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The role of bronchial washing of transforming growth factor β1 as a prognostic biomarker of advanced stages of non-small cell lung cancer

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Abstract
Lung cancer includes all malignant diseases in the lungs, both primary malignancies and metastases. Globally, there are approximately 2.2 million new lung cancer cases each year, with 1.8 million deaths recorded in 2020. In Indonesia, lung cancer is the leading cause of cancer-related deaths, accounting for 30,843 cases (13.2%), with 34,873 new cases (8.8%). The prognosis of lung cancer patients is generally poor due to late-stage diagnosis and the absence of non-invasive early detection methods. Chronic inflammation plays a crucial role in lung cancer progression, as inflammatory and tumor cells release cytokines and chemokines that influence cancer development. Transforming growth factor β1 (TGF-β1) has been identified as a potential biomarker for lung cancer prognosis. This analytical observational study employs a retrospective cohort design. TGF-β1 levels in bronchial washing fluid from non-small cell lung cancer (NSCLC) patients diagnosed at Dr. Wahidin Sudirohusodo Hospital, Makassar, were analyzed. Patients were followed for one year to assess disease progression and survival. Samples collected from June 2022 to May 2023 were stored at -80°C before analysis at the Hasanuddin University Medical Research Unit. TGF-β1 levels in bronchial washing showed a cut-off value of 1638.22 pg/ml with 62.1% sensitivity and 95.5% specificity, indicating its potential as a prognostic biomarker. Higher TGF-β1 levels correlated with advanced disease stages and shorter survival rates. Increased TGF-β1 levels in NSCLC patients are associated with poor prognosis and reduced survival. Thus, TGF-β1 serves as a valuable prognostic biomarker in NSCLC.

Introduction

Lung cancer is a malignant disease in the lungs, including malignancy originating from the lungs (primary) and malignancy from outside the lung (metastasis). Globally, lung cancer is estimated to be as many as 2.2 million new cases each year and 1.8 million deaths in 2020. Lung cancer in Indonesia is the leading cause of death due to cancer, which is 30,843 cases (13.2%), with the number of new cases reaching 34,873 cases (8.8%), the third most after breast cancer and cervical cancer.[1,2] Deaths from lung cancer are estimated to increase by 18.3% between 2006 and 2016. New statistical data from 2019 shows that lung cancer ranks as the seventeenth largest cause of disability-adjusted life years for all ages, especially in individuals aged 50 years and older.[1,2]

Most people with lung cancer are diagnosed at an advanced stage, having a poor prognosis for long-term survival. This is due to the limitations of non-invasive clinical trials for early diagnosis and screening of lung cancer patients. This reason makes the discovery of specific biomarkers essential to obtain an accurate and rapid diagnosis.[4] Epidemiological studies show that chronic inflammation plays a role not only in the process of tissue damage but also in carcinogenesis.[3,4] This applies in the early stages of carcinogenesis as well as in the advanced stages. Innate immune responses and adaptive immune responses play a role in describing the functional relationship between inflammation and cancer. Inflammatory cells and tumor cells release cytokines and chemokines that function to increase cellular and humoral system activity.[3,5]

Cytokines are compounds that function as dissolved chemical messengers that are very important in stimulating the immunological response to infections, autoimmune disorders, and cancer.[3,6] Recent advances in understanding the molecular pathways of lung cancer reveal several tumor function mediators that can be used as biomarkers.[6-9] Several studies have shown benefits in detecting specific cytokines from bronchial flushing fluid or blood serum in the differential diagnosis of lung cancer.[4,5,10] Interleukin (IL)-1β, IL-6, IL-8, and transforming growth factor β (TG-β) are cytokines involved in the pathogenesis of lung cancer that have the potential to serve as biomarkers for the diagnosis, prognosis, and evaluation of treatment response.[6,8,9]

TG-β is a cytokine protein that is secreted to regulate the proliferation, differentiation, and death of various cell types. All types of immune cells, including B cells, T cells, dendritic cells, and macrophages, secrete TGF-β. TG-β has the isomers TGF-β1, TGF-β2, and TGF-β3, which have important roles as key immunosuppressors related to autoimmune, inflammation, and cancer. TG-β1 has oncogenic effects on various malignancies. The role of TGF-β1 as a tumor promoter is very closely related to the progression of lung cancer.[11,12]

Various studies have been conducted with results supporting TGF-β1 as a prognostic biomarker in lung cancer cases. A meta-analysis study conducted by Li *et al.* and Suti *et al*. found that high expression of TGF-β1 can significantly predict poor prognosis in lung cancer patients with non-small cell lung cancer (NSCLC) type.[11,12] Ji-Won Kim *et al*. showed that increased TGF-β1 levels in the serum of patients with NSCLC type lung tumors were significantly related to shorter progression-free survival. In NSCLC patients, TGF-β1 can be detected in respiratory secretions, serum, and malignant pleural effusion, but with the same specimen, TGF-β1 cannot be detected in patients with benign lung tumors.[11,13,14]

Further research is needed to confirm some of these findings and to understand the function that these cytokines perform in lung cancer. Determining the prognosis of patients with NSCLC is important as an evaluation of the management given to patients. Therefore, this study will discuss TGF-β1 as a prognostic biomarker of NSCLC patients as the most common type of pulmonary malignancy when compared to other types.[8,15,16]

The general purpose of this study is to determine the role of TGF-β1 as a prognostic biomarker in NSCLC. To find out the comparison of TGF-β1 levels of bronchial washing in lung and non-lung cancer. Analyzing the difference in TGF-β1 levels of bronchial washing in NSCLC patients based on the clinical picture, and to analyze the relationship between TGF-β1 levels of bronchial flushes on the prognosis and survival of NSCLC patients.

