Original Study

Characterization of Melanoma-Associated Antigen-A Genes Family Differential Expression in Non-Small-Cell Lung Cancers

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Abstract

Lung cancers are among the most formidable cancers due to a lack of screening biomarkers and definite treatment. Our study has verified the melanoma-associated antigen-A gene expression in both tumoral and nontumoral tissue of non-small-cell lung cancer. Thus, this research would be useful for early melanoma-associated antigen utilization, especially for lung cancer prevention in the clinical field.

Background: The melanoma-associated antigen (MAGE) genes families are found in different cancers, including non-small-cell lung cancers. These genes are silent in normal tissues, except for the testis. The goal of this study was to investigate the differentially expressed profile of the different MAGE genes subclass in non-small-cell lung cancer (NSCLC) tumoral tissue. **Methods:** Formalin-fixed paraffin embedded NSCLC resected tissues were collected from 31 patients hospitalized in our referral hospital, and 29 patients were diagnosed with either squamous cell carcinoma (SCC) or adenocarcinoma (ADC). We used a nested reverse transcriptase–polymerase chain reaction, which comprised independent amplification of MAGE-A1, MAGE-A2, MAGE-A3/6, MAGE-A4, and MAGE-A12, to detect expression frequency of the MAGE-A family in lung tissue biopsies at both tumoral and nontumoral parts of patients' tissues. **Results:** From 29 patients with diagnosis of either SCC (n = 16) or ADCs (n = 13), 58 samples were prepared. From 58 blocks sampled for this experiment, 37 tumoral tissue samples and 22 nontumoral tissue samples expressed at least one of the MAGE-A genes. MAGE-A4 gene had the highest incidence among all MAGE-A genes in both tumoral and nontumoral gens. In SCC and ADCs, the data showed expression of at least one of the MAGE-A genes in 59.4% and 69.2% of tumoral and nontumoral tissues, respectively. **Conclusion:** The detection of the MAGE-A genes expression could be a molecular tumor marker for early diagnosis and potential targets for active immunotherapy in NSCLC, particularly in ADCs and SCC. Besides, the frequency of different subtypes of MAGE genes may vary in different regions of world.

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Introduction

Lung cancers are one of the most common and formidable cancers,[1] with high mortality worldwide[2] and in Iran,[3] whereby non-small-cell lung carcinomas (NSCLC) constitute 80% of all lung tumors.[4] Due to the high mortality rate and poor prognosis of lung cancers, many potential screening biomarkers have been tested, but none has been validated for reliable early detection of lung cancers. Melanoma-associated antigen (MAGE) is a proto-oncogen that belongs to cancer/testis antigens family, with poorly understood biologic function. The role of MAGEs in oncogenesis and inhibition of apoptosis through blocking the caspase cycle has been defined.[5] MAGE proteins are mainly classified into 2 types based on differ-

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ences in tissue-specific gene expression and gene structure.[6] Type 1 MAGE (MAGE-A, MAGE-B, and MAGE-C) expression is restricted to germ-line tissues, such as spermatocytes,[6] placenta, and certain stages of the embryonal development[7]; type 2 MAGE (MAGE-D) genes are almost universally expressed in body tissues.[8] However, type 1 MAGE expression has been detected in a broad variety of malignancies,[9,10] especially lung cancers.[11] Scientists have speculated that MAGE gene family expression could be a sensitive marker for the early diagnosis of many different types of cancers, including lung cancer.[10,12,13] Recently, researchers have suggested that overexpression of the MAGE gene family in lung cancers could be not only the target genes of early diagnosis and screening of lung cancer but also become target genes for lung cancer adjuvant immunotherapy.[14,15]

The MAGE-A genes family is mainly expressed in NSCLCs[16]; MAGE-A3 is known to be the most prevalent one expressed in 30%-60% of NSCLC.[17] Interestingly, MAGE genes are both expressed in tumoral and non-tumoral tissues of patients with lung cancer.[18] In this study, we attempted to illustrate differential expression rates and proportions of MAGE genes in both tumoral and nontumoral samples of lung tissue of patients with proven NSCLC. The purpose of this methodology was to embark on the reliability of the MAGE genes family detection by nested reverse transcriptase–polymerase chain reaction (RT-PCR) in nontumoral tissues in NSCLCs and compare that with tumoral tissue of the same patient.

