Gene expression of MAGE-A3 and PRAME tumor antigens and EGFR mutational status in Taiwanese non–small cell lung cancer patients

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Abstract

Aim: To determine the frequency of expression of the tumor-associated antigens (TAAs) melanoma-associated antigen A3 (MAGE-A3) and preferentially expressed antigen of melanoma (PRAME) and the rate of EGFR mutations in a Taiwanese non–small cell lung cancer (NSCLC) population including only adenocarcinomas and squamous cell carcinomas. Furthermore, to investigate associations between TAA expression and EGFR mutations and to evaluate these TAAs as prognostic markers for overall survival. The occurrence of single nucleotide polymorphisms in MAGEA3 and PRAME was also assessed.

Methods: Archival fresh-frozen tumor tissue specimens were tested by quantitative reverse transcription polymerase chain reaction assays to detect MAGE-A3 and PRAME expression. EGFR mutations were detected by mass spectroscopy and single nucleotide polymorphisms by gene sequencing.

Results: Of the 156 adenocarcinomas examined, 3.3% expressed MAGE-A3, 32.2% expressed PRAME and 62.8% had EGFR mutations. Of the 128 squamous cell carcinomas, 29.8% expressed MAGE-A3, 59.2% expressed PRAME and 20.5% harbored EGFR mutations. TAA expression was similar across subgroups determined by patient or tumor characteristics. There was no association between TAA expression and EGFR mutation status and TAA expression was found not to be a prognostic marker for survival. Single nucleotide polymorphisms were identified, one of which with a possible impact on MAGE-A3 expression.

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Conflicts of interest: Nicolas Delahaye, Olivier Gruselle and Jamila Louahed are employees of the GSK group of companies. Nicole Kusuma, Bart Spiessens and Aung Myo were employees of the GSK group of companies at the time of the study and manuscript development. Jamila Louahed and Bart Spiessens own stock/stock options in GSK group of companies. Szu-Hua Pan, Kang-Yi Su Gee-Cheng Chang, Sung-Liang Yu and Pan-Chyr Yang declare that they have no conflict of interest.

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**Conclusions:** In this NSCLC population, expression of MAGE-A3 and PRAME was more frequent in squamous cell carcinomas than in adenocarcinomas tumors. EGFR mutations were not associated with TAA expression for either histology and were three times more frequent in adenocarcinomas than in squamous cell carcinomas tumors.

**Key words:** adenocarcinoma, EGFR mutation, MAGE-A3 antigen, non–small cell lung cancer, PRAME antigen, squamous cell carcinoma

**INTRODUCTION**

Over the last several decades, the major therapeutic advance in non–small cell lung cancer (NSCLC), which accounts for more than 80% of all lung cancers, has been the development of pharmaceutical agents that affect known molecular targets in the tumor cells. The first targeted therapies developed were certain tyrosine kinase inhibitors (TKIs), which affect the downstream signaling of the epidermal growth factor receptor (EGFR) gene.\(^1\) The EGFR-TKIs prevent phosphorylation of the receptor at the tyrosine kinase domain and interfere with cell proliferation, differentiation, migration and survival and induce cell apoptosis.\(^2\)

A set of activating EGFR mutations have been established as predictive biomarkers for response to EGFR-TKIs such as gefitinib or erlotinib.\(^3\) A number of studies with only EGFR mutant NSCLC patients comparing EGFR-TKIs to chemotherapy uniformly showed that the patients receiving the TKIs have higher response rates, progression-free survival (PFS) and quality of life than those treated with chemotherapy.\(^4,5\) However, no benefit in overall survival (OS) was observed even in studies with relatively mature follow-up data.\(^6–13\) but the interpretation of this funding must take into consideration that many chemotherapy patients in these trials crossed over to EGFR-TKI after progression.\(^11–13\) A persistent problem is that patients inevitably develop acquired resistance to the EGFR-TKIs after a median PFS time of 14 months.\(^5\)

Ongoing research studies indicate that acquired resistance to EGFR-TKIs may be reducible or preventable by elaborating improved therapeutic strategies.\(^4,5\) and recent studies have reported the resistance mediated by the T790M mutation (responsible for 60% of EGFR-TKI resistance)\(^14\) may overcome with third-generation EGFR-TKIs like osimertinib and rociletinib.\(^15–18\) However, currently there is no targeted therapy available for the large majority of patients with advanced NSCLC whose tumors do not harbor these activating EGFR mutations.\(^5\)