Materials and Methods

This study is an analytical observational study with a retrospective cohort design. Observation was carried out by taking data on the examination of TGF-β1 levels in the bronchial washing fluid of patients with NSCLC, then looking at the progression of the patient's disease by monitoring one-year survival (survival analysis). The research was conducted at Dr. Wahidin Sudirohusodo Hospital in Makassar. Data collection and sample examination was carried out from June to October 2024. The samples examined are samples collected from June 1, 2022, to May 31, 2023, and stored in the Hasanuddin University Medical Research Unit Sample Bank at the Department of Pulmonology and Respiratory Medicine, Dr. Wahidin Sudirohusodo Hospital, Makassar. Samples were stored in the Sample Bank at a temperature of -80°C, then TGF-β1 levels were checked at the Hasanuddin University Medical Research Unit. The population in this study is all NSCLC patients diagnosed at Dr. Wahidin Sudirohusodo Hospital, Makassar. The population in this study is all patients with NSCLC who have undergone diagnostic measures starting on June 1, 2022. The sample in this study is the entire affordable population that meets the inclusion and exclusion criteria. Sampling technique using consecutive sampling. The research sample was divided into two groups, namely the NSCLC group and the control group. Then a follow-up was carried out for 12 months for the NSCLC group. Collection of patient identity data that meets the inclusion and exclusion criteria. Each patient is given a full explanation of the medical measures to be performed. Patients who agree are asked to fill out and sign an informed consent form. The study subjects who met the inclusion criteria were swabbed for bronchial washing during the diagnostic bronchoscopy procedure as much to 3 mL. Washing the bronchi is centrifuged for 10 minutes at a speed of 2000 rpm. The sample is then stored in a -80°C freezer. The sample is a bronchial washing that is obtained when the research subject undergoes bronchoscopy. The sample is stable for 24 hours at a temperature of 2-8°C. The tools used are refrigerators, deep freezers (-80°C), spectrophotometers, incubators, enzyme-linked immunosorbent assay (ELISA) plates, ELISA readers, centrifuges, spinners, sterilizers, microtubes of 1.5 mL, as well as micropipettes and tips. Meanwhile, the materials used are distilled water, ice, and human ELISA kits from BioSciences Inc. This test uses an ELISA with a biotin double antibody sandwich method to measure TGF-β1 levels. TGF-β1 is added to the well that has been coated with TGF-β1 antibodies and then incubated. Added an antibody TGF-β1 labelled with biotin to react with streptavidin-HRP, which forms the immune complex. Enzymes that are not bound after incubation are removed by washing, then substrates A and B are added. The color level of the solution and the level of TGF-β1 were positively correlated.

Results

This study aims to determine the role of TGF-β1 as a diagnostic and prognostic biomarker in NSCLC. The research sample was taken from a collection of samples that began on June 1, 2022, and ended on May 31, 2023. This study is an analytical observational study with a cohort design, using primary data of NSCLC patients who underwent prodiagnostic examinations at the Dr. Wahidin Sudirohusodo Central General Hospital, Makassar City. The research sample was obtained from the bronchial washing of the research subjects using a consecutive sampling method that was filtered based on inclusion and exclusion criteria. This study used 88 samples to check TGF-β1 levels, consisting of 66 samples from NSCLC patients and 22 samples from control patients. All samples have gone through histopathological examination at the Anatomy Pathology Laboratory of Dr. Wahidin Sudirohusodo Hospital.

This study uses patient samples that have been collected from June 1, 2022, to May 31, 2023. Based on the data collected, the characteristics of the research subjects include age, gender, ethnicity, occupation, smoking, and comorbidities, as stated in Table 1. The age grouping of the study subjects was divided into three categories based on the age limit of high risk and progressivity of lung cancer, namely ≤45 years, >45 to <60 years, and ≥60 years. In the age group ≤45 years, 13 subjects were found, consisting of 10 (76.9%) subjects from the NSCLC group and 3 (23.1%) subjects from the control group. In the age range >45 years to <60 years, there were 41 research subjects, consisting of

30 (73.2%) subjects from the NSCLC group and 11 (26.8%) subjects from the control group. In the age group of ≥60 years, 34 research subjects were recorded above 26 (76.5%) from the NSCLC group and 8 (23.5%) subjects from the control group. The results of statistical analysis found no significant difference between the age distribution in the NSCLC group and the control group (p=0.933).

Table 1 presents the distribution of research subjects by gender, showing that there were 36 male research subjects in the NSCLC group and 14 research subjects in the control group. In the female gender, there were 30 subjects in the NSCLC group and 8 subjects in the control group. The results of the statistical analysis test showed that there was no significant difference in distribution based on sex between the NSCLC group and the control group (p=0.620).