Patients and Materials

Patient Selection and Tumor Sample Collection

All patients enrolled in this study gave informed consent according to the university hospital ethical board committee in the department of pathology at the Masih Daneshvari Referral Hospital. Retrospectively, paraffinized blocks of lobectomy or pneumonectomy samples from 31 patients with resected NSCLCs were selected after reviewing hematoxylin and eosin and immunohistochemistry stained slides to confirm that the tumoral and nontumoral paraffinized blocks were separated in the same patient. According to routine standard procedure of macroscopic examination of lung cancers, we had to cut sections from non-neoplastic lung tissue far from the tumor. The patients with simultaneous testicular cancer were excluded from the study. The presence or absence of tumoral tissue in formalin-fixed and paraffin-embedded tissue samples was confirmed by S. Karimi and F. Mohammadi, 2 expert pathologists. The samples were selected from both tumoral and nontumoral samples of each patient's lung tissue and were sent to PCR testing. Records of demographic data, including age and sex, were collected from patient records at the department of pathology and surgery.

RNA Extraction From Paraffinized Tissue Specimen

Materials used in this study included the following: Trizol reagent (Invitrogen, Grand Island, NY), RNase-free water (Invitrogen), RNA extraction kit (Qiagen, Hilden, Germany), randome hexamer (Invitrogen), Taq DNA polymerase (Invitrogen). Formalin-fixed paraffin-embedded blocks of tissue specimen were scratched by using a sterile clean surgical blade to minimize RNA contamination. The tissues were cut into 5-μm slices; dissection was performed by using a microtome. The samples were collected in sterile PCR microcentrifuge tubes. For deparaffinization of samples, xylole was used in the tubes, and then the samples were washed with ethanol. For RNA extraction, the tissue samples were processed by using the RNeasy FFPE extraction kit according to the manufacturer's instructions. The RNA samples were stored at -80°C until further processed for experimental assays. Tissue processing, RNA extraction, RT-PCR assay setup, and post–RT-PCR product analysis were performed in separate designated rooms and facilities to prevent cross contamination.

RT-PCR Analysis

We used a nested RT-PCR, which comprised independent amplification of *MAGE-1*, *MAGE-2*, *MAGE-3/6*, *MAGE-4*, and *MAGE-12*, to detect expression frequency of the *MAGE-A* family in tumoral and nontumoral lung tissue biopsy samples in these cases. For RT reaction, randome hexamer was used for complementary DNA synthesis. For nested PCR to detect the MAGE-A genes family, we used the method previously described,[19] with minor modifications. The PCR products for inner primers were 143, 119,149, 118, and 181, for MAGE-1, MAGE-2, MAGE-3/6, MAGE-4, and MAGE-12, respectively. The PCR products for outer primers were 149, 215, 161, 185, and 343 for MAGE-1, MAGE-2, MAGE-3/6, MAGE-4, and MAGE-12, respectively. After PCR analysis, the samples were loaded onto 3% gel electrophoresis to be illuminated with UV light. It is important to note that, because we performed RT-PCR on paraffin-embedded blocks, we could not recognize which cells expressed MAGE family proteins based on this study. But, based on the aforementioned literature, MAGE family expression is detected almost exclusively in testis, placenta, and embryonal tissue as well as tumoral epithelial cells in a variety of cancers. If we want to recognize the exact localization of MAGE positivity in tissue, we should use monoclonal MAGE antibody with immunohistochemistry.