Immunotherapy is an emerging alternative therapeutic option. One approach to active immunotherapy is targeting tumor-associated antigens (TAAs) with immunotherapeutic agents based on whole TAA proteins. These agents can be taken up by antigen-presenting cells and endogenously processed into presentation to cytotoxic T cells and also induce a long-lasting immune memory that will limit or preclude tumor recurrence.\(^5\) Melanoma-associated antigen A3 (MAGE-A3) and preferentially expressed antigen of melanoma (PRAME) are TAAs expressed in NSCLC tumors. MAGE-A3 and PRAME are expressed in 35–50% and 46–78% of NSCLC tumors, respectively.\(^19,20\) The prevalence of EGFR mutations in NSCLC varies with tumor histology and patient characteristics such as ethnicity, gender, and smoking status.\(^21\) However, little is known about the association between EGFR mutations and expression of MAGE-A3 and PRAME in NSCLC tumors. In addition, the prognostic value of TAA expression for the patients’ OS has not yet been determined, although a high MAGE-A3 expression was a negative prognostic marker for OS in a study in which surgical resection and core needle biopsy specimens from patients with NSCLC adenocarcinomas (ACs) were analyzed.\(^22\)

This study aimed to further assess these associations. The study analyses were performed on archival tumor specimens from a sample of Taiwanese NSCLC patients, partly in a large phase III study of an immunotherapeutic targeting MAGE-A3, the prevalence of MAGE-A3 expression NSCLC tumors was low in Taiwanese patients compared to other East Asians. The patients’ MAGEA3 and PRAME genes were, therefore, also examined for the presence of single nucleotide polymorphisms (SNPs) to aid interpretation of any potential unusual TAA expression results.

**MATERIALS AND METHODS**

This retrospective study was carried out at the National Taiwan University Hospital and the Taichung Veterans General Hospital and was based on examination of archival fresh-frozen tumor tissue specimens from NSCLC patients. The patients had not received neoadjuvant therapy prior to surgery. The study protocol was
Duplicates were performed for all PCR amplifications. The 7900 ABI system (ThermoFischer) in 96-well plates. MAGEA3 RT-PCR assays 60 min at 42°C and RNAse inhibitor (Promega), 2 μM of dNTP, 10 mM of dithiothreitol, 20 Unit (U) of DNase-treated total RNA was converted to complementary DNA (cDNA) using random priming. Synthesis was performed in 20-μL volumes containing 1 x first-strand buffer, 0.5 mM of each deoxyribose nucleoside triphosphates (dNTP), 10 mM of dithiothreitol, 20 Unit (U) of RNase inhibitor (Promega), 2 μL of random primers, and 1 μL of M-MLV Reverse Transcriptase (Invitrogen) for 60 min at 42°C and then for 15 min at 70°C.

RT-PCR assays

MAGEA3, PRAME, and β-actin transcripts were amplified by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using TaqMan chemistry on the 7900 ABI system (ThermoFischer) in 96-well plates. Duplicates were performed for all PCR amplifications. To verify successful removal of gDNA, a PCR without reverse transcriptase (replaced by water) was performed. All primer and probe sequences are listed in Table S1 in the Supplementary Material. cDNA corresponding to 50 ng of total RNA was amplified by PCR in a 25 μL mixture containing 1 x TaqMan buffer, 5 mM of MgCl₂, 0.4 mM of dUTP, 0.2 mM of dATP, 0.2 mM of dGTP, 0.2 mM of dCTP, 0.625 U of Ampli Taq Gold DNA polymerase, 0.05 U of UNG, 0.2 mM of each oligonucleotide primer and 0.2 mM of TaqMan minor groove binder probe. The amplification profile was one cycle of 2 min at 50°C, one cycle of 12 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. The fluorescent signal generated by the degradation of the TaqMan probe was detected in real time during all elongation steps at 60°C.

The following positive controls were included: cDNA corresponding to 50 ng (100%) and to 0.5 ng (1.0%) of total RNA from a gene expression reference level (GERL) melanoma cell line (MZ2-MEL3.0, positive for MAGE-A3 and PRAME, obtained from the Ludwig Institute for Cancer Research). Negative controls consisted of water exposed to reverse transcriptase followed by PCR and water exposed only to PCR steps.

