue Selection

We sequentially selected 95 patients diagnosed with lung cancer between 2013 and 2019 in the State of Ceará, using available material (paraffin blocks) and follow-up information from an approximate cohort of 450 newly diagnosed patients from the same period. All patients had clinically and pathologically confirmed tumors as primary lung cancer. The Ethics Review Board reviewed and approved this research study. Cases were diagnosed by a single Thoracic Pathologist based on the current WHO criteria (31, 32). The inclusion criteria consisted of non-small cell lung cancer with enough tissue sample (paraffin blocks) and availability for additional immunohistochemical studies. All patients with secondary tumors of the lung were excluded from the study. Epidemiology and clinicopathological characters (such as gender, age, smoking status, comorbidities, TNM stage, survival, molecular mutations) were obtained from medical records or by contacting the patients.

Tumor necrosis was defined as any amount of necrotic tumor, as an area of increased eosinophilia without tumor cell nuclei, only the shadow of membranous tumor cells or with nuclear shrinkage or fragmentation.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on histological sections of tumors obtained from biopsies or resections. The sections were cut and mounted on electrically charged glass slides. GSK-3β (Cell Signaling, clone 27C10) and PTEN (Clone SP218; Roche Diagnostics Limited, Burgess Hill, UK) IHC analyses were performed on the Ventana® platform (BenchMark ULTRA IHC/ISH Staining Module, Ventana Medical Systems, Tucson, AZ). The PD-L1 IHC analysis (22C3 pharmDx, Agilent, clone 22c3) was recovered using PT-Link (Dako PT100), followed by target recovery with EnVision™ FLEX pH 6.0 buffer, using the Agilent Technologies®, USA visualization system in Autostainer Link 48® equipment.

GSK-3β was recorded according to the cytoplasmic and membranous staining intensity as 0 (negative), 1+(weak), 2+ (moderate), and 3+ (strong), and the percentage of stained tumor cells. A final histological score was established (% of weak
staining × 1] + [% of moderate staining × 2] + [% of strong staining × 3]) to determine the overall percentage of GSK-3β positivity across the entire stained tumor sample, yielding a range from 0 to 300. Any tumor with a score >200 was considered as showing high GSK-3β expression.

PD-L1 was assessed as membranous positivity in tumor cells, following the Tumor Proportion Score (TPS) as practiced currently (33). We tried different cutoff points to reflect clinical scenarios: <1%, 1%–49%, ≥50%. In investigating the correlation with PTEN, we use yet another method of quantification, characterizing PD-L1 as positive or negative expression if tumor cells had a positivity above 1%.

PTEN protein was considered lost if the cytoplasmic and nuclear staining intensity was markedly decreased or entirely negative across >10% of tumor cells when compared to surrounding benign tissue, which provides internal positive controls. Other molecular data (EGFR, ALK, BRAF, and KRAS status) were retrieved from medical records, and correlation with the protein expression findings was attempted.

**Statistical Analysis**

Patients were followed for up to 68.3 months (mean follow-up of 18.8 months, SD 14.3 months). Distant metastasis was defined as recurrence at any site other than the abovementioned ones and was confirmed by imaging studies and histopathological evidence, when necessary. Overall survival (OS) was defined as the interval between the initial diagnosis and death (event) or the last follow-up date.

Statistical evaluation was performed by Stata® version 13 statistical software (StataCorp LP, College Station, TX, USA). Initially, a descriptive analysis of the study population variables was performed, calculating absolute and relative frequencies. Subsequently, bivariate analysis was performed using Pearson’s or Fisher’s chi-square test (when expected values of the contingency table cell were <5), with their respective 95% confidence intervals and statistical significance (p <0.05).

Also, multiple regression was run to predict the overall survival based on the variables age, gender, smoking, histology, treatment (QT), clinic stage, EGFR, PD-L1, and GSK3β.
expression. The assumptions of linearity, independence of errors, homoscedasticity, unusual points, and normality of residuals were tested using SPSS 24.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Patient Demographics and Treatment

We evaluated 95 patients with primary lung cancer (90 samples from lung parenchyma and five from pleural tissue). Most patients were female (52.6%), and the majority had a positive smoking history (77.7% of the males and 52% of females). The median age at diagnosis was 68.3 years (range, 32.1–94.9 years), with individuals over 60 years accounting for 82.1%. All data regarding the clinical and pathological factors are presented in Table 1.