Statistical Analysis

Each value represented the mean (SD). One-way repeated measures analysis of variance, followed by the Tukey post hoc test multiple group comparison, was used to analyze group differences of the resultant data. The correlation ratio was measured by the Spearman correlation coefficient for nonparametric variables. The threshold for statistical significance was considered $P < .05$ in this study (Table 1).

Results

Demographic and Clinical Data

Thirty-one patients were selected, and 2 samples, one from tumoral tissue and one from nontumoral tissue around the tumoral tissue, were analyzed for expression of MAGE-A genes. The patients were 22 men (71%) and 9 women (29%); age range, 25-81 years and mean (SD) of 61.4 $\pm$ 12.7 years. The pathologic diagnosis were squamous cell carcinoma (SCC) in 16 cases (52%), adenocarcinoma (ADC) in 13 cases (41.9%), adenosquamous in 1 case (3.2%), and large-cell undifferentiated carcinoma in 1 case (3.2%). Demographic data of patients are listed in Table 2. All the patients with SCC were men, but ADCs samples were from 5 male and 8 female patients. In all experimental assays, control negative selected from biopsy sample of patient with negative tumoral result. All of the specimens were resected, and the malignant epithelial tumoral tissue showed glandu-

Table 1 Characteristics of the Melanoma-Associated Antigen-A (MAGE) Genes Family and GAPDH Oligonucleotide Probes

MAGE-1	
Outer sense	GTA GAG TTC GGC CGA AGG AAC
Outer antisense	CAG GAG CTG GGC AAT GAA GAC
Inner sense	TAG AGT TCG GCC GAA GGA AC
Inner antisense	CTG GGC AAT GAA GAC CCA CA
MAGE-2	
Outer sense	CAT TGA AGG AGA AGA TCT GCC T
Outer antisense	GAG TAG AAG AGG AAG AAG CGG T
Inner sense	CAT TGA AGG AGA AGA TCT GCC T
Inner antisense	CAG GCT TGC AGT GCT GAC TC
MAGE-3/6	
Outer sense	GAA GCC GGC CCA GGC TCG
Outer antisense	GAT GAC TCT GGT CAG GGC AA
Inner sense	GGC TCG GTG AGG AGG CAA G
Inner antisense	GAT GAC TCT GGT CAG GGC AA
MAGE-4	
Outer sense	CAC CAA GGA GAA GAT CTG CCT
Outer antisense	TCC TCA GTA GTA GGA GCC TGT
Inner sense	CAC CAA GGA GAA GAT CTG CCT
Inner antisense	CAG GCT TGC AGT GCT GAC TCT
MAGE-12	
Outer sense	TCC GTG AGG AGG CAA GGT TC
Outer antisense	ATC GGA TTG ACT CCA GAG AGT A
Inner sense	TCC GTG AGG AGG CAA GGT TC
Inner antisense	GAG CCT GCG CAC CCA CCA A
GAPDH	
Reverse	GTCCACCACCCTGTTGCTGTAG
Forward	CAAGGTCATCCATGACAACTTTG

lar differentiation or mucin production in the cases of ADC and keratin formation or intercellular bridges in the cases of SCC; there was no need to perform immuno histochemistry for making these diagnoses.

Expression Rate of MAGE Family in Different NSCLC Pathologies

Twenty-nine patients were diagnosed with either SCC or ADCs. From 29 patients with a diagnosis of either SCC (n = 16) or ADCs (n = 13), 58 samples were prepared, and, from those 58 blocks sampled for this experiment, 37 of tumoral tissue samples and 22 of nontumoral tissue samples expressed at least one of MAGE-A genes (Table 3). MAGE-A genes expression rate in ADCs and SCC was significantly higher than other type of NSCLCs ($P = .03$). Among positive cases, 55% showed single expression and 45% showed multimarker expression of the *MAGE-A* family. In nontumoral samples from ADCs and SCC, the expression rate of MAGE genes expression was 38.5% and 31.2%, respectively, and, in tumoral samples, the rates were 53.8% and 37.5%, respectively. There was no significant difference between expression of different *MAGE-A* genes between ADCs and SCC in tumoral and nontumoral tissues ($P = .77$).