The distribution of research subjects based on ethnic background consisted of Bugis, Makassar, Toraja, Mandar, and other tribes. In the NSCLC group, there were 20 subjects from the Bugis tribe, 13 subjects from the Makassar tribe, 12 subjects from the Toraja tribe, 11 subjects from the Mandar tribe, and 10 subjects from other tribes. The control group consisted of 7 subjects from the Bugis tribe, 7 subjects from the Makassar tribe, 3 subjects from the Toraja tribe, 2 subjects from the Mandar tribe, and 3 subjects from other tribes. The results of statistical analysis did not show a significant relationship in ethnic distribution between the NSCLC group and the control group (p=0.746). The job classification of research subjects includes civil servants (PNS), self-employed, farmers, housewives, retirees, and other categories. The NSCLC group consists of 11 civil servant subjects, 20 self-employed subjects, 6 farmer subjects, 20 housewife subjects, 8 retired subjects, and 1 other category subject. The control group included 5 civil servant subjects, 10 self-employed subjects, 2 farmer subjects, 4 housewife subjects, and 1 other category subject. The results of statistical analysis showed no significant difference in the distribution of work between the two groups (p=0.340).

Smoking status was analyzed as a major risk factor for lung cancer by classification of non-smokers, passive smokers, light smokers, moderate smokers, and heavy smokers. The NSCLC group consisted of 15 non-smoking subjects, 20 passive smoking subjects, 14 light smoker subjects, 9 moderate smokers, and 8 heavy smokers. The control group included 5 non-smoking subjects, 7 passive smoking subjects, 5 light-smoking subjects, and 5 moderate smoking subjects. Statistical analysis did not show a significant relationship in the distribution of smoking status between the NSCLC group and the control group (p=0.470).

Comorbidity analysis in the NSCLC group showed 32 subjects without comorbidities, 1 subject with tuberculosis, 14 subjects with chronic obstructive pulmonary disease (COPD), 9 subjects with cardiovascular disease, 4 subjects with hypertension, and 6 subjects with diabetes mellitus. The control group consisted of 12 subjects without comorbidities, 6 subjects with Tuberculosis, 2 subjects with COPD, and 2 subjects with Hypertension. The results of statistical analysis showed a significant relationship between comorbidities and the grouping of study subjects. The NSCLC group had a higher prevalence of COPD, Hypertension, and Diabetes Mellitus comorbidities than the control group (p=0.001).

Comparative analysis of TGF-β1 levels of bronchial washing of the NSCLC group with controls based on the characteristics of the study subjects is seen in Table 2. Bronchial washing TGF-β1 levels showed significant differences between the NSCLC group and controls in specific characteristics. These significant differences were seen in the age group of 45-60 years (p=0.007), male subjects (p=0.019), female subjects (p=0.020), Makassar tribal subjects (p=0.043), Toraja tribal subjects (p=0.030), other tribal subjects (p=0.043), passive smokers (p=0.004), and subjects without comorbidities (p=0.004). Analysis of TGF-β1 levels of bronchial washing showed higher values in the NSCLC group compared to the control group. Stratification by age showed the highest levels of TGF-β1 bronchial washing at the age of >45-<60 years, followed by the NSCLC group <45 years and >60 years old. In the control group, it was found that at the age of >60 years, it was slightly higher than the age group of <45 years and >45-<60 years. In the results of the analysis, there was no significant difference in TGF-β1 levels of bronchial washing between the age groups of ≤45 years and ≥60 years. The age group of >45-<60 years showed significantly higher levels of TGF-β1 bronchial washing in the NSCLC group compared to the control group (p=0.007). The level of TGF-

