RESEARCH ARTICLE

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# Predictive and Prognostic Value of *TUBB3*, *RRM1*, *APE1*, and Survivin Expression in Chemotherapy-Receiving Patients with Advanced Non-Small Cell Lung Cancer

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## Abstract

**Background:** This study aimed to evaluate the expression of class III β-tubulin (*TUBB3*), ribonucleoside-diphosphate reductase 1 (*RRM1*), apurinic/apyrimidinic endonuclease 1 (*APE1*), and survivin in patients with advanced non-small cell lung cancer (NSCLC) to predict response to chemotherapy. **Methods:** *TUBB3*, *RRM1*, *APE1*, and survivin expression levels were determined using immunohistochemistry. Protein expression was validated in Car/Pac-resistant human H1792 and A549 cells. This study included 86 patients, among whom 34 received cisplatin (Cis)/gemcitabine (Gem) and 52 received carboplatin (Car)/paclitaxel (Pac). **Results:** Patients with low *TUBB3* expression and high *RRM1* and survivin expression had higher response rates than those with low *RRM1* and survivin expression and high *TUBB3* expression in the Car/Pac regimen. The multivariate analysis indicated that *TUBB3* and *RRM1* were significant independent predictive biomarkers for the Car/Pac regimen; however, there was no association between any protein and overall response in patients treated with this regimen. In the Cis/Gem regimen, only high *TUBB3* expression was associated with poor overall survival; however, it did not exhibit a prognostic ability. **Conclusion:** The expression levels of *TUBB3* and *RRM1* in NSCLC cells are potential predictive biomarkers, but not prognostic factors, of response to chemotherapy in patients with NSCLC receiving the Car/Pac regimen.

**Keywords:** *TUBB3*- *RRM1*- *APE1*- survivin- response to chemotherapy

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## Introduction

Lung cancer is the most common cancer and the leading cause of cancer-related deaths worldwide, accounting for 11.6% of all new cancer cases and 18.4% of all deaths in 2021 (Bray et al., 2018). Lung cancer is classified into two main histological categories: non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma. NSCLC accounts for more than 80-85% of lung cancer cases, and patients with advanced NSCLC have a poor five-year survival rate of 5% (Schabath and Cote, 2019). Although targeted therapy and immunotherapy have considerably improved the survival of patients with NSCLC, they are highly costly and do not benefit patients with NSCLC who lack driver mutations. Therefore, chemotherapy remains the standard of care for these patients. Patients with locally advanced (stage III) or advanced (stage IV) NSCLC and good performance status are treated with a platinum-based doublet regimen containing a platinum agent (cisplatin [Cis] or carboplatin [Car]) and another type of chemotherapeutic drugs such as etoposide, gemcitabine (Gem), and paclitaxel (Pac) (Spira and Ettinger, 2004). However, there are no differences in the response and survival rates across these different chemotherapy regimens (Schiller et al., 2002), which could be attributed to a lack of guided markers for regimen selection. Therefore, predictive molecular biomarkers specific to each treatment regimen are needed.

Alterations in the expression of proteins associated with chemotherapy resistance have been reported. Class III β-tubulin (*TUBB3*) plays a crucial role in the inhibition of microtubule activity, resulting in cell cycle arrest and subsequently apoptosis (Burkhart et al., 2001). Low *TUBB3* expression is associated with a good response rate in patients with NSCLC receiving Pac-based chemotherapy (Seve et al., 2005; Li et al., 2014). Ribonucleotide reductase M1 (*RRM1*) is an enzyme involved in DNA synthesis, whose high expression is associated with resistance to Gem-based chemotherapy in colon adenocarcinoma (Cao et al., 2003) and NSCLC

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(Rosell et al., 2004; Ceppi et al., 2006). Apurinic/apyrimidinic endonuclease 1 (*APE1*; also known as Ref-1) functions in both DNA repair and transcriptional regulation (Li and Wilson, 2014). High *APE1* expression is associated with a poor survival rate in patients with osteosarcoma (Wang et al., 2004) and head-and-neck cancer (Koukourakis et al., 2001), and it is correlated with a low response rate in patients receiving Pac-based chemotherapy (Li et al., 2014). Survivin, an anti-apoptotic protein, plays a key role in the cell cycle, mitosis, and apoptosis (Jaiswal et al., 2015). Increased survivin mRNA expression is associated with enhanced resistance to treatment with Cis and 5-FU in esophageal cancer (Kato et al., 2001). In contrast, high nuclear survivin expression is correlated with a high response to taxane-based chemotherapy in NSCLC (Wu et al., 2014). Although these proteins have been indicated as potential predictive or prognostic biomarkers for chemotherapy, limited studies have assessed the association between both their expression and responsiveness to each treatment regimen (Car/Pac or Cis/Gem regimens). Additionally, some proteins have yielded inconsistent results. Therefore, this study aimed to investigate the predictive and prognostic values of the expression levels of *TUBB3*, *RRM1*, *APE1*, and survivin in various chemotherapeutic regimens for NSCLC. This study may help in the appropriate selection of regimens and clinical decision-making.

## Materials and Methods

*Patient selection*

The study included patients with histologically confirmed NSCLC stage III or IV who had received either the Cis/Gem or Car/Pac regimen at the Songklanagarind Hospital between January 2015 and December 2017. Patients were evaluated for performance status using an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1. Data on the clinical characteristics were obtained from hospital-based cancer registries.

*Evaluation of response to chemotherapy*

Baseline tumor measurements were performed using computed tomography (CT) before treatment. After treatment, all patients were followed-up every 3 weeks by physical examination and blood testing. Chest radiography and CT were performed to evaluate the response to chemotherapy. Tumor responses were categorized according to Response Criteria in Solid Tumors (RECIST) version 1.1: complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) (Eisenhauer et al., 2009).