Differential Expression of MAGE Genes and Clinical Characteristics

The study samples showed frequent and heterogeneous *MAGE* expression, and this heterogenicity in expression of the MAGE genes family is more prominent in subgroups of tumoral lesions. From all the MAGE-A family analyzed in this study, MAGE-A4 expression was significantly higher among all genes and in both tumoral tissue samples (41.9%) ($P = .02$) and nontumoral samples (32.3%) ($P = .043$) (Table 4). Interestingly, among the most common type of NSCLC in this study, ADCs and SCC, MAGE-A4 expression was significantly higher than other MAGE-A genes family (46.2% and 34.4%, respectively) ($P = .038$ and .05, respectively) (Figure 1A, B). The lowest expression among all in both tumoral and nontumoral samples belonged to MAGE-A2. MAGE-A3/6 frequency of expression was 30.8% and 31.2% in tumoral samples and 7.7% and 31.2% in nontumoral samples of ADCs and SCC, respectively (Figure 1).

We also evaluated the coexpression of the MAGE-A genes family in both tumoral and nontumoral samples of the same patients at the same time (Figure 1C). In 41.9% of cases, at least one MAGE gene in tumoral and nontumoral tissues was coexpressed. Also, coexpression of the MAGE-A genes family was significantly higher in ADCs than all other pathologies ($P = .023$). The coexpression of MAGE-A4 (19.4%) in total samples was significantly ($P = .045$) than other MAGE-A gene, which was the higher coexpressed in both ADCs and SCC (Figure 1).

Number of MAGE Genes Expression and Clinical Characteristics

We next analyzed the number of MAGE genes expression in tumoral and nontumoral tissue samples. The interesting results showed variations of the number of expression (1 to 3) categorized based on tumoral and nontumoral classifications in ADCs and SCC samples (Figure 2).

In 38.7% of tumoral and 32.3% of nontumoral samples of lung tissue in a total of 62 samples from patients with NSCLC, one of the MAGE genes was expressed. In 29% of tumoral and 16.1% of nontumoral lung tissue samples from patients, 2 MAGE genes were expressed. Coexpression of MAGE-A4 was significantly higher in both ADCs and SCC compared with other MAGE-A genes ($P = .02$).

In tumoral blocks, when MAGE-A1 was expressed, pathologic diagnosis in 37.5% of cases was ADCs; in 50% of cases, it was SCC; and in nontumoral blocks, 60% of cases were ADCs and 40% were SCC. In nontumoral cases, MAGE-A4 genes were expressed in 53.8% of ADCs and 46.2% of SCC pathologies, and, in tumoral samples, MAGE-A4 expression was expressed in 50% of both pathologies. It is important to note that, in our results, there was no significant difference in MAGE-A4 expression in both pathologies ($P = .12$).

Table 2 Demographic Characteristics (Age and Sex) in Different Pathologic Diagnosis in Patients With Lung Cancer in Our Study (n = 31)

	ADCs (n = 13)	AdenoSCC (n = 1)	L-C-Und-CA (n = 1)	SCC (n = 16)
Age, Year, Mean (SD)	56.2 ± 13.3	78 ± 17.2	58 ± 11.8	64.8 ± 11.2
Sex, No. (%)				
Women	8 (61.5)	1 (100)	0 (0)	–
Men	5 (38.5)	0 (0)	1 (100)	16 (100)

Abbreviations: ADC = adenocarcinoma; AdenoSCC = adeno-squamous cell carcinoma; LC-Und-CA = large-cell undifferentiated carcinoma; SCC = squamous cell carcinoma.