β1 bronchial washing for the male sex in the NSCLC group was 1614.52 pg/mL, while the control group was 1202.35 pg/mL. The TGF-β1 level of bronchial washing for the female sex in the NSCLC group was 2492.27 pg/mL, while in the control group, it was 1263.94 pg/mL. The results of the sex analysis showed that the TGF-β1 levels of bronchial washing in the NSCLC group were significantly higher than in the control group, both in male subjects ($p = 0.019$) and female subjects ($p = 0.020$).
Table 2 shows how the study subjects were divided into 5 different tribes, with TGF-β1 levels of bronchial washing always being greater in the NSCLC group. The average level of TGF-β1 bronchial washing was highest in the Mandar tribe, followed by the Toraja tribe, the Lain tribe, the Makassar tribe, and the Bugis tribe. The distribution by tribe showed significant differences in the Makassar ($p=0.043$), Toraja ($p=0.30$), and other tribes ($p=0.43$). The other ethnic groups did not show a difference in TGF-β1 levels of bronchial washing between the NSCLC group and the control group. The highest TGF-β1 levels of bronchial washing were obtained in the NSCLC group of housewives, which reached an average of 2784.52 pg/mL, and farmers reached an average of 1427.73 pg/mL in the control group. Analysis of the occupational data showed no difference in TGF-β1 levels of bronchial washing between the two groups. The study subjects of passive smokers in the NSCLC group had the highest average levels of TGF-β1 bronchial washing for smoking status characteristics, followed by mild smokers, heavy smokers, non-smokers, and the lowest in those who were moderate smokers. Smoking status showed significantly higher levels of TGF-β1 bronchial washing in the NSCLC group compared to the control group for the passive smoking category ($p=0.004$). Subjects without comorbidities (2375.8 pg/mL) in the NSCLC group had the highest average levels of TGF-β1 bronchial washing compared to all the comorbidities groups. Subjects without comorbidities showed significantly higher average levels of TGF-β1 bronchial washing in the NSCLC group than in the control group ($p=0.004$). Some variables could not be analyzed due to the absence of research subjects on certain characteristics, including occupational categories (retirees and others), heavy smoker status, and comorbidities (tuberculosis, cardiovascular, and diabetes mellitus). The variable obtains the information NA (not analyzed).
Analysis using the Youden Index method was used to determine the cut-off value of TGF-β1 levels of bronchial washing between the NSCLC group and the control. This cut-off value is a certain limit value that can be used to separate the NSCLC and control (diagnostic) categories. The determination of the optimal cut-off point value is done taking into account the balance between sensitivity and specificity. The results of the calculation of the Youden Index (YI) for TGF-β1 bronchial washing are shown in Table 3.
Table 3 presents the coordinate analysis of the TGF-β1 curve of bronchial washing. TGF-β1 analysis of bronchial washing showed a cut-off point value of 1638.22 pg/mL with a sensitivity of 62.1% and a specificity of 95.5%, resulting in a Youden Index of 0.576. The cut-off point value of 1659.90 pg/mL showed a sensitivity of 60.6% and a specificity of 95.5% with a Youden Index of 0.561. The cut-off point of 1620.46 pg/mL yielded a sensitivity of 63.6% and a specificity of 90.9% with a Youden Index of 0.545. The cut-off point value best used as a cut-off of TGF-β1 levels of bronchial washing as a diagnostic is 1638.22 pg/mL with a sensitivity value of 62.1% and a specificity of 95.5%.
Table 4 presents histological type analysis at diagnosis, showing the distribution of 26 subjects (39.4%) for adenocarcinoma, 30 subjects (45.5%) for squamous cell carcinoma, and 10 subjects (15.2%) for adenosquamous. Stratification based on tumor size showed 30 subjects (45.5%) T3 and 36 subjects (54.5%) T4. Regional lymph node (KGB) involvement was distributed with 8 subjects (12.1%) at N0, 54 subjects (81.8%) at N1, and 4 subjects (6.1%) at N2. Metastatic status showed 17 subjects (25.8%) without metastasis, 15 subjects (22.7%) M1a, and 34 subjects (51.5%) M1b. Tumor size, regional KGB involvement, and metastases affected the rate of lung cancer stages. The distribution of stages showed 17 subjects (25.8%) stage III and 49 subjects (74.2%) stage IV. The method of diagnosis enforcement in the NSCLC group consisted of 29 subjects (43.9%) through bronchoscopy specimens and 37 subjects (56.1%) through TTNA/core biopsy. Pre-diagnostic CT-scan results showed 26 subjects (39.4%) with central lesions and 40 subjects (60.6%) with peripheral lesions. Bronchoscopy images showed 11 subjects (16.7%) with intralumen lesions and 55 subjects

(83.3%) without intralumen masses. Administrative data and outputs show that among the 66 research subjects of the NSCLC group, there are 18 (27.3%) research subjects who do not receive therapy until they die. The study subjects who received therapy consisted of 24 subjects (36.4%) who received TKI and 24 subjects (36.4%) who received systemic chemotherapy. The study subjects who achieved one-year survival were 12 subjects (18.2%), and 54 subjects (81.8%) died within less than one year.

Table 5 presents an analysis of the clinical characteristics of the NSCLC group, including histological type, primary tumor, regional lymph nodes, metastasis, stages, computed tomography scan images, bronchoscopy images, diagnostic methods, molecular examinations, treatment modalities, and survival, along with the average TGF-β1 levels of bronchial washing. The results of statistical tests showed significant differences in TGF-β1 levels of bronchial washing on several clinical variables, including regional lymph node stage ($p=0.003$), metastatic status ($p<0.001$), clinical stage ($p<0.001$), diagnostic method ($p=0.001$), and survival ($p=0.001$). The results of the analysis of the clinical characteristics of NSCLC and the relationship with TGF-β1 levels of bronchial washing showed several important findings. The TGF-β1 levels of bronchial washing show variation based on the type of histopathology. Adenocarcinoma showed the highest average levels (2456.51±4138.30 pg/mL) compared to squamous (1822.21±507.17 pg/mL) and adenosquamosa (1435.56±539.09 pg/mL), although the difference was not statistically significant ($p=0.212$). TGF-β1 levels of bronchial washing based on the size of primary tumors, T3 classification showed an average level of TGF-β1 of bronchial washing of 1566.01 pg/mL, while T4 of an average TGF-β1 level of bronchial washing was 2386.41 pg/mL ($p=0.055$). This difference is not statistically significant.

In the regional KGB category, it was found that the level of TGF-β1 of bronchial washing in the N0 group averaged 1075.89 pg/mL, N1 averaged 2214.10 pg/mL, and N2 averaged 1180.61pg/mL with a value of $p=0.003$, which indicated a statistically significant difference. Metastatic status showed a very significant difference ($p<0.001$) with TGF-β1 levels of bronchial washing M0 with an average of 1198.06 pg/mL, M1a with an average of 1981.37 pg/mL, and M1b with an average of 2435.40 pg/mL. Table 5 shows that the average level of TGF-β1 bronchial washing in stage IV NSCLC subjects was significantly higher than in stage III. A positive correlation was identified between increased regional lymph node stage and metastatic status with TGF-β1 levels. These results indicate that an increase in the TGF-β1 level of bronchial washing is directly proportional to the severity of the disease.