*Immunohistochemical analysis*

Immunoperoxidase labeling was performed using an automated immunostainer (Leica BONDMAX; Leica Biosystems, Wetzlar, Germany). Antigen retrieval was performed in an EDTA-based pH 9 epitope retrieval solution (Bond Epitope Retrieval Solution 2, Leica Biosystems) in a pressure cooker at 95°C for 4 min. Sections were incubated with a bond peroxidase-blocking reagent (Bond Polymer Refine Detection, Leica Biosystems) and then incubated with primary antibodies against *TUBB3* (2G10, mouse monoclonal antibody sc-80005, dilution 1:500), *RRM1* (A-10, mouse monoclonal antibody sc-377415, dilution 1:500), *APE1* (C-4, mouse monoclonal antibody sc-17774, dilution 1:10,000), and survivin (D-8, mouse monoclonal antibody sc-17779, dilution 1:100). All antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Detection was performed using the Bond Polymer Refine Detection Kit (Leica Biosystems). Diaminobenzidine (DAB, Leica Biosystems) was used as a chromogen for color development, and the slides were counterstained with EnVision FLEX hematoxylin (Leica Biosystems).

*Evaluation of protein expression*

Immunostaining was qualitatively evaluated by quantifying the intensity of staining and the percentage of positively stained tumor cells. The staining intensity was scored as 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (intense staining). The percentage of positively stained tumor cells (0-100%) was estimated from the total number of tumor cells on the slide. The final immunohistochemistry (H) score (0-300) was calculated by multiplying the intensity and percentage of positively stained cells. Evaluation of immunostaining was performed by the senior pathologist who was blinded to the cliniclae data and outcomes.

*Cell culture*

A549 and H1792 lung adenocarcinoma-derived human NSCLC cell lines were purchased from American Type Culture Collection (Manassas, HI, USA). Cells were grown in RPMI-1640 media (Sigma-Aldrich; St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and maintained at 37°C in a 5% $CO_2$ atmosphere.

*Induction of resistance in NSCLC cells*

Pac- and Car-resistant NSCLC cells were produced from each parental cell line during a 12-month period by continuous culturing in drug-containing media as described by Mon et al. (Mon et al., 2021). To generate resistant cells, the initial dose at $IC_{70}$ values was determined using dose-response curves of Pac (15-1,000 nM) and Car (6-400 μM) (Sigma-Aldrich) over 72 h. A549 cells were initially treated with Pac and Car at a concentration of 50 nM and 20 μM, respectively. H1792 cells were treated with Pac and Car at a concentration of 30 nM and 20 μM, respectively. The medium was removed after drug treatment for 3 days, and the cells were allowed to recover for a further 3 days. Pac-resistant A549 (A549/Pac), Car-resistant A549 (A549/Car), Pac-resistant H1792 (H1792/Pac), and Car-resistant H1792 (H1792/Car) cells were established using stepwise selection.

*Cell viability measurement*

Parental and resistant cells were seeded at a density of $1.5\times10^4$ cells/well into 96-well plates and allowed to grow overnight at 37°C in a 5% $CO_2$ atmosphere at a growth

rate of 50-60%. After 72 h of treatment with either Pac or Car, 100 µL of an MTT solution (0.5 mg/ml; Gibco) was added to each well and incubated at 37 °C for 2 h. The MTT solution was removed, and 100 µL of dimethyl sulfoxide (Gibco) were added. After 30 min of incubation in the dark at room temperature, the optical densities at 550 nm and 650 nm were measured using a microplate reader (Molecular Devices, San Jose, CA, USA). The concentration at which a substance exerted half of its maximal inhibitory effect ($IC_{50}$ value) was calculated using the $IC_{50}$ online calculator (https://www.aatbio.com/tools/ic50-calculator). $IC_{50}$ values were obtained using a four-parameter logistic regression model. Each condition was tested in duplicate and five independent MTT assays were performed. The relative resistance index was defined as the $IC_{50}$ value of the resistant cells divided by the $IC_{50}$ value of the parental cells.

*Western blot analysis*

RIPA buffer (pH 7.4) containing a protease inhibitor cocktail (MilliporeSigma, Burlington, MA, USA) was used to lyse the cells. Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) was used to quantify the total protein content. Proteins (50 µg/well) were electrophoretically separated on a 12% sodium dodecyl sulfate-polyacrylamide gel and then transferred to a nitrocellulose membrane (Bio-Rad Laboratories). The membranes were probed with mouse monoclonal antibodies against *TUBB3*, *RRM1*, *APE1*, and survivin (1:2,000 dilution; Santa Cruz Biotechnology) and rabbit polyclonal antibodies against β-actin (1:3,000 dilution; Cell Signaling Technology, Danvers, MA, USA) at 4 °C overnight after blocking with 3% bovine serum albumin (Sigma-Aldrich) for 2 h at room temperature. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:3,000; Cell Signaling Technology) at room temperature for 2 h. ECL chemiluminescent substrate (Bio-Rad Laboratories) was used to detect bands. An Image Quant TM LAS 4000 digital imaging system (GE Healthcare, Chicago, IL, USA) was used to capture the images. The relative expression of each protein was calculated using β-actin as a loading control. The expression levels are presented as mean ± standard deviation (S.D.).

*Statistical analysis*

Descriptive data for categorical variables were reported as percentages, and continuous variables were reported as mean ± S.D. The associations between clinicopathological variables, protein expression, and chemotherapy response status were analyzed using the chi-squared test or Fisher's exact test, as appropriate. The immunohistochemistry score (0-300) of each protein was dichotomously classified into low and high expression for further analysis, using an optimum cutoff point corresponding to the value with the best sum of sensitivity and specificity in the receiver operating characteristic (ROC) curve analysis. Logistic regression was used to determine factors associated with chemotherapy response. For survival analysis, the Kaplan-Meier curve was constructed, and the difference in overall survival among different categories of variables was tested using the log-rank test. The independent prognostic value of the variables was obtained using a multivariate Cox proportional hazards model. Differences in quantitative values between the experimental groups were tested using an unpaired t-test. Statistical significance was set at $P < 0.05$. All statistical analyses were performed using the R statistical software version v.4.0.3 (R Foundation for Statistical Computing, Vienna, Austria).

## Results

*Expression of TUBB3, RRM1, APE1, and survivin proteins in NSCLC tissues*

A total of 86 patients were included in this study, and the clinicopathological characteristics of the patients are shown in Table 1. The mean age of the patients was 64 years, and more than half (73.3 %) of the patients had adenocarcinoma (ADC). Thirty-four patients had been treated with Cis/Gem and fifty-two had been treated with Car/Pac. Additionally, 30 patients were categorized as responders (1 patient with CR and 29 with PR), whereas 56 patients were non-responders (29 patients with PD and 27 patients with SD).