Seventy-seven patients (81.1%) had advanced clinical-stage tumors at diagnosis (Stages III–IV). Treatment consisted of systemic therapy (including cytotoxic therapy, immunotherapy, or both) in 70.5% patients, lobectomy in 25.2% patients, segmentectomy in 2.1%, and pneumonectomy in 2.1%. Patients following chemotherapy standard protocol represented 85.5% of the cohort, with an additional 25.5% undergoing anti-PD1 immunotherapy regimens. Tyrosine kinase inhibitors corresponded to treatment in 20.2% of cases. Antiangiogenics were found as part of therapy in only 10% of patients. Radiation therapy was administered as a therapeutic modality in 51.1% of patients. It was used to control bone pain, definitive therapy concomitant with chemotherapy, or finally approach brain metastasis. Tumors were in the right upper lobe in 27.4%, followed by the left superior in 25.3%, left inferior in 18.9%, right inferior in 17.9%, and the middle lobe in 5.3%.

Pathological Classification

The predominant histological subtype was adenocarcinoma (69.5%) followed by squamous cell carcinoma (20.0%). The remainder were unclassifiable non-small cell carcinoma with characteristics of large-cell carcinoma (WHO recommendation). Invasive adenocarcinomas with predominant acinar and solid patterns of growth were the most often identified (39.4% and 37.9%, respectively). There were seven lepidic-predominant (10.6% of adenocarcinomas), one mucinous, and one minimally invasive adenocarcinoma (1.5% each). Most squamous cell carcinomas were moderately differentiated non-keratinizing, tumors, showing the usual morphology. Non-small cell, not otherwise specified carcinomas, did not show glandular, squamous or neuroendocrine differentiation either by light microscopy morphology or immunohistochemistry. Tumor necrosis was present at least focally in 40% of cases (38 cases) and showed a strong correlation with solid growth pattern [high-grade, \(R=0.335, p=0.012\)].

GSK-3 Beta Protein Expression

GSK-3β was identified in 75 tumors (78%), being considered high in 17 cases (22.7%) and low in 58 cases (77.3%) (Figures 2 and 3). There was no difference in GSK-3β positivity when comparing gender, age, or smoking history. GSK-3β-positive tumors were more prevalent in advanced-stage tumors. Among stage III/IV tumors, 84% showed GSK3 positivity, in contrast with 55.6% of stage I/II tumors, with statistical significance in the univariate and multivariate analyses \((p = 0.007)\) (Table 2).

Interestingly, when evaluating by size alone, we identified a greater number of patients with GSK-3β expression in T1/T2 tumors (95%CI 0.86–1.32, \(p = 0.523\)), compared to T3/T4 cancers, but without statistical significance.

We found no statistically significant differences regarding GSK-3β and driver mutations, such as EGFR, ALK, ROSI, or BRAF (Table 1). Only 11 patients were tested for KRAS mutations. Three cases were KRAS mutated (27.2%), while eight were KRAS wild type (72.8%). There was no direct correlation between GSK-3β expression and PD-L1 positivity or PD-L1 percentage. When considering the PDL1 score most often used in clinical trials and treatment guidelines (negative, 1%–49% and > 50%), no correlation was found. The data is summarized in Table 1. Tumor necrosis was also found to be statistically significant with PD-L1 expression regardless of PD-L1 cutoff levels \((p<0.001)\), but there was no correlation between tumor necrosis and GSK-3β expression in the cohort.

Multivariate logistic regression analysis further showed that overall survival was related to EGFR gene mutation \((p=0.023)\), final clinical staging \((p=0.033)\) and GSK-3β expression \((p=0.035)\). These results indicate that the expression of GSK-3β could be potentially a marker of overall prognosis independent of driver mutation status and is correlated with smoking status and clinical stage (Table 3).