Calculation of gene expression level

Gene expression cut-off values were calculated on the basis of positive GERL cell RNA. The MAGEA3 expression level of the 1.0% GERL (equivalent to 0.5 ng of RNA) and PRAME expression level of 0.3% GERL, each normalized by the β-actin expression level of the 100% GERL (corresponding to 50 ng of RNA), were set as the cut-off values and calculated by the formula:

\[ \text{Cut - off value} = 2^{(a-b)} \]

where \( a \) is the β-actin cycle threshold (Ct) value obtained from the equivalent of 50 ng (100%) of GERL RNA and \( b \) is the gene \( C_i \) value obtained from the equivalent of 0.5 ng (1.0%) of GERL RNA.

A tumor specimen was considered gene-positive when the expression level after normalization by β-actin was equal to or above the cut-off value determined as described. The expression levels for the tumor specimens were calculated by the formula:

\[ \text{gene expression level} = 2^{(c-d)} \]

where \( c \) is the β-actin \( C_i \) value obtained from the equivalent of 50 ng of tumor RNA, and \( d \) is the gene \( C_i \) value obtained from the equivalent of 50 ng of tumor RNA.
In addition, the following conditions had to be met for a tumor specimen to be categorized as positive for the expression of MAGEA3 or PRAME: (1) absence of PCR contamination using the two negative controls; (2) the two positive controls had to be in an appropriate range (i.e., mean ± three standard deviations); (3) the β-actin C<sub>t</sub> value obtained for the specimen had to be <25.73; if the C<sub>t</sub> value exceeded this threshold, the quality of the RNA was considered to be too degraded to perform the assay; (4) absence of gDNA contamination by requiring that ΔC<sub>t</sub> (C<sub>t</sub> of PCR without RT minus the C<sub>t</sub> of PCR with RT) > 3.

**EGFR mutation status**

The EGFR mutation status was tested by using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) based on MassARRAY system (SEQUENOM) and according to our previous publications. All assays were performed in ISO15189 Medical Laboratory certified Pharmacogenomics Laboratory (no. 2695) of the National Taiwan University Hospital. After amplification of the region (exons 18–21) containing EGFR driver mutations by PCR reactions, single nucleotide extension was performed through detection probes followed by MALTI-TOF MS analysis. The assays used were SEQUENOM’s iPLEX assays with primers and probes designed by the investigators (Supplementary Material, Tables S2 and S3).

For the PCR amplification, a final volume of 5 μL reagent was made, containing 10 ng of the patient's genomic DNA, 0.5 unit HotStar Taq, 500 μM dNTP, 100 nM primers, 1.25 × of 10 × HotStar buffer and 1.625 mM MgCl2. Shrimp alkaline phosphate (SAP) treatment for dNTP neutralization was performed as follows: 0.5 unit SAP with 1.7 × SAP buffer was prepared into a final 2 μL mix and then added to the PCR product for incubation and subsequent inactivation. The final step was probe single nucleotide extension by using Typlex Reagent Kit containing Sequenase 0.0205 μL, termination mix 0.1 μL, 10× Typlex buffer 0.2 μL and multiplex extension primers with a final concentration of 7–14 mM in a total of 2 μL reagent.

After SpectroClean Resin clean-up, samples were loaded onto the matrix of SpectroCHIP by Nanodispenser (Matrix) and then analyzed by Bruker Autoflex MALDI-TOF MS. Data were collected and analyzed by Typer4 software with polymorphism parameters. The PCR primers (Supplementary Material, Table S5) and extension probes (Supplementary Material, Table S6) used for this detection were designed by the Mysequenom online designing tool (https://www.mysequenom.com/default.aspx).

**Statistical methods**

The sample size was not determined on the basis of a formal calculation building on prespecified hypotheses, as the study was entirely exploratory with no inferential analyses and presenting only descriptive statistics. The proportions of tumors with an antigen expression level above the cut-off value were estimated relative to the number of tumors with a valid assay result for the respective antigen excluding tumors with missing or invalid expression results.