Table 3 Expression of at Least 1 Melanoma-Associated Antigen-A (MAGE) gene Classified According to Sex Age and No. Patients (%)

At Least 1 MAGE Expression	Total (n = 62)	Tumor (n = 31)	Nontumor (n = 31)
Nonstratified	40 (64.5%)	24 (77.4%)	16 (51.6%)
Sex			
Men	28 (63.6%)	17 (77.3%)	11 (50%)
Women	12 (66.7%)	7 (77.8%)	5 (55.6%)
Age			
≤40 y	2 (40%)	1 (50%)	1 (33.3%)
41-50 y	4 (80%)	3 (100%)	1 (50%)
51-60 y	9 (56.2%)	5 (62.5%)	4 (50%)
61-70 y	15 (75%)	8 (80%)	7 (70%)
71-80 y	8 (66.7%)	5 (83.3%)	3 (50%)
≥81 y	2 (50%)	2 (100%)	0 (0%)

Table 4 Differential of MAGE Genes Expression in Tumoral and Nontumoral Lung Tissue Samples in ADC (n = 29) and SCC (n = 29)

	Total Samples		Tumor		Nontumor	
	ADCs	SCC	ADCs	SCC	ADCs	SCC
MAGE-1	6	6	3	4	3	2
MAGE-2	1	0	1	0	0	0
MAGE-3/6	5	9	4	5	1	5
MAGE-4	12	11	7	6	5	5
MAGE-12	5	3	4	3	1	0

Abbreviations: ADC = adenocarcinoma; MAGE = melanoma-associated antigen-A; SCC = squamous cell carcinoma.

Discussion

MAGE genes family expression in lung cancer could be a suitable target for both early detection and immunotherapy.[20] Here, in this study, we used a valid method of nested RT-PCR to detect MAGE-A genes differential expression in patients with lung cancer; we also attempted to understand the most prevalent type of MAGE-A genes family in NSCLC. Our results showed that MAGE-A genes were expressed in both tumoral and normal-looking tissues around tumoral tissues in patients with lung cancer. Although there was slight variation in expression distribution of MAGE genes in tumoral and nontumoral tissues, overall, the expression patterns in tumoral and nontumoral tissues were almost close to each other. Other studies, showed the same results, that MAGE-A2, MAGE-A3, MAGE-B3 genes were detected by RT-PCR and in situ hybridization, not only in NSCLC tissues but also in neighboring healthy tissues around a lesion, and also in bronchial cells of patients with risk factors of lung cancer.[9] In that study in NSCLC tumoral tissue samples, 70% of samples expressed MAGE-A1 and 85% expressed MAGE-A3, and neighboring nontumoral part revealed expression of MAGE-A1 and MAGE-A3 in 65% and 75% of samples.

However, MAGE-A gene expressions showed subgroup variation in their expression in different tumoral pathologies in our study, and

Figure 1 Proportion of Differential Expression of Melanoma-Associated Antigen-A Genes (MAGE) Genes in Non-Small-Cell Lung Cancer (NSCLC) Tumoral (A) and Nontumoral (B) Samples, Categorized Based on the Pathology of Tumoral Sections. MAGE 4 Had the Highest Expression in Both Adenocarcinoma (AdenoCA) and Squamous Cell Carcinoma (SCC) Tumoral and Nontumoral Samples. Coexpression of the MAGE-A Genes Family in Both Tumoral and Nontumoral Tissues Simultaneously in AdenoCA and SCC Samples (C)