*Association of clinicopathological variables and protein expression with response to chemotherapy*

To determine the expression of the proteins *TUBB3*,

Table 1. The Clinicopathological Characteristics of Patients with NSCLC Receiving Chemotherapy who were Included in This Study.

| Variables | Category | Number of cases (%) |
|---|---|---|
| Age (years) | ≥60 | 52 (60.5) |
| | <60 | 34 (39.5) |
| Sex | Female | 28 (32.6) |
| | Male | 58 (67.4) |
| Smoking | Never | 18 (20.9) |
| | Habitual | 34 (39.5) |
| | Unknown | 34 (39.5) |
| Alcohol drinking | Never | 22 (25.6) |
| | Habitual | 5 (5.8) |
| | Unknown | 59 (68.6) |
| Histology | Unspecified NSCLC | 2 (2.3) |
| | ADC | 63 (73.3) |
| | SCC | 21 (24.4) |
| Clinical stage | III | 9 (10.5) |
| | IV | 77 (89.5) |
| Regimen | Cis/Gem | 34 (39.5) |
| | Car/Pac | 52 (60.5) |
| CT Response | CR | 1 (1.2) |
| | PR | 29 (33.7) |
| | PD | 29 (33.7) |
| | SD | 27 (31.4) |

ADC, adenocarcinoma; SCC, squamous cell carcinoma; NSCLC, non-small cell lung cancer; Cis, cisplatin; Gem, gemcitabine; Car, carboplatin; Pac, paclitaxel; CT, computed tomography; CR, complete response; PR, partial response; PD, progressive disease; SD, stable disease.

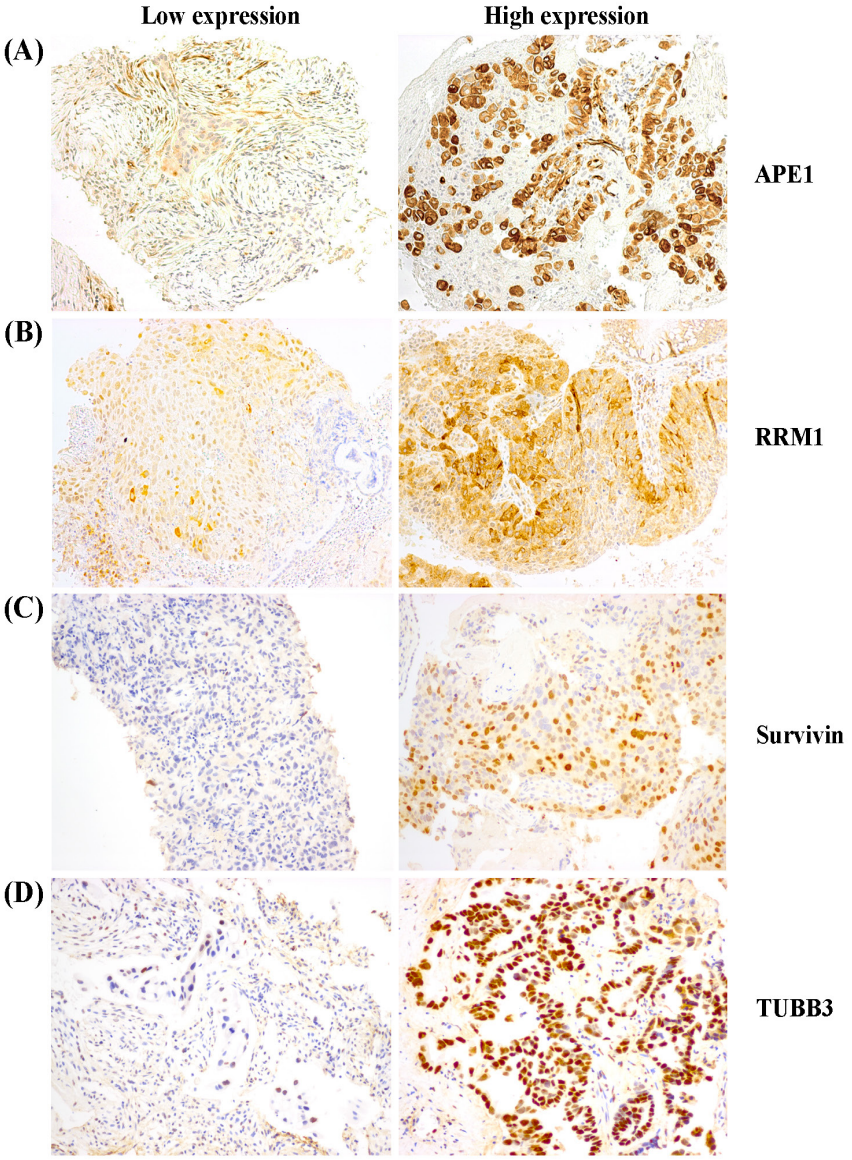


Figure 1. Immunohistochemical Staining of the Four Proteins with Low and High Expression Levels in NSCLC Cancer Tissues. (A) *APE1,* (B) *RRM1*, (C), survivin, and (D), and *TUBB3*. Original magnification, 200x. NSCLC, non-small cell lung cancer; *TUBB3*, class III β-tubulin; *RRM1*, ribonucleotide reductase M1; *APE1*, apurinic/apyrimidinic endonuclease.

*APE1*, and survivin, nuclear staining was performed, whereas cytoplasmic staining was performed to determine *RRM1* expression (Fig.1). The median interquartile range (IQR) H-score was 210 (35,300), 180 (35,300), 180 (106,200), and 5.00 (220) for *TUBB3*, *RRM1*, APE, and survivin, respectively. Protein expression was categorized as high and low according to the cutoff H-score based on a ROC curve analysis, which was 67.5, 125, 165, and 5.5 for *TUBB3*, *RRM1*, *APE1*, and survivin, respectively. The associations between clinicopathological variables and response to chemotherapy are shown in Table 2. For the Car/Pac regimen, high *TUBB3* expression was significantly associated with no response (P= 0.043). In contrast, high *RRM1* (P = 0.010) and survivin expression levels (P = 0.047) were associated with responders. No significant associations between response to chemotherapy response and any clinicopathological variables were observed in patients receiving the Cis/Gem regimen.

