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Expression of MAGE-A3 in and histopathological analysis of forceps biopsy specimens of non–small-cell lung carcinoma patients

Isnin Anang Marhana[1,*], Muhammad Amin[1], Gondo Mastutik[2], Mokhammad Mukhlis[2]

[1]Department of Pulmonology and Respiratory Medicine, Faculty of Medicine, Airlangga University, Surabaya, Indonesia
[2]Department of Pathology Anatomy, Faculty of Medicine, Airlangga University, Surabaya, Indonesia

Abstract

English:

Non–small-cell lung cancer (NSCLC) is a type of epithelial lung cancer and associated with cigarette smoking (passive or active). Melanoma-associated antigen 3 (MAGE-A3) is widely expressed in various types of tumours, including NSCLC. This study aimed to examine the MAGE-A3 expression in forceps biopsy specimens as a tumour biomarker to be used for early diagnosis and screening of lung cancer. This study was an observational, analytical study with a cross-sectional study design. The sample size was determined based on Ronald Fisher's classic z transformation formula, and samples were selected using consecutive sampling. The study population included 14 lung tumour patients. Samples were obtained by forceps biopsy with bronchoscopy guidance. Histopathological analysis was carried out using Giemsa staining. The expression of MAGE-A3 was determined using RT-PCR. All data were analysed using SPSS statistics software (IBM SPSS Statistics, IBM® SPSS® Statistics is a powerful statistical software platform RRID: SCR_019096). In this study, there were 6 subjects (42.9%) with NSCLC adenocarcinoma and 8 subjects (57.1%) with squamous cell carcinoma. The positive MAGE-A3 expression was found in 5 (35.7%) of the total research subjects, and the expression on RT-PCR analysis was at 569 bp. We found that MAGE 3 gene was mostly expressed in adenocarcinoma of NSCLC, even though there was no statistical correlation with histopathological results (P > 0.05). MAGE-A3 expression in forceps biopsy specimens of NSCLC was mostly found in the adenocarcinoma type at 569 bp. Therefore, it could be used as a tumour biomarker for early diagnosis and screening of lung cancer.

Keywords

Forceps biopsy • lung cancer • melanoma-associated antigen 3 • non–small-cell lung cancer

Expresia MAGE-A3 și examenul histopatologic al probelor de biopsie cu forceps în cazul pacienților cu carcinom pulmonar cu celule non small

Rezumat

Romanian:

Cancerul pulmonar cu celule non-small (NSCLC) este un tip de cancer pulmonar de natură epitelial asociat cu fumatul (pasiv sau activ). Antigenul 3 asociat cu melanomul (MAGE-A3) este larg exprimat în diverse tipuri de țesuturi tumorale, inclusiv în NSCLC. Această studiu și-a propus să examineze expresia MAGE-A3 din piesele de biopsie, ca biomarker tumoral ce poate fi utilizat pentru diagnosticul precoce și screening-ul cancerului pulmonar. Acest studiu a fost un studiu analitic observațional utilizând un design transversal. Dimensiunea eșantionului a fost determinată pe baza formulei de transformare clasică „z" a lui Ronald Fisher, folosind o selecție consecutivă. Populația de studiu a inclus 14 pacienți cu tumori pulmonare. Eșantionul a fost obținut prin biopsie în cadrul bronhoscopiei. Analiza histopatologică s-a efectuat utilizând colorația Giemsa. Expresia MAGE-A3 s-a determinat prin analiza RT-PCR. Toate datele au fost analizate utilizând software-ul de statistică SPSS (IBM SPSS Statistics, RRID: SCR_019096). Au fost identificați 6 subiecți (42,9%) cu adenocarcinom NSCLC și 8 subiecți (57,1%) cu carcinom cu celule scuamoase. Expresia pozitivă a MAGE-A3 a fost găsită la 5 subiecți din totalul subiecților (35,7%), iar expresia din analiza RT-PCR a fost la 569 bp. Am constatat că gena MAGE-A3 este cel mai mult exprimată în adenocarcinomul NSCLC, chiar dacă nu există o corelație statistică cu rezultatele histopatologice (P >0,05). Expresia MAGE-A3 din mostrele obținute prin biopsia tumorilor NSCLC a fost cea mai frecventă în tipul adenocarcinom 569 bp. Prin urmare, poate fi utilizată ca biomarker tumoral pentru diagnosticul precoce și screening-ul cancerului pulmonar.