Table 5 presents an analysis based on diagnostic methods, showing that NSCLC subjects with diagnosis through TTNA/core biopsy had an average TGF-β1 level of bronchial washing 2023.49 pg/mL, which is significantly higher than the diagnosis through bronchoscopy of 2000.76 pg/mL ($p=0.001$). The EGFR mutation group showed the highest average of 2330.20 pg/mL, followed by the group without molecular examination of 1833.35 pg/mL, and the group without EGFR mutations of 1709.92 pg/mL. No significant differences in TGF-β1 levels of bronchial washing were found based on the results of EGFR examinations, radiological images, bronchoscopy images, histopathological types, and therapy modalities given. The treatment modality of tyrosine kinase inhibitors showed the highest average of 2330.20 pg/mL, followed by systemic chemotherapy of 1768.11 pg/mL, and the untreated group of 1851.76 pg/mL. Survival of less than 1 year dominated with an average of 2198.98 pg/mL compared to survival of more than 1 year of 1178.86 pg/mL. The results of the survival analysis showed that NSCLC subjects who died <1 year of the monitoring period had significantly higher levels of TGF-β1 bronchial flush compared to NSCLC subjects who survived more than 1 year ($p=0.001$).

The results of the calculation of the average survival time of the TGF-β1 group of low bronchial washing levels (<1028.47 pg/mL) reached 10.33 months. The TGF-β1 group of high bronchial washing (≥1028.47 pg/mL) showed an estimated 2.895 months. The mean survival time of the TGF-β1 group with high bronchial washing showed a shorter duration than the low level group. Statistical calculations indicated a correlation between the TGF-β1 levels of bronchial washing and the survival prognosis of the study subjects. Table 6 presents the results of the analysis of the mean and median

survival time based on the TGF-β1 level of bronchial washing, using the Kaplan-Meier method. The data was analyzed based on the cut-off point of TGF-β1 levels at the level of 1028.47 pg/mL.
Table 6 presents the group with TGF-β1 levels of bronchial washing <1028.47 pg/mL; the estimated survival time was 10.333 months with a 95% confidence interval ranging from 8.290 to 12.376 months. Meanwhile, in the group with a bronchial washing TGF-β1 level of ≥1028.47 pg/mL, the estimated survival time was 2.895 months with a 95% confidence interval ranging from 1.958 to 3.831 months. Overall, the median survival time was 3,905 months with a 95% confidence interval between 2,852 to 4,958 months. Statistical analysis using the Log Rank and Cox Coat tests yielded a p-value of 0.001, indicating a statistically significant difference in survival time between the two groups of bronchial washing TGF-β1 levels. These findings suggest that patients with lower bronchial washing TGF-β1 levels (<1028.47 pg/mL) had a significantly longer survival time compared to patients with higher bronchial washing TGF-β1 levels (≥1028.47 pg/mL), with an estimated survival time difference of approximately 7,438 months.

Discussion

The subject of this study is the lung tumor patient who underwent pro diagnostic bronchoscopy at Dr. Wahidin Sudirohusodo Hospital, Makassar. The samples collected were from April 2023 to March 2024. TGF-β1 levels of bronchial washing were checked in 66 NSCLC samples and 22 control samples. The examination was carried out by the ELISA method using a spectrometer with a wavelength of 450 nm. The bronchial washing sample is homogenized first to ensure that the components in the bronchial washing are evenly distributed before the measurement is taken. Statistical test analysis was carried out with the help of SPSS version 25.0 software. In general, in this study, the TGF-β1 levels of bronchial washing in the NSCLC group were higher than controls in almost all categories. These results are in line with those of Chen *et al*., showing that TGF-β levels of bronchial washing increased in NSCLC patients.[5] Bronchial washing is a bioactive material that includes cellular and non-cellular components in biological processes such as inflammation and infection in the bronchi. Cytokine changes in bronchial washing fluid reflect the pulmonary immunological reaction to malignancy. Domagala-Kulawik *et al*. showed a significant increase in TGF-β1 in the bronchial washing of the NSCLC group compared to controls.[17]
Based on age, there was a significant increase in TGF-β1 levels of bronchial washing in the NSCLC group compared to controls at the age of >45-<60 years (2465.60 pg/mL *vs.* 1256.00 pg/mL, p=0.007). These findings are in line with the study of Li *et al*., which reported that increased TGF-β1 expression was more significant in middle-aged NSCLC patients and had a correlation with a poorer prognosis. Research conducted by Dana *et al*. shows that the majority of NSCLC are in the age group of 41-60 years. Similar to several other studies, it also consistently states that the incidence of lung cancer >45 increases significantly. Lung cancer is rare at the age of <45 years. Further age is associated with the risk of lung cancer caused by the continuous shortening of telomeres during repeated cell replication cycles, so the older a person is, the greater the chance of DNA damage.[50]
Based on sex, both males (1614.62 pg/mL *vs.* 1202.35 pg/mL, p=0.019) and females (2492.27 pg/mL *vs.* 1263.04 pg/mL, p=0.020) had a significant increase in TGF-β1 levels of bronchial washing in the NSCLC group compared to the controls. This study also showed that the TGF-β1 levels of bronchial washing between men and women were higher in women (2492.27 pg/mL) than in men (1614.62 pg/mL). These findings are in line with the research of Zhang *et al*., which showed that TGF-β1 expression in NSCLC has different patterns based on sex. This difference is attributed to the interaction between TGF-β1 with estrogen receptor (ER) and androgen receptor (AR), which can affect cancer cell signaling pathways. Estrogen can induce TGF-β1 expression through the ER pathway. An increase in TGF-β1 can then activate the TGF-β/Smad signaling pathway that promotes the proliferation, migration, and invasion of lung cancer cells. Androgens can inhibit TGF-β1 expression through the AR pathway.[18]
A decrease in TGF-β1 can reduce the activation of the TGF-β/Smad pathway, thereby inhibiting the EMT process and metastasis of lung cancer cells. An increase in TGF-β1 due to activation of the ER