The results of the logistic regression for response to chemotherapy are shown in Table 3. In the univariate analysis of the Car/Pac regimen, a high *TUBB3* expression level was negatively associated with response (odds ratio [OR], 0.30; 95% CI, 0.09-0.98) whereas high *RRM1* (OR, 5.16; 95% CI, 1.41-18.91), and high survivin expression levels (OR, 5.16; 95% CI, 1.41-18.91) were significantly associated with response. In the multivariate analysis, *TUBB3* and *RRM1* were independent prognostic factors for responsiveness to Car/Pac chemotherapy. However, no variables were significantly associated with the response status to the Cis/Gem regimen, likely due to the limited number of patients.

*Association of clinicopathological variables and protein expression with overall survival*

In the Kaplan-Meier analysis (Figure 2), no significant difference in the overall survival (OS) was observed

Table 2. The Association of Clinicopathological Variables and Protein Expression with Response to Chemotherapy

| Variables | Car/Pac regimen | | | Cis/Gem regimen | | |
|---|---|---|---|---|---|---|
| | R (%) | NR (%) | P-value | R (%) | NR (%) | P-value |
| Age (years) | | | 0.782 | | | 0.427 |
| ≥60 | 13 (61.9) | 18 (58.1) | | 7 (77.8) | 14 (56.0) | |
| <60 | 8 (38.1) | 13 (41.9) | | 2 (22.2) | 11 (44.0) | |
| Sex | | | 0.509 | | | 1 |
| Female | 5 (23.8) | 10 (32.3) | | 3 (33.3) | 10 (40.0) | |
| Male | 16 (76.2) | 21 (67.7) | | 6 (66.7) | 15 (60.0) | |
| Smoking | | | 0.382 | | | 0.694 |
| Never | 2 (9.5) | 4 (12.9) | | 2 (22.2) | 10 (40.0) | |
| Habitual | 11 (52.4) | 10 (32.3) | | 4 (44.4) | 9 (36.0) | |
| Unknown | 8 (38.1) | 17 (54.8) | | 3 (33.3) | 6 (24.0) | |
| Alcohol drinking | | | 0.224 | | | 1 |
| Never | 4 (19) | 2 (6.5) | | 5 (55.6) | 11 (44.0) | |
| Habitual | 2 (9.5) | 1 (3.2) | | 0 (0) | 2 (8.0) | |
| Unknown | 15 (71.4) | 28 (90.3) | | 4 (44.4) | 12 (48.0) | |
| Histopathology | | | 0.196 | | | 0.743 |
| Unspecified NSCLC | 1 (4.8) | 0 (0) | | 0 (0) | 1 (4.0) | |
| ADC | 13 (61.9) | 25 (80.6) | | 6 (66.7) | 19 (76.0) | |
| SCC | 7 (33.3) | 6 (19.4) | | 3 (33.3) | 5 (20.0) | |
| Stage | | | 0.420 | | | 0.465 |
| III | 4 (19.0) | 3 (9.7) | | 1 (11.1) | 1 (4.0) | |
| IV | 17 (81.0) | 28 (90.3) | | 8 (88.9) | 24 (96.0) | |
| *TUBB3* | | | 0.043 | | | 1 |
| Low | 11 (55.0) | 8 (26.7) | | 2 (25.0) | 6 (28.6) | |
| High | 9 (45.0) | 22 (73.3) | | 6 (75.0) | 15 (71.4) | |
| *RRM1* | | | 0.01 | | | 0.393 |
| Low | 4 (19.0) | 17 (54.8) | | 1 (11.1) | 6 (28.6) | |
| High | 17 (81.0) | 14 (45.2) | | 8 (88.9) | 15 (71.4) | |
| *APE1* | | | 0.174 | | | 1 |
| Low | 4 (19.0) | 10 (37.0) | | 2 (22.2) | 3 (15.8) | |
| High | 17 (81.0) | 17 (63.0) | | 7 (77.8) | 16 (84.2) | |
| Survivin | | | 0.047 | | | 0.678 |
| Low | 6 (35.3) | 19 (65.5) | | 3 (37.5) | 11 (55.0) | |
| High | 11 (64.7) | 10 (34.5) | | 5 (62.5) | 9 (45.0) | |

Car, carboplatin; Pac, paclitaxel; Cis, cisplatin; Gem, gemcitabine; R, responders; NR, non-responders; ADC, adenocarcinoma; SCC, squamous cell carcinoma; NSCLC, non-small cell lung cancer; TUBB3, class III β-tubulin; RRM1, ribonucleotide reductase M1; APE1, apurinic/apyrimidinic endonuclease.

with the different expression levels (high and low) of all proteins, except *TUBB3* (P = 0.030). Similarly, the Cox regression analysis revealed no significant association between protein expression and response to the Car/Pac regimen (Table 4); only sex and smoking status were significant variables. The univariate analysis for the Cis/Gem regimen revealed that stage (P = 0.036) and *TUBB3* (P = 0.030) expression were significantly associated with OS.

*Expression of TUBB3, RRM1, APE1, and survivin in drug-resistant cell lines*

To validate the association between the four proteins and response to chemotherapy in vitro, we generated chemotherapy-resistant cell lines. Pac- and Car-resistant NSCLC cells, including A549/Pac, A549/Car, H1792/Pac, and H1792/Car, were generated following continuous exposure to either Pac or Car. Compared with the parental cells, A549/Pac demonstrated a significant reduction in sensitivity to treatment with Pac, with a 70.1-fold resistance compared to their parental cells ($IC_{50}$, 3,268 nM vs. 46.6 nM; Figure 3A). Similarly, A549/Car cells demonstrated a 4.1-fold resistance to Car ($IC_{50}$, 289.1 μM vs. 69.9 μM), H1792/Pac cells had a 4.2-fold increased resistance to Pac ($IC_{50}$, 191.9 nM vs. 45.4 nM), and H1792/Car had increased resistance to Car by 2.3-fold compared to their parental cells ($IC_{50}$, 275.1 μM vs. 119.2 μM; Figure 3C-D).