Cuvinte-cheie

Biopsie cu forceps • cancer pulmonar • antigen 3 asociat melanomului • cancer pulmonar fără celule mici

*Corresponding author: Isnin Anang Marhana

E-mail: isnin.anang@fk.unair.ac.id

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Introduction

Lung cancer is the major cause of cancer-related deaths worldwide (1). Indonesia ranked fourth in the lung cancer mortality in 2018, with 30,023 new cases (8.6%) of cancers based on data from the Globocan information centre. Lung cancer is the most common cancer among men, excluding non-melanoma skin cancer (ranked by cases). Meanwhile, lung cancer deaths account for around 12.6% of all cancer deaths in Indonesia (2). In 2020, the American Cancer Society estimated that 228,820 new cases of lung cancer (116,300 in men and 112,520 in women) and 135,720 deaths from lung cancer (72,500 in men and 63,220 in women) in the United States (3). Lung cancer can be divided into two types: non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). NSCLC is a type of epithelial lung cancer including squamous cell carcinoma, large-cell carcinoma and adenocarcinoma. NSCLC is associated with cigarette smoking (passive or active) (1).

Lung cancer should be treated early when the tumour is small and localised. It is urgent to increase the cure rate and life expectancy. Currently, several therapeutic targets are widely used, but they have limited therapeutic efficacy for controlling cancer cells in the lung (4). Biomolecular developments provide an alternative for early detection in non-invasive cancer. One of them is the tumour antigen. Melanoma-associated antigen 3 (MAGE-A3) is a testicular cancer antigen and widely expressed in various types of tumour tissues (5). MAGE-A3 is expressed in about 35%–40% of NSCLC tumours. MAGE-A3 vaccines have been clinically tested for the prevention of postoperative recurrence of MAGE-A3 + NSCLC and other cancers (6). Moreover, there was limited information about using MAGE-A3 for screening of NSCLC.

Objectives

Currently, several molecular detection techniques are used to determine genetic changes. Polymerase chain reaction (PCR) is the most widely used technique. It can detect 103–104 copies of mutated genes and specific tumour antigens even in a very small sample. Compared to other molecular methods, PCR has several advantages, including being objective, fast, versatile and cost-effective when applied to small tissue samples (7,8). Moreover, the melanoma-associated antigen (MAGE) family can be detected using Reverse Transcription Polymerase Chain Reaction (RT-PCR) to detect tumour antigens (7,9). Previous research reported that MAGE expression was around 30%–50% in lung cancer (10–12). The MAGE family can be detected by RT-PCR, and it can be conducted with Messenger Ribonucleic Acid mRNA (100 ng) using superscript reverse transcriptase to produce Complementary Deoxyribonucleic Acid (cDNA) (13). Then, the cDNA integrity test using β-actin was performed (14). Therefore, the objective of this study is to examine the MAGE-A3 expression in forceps biopsy specimens as a tumour biomarker for early diagnosis and screening of lung cancer.

Materials and methods

Study design and sampling

This study was an observational, analytical or cross-sectional study using consecutive sampling methods. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethical Review Committee of the Dr. Soetomo Hospital, Surabaya, Indonesia (registered number 445/Panke.KKE/VII/2017), and informed consent was provided by all individual participants. The study was conducted in the Department of Pulmonology, Dr. Soetomo Hospital, Surabaya, for 6 months. Data are presented in tabular and graphical visualisation. The study population consisted of lung tumour patients who underwent a forceps biopsy with bronchoscopy based on the inclusion and exclusion criteria. The inclusion criteria were patients who were 25–70 years old, had at least one tumour or a tumour size of lesion >3 cm, were diagnosed with lung cancer based on a CT scan of the thorax, underwent a forceps biopsy with bronchoscopy guidelines for tissue specimen collection, had a performance score of >70%, received no previous systemic therapy for lung cancer and was willing to participate in the study (signed informed consent). The exclusion criteria were patients with primary cancer in other organs and those with lung cancer that is metastasised from other organ cancers.