pathway, as well as a decrease in TGF-β1 due to activation of the AR pathway, can affect the progressivity and aggressiveness of lung tumors in NSCLC patients. This finding is interesting because it is different from several studies, such as Yang *et al*., which found higher TGF-β1 levels in the NSCLC group in men. In their study, Zhao *et al.* also found that TGF-β1 levels did not show significant differences by sex, but were more influenced by stage and progression-free survival. Higher levels of TGF-β1 in the women in this study may correlate with differences in therapy responses, variations in prognosis, and different metastasis patterns. The study by Ahn *et al*. also shows that estrogen receptors affect TGF-β1 signaling, so that it can worsen the prognosis of NSCLC lung cancer.[19]

Regarding the tribal background, in this study, the research subjects of the NSCLC group came from various tribes in Indonesia, especially tribes from South Sulawesi and West Sulawesi. The subjects of this study were dominated by the Bugis tribe as many as 27 subjects (30.7%), although the difference in TGF-β1 levels of bronchial washing of the NSCLC group was not significant in the Bugis tribe. The TGF-β1 level of bronchial washing in the NSCLC group was highest in the Mandar tribe (3748.31 pg/mL), although it was also not significant (p=0.114) with the control (773.13 pg/mL). Significant increases in the level of TGF-β1 bronchial washing in the NSCLC group were found in the Makassar, Toraja, and other tribes. Several studies have been conducted in South Sulawesi by Wardani *et al*. and Santosos et al also found that the Bugis tribe dominates as a basic characteristic in the NSCLC. However, there has been no other research publication that specifically discusses the NSCLC incident based on tribal background in Sulawesi.[20]

Based on the work, the TGF-β1 level of bronchial washing in the NSCLC group was highest in the work of housewives (2784.52 pg/mL), although it was not significant to the control. These results are in line with the meta-analysis research of Zhang *et al.*, who said the risk of lung cancer increases in women who do not smoke due to exposure to cooking oil smoke. Consistent results were also found in a meta-analysis study Xue *et al*. stated an association between exposure to cooking oil smoke and the risk of lung cancer in Chinese women who did not smoke. This is explained by exposure to polycyclic aromatic hydrocarbons, harmful compounds in cooking oil fumes that will cause oxidative DNA damage and lipid peroxidation. Exposure to cooking oil smoke will increase oxidative stress and endoplasmic reticulum stress, which induces cytotoxicity and apoptosis in alveolar epithelial cells II. Several studies have shown that mutagens and carcinogens are released by smoke from heated cooking oil, using the Ames test or SOS chromotest. Biological experiments on human adenocarcinoma lung cancer cells found that various mutagenic compounds are produced by cooking oil fumes that cause DNA damage and oxidative damage to cells.[21]

Another study by Ramos *et al*. found that short-term exposure to wood smoke in guinea pigs led to increased expression of pro-inflammatory cytokines, gelatinase, and metalloproteinase tissue inhibitors (TIMPs). Specifically, there was an increase in the total number of cells, macrophages, neutrophils, and collagen in the bronchoalveolar lavage fluid.[22,23] In addition, the expression of mRNA TGF-β1, TNF-α, IFN-γ, IL-1β, IL-6, IL-8, MMP-2, MMP-9, TIMP-1, and TIMP-2 is increased in lung tissue. These findings suggest that short-term exposure to wood smoke can trigger inflammatory processes and extracellular matrix remodeling similar to those that occur in humans. Most of the NSCLC patients with high TGF-β1 bronchial washing (91.2%) were found to have died within one year, and only 5 subjects (8.8%) survived more than one year.[24,25] These results are in line with meta-analysis studies that state that TGF-β protein expression in patients with NSCLC has been described as correlated with survival.[26,27] Similar results were reported in the study of Ramundo *et al*. that significant levels of TGF-β expression in NSCLC patients are associated with poor survival, so TGF-β expression can predict a much worse prognosis for patients with lung cancer. Survival function curves show higher levels of bronchial washing TGF-β1 leading to poor prognosis with lower survival. These findings are in line with research conducted by Li et al. that high TGF-β1 expression is associated with increased tumor aggressiveness and decreased overall survival. In addition, the research of Duan *et al.* also showed that higher levels of TGF-β1 in bronchial washing in advanced NSCLC patients correlated with poor prognosis and lower survival rates. The study

conducted by Huang *et al.* also found that there are two factors that affect the prognosis of patients with NSCLC, namely TGF-β1 expression and lymph node metastases.[28] According to the Kaplan-Meier survival analysis used in the study, the 5-year survival rate in patients with low TGF-β1 expression was higher than in patients with excess TGF-β1 expression. High expression of TGF-β1 protein in NSCLC has been associated with poor prognosis, especially in cases with lymph node metastases. This suggests that high levels of TGF-β1 may correlate with more aggressive disease and reduced survival rates. Some experiments have found that inhibiting TGF-β1 expression shows increased survival in some cancers.[29]

These results suggest that high expression of TGF-β is associated with poorer survival in patients with NSCLC. TGF-β expression can promote tumor cell growth and differentiation through autocrine or paracrine activity, resulting in increased cell matrix interactions, inhibition of immune surveillance, or increased angiogenic activity. TGF-β can inhibit the growth of normal epithelial cells, but tumor cells have a strong resistance to TGF-β1 inhibition. TGF-β1 levels play an important role in tumor development and metastasis, contributing to the formation of cancer-like stem cells, thus associated with drug resistance and tumor recurrence. Therefore, high levels of TGF-β1 in bronchial washing may indicate a poor prognosis in lung cancer patients.[30]

Limitations

This study is the first study in Indonesia that examines the use of TGF-β1 bronchial washing as diagnostic and prognostic tools as well as the survival of NSCLC patients, which is an advantage of this research. This study did not examine all aspects of NSCLC clinicopathology, and this study used a small number of samples.