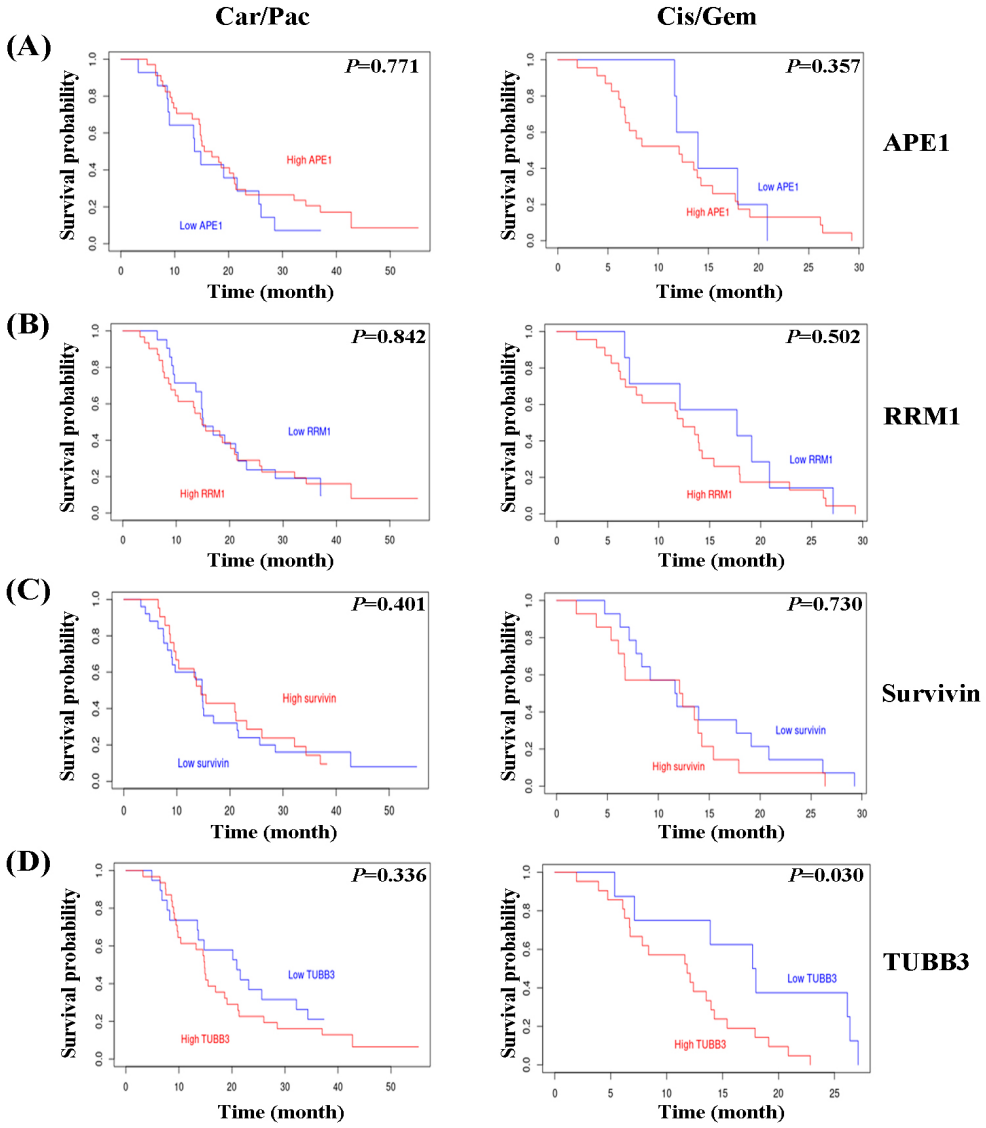


Figure 2. Kaplan-Meier Analysis of the Overall Survival of Patients with NSCLC Receiving Either Car/Pac or Cis/Gem Regimen. The survival analysis according to (A) *APE1*, (B) *RRM1*, (C) survivin, and (D) *TUBB3* expression. NSCLC, non-small cell lung cancer; Car, carboplatin; Pac, paclitaxel; Cis, cisplatin; Gem, gemcitabine; *TUBB3,* class III β-tubulin; *RRM1*, ribonucleotide reductase M1; *APE1*, apurinic/apyrimidinic endonuclease.

We then validated the expression of the investigated proteins by western blot analysis. The levels of protein expression in sensitive cells (treated parental cells) and the developed resistant cells were compared to those of naïve parental cells (Figure 4). The results showed that *TUBB3* was significantly upregulated in both resistant cell lines (A549/Pac, P<0.001; H1792/Pac, P = 0.017; and A549/Car, P = 0.037). *RRM1* was downregulated in A549(+Car) cells but was upregulated in both sensitive H1792(+Car) and resistant H1792/Car cells. Survivin was significantly downregulated in sensitive A549(+Pac) and A549(+Car) cells but increased in sensitive H1792(+Pac) (P<0.001) and H1792(+Car) (P = 0.004) cells. *APE1* expression was upregulated in both sensitive and resistant cells; however, the difference was not statistically significant.

## Discussion

In this study, we revealed that the expression levels of *TUBB3*, *RRM1*, and survivin in tumor tissues are associated with response to the Car/Pac regimen and that *TUBB3* and *RRM1* were independent predictive biomarkers, but not prognostic biomarkers, for patients treated with combined Car/Pac therapy. None of the proteins demonstrated utility as predictive biomarkers for the Cis/Gem regimen. We also revealed that the expression pattern of the proteins in the in vitro model was consistent with the results of the clinical study.

*TUBB3* plays an essential role in regulating the microtubule dynamics (Kanakkanthara and Miller, 2021). *TUBB3* overexpression induces resistance to Pac by reducing the ability of the drug to suppress microtubule stabilizations (Kavallaris, 2010). Previous studies on patients with advanced NSCLC have shown that compared to high *TUBB3* expression, low or negative *TUBB3* expression is associated with a better response to Pac-based chemotherapy and longer OS (Seve et al., 2005; Zhang et al., 2012; Li et al., 2014). Consistent with

Table 3. Results of the Logistic Regression Analysis of the Clinicopathological Variables and Response to Chemotherapy.