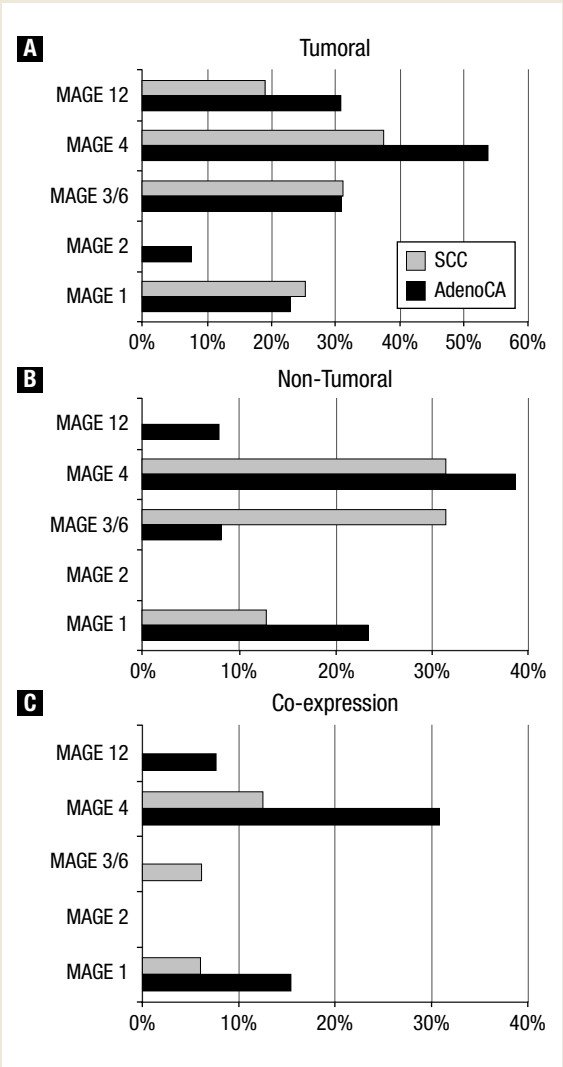
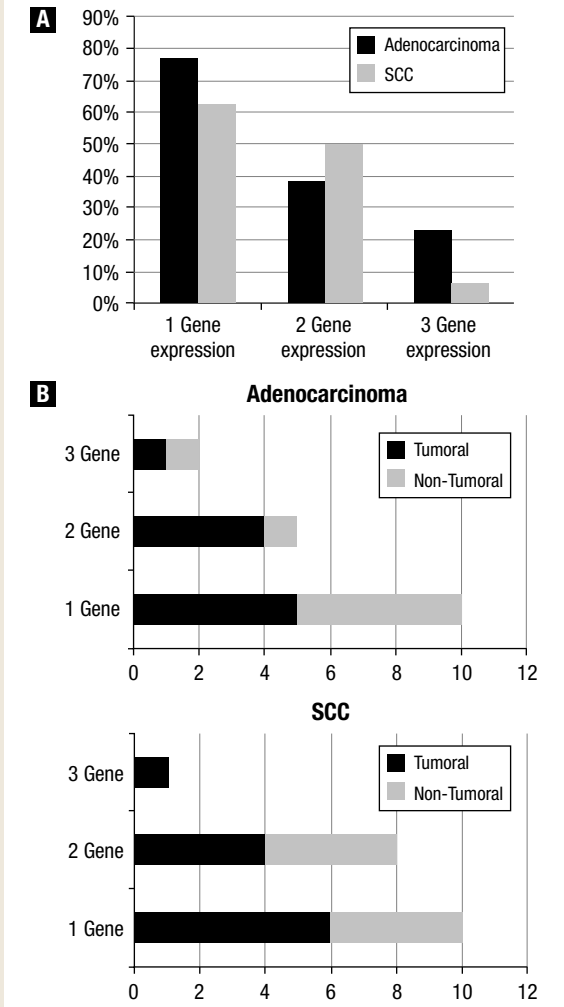