Conclusions

The following is a summary of the results of the study on the role of TGF-β1 as a prognostic biomarker in NSCL. TGF-β1 levels of bronchial washing increased in NSCLC patients, and its levels of bronchial flushes increased in line with regional KGB excretion, general stage, and distant metastases in NSCLC patients. High levels of TGF-β1 in bronchial washing have a correlation with shorter survival in NSCLC patients, so it can be a biomarker of a good prognosis in NSCLC patients. Based on the results of the study and discussion, it can be concluded that higher TGF-β1 levels in NSCLC indicate a strong association with poor prognosis (more aggressive tumor progression) and shorter survival.

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Table 1. Demographic characteristics of the research subjects.

Characteristic	Quantity (n)	Frequency (%)		p-value
		NSCLC	**Control**	
Age				
≤ 45 years old	13	10(76.9%)	3(23.1%)	0.933**
> 45 - <60 years old	41	30(73.2%)	11(26.8%)	
≥ 60 years old	34	26(76.5%)	8(23.5%)	
Gender				
Man	50	36 (72.0%)	14 (28.0%)	0.620**
Woman	38	30 (78.9%)	8 (21.1%)	
Tribe				
Bugis	27	20 (74.1%)	7 (25.9%)	0.746*
Makassar	20	13 (65.0%)	7 (35.0%)	
Toraja	15	12 (80.0%)	3 (20.0%)	
Mandar	13	11 (84.6%)	2 (15.4%)	
Miscellaneous	13	10 (76.9%)	3 (23.1%)	
Work				
Civil Servants	16	11 (68.8%)	5 (31.3%)	0.340**
Self employed	30	20 (66.7%)	10 (33.3%)	
Farmer	8	6 (75.0%)	2 (25.5%)	
Housewives	24	20 (83.3%)	4 (16.7%)	
Pensioner	8	8 (100%)	0 (0%)	
Miscellaneous	2	1 (50.0%)	1 (50.0%)	
Smoke				
No smoking	20	15 (75.0%)	5 (25.0%)	0.470**
Passive Smoker	27	20 (74.1%)	7 (25.9%)	
Light Smokers	19	14 (73.7%)	5 (26.3%)	
Moderate Smoker	14	9 (64.3%)	5 (35.7)	
Heavy Smokers	8	8 (100%)	0 (0%)	
Authoritative Diseases				
None	44	32 (72.7%)	12 (27.3%)	**0.001****
Tuberculosis	7	1 (14.3%)	6 (85.7%)	
PPOK	16	14 (87.5%)	2 (12.5%)	
Cardiovascular	9	9 (100%)	0 (0%)	
Hypertension	6	4 (66.7%)	2 (33.3%)	
Diabetes Mellitus	6	6 (100%)	0 (0%)	

*Chi-square; **Fisher Exact test.

Table 2. Comparison of TGF-β1 levels of bronchial washing control group and NSCLC by characteristics.

Characteristic	Mean ± SD (pg/ml)				p-value
	N	NSCLC (n=66)	N	Control (n=22)	
Age					
≤ 45 years old	10	1904.75±586.12	3	984.22±812.20	0.112#
>45 - <60 years old	30	2465.60±3832.25	11	1256.90±630.88	**0.007#**
≥ 60 years old	26	1533.68±511.01	8	1270.74±293.65	0.191#
Gender					
Man	36	1614.52±651.17	14	1202.35±400.35	**0.019#**
Woman	30	2492.27±3807.01	8	1263.94±756.12	**0.020#**
Tribe					
Bugis	20	1477.03±756.02	7	1414.53±807.05	0.956#
Makassar	13	1600.84±497.69	7	1164.85±197.23	**0.043#**
Toraja	12	1929.65±472.19	3	1240.37±233.58	**0.030#**
Mandar	11	3748.31±6232.31	2	773.13±952.05	0.114#
Miscellaneous	10	1815.25±408.20	3	1207.14±361.35	**0.043#**
Work					
Civil Servants	11	1757.29±789.87	5	1234.51±349.34	0.126#
Self employed	20	1678.15±600.95	10	1164.78±433.61	0.071#
Farmer	6	1483.89±885.69	2	1427.73±261.96	0.505#
Housewives	20	2784.52±4652.23	4	1300.03±1109.06	0.188#
Pensioner	8	1767.58±479.92	0	NA	NA
Miscellaneous	1	1023.60	1	1068.49	NA
Smoke					
None	15	1655.02±550.55	5	1599.86±696.82	0.694#
Passive Smoker	20	2837.44±4663.96	7	1021.43±477.55	**0.004#**
Light Smokers	14	1768.22±503.79	5	1232.82±651.36	0.079#
Moderate Smoker	9	1432.79±727.09	5	1126.22±117.79	0.463#
Heavy Smokers	8	1708.36±604.18	0	NA	NA
Authoritative Diseases					
None	32	2375.87±3705.59	12	1172.75±710.30	**0.004#**
Tuberculosis	1	732.47	6	1399.37±195.40	NA
PPOK	14	1600.94±697.38	2	1011.55±90.56	0.204#
Cardiovascular	9	1939.59±533.74	0	NA	NA
Hypertension	4	1832.44±683.72	2	1226.09±222.87	0.355#
Diabetes Mellitus	6	1488.61±635.05	0	NA	NA

Mann-Whitney test; NA = not analyzed

Table 3. Curve coordinates and Youden Index (YI) and standard deviation of cut-off point values TGF-β1 bronchial washing.