A Fisher’s exact test was performed to test the association between EGFR mutations and expression of MAGE-A3 and PRAME. For these exploratory analyses, a
Table 1  Patient and tumor characteristics

| Characteristics | AC (N = 156) | SCC (N = 128) |
|-----------------|--------------|--------------|
|                 | n | % | 95% CI | n | % | 95% CI |
| Age (years)     |   |   |         |   |   |         |
| ≤44             | 9 | 5.8 | 2.7–10.7 | 2 | 1.6 | 0.2–5.5 |
| 45–54           | 31 | 19.9 | 13.9–27.0 | 8 | 6.3 | 2.7–11.9 |
| 55–64           | 37 | 23.7 | 17.3–31.2 | 30 | 23.4 | 16.4–31.7 |
| 65–74           | 47 | 30.1 | 23.1–38.0 | 52 | 40.6 | 32.0–49.7 |
| ≥75             | 32 | 20.5 | 14.5–27.7 | 36 | 28.1 | 20.5–36.8 |
| Gender          |   |   |         |   |   |         |
| Female          | 85 | 54.5 | 46.3–62.5 | 10 | 7.8 | 3.8–13.9 |
| Male            | 71 | 45.5 | 37.5–53.7 | 118 | 92.2 | 86.1–96.2 |
| Tumor stage     |   |   |         |   |   |         |
| I               | 84 | 53.8 | 45.7–61.8 | 62 | 48.4 | 39.5–57.4 |
| II              | 24 | 15.4 | 10.1–22.0 | 36 | 28.1 | 20.5–36.8 |
| III             | 40 | 25.6 | 19.0–33.2 | 27 | 21.1 | 14.4–29.2 |
| IV              | 8 | 5.1 | 2.2–9.9 | 3 | 2.3 | 0.5–6.7 |
| Smoking status  |   |   |         |   |   |         |
| Nonsmoker       | 122 | 78.2 | 70.9–84.4 | 66 | 51.6 | 42.6–60.5 |
| Smoker          | 34 | 21.8 | 15.6–29.1 | 62 | 48.4 | 39.5–57.4 |

AC, adenocarcinoma; SCC, squamous cell carcinoma; N, number of patients / tumors; n, number of patients / tumors in a given category; % = (n / N) × 100; CI, confidence interval; nonsmoker, patient who had smoked ≤ 100 cigarettes over his/her entire lifetime; smoker, patient who had smoked > 100 cigarettes over his/her entire lifetime.

P-value below 0.05 was used to highlight potential differences. However, given that no correction for multiplicity has been made and that the clinical relevance of potential differences was not taken into account in the planning of these exploratory analyses, any such differences must be interpreted with caution.

Kaplan–Meier (KM) OS curves were compared using the log-rank test. Unadjusted hazard ratios (HRs) were estimated by means of the Cox proportional hazard regression model.

RESULTS

A total of 284 specimens, 156 ACs and 128 SCCs, were examined. Patient and tumor characteristics are presented in Table 1. Among SCC and ACC tumor patients, 92.2% and 45.5% were male, respectively; 48.4% of the SCC and 21.8% of the AC patients were smokers.

Antigen expression

Out of the 284 tumor specimens collected with the right histology, 195 and 198 were respectively tested for MAGE-A3 and PRAME expression, 138 (70.8%) and 192 (97.0%) gave valid MAGE-A3 or PRAME results, respectively.

Of the AC tumors, 3.3% expressed MAGE-A3, 32.2% expressed PRAME (Table 2); 3.8% expressed both MAGE-A3 and PRAME and 32.9% expressed at least one of the antigens (data not shown). The results of the exploratory analyses of associations between antigen expression and patient/tumor characteristics are presented in Table 2. As only three AC tumors were found to express MAGE-A3, subset analysis was precluded. There was no difference in PRAME expression according to the subgroups of the characteristics investigated except for tumor stage. However, the large differences observed between the tumor stage categories did not suggest any systematic association (Table 2).

Of the SCC tumors, 29.8% expressed MAGE-A3 and 59.2% expressed PRAME (Table 2); 15.8% expressed both MAGE-A3 and PRAME and 63.2% expressed at least one of these antigens (data not shown). Both MAGE-A3 and PRAME expression was similar across the subgroups compared for the patient and tumor characteristics investigated.