Forceps biopsy procedure

Samples were obtained by forceps biopsy with bronchoscopy guidance. A forceps biopsy has a significantly higher diagnostic sensitivity (88.18%) to lung cancer than transbronchial needle aspiration in the endobronchial lesions (71.65%) ($P < 0.01$) (15). The sample size was determined based on Ronald Fisher's classic *z* transformation formula, requiring a minimum of 13 research samples. The success of molecular targeted therapies in NSCLC increases adequate specimens using forceps biopsy with bronchoscopy guidance for accurate tumour subtyping to minimise invasive procedures.

Giemsa staining

On a clean, dry microscopic glass slide, a thin film of the specimen (blood) was made and left to air dry. Then, the smear was fixed by dipping (2–3 times) the slide into pure methanol and left to air dry for 30 s. After that, the slide was

flooded with 5% Giemsa stain solution and kept for 20–30 min, and then washed with tap water and left to dry.

RNA extraction from tissue specimens

Materials used in this study were TRIzol reagents (Invitrogen, Grand Island, USA), RNase-free water (Invitrogen), Ribonucleic Acid RNA extraction kits (Qiagen, Hilden, Germany), hexamer random primers (Invitrogen) and Taq DNA polymerase (Invitrogen). Tissue specimens were collected in sterile PCR microcentrifuge tubes. Xylol was added to the tubes. Then, the samples were washed with ethanol solution. For RNA extraction, the tissue samples were processed using the FFN RNeasy extraction kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. The RNA samples were stored at 15–25°C for at least 9 months until further analysis. Tissue preparation, RNA extraction, RT-PCR test arrangements and post-RT-PCR product analysis were conducted in designated rooms and facilities, separately, to prevent cross-contamination (16).

Frozen RNA from specimens (up to 30 mg, depending on tissue type) in a stable tissue were broken down in the RLT buffer and homogenised. Ethanol solution was added to the lysate for creating conditions that is suitable for the selective binding of RNA to the RNeasy membrane column. The sample was applied to the Mini RNeasy spin column. Total RNA bound to the membrane, contaminants were washed away efficiently, and high-quality RNA was eluted in RNase-free water (17).

RT-PCR analysis

The RT-PCR tests were performed using MMRP 3 primer and MAGE-A3. Independent MAGE-A3 was amplified to detect MAGE-A family expression in the biopsy samples of lung tumour tissue. For RT reactions, the hexamer random was used for cDNA synthesis. The PCR product size for primary primers was 149 bp for MAGE-A3, while PCR products size for external primers was 161 bp for MAGE-A3. After PCR analysis, the sample was placed in 3% electrophoresis gel for visualisation using ultraviolet light.

Statistical analysis

Data were analysed using chi-square analysis and a contingency coefficient to determine the strength of the relationship test using SPSS statistics software (IBM SPSS Statistics, RRID: SCR_019096).

Results

Characteristics of research samples

The study sample was obtained from 14 patients with lung tumours, especially NSCLC-type adenocarcinoma and squamous cell carcinoma, confirmed by chest CT scans, who underwent forceps biopsy by fibre optic bronchoscopy (FOB) guidance for specimen collection. Table 1 presents sample characteristics. Most of the subjects were male (85.7%), with an average age of 57.81 ± 7.44 years, and 12 samples were smokers (85.7%) (Table 1).

Histopathology of forceps biopsy specimens in lung cancer patients

As shown in Table 2, there are six subjects (42.9%) with NSCLC adenocarcinoma and eight subjects (57.1%) with squamous cell carcinoma. The histopathology of the samples were examined using Giemsa staining (Figure 1). Figure 1A shows the histopathological type of adenocarcinoma, and Figure 1B shows the histopathological type of squamous cell carcinoma. All samples were obtained by forceps biopsy during the bronchoscopy procedure, and the Giemsa-stained samples were observed at 400x magnification under a microscope.

MAGE-A3 expression in forceps biopsy specimens of lung cancer patients

Furthermore, we obtained positive MAGE-A3 expression in five (35.7%) of the total research subjects (Table 2). Figure 2 shows melanoma antigen MAGE-A3 expressions on RT-PCR at 569 bp, with expression at sample codes L, N and O.