| Variables | Car/Pac regimen | | | | Cis/Gem regimen | | | |
|---|---|---|---|---|---|---|---|---|
| | Univariate analysis | | Multivariate analysis | | Univariate analysis | | Multivariate analysis | |
| | Crude OR (95%CI) | P- value | Adjusted OR (95%CI) | P-value | Crude OR (95%CI) | P-value | Adjusted OR (95%CI) | P-value |
| Age (years) | | | | | | | | |
| <60 | 1 | | 1 | | 1 | | 1 | |
| ≥60 | 1.17 (0.38,3.65) | 0.782 | 0.05 (0,2.47) | 0.134 | 2.75 (0.47,15.96) | 0.260 | 5.92e+35 (0,Inf) | 0.998 |
| Sex | | | | | | | | |
| Female | 1 | | 1 | | 1 | | 1 | |
| Male | 1.52 (0.43,5.35) | 0.511 | 3.48 (0.13,94.16) | 0.459 | 1.33 (0.27,6.61) | 0.725 | 0 (0,Inf) | 0.998 |
| Smoking | | | | | | | | |
| Never | 1 | | 1 | | 1 | | 1 | |
| Habitual | 2.20 (0.33,14.73) | 0.416 | 0.02 (0,2.33) | 0.103 | 2.22 (0.33,15.18) | 0.415 | 7.55e+228 (0,Inf) | 0.998 |
| Unknown | 0.94 (0.14,6.25) | 0.950 | 0.30 (0.01,8.95) | 0.487 | 2.50 (0.32,19.53) | 0.382 | 2.01e+126 (0,Inf) | 0.998 |
| Alcohol drinking | | | | | | | | |
| Never | 1 | | 1 | | 1 | | 1 | |
| Habitual | 1 (0.05,18.91) | 1 | 0.01 (0,8.04) | 0.165 | 0 (0,Inf) | 0.995 | 0 (0,Inf) | 0.999 |
| Unknown | 0.27 (0.04,1.64) | 0.154 | 0 (0,0.39) | 0.026 | 0.73 (0.16,3.45) | 0.695 | 0 (0,Inf) | 0.999 |
| Histopathology | | | | | | | | |
| SCC | 1 | | 1 | | 1 | | 1 | |
| ADC | 0.45 (0.12,1.60) | 0.216 | 1.73 (0.05,61.90) | 0.763 | 0.53 (0.10,2.88) | 0.995 | 0 (0,Inf) | 0.999 |
| Unspecified NSCLC | 4.93e+06 (0,Inf) | 0.992 | 1.78e+10 (0,Inf) | 0.992 | 0 (0,Inf) | 0.695 | 3.27e+77 (0,Inf) | 0.999 |
| Stage | | | | | | | | |
| III | 1 | | 1 | | 1 | | 1 | |
| IV | 0.46 (0.09,2.29) | 0.339 | 29.74 (0.33,2715.76) | 0.141 | 0.33 (0.02,5.97) | 0.455 | 8.62e+17 (0,Inf) | 0.999 |
| TUBB3 | | | | | | | | |
| Low | 1 | | 1 | | 1 | | 1 | |
| High | 0.30 (0.09,0.98) | 0.047 | 0 (0,0.47) | 0.023 | 1.20 (0.19,7.70) | | 2.87e+118 (0,Inf) | 0.998 |
| RRM1 | | | | | | | | |
| Low | 1 | | 1 | | 1 | | 1 | |
| High | 5.16 (1.41,18.91) | 0.013 | 24.41 (1.05,567.20) | 0.047 | 3.20 (0.33,31.42) | 0.848 | 2.59e+18 (0,Inf) | 0.999 |
| APE1 | | | | | | | | |
| Low | 1 | | 1 | | 1 | | 1 | |
| High | 2.50 (0.65,9.55) | 0.180 | 17.62 (0.61,511.55) | 0.095 | 0.66 (0.09,4.84) | 0.318 | 5.97e+53 (0,Inf) | 0.999 |
| Survivin | | | | | | | | |
| Low | 1 | | 1 | | 1 | | 1 | |
| High | 3.48 (0.99,12.22) | 0.051 | 6.34 (0.39,103.43) | 0.195 | 2.04 (0.38,10.94) | 0.679 | 0 (0,Inf) | 1 |

Car, carboplatin; Pac, paclitaxel; Cis, cisplatin; Gem, gemcitabine; OR, odds ratio; CI, confidence interval; ADC, adenocarcinoma; SCC, squamous cell carcinoma; NSCLC, non-small cell lung cancer; TUBB3, class III β-tubulin; RRM1, ribonucleotide reductase M1; APE1, apurinic/apyrimidinic endonuclease.

these findings, our results indicated a correlation between high *TUBB3* expression and poor response to Car/Pac, but not the Cis/Gem regimen. This was substantiated by our in vitro study, which demonstrated high *TUBB3* expression in resistant A549 and H1792 cell lines. These results suggest that *TUBB3* is a potential marker to predict clinical response in patients with advanced NSCLC following Pac-based therapy.

*RRM1* is a key enzyme that converts ribonucleotides into deoxyribonucleotides for DNA synthesis (Elnaggar et al., 2012). *RRM1* has been proposed as a predictive and/or prognostic biomarker for NSCLC in various chemotherapy regimens. In platinum-based therapy, Wang et al. have found that positive *RRM1* expression was associated with poor response (Wang et al., 2010), whereas Liang et al. have found that it was associated with a better response (Liang et al., 2014). In Gem-based therapy, most studies have revealed an association between increased or positive *RRM1* expression and poor response (Reynolds et al., 2009; Lee et al., 2010; Gao et al., 2011), as well as shorter progression-free survival (Rosell et al., 2004; Gao et al., 2011) and OS (Rosell et al., 2004; Ceppi et al., 2006; Lee et al., 2010; Gao et al., 2011) in NSCLC. In the current study, we demonstrated that high *RRM1* expression was associated with a good response to Car/Pac therapy, but not Cis/Gem therapy. However, the in vitro experiments revealed that *RRM1* expression was increased in both sensitive and resistant H1792 cells exposed to