Figure 2 Number of the Melanoma-Associated Antigen-A (MAGE) Genes Family Expression in Adenocarcinoma (AdenoCA) and Squamous Cell Carcinoma (SCC) (A). In the Right Panel: (B) the Number of MAGE Gene Expression in AdenoCA and SCC Has Been Stratified Based on Expression in Tumoral or Nontumoral Tissue Sample



the highest expression number was described in ADCs and SCC. In the study by Lee et al,[21] 80% of lung ADCs tumors showed one of the MAGE-A family expressions; in our study, this number was 84.6%; also, MAGE-A genes expression in SCC was 68.8% in the study by Lee et al[21] and 89.6% in our study. The close relation of our results and study by Lee et al[21] indicates the very high chance of MAGE-A gene family association with NSCLC, particularly ADCs and SCC. In this study, 51.6% of a nontumoral sample of lung tissues in patients with NSCLC expressed at least one of the MAGE-A genes family. In Lee et al,[21] in the case of negative cytology of lavage, 66.7% of cases were positive for MAGE-A or SSX4 gene expression.[21] The 15% difference between our results and study by Lee et al[21] might be the result of an increase in sensitivity when using both MAGE and SSX4 probes in their study. The number of ADC cases was almost the same in both studies, but the number of SCC cases was higher in the study by Lee et al.[21] Although we could not definitively answer the relationship between subtype and predominant pattern, in future studies, with a larger sample size, we could subtype ADC cases and look for any relationship between subtype, predominant pattern, and MAGE family expression.

MAGE-A4 showed the highest expression in NSCLC in our study, in contrast to other studies, in which MAGE-A1 or MAGE-A3 were found to have the highest expression.[22,23] With regard to this observation, one could speculate that ethnicity also is an important factor in differential expression of MAGE-A genes. When considering that these genes have already been potential candidates for clinical trials immunotherapy in lung cancers, we suggest future studies in different geographical areas and ethnicities to clarify the distribution pattern of MAGE-A among different regions of the world so to achieve higher effectiveness in future immunotherapy.

Altogether, finding a potential preneoplastic gene family is an attractive research topic in clinical lung cancer field. In the future, one of the MAGE gene families might play a key role in screening and immunotherapy. It is hoped that our results in proving the feasibility and accessibility of nested RT-PCR in the detection of MAGE genes in NSCLC, particularly ADCs and SCC, are satisfactory and will help researchers to expand this model to find easier sampling ways, such as sputum analysis or bronchial alveolar fluid. However, the MAGE expression rate would be low due to RNA extraction from the paraffin block and RNA would be degraded during the waiting time before preparation of paraffin block. Also, targeting these genes for immunotherapy may be another prospective advantage of these genes in lung cancer, because MAGE genes attract CD8 T cells for induction of their antitumor function. Other clinical trials have already had some positive results with MAGE-A–derived immunogens, especially MAGE-A3,[24,25] which acts through recruiting dendritic cells to the cancer tissue.[26] Attempts at targeting these genes for immunotherapy will open the new era of science and treatments to the researchers and clinicians to the lung cancer therapies, especially in NSCLC.

This study showed that MAGE-A gene family detection by nested RT-PCR could be potential targets for a molecular tumor marker for the early diagnosis of NSCLC and active immunotherapy, and its detection methods are not limited to protein-dependent methods and sampling difficulties.

Clinical Practice Points

- MAGE-A genes family expression had been studied by many groups; here we present an interesting study on MAGE-A gene expression in NSCLC, particularly in ADCs and SCC.
- MAGE genes family expression in lung cancer could be a suitable target for both early detection and immunotherapy.
- MAGE-A gene expression showed subgroup variations in their expression in different tumoral pathologies in our study, and the highest expression number was described in ADCs and SCC.
- In our study, the MAGE-A4 gene had the highest incidence among all MAGE-A genes in both tumoral and nontumoral genes, in contrast to other studies in which they found MAGE-A1 or MAGE-A3 to have the highest expression.
- In SCC and ADCs, analysis of the data showed expression of at least one MAGE-A gene in 59.4% and 69.2% of both tumoral and nontumoral tissues, respectively.
- This research would be useful for early MAGE utilization, especially for lung cancer prevention in the clinical field.

Disclosure

The authors of this article declare no conflict of interest, no financial, consulting, and personal relationships with any other people or organizations that could influence (bias) the authors' work.

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