EGFR mutations

All the tumor specimens but one gave a valid EGFR mutation result (Table 3). Among the AC tumors, 62.8% bore at least one EGFR mutation and 54.5% were TKI sensitive; the most common mutations were L858R
Table 2  Rates of MAGE-A3 and PRAME expression overall and according to age, gender, smoking status and tumor stage

AC (Total = 156)

| Subset          | MAGE-A3 expression | PRAME expression |
|-----------------|--------------------|------------------|
|                 | N     | n     | %     | 95% CI | N     | n     | %     | 95% CI |
| Overall         | 91    | 3     | 3.3   | 0.7–9.3| 121   | 39    | 32.2  | 24.0–41.3|
| Age (years)     |       |       |       |        |       |       |       |        |
| ≤ 44            | 5     | 0     | 0     | 0.0–52.2| 8     | 6     | 75.0  | 34.9–96.8|
| 45–54           | 20    | 0     | 0.0   | 0.0–16.8| 24    | 5     | 20.8  | 7.1–42.2|
| 55–64           | 20    | 0     | 0.0   | 0.0–16.8| 30    | 10    | 33.3  | 17.3–52.8|
| 65–74           | 30    | 1     | 3.3   | 0.1–17.2| 35    | 12    | 34.3  | 19.1–52.2|
| ≥ 75            | 16    | 2     | 12.5  | 1.6–38.3| 24    | 6     | 25.0  | 9.8–46.7|
| Gender          |       |       |       |        |       |       |       |        |
| Female          | 47    | 1     | 2.1   | 0.1–11.3| 68    | 24    | 35.3  | 24.1–47.8|
| Male            | 44    | 2     | 4.5   | 0.6–15.5| 53    | 15    | 28.3  | 16.8–42.3|
| Smoking status  |       |       |       |        |       |       |       |        |
| Nonsmoker       | 70    | 1     | 1.4   | 0.0–7.7 | 90    | 28    | 31.1  | 21.8–41.7|
| Smoker          | 21    | 2     | 9.5   | 1.2–30.4| 31    | 11    | 35.5  | 19.2–54.6|
| Tumor stage     |       |       |       |        |       |       |       |        |
| I               | 47    | 2     | 4.3   | 0.5–14.5| 68    | 19    | 27.9  | 17.7–40.1|
| II              | 17    | 1     | 5.9   | 0.1–28.7| 16    | 9     | 56.3  | 29.9–80.2|
| III             | 23    | 0     | 0.0   | 0.0–14.8| 31    | 8     | 25.8  | 11.9–44.6|
| IV              | 4     | 0     | 0.0   | 0.0–60.2| 6     | 3     | 50.0  | 11.8–88.2|

SCC (Total = 128)

| Subset          | MAGE-A3 expression | PRAME expression |
|-----------------|--------------------|------------------|
|                 | N     | n     | %     | 95% CI | N     | n     | %     | 95% CI |
| Overall         | 47    | 14    | 29.8  | 17.3–44.9| 71    | 42    | 59.2  | 46.8–70.7|
| Age (years)     |       |       |       |        |       |       |       |        |
| ≤ 44            | 1     | 1     | 100   | 2.5–100 | 1     | 1     | 100   | 2.5 - 100 |
| 45 - 54         | 5     | 0     | 0.0   | 0.0–52.2| 4     | 1     | 25.0  | 0.6– 80.6 |
| 55 - 64         | 11    | 3     | 27.3  | 6.0–61.0 | 19    | 10    | 52.6  | 28.9–75.6 |
| 65 - 74         | 19    | 6     | 31.6  | 12.6–56.6| 28    | 19    | 67.9  | 47.6–84.1 |
| ≥ 75            | 11    | 4     | 36.4  | 10.9–69.2| 19    | 11    | 57.9  | 33.5–79.7 |
| Gender          |       |       |       |        |       |       |       |        |
| Female          | 4     | 1     | 25.0  | 0.6–80.6 | 5     | 0     | 0.0   | 0.0–52.2|
| Male            | 43    | 13    | 30.2  | 17.2–46.1| 66    | 42    | 63.6  | 50.9–75.1|
| Smoking status  |       |       |       |        |       |       |       |        |
| Nonsmoker       | 19    | 7     | 36.8  | 16.3–61.6| 27    | 17    | 63.0  | 42.4–80.6|
| Smoker          | 28    | 7     | 25.0  | 10.7–44.9| 44    | 25    | 56.8  | 41.0–71.7|
| Tumor stage     |       |       |       |        |       |       |       |        |
| I               | 23    | 8     | 34.8  | 16.4–57.3| 38    | 22    | 57.9  | 40.8–73.7|
| II              | 13    | 5     | 38.5  | 13.9–68.4| 19    | 11    | 57.9  | 33.5–79.7|
| III             | 9     | 1     | 11.1  | 0.3–48.2 | 12    | 8     | 66.7  | 34.9–90.1|
| IV              | 2     | 0     | 0.0   | 0.0–84.2 | 2     | 1     | 50.0  | 1.3–98.7 |