Of note, we identified a statistical correlation between GSK3 and PTEN (95% CI 0.72–28.73, \(p = 0.021\)) with the greater number of PTEN positivity in cases where GSK3 intensity score was \(= 2\) (95%CI 0.48–89.7, \(p = 0.001)\) (Table 2).

Survival Analysis

Patients were followed for up to 68.3 months (median 14.1, range 1.0–68.3). Patients with adenocarcinomas survived on average 19.6 months and patients with squamous cell carcinomas, 14.5 months. Solid-predominant tumors had a poor survival rate when compared to the remainder \((p<0.01)\). There was a significant difference when comparing the survival of patients with high GSK expression with patients with low or absent GSK \((p<0.0062; Figure 4, Kaplan-Meier)\). We also found a significant difference in survival in patients presenting with higher stage and lymph node metastasis, as expected \((p=0.010)\). There was no statistical difference regarding survival when comparing PD-L1, ALK, or EGFR status alone or in combination with GSK-3β. Patients with PTEN positivity had a better overall survival \((p=0.026)\).

DISCUSSION

Immune manipulation is a chapter of extensive research in oncology. Knowledge about biomarkers predicts better outcomes and results. Our article studied GSK-3 beta protein expression, tumor prognosis, and possible interactions in non-small cell lung cancer. The high expression of GSK-3 was correlated to a worse prognosis, underscoring the importance of studies hypothesizing that blocking GSK can be a potential therapeutic target in non-small lung cancer. After understanding their
TABLE 1 | Analysis of clinico-pathologic characteristics of studied population by GSK-3β expression, with bivariate and multivariate analysis.