Relationship of MAGE-A3 expression and histopathology of forceps biopsy specimens of NSCLC patients

As shown in Table 2 shows four subjects (66.7%) with adenocarcinoma and a subject (12.5%) with squamous cell carcinoma have positive *MAGE-A3* gene expression. In present study, we found two subjects (33.7%) who had adenocarcinoma and seven subjects (87.5%) who had squamous cell carcinoma with negative MAGE-A3 expression. There was no significant relationship between MAGE-A3

Table 1. Characteristics of research samples

	N	%
Age (average ± standard deviation)	(59.46 ± 6.591)	
< 60 years	6	42.9
≥60 years	8	57.1
Gender		
Male	12	85.7
Female	2	14.3
Smoking status		
Smoker	12	85.7
Non-smoker	2	14.3
Histopathology		
Adenocarcinoma	6	42.9
Squamous cell carcinoma	8	57.1

Table 2. Relationship of MAGE-A3 expression with histopathology types

Histopathology type		Adenocarcinoma	Squamous cell carcinoma	Total	*P* value of Fischer's exact test
MAGE-A3	Positive	4 (66.7%)	1 (12.5%)	5 (35.7%)	0.063
	Negative	2 (33.3%)	7 (87.5%)	9 (64%)	
Total		6 (100%)	8 (100%)	14 (100%)	

MAGE-A3, melanoma-associated antigen 3.

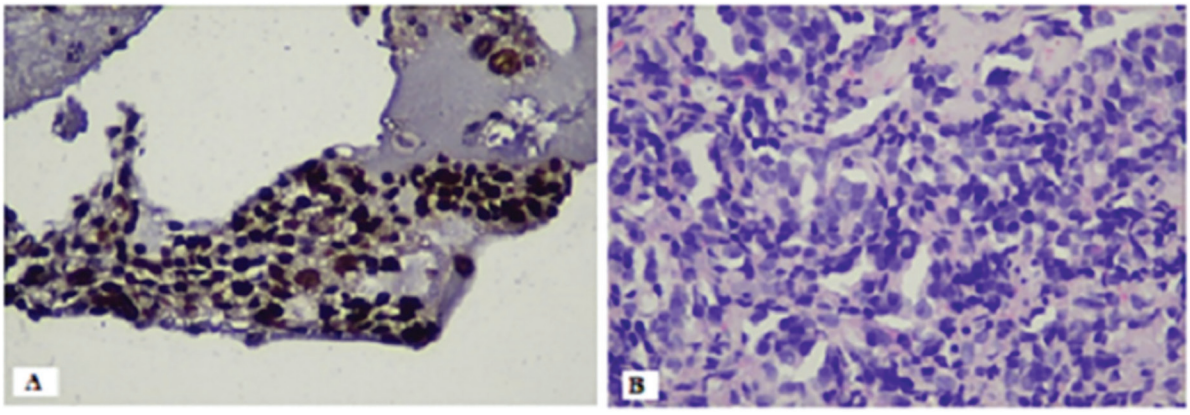

Figure 1. Histopathological imaging of adenocarcinoma **(A)** and squamous cell carcinoma **(B)** using Giemsa staining at a magnification of 400x.