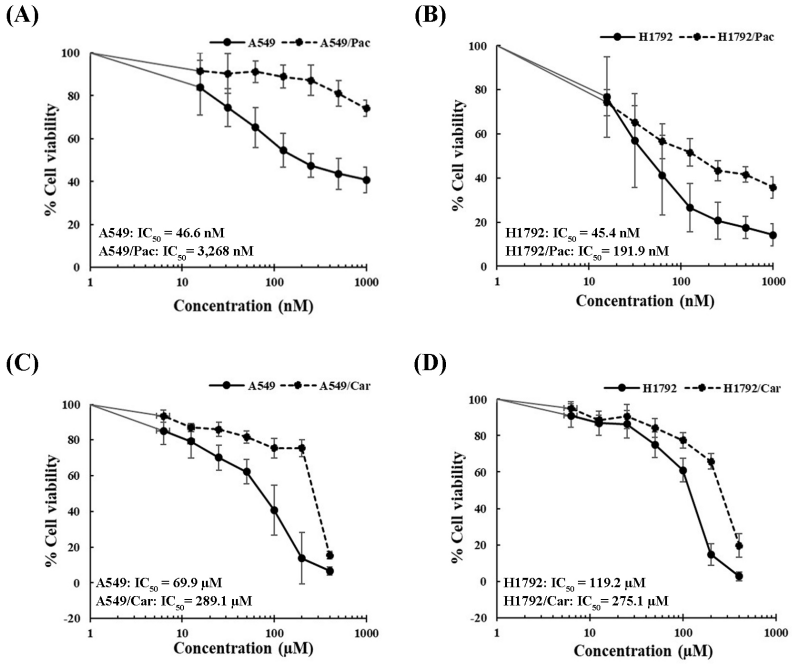


Figure 3. Cytotoxic Effects of Paclitaxel and Carboplatin in Sensitive and Resistant Cells. (A and C) Cell viability of parental A549 and A549/Pac treated with various concentrations of Pac (0-1,000 nM) and carboplatin (0-400 µM), respectively. (C and D) Cell viability of parental H1792 and H1792/Pac cells treated with various concentrations of Pac (0-1,000 nM) and carboplatin (0-400 µM), respectively. A549/Pac, paclitaxel-resistant A549 cell; A549/Car, carboplatin-resistant A549 cell; H1792/Pac, paclitaxel-resistant H1792 cell; H1792/Car, carboplatin-resistant H1792 cell.

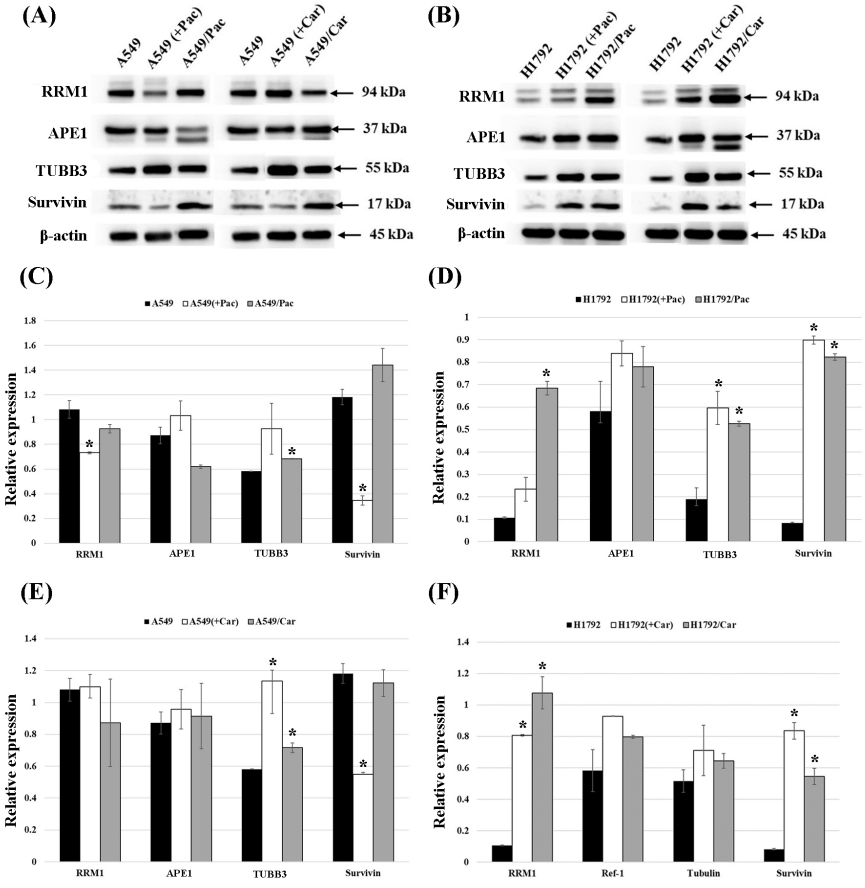


Figure 4. Validation of the Expression of *RRM1, APE1, TUBB3*, and Survivin in Car- or Par- Sensitive/Resistant Cells. Western blot analysis of the expression of RRM1, APE1, TUBB3, and survivin in (A) parental A549, A549(+Pac), A549/Pac, A549(+Car), and A549/Car cells and (B) in parental H1792, H1792(+Pac), H1792/Pac, H1792(+Car), and H1792/Car cells. (C-F) Quantification of the western blotting results. Data are presented as the mean ± S.D. from either two-or-three- independent experiments. *P ≤ 0.05 vs. either parental A549 or H1792 cells.

Table 4. Results of the Cox Regression Analysis of the Overall Survival of Patients with NSCLC Receiving Chemotherapy.