|                          | GSK                 | Bivariate          | Multivariate       |
|--------------------------|---------------------|--------------------|--------------------|
|                          | Total N (%)         | Negative N (%)     | Positive N (%)     | RP     | CI95%           | P-value |
| Gender                   | Female 50 (52.6)    | 8 (16.0)           | 42 (84.0)          | 1.14   | 0.92–1.42       | 0.203   |
|                          | Male 45 (47.4)      | 12 (26.7)          | 33 (73.3)          | Ref    |                  |         |
| Ethnic group             | Caucasian 42 (44.2) | 6 (14.3)           | 36 (85.7)          | Ref    |                  |         |
|                          | African-Brazilian 2 | 2 (100)            |                  | Ref    |                  |         |
|                          | Multiracial 51 (53.7)| 12 (23.5)         | 39 (76.5)          | 0.89   | 0.73–1.08       | 0.261   |
| Age (years)              | <60 17 (17.9)       | 4 (23.5)           | 13 (76.5)          | Ref    |                  |         |
|                          | ≥60 78 (82.1)       | 16 (20.5)          | 62 (79.5)          | 1.04   | 0.78–1.38       | 0.782   |
| Smoking                  | No 34 (35.8)        | 4 (11.8)           | 30 (88.2)          | Ref    |                  |         |
|                          | Yes 61 (64.2)       | 16 (26.2)          | 45 (73.8)          | 0.83   | 0.69–1.01       | 0.097   |
| Site (lobe)              | Inferior Right 17 (17.9)| 5 (29.4)         | 12 (70.6)          | Ref    |                  |         |
|                          | Inferior Left 18 (18.9)| 4 (22.2)          | 14 (77.8)          | 1.10   | 0.74–1.63       | 0.628   |
|                          | Middle 5 (5.3)      | 5 (100)            |                  | Ref    |                  |         |
|                          | Superior Right 26 (27.4)| 6 (23.1)          | 20 (76.9)          | 1.09   | 0.75–1.58       | 0.842   |
|                          | Superior Left 24 (25.3)| 5 (20.8)         | 19 (79.2)          | 1.12   | 0.77–1.62       | 0.529   |
| Histology                | Adenocarcinoma 66 (69.5)| 13 (19.7)        | 53 (80.3)          | 1.34   | 0.79–2.25       | 0.151   |
|                          | Squamous Cell Carcinoma 19 (20.3)| 3 (158)     | 16 (84.2)          | 1.40   | 0.82–2.41       | 0.148   |
|                          | Other 10 (10.5)     | 4 (40.0)           | 6 (60.0)           | Ref    |                  |         |
| Adenocarcinoma subtype   | Acinar 26 (39.4)    | 3 (11.5)           | 23 (88.5)          | 2.06   | 0.87–4.91       | 0.009   |
|                          | Lepidic 7 (10.6)    | 4 (57.1)           | 3 (42.9)           | Ref    |                  |         |
|                          | Minimally invasive 1 (1.5) | 1 (100)         |                  | Ref    |                  |         |
|                          | Invasive mucinous 1 (1.5) | 1 (100)         |                  | Ref    |                  |         |
|                          | Solid 25 (37.9)     | 5 (20.0)           | 20 (80.0)          | 1.87   | 0.78–4.49       | 0.053   |
| T (primary tumor)        | 1 + 2 51 (56.7)     | 9 (17.6)           | 42 (82.4)          | 1.07   | 0.86–1.32       | 0.523   |
|                          | 3 + 4 39 (43.3)     | 9 (23.1)           | 30 (76.9)          | Ref    |                  |         |
| N (regional lymph node)  | 0 + 1 41 (45.1)     | 11 (26.8)          | 30 (73.2)          | Ref    |                  |         |
|                          | 2 + 3 50 (54.9)     | 8 (16.0)           | 42 (84.0)          | 1.15   | 0.92–1.43       | 0.206   |
| TNM Stage                | I + II 18 (18.9)    | 8 (44.4)           | 10 (55.6)          | Ref    |                  | 4.59    |
|                          | III + IV 77 (81.1)  | 12 (15.6)          | 65 (84.4)          | 1.51   | 0.99–2.32       | 0.007   |
| EGFR                     | Inconclusive 2 (2.1) | 1 (50.0)           | 1 (50.0)           | Ref    |                  |         |
|                          | Negative 45 (47.4)  | 10 (22.2)          | 35 (77.8)          | 1.55   | 0.38–6.27       | 0.364   |
|                          | Positive 20 (21.1)  | 2 (10.0)           | 18 (90.0)          | 1.80   | 0.45–7.25       | 0.116   |
| ALK                      | Negative 55 (57.9)  | 11 (20.0)          | 44 (80.0)          | Ref    |                  |         |
|                          | Positive 6 (6.3)    | 1 (16.7)           | 5 (83.3)           | 1.04   | 0.71–1.52       | 0.845   |
| PDL1                     | Negative 62 (65.3)  | 14 (22.6)          | 48 (77.4)          | Ref    |                  |         |
|                          | Positive 29 (30.5)  | 5 (17.2)           | 24 (82.8)          | 1.07   | 0.86–1.32       | 0.559   |
| PDL1 score               | <1% 61 (64.2)      | 14 (22.9)          | 47 (77.1)          | Ref    |                  |         |
|                          | 1-49% 17 (17.9)    | 3 (17.6)           | 14 (82.4)          | 1.07   | 0.82–1.38       | 0.639   |
|                          | ≥50% 13 (13.7)     | 2 (15.4)           | 11 (84.6)          | 1.09   | 0.84–1.44       | 0.547   |
| ROS1                     | Uncertain 1 (1.1)   | 1 (100)            |                  | Ref    |                  |         |
|                          | Inconclusive 2 (2.1) | 1 (50.0)          | 1 (50.0)           | Ref    |                  |         |
|                          | Negative 26 (27.4)  | 4 (15.4)           | 22 (84.6)          | 1.69   | 0.42–6.38       | 0.281   |
| BRAF                     | Negative 15 (15.8)  | 4 (26.7)           | 11 (73.3)          | Ref    |                  | 0.551   |
|                          | Positive 1 (1.1)   | 1 (100)            |                  | Ref    |                  |         |
intracellular interactions, GSK3 inhibitors have been researched in the treatment of cancer (34). Many types of drugs have been developed (35) and are being tested in pre-clinical and phase I studies, with promising results (36).