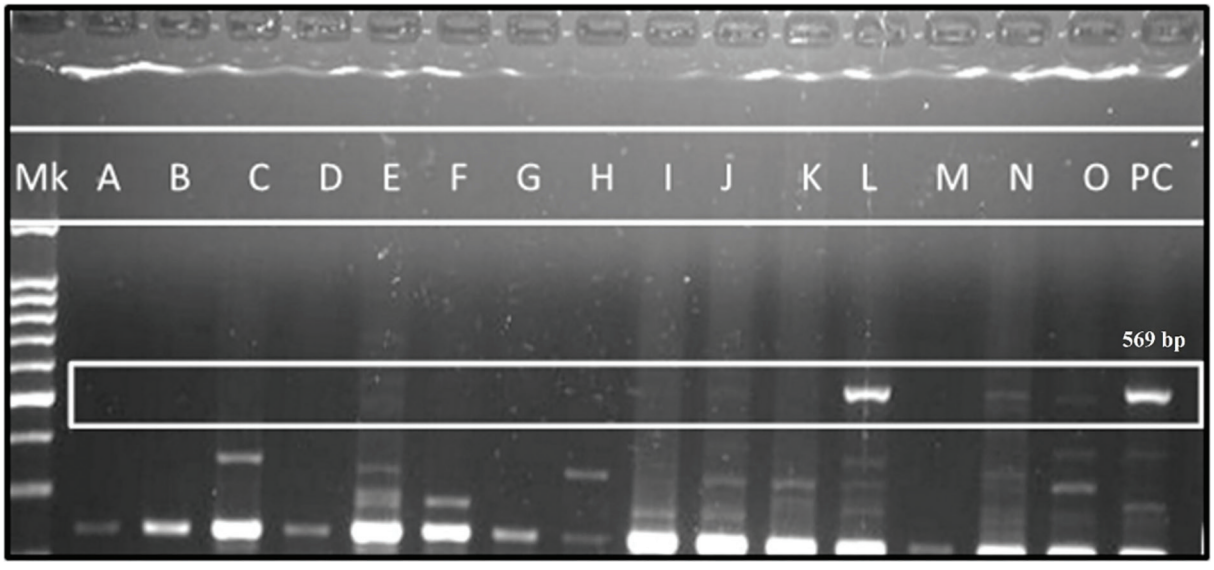

Figure 2. Expression analysis of MAGE-A3 in forceps biopsy specimens with an amplicon size of 569 bp by PCR. A–O, sample codes; MAGE-A3, melanoma-associated antigen 3; Mk, DNA ladder; PC, positive control; PCR, polymerase chain reaction.

expression and histopathological type ($P > 0.05$) based on Fischer's exact test, but there was a tendency toward positive *MAGE-A3* gene expression in NSCLC lung cancer patients with adenocarcinoma. However, in lung cancer patients with squamous cell carcinoma had negative MAGE-A3 expression results.

Table 3. Smoker status and MAGE-A3 expression

Histopathology type		Positive	Negative	Total	*P* value of Fischer's exact test
Smoking status	Yes	5 (41.7%)	7 (58.3%)	12 (100%)	0.396
	No	0 (0%)	2 (100%)	2 (100%)	
Total		5 (35.7%)	9 (64.3%)	14 (100%)	

Relationship between smoker status and MAGE-A3 expression

As shown in Table 3, we found that five subjects (41.7%) had positive MAGE-A3 expressions, while seven subjects (58.3%) had negative MAGE-A3 expressions. None of the non-smokers had positive MAGE-A3 expressions, while two subjects (100%) had negative MAGE-A3 expressions. Fisher's exact test results showed a significance value of 0.396 ($P > 0.05$), indicating that there was no significant relationship between the smoking status and MAGE-A3 expression. However, the data showed a higher rate of MAGE A3 expression in the smoker group.

Discussions and conclusions

In the present study, we demonstrated that *MAGE-A3* gene expression varied in different subgroups of tumour pathologies. The highest number of expressions occurred in adenocarcinoma and squamous cell carcinoma subgroups. In a study by Shin et al. (18), MAGE-A3 expression was identified in 21 patients (8%) with pulmonary adenocarcinoma tumours, showed one expression of the MAGE-A3 family. The number of adenocarcinoma and squamous cell carcinoma (NSCLC) lung cancer cases in this study was similar: 6 cases (42.9%) of adenocarcinoma and 8 cases (57.1%) of squamous cell carcinoma. Research conducted by Shin et al. (18) found that the number of cases is similar in each type of NSCLC histopathology.

In the present study, there were 12 (85.7%) male patients and 2 (14.3%) female patients. Our study research was in accordance with previous studies. Lung cancer is commonly found among men due to their prolonged smoking habits compared to women (19). Kim et al. (9) reported that MAGE-A3 expression in positive lung cancer is significantly higher in men than in women. The risk of lung cancer is higher in individuals older than 60 years (20). A similar finding was reported by a previous study. In our study, there were eight people older than 60 years, with an average age of 59.46 ± 6.591 years.