| Variables | Car/Pac regimen | | | | Cis/Gem regimen | | | |
|---|---|---|---|---|---|---|---|---|
| | Univariate analysis | | Multivariate analysis | | Univariate analysis | | Multivariate analysis | |
| | Crude HR (95% CI) | P-value | Adjusted HR (95% CI) | P-value | Crude HR (95% CI) | P-value | Adjusted HR (95% CI) | P-value |
| Age (years) | | | | | | | | |
| <60 | 1 | | 1 | | 1 | | 1 | |
| ≥60 | 1.74 (0.93,3.24) | 0.082 | 1.83 (0.72,4.64) | 0.202 | 1.14 (0.56,2.29) | 0.723 | 0.36 (0.09,1.45) | 0.150 |
| Sex | | | | | | | | |
| Female | 1 | | 1 | | 1 | | 1 | |
| Male | 1.59 (0.80,3.15) | 0.185 | 7.09 (1.66,30.37) | 0.008 | 0.99 (0.48,2.03) | 0.970 | 0.11 (0,5.18) | 0.258 |
| Smoking | | | | | | | | |
| Never | 1 | | 1 | | 1 | | 1 | |
| Habitual | 0.74 (0.29,1.89) | 0.523 | 0.18 (0.03,1.01) | 0.051 | 0.79 (0.35,1.80) | 0.580 | 3.52 (0.07,173.12) | 0.527 |
| Unknown | 0.90 (0.36,2.25) | 0.820 | 0.71 (0.19,2.63) | 0.604 | 0.90 (0.37,2.17) | 0.812 | 0.14 (0,4.94) | 0.277 |
| Alcohol drinking | | | | | | | | |
| Never | 1 | | 1 | | 1 | | 1 | |
| Habitual | 0.81 (0.16,4.19) | 0.799 | 0.90 (0.11,7.45) | 0.920 | 1.68 (0.37,7.54) | 0.501 | 0.78 (0.13,4.50) | 0.778 |
| Unknown | 0.97 (0.38,2.46) | 0.942 | 0.46 (0.11,1.85) | 0.275 | 1.15 (0.56,2.33) | 0.707 | 1.58 (0.18,13.84) | 0.682 |
| Histopathology | | | | | | | | |
| SCC | 1 | | 1 | | 1 | | 1 | |
| ADC | 2.62 (0.22,2.82) | 0.625 | 3.52 (0.46,7.08) | 0.998 | 0.51 (0.01,1.42) | 0.506 | 1.35 (0.17,1.95) | 0.998 |
| Unspecified NSCLC | 2.64 (0.22,3.15) | 0.607 | 3.50 (0.46,6.50) | 0.998 | 3.66 (0.59,7.67) | 0.891 | 4.70 (0.21,8.21) | 0.780 |
| Stage | | | | | | | | |
| III | 1 | | 1 | | 1 | | 1 | |
| IV | 0.96 (0.40,2.27) | 0.919 | 0.32 (0.09,1.18) | 0.087 | 0.20 (0.04,0.90) | 0.036 | 0.13 (0.01,1.66) | 0.116 |
| TUBB3 | | | | | | | | |
| Low | 1 | | 1 | | 1 | | 1 | |
| High | 1.37 (0.72,2.59) | 0.336 | 0.68 (0.26,1.76) | 0.431 | 3.03 (1.12,8.25) | 0.030 | 4.31 (0.50,37.28) | 0.184 |
| RRM1 | | | | | | | | |
| Low | 1 | | 1 | | 1 | | 1 | |
| High | 1.06 (0.58,1.94) | 0.842 | 1.26 (0.58,2.72) | 0.560 | 1.34 (0.57,3.17) | 0.502 | 1.97 (0.40,9.68) | 0.402 |
| APE1 | | | | | | | | |
| Low | 1 | | 1 | | 1 | | 1 | |
| High | 0.75 (0.39,1.46) | 0.401 | 0.46 (0.16,1.31) | 0.146 | 1.19 (0.45,3.18) | 0.730 | 1 (0.14,7.04) | 0.995 |
| Survivin | | | | | | | | |
| Low | 1 | | 1 | | 1 | | 1 | |
| High | 0.95 (0.51,1.77) | 0.877 | 0.96 (0.32,2.92) | 0.944 | 1.43 (0.67,3.09) | 0.357 | 1.30 (0.20,8.44) | 0.787 |

Car, carboplatin; Pac, paclitaxel; Cis, cisplatin; Gem, gemcitabine; HR, hazard ratio; CI, confidence interval; ADC, adenocarcinoma; SCC, squamous cell carcinoma; NSCLC, non-small cell lung cancer; TUBB3, class III β-tubulin; RRM1, ribonucleotide reductase M1; APE1, apurinic/apyrimidinic endonuclease.

either Car or Pac. Therefore, our findings contradict those of previous studies, except for those of the study by Liang et al., (2014). These discordances indicate that *RRM1* expression is heterogeneously distributed within NSCLC (Jakobsen et al., 2013) and may vary depending on the chemotherapy regimen. Therefore, although we demonstrated the predictive role of *RRM1* in patients undergoing Pac-based therapy, *RRM1* may not be suitable for assisting with regimen selection.

Survivin is an apoptotic inhibitor that plays a significant role in promoting the cell cycle and inhibiting apoptosis (Nogueira-Ferreira et al., 2013). Positive survivin expression was associated with a poor response in patients with breast cancer receiving docetaxel (Yuan et al., 2012). Similarly, increased survivin expression in tumor tissues was associated with a poor clinical response to platinum-based therapy in NSCLC (Karczmarek-Borowska et al., 2005; Chen et al., 2010). In contrast, Zhou et al., (2004) reported that the expression level of survivin was reduced in taxol-resistant ovarian cancer cells, indicating an association with taxol resistance. Wu et al., (2014) reported that higher survivin expression is associated with a greater response to taxol (Pac or docetaxel)-based chemotherapy and OS in NSCLC. Our results are in line with the studies by Zhou et al., (2004) and Wu et al., (2014) revealing that high survivin expression is associated with a better response than low survivin expression in patients with NSCLC treated with Car/Pac therapy. These

findings were also validated in our in vitro experiments, which revealed an increase in survivin levels in sensitive H1792 cells treated with either Pac or Car. Collectively, these findings suggest a relationship between survivin and Pac-based chemotherapy resistance. However, our findings did not demonstrate an independent predictive ability for survivin.

The present study was limited by the small number of patients receiving either the Car/Pac (n=52) or Cis/Gem (n=34) regimens, which may result in a non-significant association between the variables and protein expression in response to chemotherapy and OS, particularly in the Cis/Gem regimen. Furthermore, we used two human NSCLC cell lines with distinct properties, A549 and H1792. A549 cells were derived from a primary lung tumor, whereas H1792 cells were derived from metastatic sites. Therefore, the use of these cells may lead to conflicting results across all four protein expression levels in cells sensitive or resistant to Pac and Car.

In conclusion, our study demonstrated that *TUBB3* and *RRM1* may be used as predictive biomarkers, but not as prognostic biomarkers, for the Car/Pac regimen in advanced NSCLC. None of the investigated proteins were predictive biomarkers for the Cis/Gem regimen; however, further studies with a larger number of patients are required. This study may help in clinical decision-making to select an effective treatment for patients with advanced NSCLC.

## Author Contribution Statement

Conceptualization: P.R., S.L.-G. and P.T.; Data Curation: S.L.-G.; Formal Analysis: J.J. and P.T.; Investigation: P.R. and S.T.; Validation: P.R.; Visualization: P.R.; Writing – Original Draft Preparation: P.R.; Writing – Review & Editing: P.R. and P.T..

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*Ethics approval*

This study was approved by the Ethics Committee on Human Research, Faculty of Medicine and Prince of Songkla University (REC:61-045-4-2). Patient consent was waived due to using paraffin-embedded blocks in pathology.

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