We identified a greater number of advanced-stage III/IV tumors in patients with high GSK3 expression (p=0.007). This translates into a possible more aggressive biological component in this population, or even an association with different genotypes, since higher tumor mutation burden (TMB) has been associated with advanced lung tumors. In contrast, recent studies have shown that tumors with high TMB may respond better to nivolumab and pembrolizumab (37). Somatic mutations can produce neoantigens, immunogenic peptides that activate the immune response. TMB and PDL1 have the same clinical applicability. However, TMB represents a complement and not a substitute for PDL1 (38). Another interesting finding in the current study is the correlation of tumor necrosis and PD-L1 expression. These are in accordance to other studies that showed tumors with higher PD-L1 expression with a greater tendency to necrosis, a more aggressive tumor phenotype and higher proliferation rate (39, 40).

Tumor cells have developed several mechanisms to escape immune surveillance. GSK3 stands out in the regulation of the PD-1/PD-L1 inhibitory checkpoint. This immune activation process can occur through PD-1 interaction or modulation in the tumor molecule PD-L1. Li et al. demonstrated that GSK-3β correlates to PD-L1 and induces phosphorylation-dependent degradation of PD-L1 by b-TrCP (41). The inhibition of GSK3 also acts to promote PD1 downregulation. When associated with anti-PD1 or anti-PDL1 block, it enhances the cytotoxic capacity of T cells (42). Taylor et al. demonstrated that GSK-3 participates in the regulation of PD-1 transcription. GSK-3 inhibition increases Tbet activity, reducing PD-1 transcription, with a further intensification of T lymphocyte cytolytic action (28). Despite the theoretical rationale supporting this association, we did not identify a relationship between PDL1 and GSK-3β scores in our cohort. A recent publication about the positivity of PDL1 in the same region of northeastern Brazil showed that 59.5% of patients were PDL1 negative (43).
Matsuo et al. studied the importance of AKT, mTOR, and GSK3 in the occurrence of lymph node involvement in oral cavity squamous cell carcinomas. Elevated expression of GSK3 and pGSK3-βSer9 was associated with metastasis in cervical lymph nodes (p = 0.004 and p = 0.03) (44) and advanced stages (cTNM), suggesting the relationship of GSK3 with tumor invasion and metastases. In our study, of the few squamous cell carcinomas studied, the expression score of GSK3 was more evident in poorly differentiated tumors and advanced stages. Blocking GSK-3β reduced cell proliferation, stimulated apoptosis, maintained cells in the GO/G1 phase and increased cell invasion. A study published by Zeng et al. focused on the association between GSK3 and survival. The positive expression of GSK3 by immunohistochemistry was also related to a worse prognosis (45). Therefore, the relationship between GSK3 and direct survival has been hypothesized.

Cigarette exposure corresponds to a cluster of toxic substances that promote damage to alveolar cells (46). The mechanisms involved are still not fully understood. Besides, it induces beta-catenin accumulation (47). Nagahori et al. analyzed a polymorphism of the GSK3 gene and its connection with smoking habits. In a cohort of 384 patients, rs334558 was associated with smoking in genotype and allelic frequency (48). Numajiri M et al. also published an article describing the relationship between a polymorphism of the GSK3 gene and its relationship with nicotine dependence (49). Despite the possible inhibition of GSK3 by smoking, we found no statistical basis for this association in our population (p=0.097).

Anaplastic lymphoma kinase (ALK) is a tyrosine kinase with altered expression in several tumors. In lung cancer, ALK-EML4 rearrangement occurs in about 3%–5% of cases (50), allowing the use of target drugs and achieving better survival results (51). Malagon et al. analyzed the relationship between ALK and GSK3

## Table 2: Bivariate analysis and correlations of GSK-3β expression and PTEN status in lung tumors.

| GSK-3β | PTEN | Bivariate |
|--------|------|-----------|
|        | Total n (%) | PTEN Negative N (%) | PTEN Positive N (%) | RP | CI95% | P-value |
|        |             | Ref | 0.72–28.73 | 0.021 |
|        |             | 0.27–19.8 | 0.416 |
|        |             | 1.04–39.7 | 0.001 |