The incidence of lung cancer is closely related to the smoking history. Thus, the risk of lung cancer is related to the number of cigarettes smoked, the age when the patient started smoking and the smoking duration. The risk of lung cancer in active smokers is 20 times higher than that in non-smokers (1,18). Among the study subjects, 75% had a long history of smoking. In Jang et al.'s (13) study, of the 20 specimens analysed, 7 (35%), 10 (50%) and 12 (60%) specimens showed MAGE-A1, -A3, and -B2 expressions, respectively. The chronic inflammatory state in premalignant lesions was found to be induced by smoking. It triggers the development of tumour-specific immune responses. Smoking contains multiple toxic compounds that directly activate oncogenes and cause genomic changes in epigenetic including expression of tumour antigen MAGE A3.

Forceps biopsy is a procedure of examining solid samples of tissue removed using a forceps device with bronchoscopy guidance. Operators can use the forceps tool directly in the lesion area, which can be visualised by FOB (21). The forceps biopsy procedure can be conducted with FOB guidance to diagnose lung tumours and performed on central lung masses. It is considered a safe procedure with a high diagnostic success rate. This procedure can be performed on lung tumours with central lesions. In this study, there were no research subjects who had complications of pneumothorax, pulmonary haemorrhage, haemoptysis and subcutaneous haematoma.

The sensitivity of forceps biopsy is approximately 88.18%. The most common complications of forceps biopsy are pneumothorax and pulmonary haemorrhage; haemoptysis is also reported in several studies (22). Casoni et al. (23) stated that pneumothorax complications due to forceps biopsy are relatively low (8%). Furthermore, FOB can be used to determine any post-procedural complications. If the tumour lesion is visible and there is no change in the location, shape or size post-procedure, it indicates no pneumothorax (24). In line with this study, samples obtained were quite small because it was difficult to obtain adequate sample by bronchoscopy. Other studies also faced the same challenge. The study by Kim et al. (25) involved 28 cancer patients and the study by Leaker et al. (26) was carried out in eight smokers, and both studies provide reliable and insightful ways to improve the diagnosis of lung cancer.

In this study, of the 14 subjects with NSCLC lung cancer who underwent forceps biopsy, positive MAGE-A3 expression was found in 5 (35.7%) subjects. The histopathological analysis of forceps biopsy specimens showed a relatively high diagnostic success rate (>90%). Our study results were in line with

previous studies by Mezzone et al. (24), who showed that the average diagnostic success rate of endobronchial biopsy was 80%, with a range from 51% to 97%.

MAGE is a proto-oncogene belonging to the family of cancer testicular antigen (CTA). The biological function is not widely understood. MAGE plays an important a role in oncogenesis and apoptosis inhibition by blocking the caspase cycle. It acts as a signal to induce an apoptotic process in a cell from the intracellular or extracellular pathways (24). The *MAGE* gene is widely expressed in lung tissues of patients with NSCLC. The expression is a consequence of the presence of a tumour or indication of pulmonary tumorigenesis.The non-smoker participant without evidence of lung cancer was enrolled in a clinical chemoprevention trial. Among the 14 specimens analysed, positive MAGE-A3 expression was found in 5 subjects (41.7%).

We further verify that the *MAGE* gene expression originates from pulmonary epithelial cells. An in situ mRNA hybridisation process with a specific RNA probe for each MAGE mRNA species was carried out. Previous studies from Asia, Europe and America reported that *MAGE* gene expression could be found only in 30–50% of lung tumour tissue (23). However, the expression of MAGE-A3 on NSCLC varied. Karimi et al. (27) reported differences in the *MAGE* gene expression of NSCLC, with 70% of samples expressing MAGE-A1 and 85% of samples expressing MAGE-A3.