Variable	Cut-off point value	Sensitivity	Specificity	Youden Index
TGF-β1 Bronchial Washing	**1638.22**	**62.1%**	**95.5%**	**0.576**
	1659.90	60.6%	95.5%	0.561
	1620.46	63.6%	90.9%	0.545

Table 4. Curve coordinates and Youden Index (YI) and standard deviation of cut-off point values TGF-β1 bronchial washing.

Clinical Picture	Frequency n (%)
Types of Histology	
Adenocarcinoma	26 (39.4%)
Squamous	30 (45.5%)
Adenoskuamosa	10 (15.2%)
Primary Tumor	
T3	30 (45.5%)
Q4	36 (54.5%)
Regional Lymph Nodes	
N0	8 (12.1%)
N1	54 (81.8%)
N2	4 (6.1%)
Metastasis	
M0	17 (25.8%)
M1a	15 (22.7%)
M1b	34 (51.5%)
Stadium	
Stage III	17 (25.8%)
Stage IV	49 (74.2%)
CT-scan overview	
Central Lesions	26 (39.4%)
Peripheral Lesions	40 (60.6%)
Overview of Bronchoscopy	
Visible Intralumen lesions	11 (16.7%)
No visible intralumen lesions	55 (83.3%)
Diagnostic	
Bronchoscopy	29 (43.9%)
TTNA/*Core Biopsy*	37 (56.1%)
Molecular Examination	
EGFR mutation	24 (36.4%)
No EGFR mutase	10 (15.2%)
No molecular examination	32 (48.5%)
Governance	
Tyrosine Kinase Inhibitor	24 (36.4%)
Systemic Chemotherapy	24 (36.4%)
Haven't Gotten Therapy	18 (27.3%)
Progression-free Survival	
Haven't done RECIST yet	41 (62.1 %)
RECIST 1	11 (16.7%)
RECIST 2	7(10.6)
RECIST 3	7 (10,6)
Survival	
< 1 year	54 (81.8%)
≥ 1 year	12 (18.2%)

Table 5. Clinical picture of the NSCLC group and the average TGF-β1 level of bronchial washing.

Clinical Picture	N	Red ± SD	p-value
Types of Histology			
Adenocarcinoma	26	2456.51 ± 4138.30	0.212**
Squamous	30	1822.21 ± 507.17	
Adenoskuamosa	10	1435.56 ± 539.09	
Primary Tumor			
T3	30	1566.01 ± 586.53	0.055*
Q4	36	2386.41 ± 3491.88	
KGB Regional			
N0	8	1075.89 ± 598.52	**0.003****
N1	54	2214.10 ± 2850.05	
N2	4	1180.61 ± 958.70	
Metastasis			
M0	17	1198.06 ± 493.12	**<0.001****
M1a	15	1981.37 ± 469.37	
M1b	34	2435.40 ± 3581.97	
Stadium			
Stage III	17	1198.06 ± 493.12	**<0.001***
Stage IV	49	2296.41 ± 2988.30	
CT-scan overview			
Central Lesions	26	1888.15 ± 425.52	0.131*
Peripheral Lesions	40	2094.98 ± 3368.60	
Overview of Bronchoscopy			
Visible Intralumen lesions	11	1983.32 ± 315.65	0.171*
No visible intralumen lesions	55	2019.54 ± 2876.29	
Diagnostic			
Bronchoscopy	29	2000.76 ± 398.24	**0.001***
TTNA/*Core Biopsy*	37	2023.49 ± 3509.13	
Molecular Examination			
EGFR mutation	24	2380.20 ± 4321.84	0.252**
No EGFR mutations	10	1709.92 ± 648.63	
No molecular examination	32	1833.35 ± 544.12	
Anticancer			
Tyrosine Kinase Inhibitor	24	2380.20 ± 4321.84	0.280**
Systemic Chemotherapy	24	1768.11 ± 533.19	
Haven't Gotten Therapy	18	1851.76 ± 616.92	
Progression-free Survival			
Haven't done RECIST yet	41	2389.48 ± 3258.06	0,507**
RECIST 1	11	1467.75 ± 445.32	
RECIST 2	7	1076.71 ± 556.35	
RECIST 3	7	1605.75 ± 699.37	
Survival			
< 1 year	54	2198.98 ± 2860.12	**0.001***
≥ 1 year	12	1178.86 ± 601.51	

*Mann-Whitney test; **Kruskal-Wallis test

Table 6. Mean and median survival time based on bronchial flush TGF-β1 levels.

TGF-β1 levels	Estimate (Month)	95% CI	Frequency	p-value (Log Rank, Cox Coat)
<1028.47 pg/ml	10.333	8.290 – 12.376	6	<0.001
≥1028.47 pg/ml	2.895	1.958 – 3.831	60	
Overall	3.905	2.852 – 4.958	66	

*Kaplan-Meier