**Variables**

| Variables | Unstandardized Coefficients | Standardized Coefficients | t | Sig. |
|-----------|-----------------------------|---------------------------|---|------|
| Death (Constant) | 3.994 | 0.917 | 4.353 | 0.000 |
| Age | -0.005 | 0.006 | -0.126 | -0.895 | 0.376 |
| Gender | -0.149 | 0.148 | -0.161 | -1.010 | 0.319 |
| Smoking | 0.147 | 0.146 | 0.155 | 1.006 | 0.321 |
| Histology | 0.111 | 0.150 | 0.119 | 0.741 | 0.463 |
| Treatment | -0.187 | 0.261 | -0.123 | -0.719 | 0.477 |
| PD1 | -0.200 | 0.142 | -0.203 | -1.411 | 0.167 |
| EGFR | -0.453 | 0.192 | -0.446 | -2.364 | 0.023 |
| Clinic stage | -0.185 | 0.084 | -0.354 | -2.207 | 0.033 |
| GSK-3β | -0.375 | 0.171 | -0.328 | -2.189 | 0.035 |

*Bold text indicates significant differences (p < 0.05).*
in neuroblastomas and neural crest cells. They suggest a positive regulation of GSK3 via ALK tyrosine kinase (52). McDonnell SRP et al. studied anaplastic large-cell lymphoma, a pediatric disease, in which the NPM-ALK alteration is present in 70%–80% of cases. They revealed that ALK activity acts on the phosphorylation of S9-GSK-3 via PI3K/AKT, making GSK3 an important regulator of ALK carcinogenesis (53).

EGFR is a surface receptor, a HER family component, also known as erbB1, HER1, or even as EGFR (54). Its activation triggers intracellular cascades, promoting cell growth and oncogenesis (55). It is especially important in lung tumors, acting as a predictor of response to tyrosine kinase inhibitors (56). Fitzgerald et al. studied the role of EGFR in the intracellular signaling pathways of pancreatic tumors. EGFR activates the Ras/Raf/MEK/ERK pathway. Consequently, it stimulates the ETS transcription factor that binds to the GSK-3beta promoter and induces the expression of GSK-3 and IKK. Subsequently, this regulates the production of NF-kappa-B, leading to gene transcription and cell proliferation (57). This exemplifies a possible relationship between EGFR mutations and GSK3 expression.

In the current study, an additional analysis in a subset of 27 resection samples demonstrated a statistically significant association between GSK-3β and PTEN analysis by immunohistochemistry (p = 0.021). This relationship was more evident in cases with strong GSK-3β expression or the expression in > 50% of the tumor. Zhengyu H et al. demonstrated that PTEN overexpression reduces fibroblast proliferation by inhibiting the PI3K/AKT/GSK3 pathway (26). Gao C. et al. showed by measuring the levels of GSK-3β, PTEN, and AKT in breast tumor cell lines, that the PTEN/PI3K/AKT pathway can be regulated by GSK3 (58). PTEN inactivation is associated with lower survival and resistance to systemic treatment (59). These findings underscore the importance of GSK activation in tumor survival and growth regulation (60).

Although previously identified as a tumor suppressor, PTEN is not currently used in clinical practice as a lung cancer biomarker. While active PTEN has anti-tumor action, resulting in better outcomes (18, 61, 62). This is in contrast to our results, which shows a positive correlation. Further research involving GSK3 and PTEN blockers in lung cancer is necessary for better clarification and therapeutic use to manipulate these intracellular checkpoints.

In conclusion, GSK-3β is a molecule involved in multiple signaling pathways. Positive tumor expression was associated with a more advanced tumor stage and worse overall survival in lung cancer. To the best of our knowledge, this is the first study to identify the expression of GSK-3β as a potential marker for overall survival and establish a simple histological score to be measured in resected tissues. The use of GSK-3β expression as an immune response biomarker remains a challenge. Future studies will seek to explain the role of its interaction with PTEN.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article supplementary material. Further inquiries can be directed to the corresponding author.

**ETHICS STATEMENT**

The studies involving human participants were reviewed and approved by Federal University of Ceará. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.
AUTHOR CONTRIBUTIONS

MA, BC, and FT designed the study. DB, AK, and JS performed all molecular studies and immunohistochemical studies. MA and FM reviewed patients charts and follow-up information. Slides were evaluated by pathologists FT, CN, and AO. MA, FT, and BC wrote and reviewed the final manuscript. FT is the lead investigator. All authors contributed to the article and approved the submitted version.

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