In two previous studies, there were some differences in the incidence of MAGE expression in lung cancer. Weynants et al. (28) reported that the expression of MAGE-A1 and A3 expression was observed in 35% and 33%, respectively, while Shichijo et al. (29) reported that the observed expression of MAGE-A1 and A3 was 11% and 38%, respectively. There was no difference between the results of the study conducted by Weynant et al. (28) and those of the present study. The reason is still unknown, but the fact that the MAGE family gene has a great similarity in sequence (MAGE-3 and MAGE-6 were 98% similar in the nucleotide composition) may be contributed to results of the present study. These data indicate that tumour cells express MAGE-3 and HLA-A2 in vivo by specific MAGE-3 CTL. However, these findings suggest that specific immunotherapy, directed at MAGE-3 antigens, may apply to about 10% of Japanese patients with lung cancer.

A positive MAGE-A3 expression was found in five subjects (38.5%). Previous studies were conducted in 34 countries (Europe, America and Asia Pacific): of 12,820 valid samples, only 4210 (33%) showed positive MAGE-A3 expression (28). Some previous studies showed that MAGE-A3 expression was the highest. Therefore, it was used as a vaccination target gene (30). MAGE-A3 can be used as a diagnostic and prognostic biomarker in NSCLC patients. Moreover, it is associated with worsening clinical symptoms and tumour progression (31).

In a present study, 14 patients were diagnosed with NSCLC: six subjects had adenocarcinoma (42.9%) and eight subjects (57.1%) had lung cancer with squamous cell carcinoma. The *MAGE* gene expression rate was 35.7% in tumour samples from adenocarcinoma and squamous cell carcinoma. There was no significant relationship between the histopathology types (adenocarcinoma and squamous cell carcinoma) and MAGE-A3 expression ($P > 0.05$). It is because only 5 (33.3%) of the 14 subjects were diagnosed with NSCLC and showed positive MAGE-A3 expression. Tsai et al. (20) found the *MAGE* gene expression rate in NSCLC was 88%, but the MAGE-A3 expression rate was only 13%.

These findings were different from those the study by Yanagawa et al. (32), who examined the relationship between MAGE-A1 and MAGE-A3 with histopathological types in NSCLC and reported positive MAGE-A3 expression in 26 (38.8%) of 67 NSCLC samples. There was a relationship between MAGE-A3 expression and histopathological type ($P = 0.003$), and positive MAGE-A3 expression was higher in squamous cell carcinoma (20,32). In our study, we only examined the MAGE-A3 expression.

Study from East and Southeast Asia (South Korea, Taiwan, Singapore and Thailand) showed adenocarcinoma as dominant among 377 sample of NSCLC patients. We found that a higher MAGE-A3 expression was found in squamous cell carcinoma than in adenocarcinoma, suggesting a possible relationship between MAGE-A3 expression and tumour histopathology ($P = 0.05$). Differences in the characteristics of NSCLC patients in East and West Asia led to differences in the percentage of MAGE-A3 expression (33). The limitations of specific anti-MAGE antibodies to distinguish different MAGE proteins cause previous researchers used many microanalysis techniques, RT hybridisation-PCR and RNA in-situ to recognise *MAGE* gene expression.

In cancer, the distribution of the cytoplasm and nucleus varies. It suggests that differences in members of the MAGE-A family induce differences in localisation or control mechanisms in certain tumour cells. Localisation can be a factor in explaining the MAGE expression, which depends on the localisation of the MAGE target or relevant interacting protein (33,34).

The MAGE-A3 expression in forceps biopsy specimens of NSCLC was mostly identified in the adenocarcinoma type at 569 bp. Therefore, it could be a tumour biomarker for the early diagnosis and screening of lung cancer.

Limitation

There are several limitations to this study. It was performed in a single institution in a homogeneous Indonesian ethnic group, and we used only one marker of MAGE A3. Increasing

the sample size and including different populations and using several MAGE genes expression from different MAGE genes family would improve the research quality.

Recommendation

Further research is needed on MAGE expression in non–small-cell lung cancer including several types of MAGE (A1–A6). A wider scope of research would provide more accurate information about the correlation of MAGE expression in patients with lung cancer.

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Conflict of interest

The authors declare that there is no conflict of interest.

Ethical approval and informed consent

The study was approved by Ethical Review Committee of the Dr. Soetomo Hospital, Surabaya, Indonesia (registered number 445/Panke.KKE/VII/2017), and informed consent was provided by all individual participants.

Data availability

The data supporting this research are available from the authors on reasonable request.

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