AC, adenocarcinoma; SCC, squamous cell carcinoma; N, number of specimens in this category with a valid test result for the respective antigen; n, number of specimens in a given category expressing the respective antigen; % = (n/N) × 100; CI, confidence interval; nonsmoker, patient who had smoked ≤ 100 cigarettes during his/her entire life; smoker, patient who had smoked > 100 cigarettes during his/her entire life.
Table 3  EGFR mutation results, overall and per type of mutation

| Mutations       | AC (N = 156) |          |         | SCC (N = 128) |          |         |
|-----------------|--------------|----------|---------|---------------|----------|---------|
|                 | n  | %     | 95% CI  | n   | %     | 95% CI  |
| All             |    |        |         |    |        |         |
| WT              | 58 | 37.2   | 29.6–45.3 | 101 | 79.5  | 71.5–86.2 |
| Mutated         | 98 | 62.8   | 54.7–70.4 | 26  | 20.5  | 13.8–28.5 |
| Missing         | 0  | –      | –       | 1   | –     | –       |
| Exon 19 deletions|     |        |         |    |        |         |
| Yes             | 30 | 19.2   | 13.4–26.3 | 2   | 1.6   | 0.2–5.6  |
| L858R           |     |        |         |    |        |         |
| Yes             | 55 | 35.3   | 27.8–43.3 | 12  | 9.4   | 5.0–15.9 |
| T790M†         |     |        |         |    |        |         |
| Yes             | 13 | 8.3    | 4.5–13.8 | 12  | 9.4   | 5.0–15.9 |
| TKI sensitivity |    |        |         |    |        |         |
| TKI resistant   | 71 | 45.5   | 37.5–53.7 | 113 | 89.0  | 82.2–93.8 |
| TKI sensitive   | 85 | 54.5   | 46.3–62.5 | 14  | 11.0  | 6.2–17.8 |
| Missing         | 0  | –      | –       | 1   | –     | –       |

†Note: Of the 13 T790M mutated tumors detected in the AC cohort, 10 were L858R/T790M double mutants and two were Exon 19 deletions/T790M double mutants. Of the 12 T790M mutated patients detected in the SCC cohort, five were L858R/T790M double mutants and one was L858R/Exon 19 deletions/T790M triple mutant. To simplify the result interpretation, these double and triple mutants were considered as T790M mutants with a TKI-resistant status.

AC, adenocarcinoma; SCC, squamous cell carcinoma; WT, wild type; TKI resistant, T790M (including the double and triple mutants) + WT; TKI sensitive, Exon 19 deletions, L858R; N, number of patients/samples; n, number of patients/samples in a given category; %, n/number of patients/specimens with available results × 100; CI, confidence interval.

Table 4  EGFR mutation status of AC tumors according to their antigen expression

| Mutation status | MAGE-A3-negative N = 88 |          | MAGE-A3-positive N = 3 |          | P-value |
|-----------------|--------------------------|----------|------------------------|----------|---------|
|                 | n  | %     | 95% CI  | n   | %     | 95% CI  |          |
| AC tumors with valid MAGE-A3 test (N = 91) |    |        |         |    |        |         |          |
| WT              | 36 | 40.9   | 30.5–51.9 | 0  | 0.0   | 0.0–70.8 | 0.27     |
| Mutated         | 52 | 59.1   | 48.1–69.5 | 3  | 100   | 29.2–100 |          |
| AC tumors with valid PRAME test (N = 121) |    |        |         |    |        |         |          |
| WT              | 33 | 40.2   | 29.6–51.7 | 14 | 35.9  | 21.2–52.8 | 0.69     |
| Mutated         | 49 | 59.8   | 48.3–70.4 | 25 | 64.1  | 47.2–78.8 |          |
| SCC tumors with valid MAGE-A3 test (N = 47) |    |        |         |    |        |         |          |
| WT              | 26 | 78.8   | 61.1–91.0 | 12 | 85.7  | 57.2–98.2 | 0.70     |
| Mutated         | 7  | 21.2   | 9.0–38.9  | 2  | 14.3  | 1.8–42.8  |          |
| SCC tumors with valid PRAME test (N = 71) |    |        |         |    |        |         |          |
| WT              | 22 | 75.9   | 56.5–89.7 | 33 | 78.6  | 63.2–89.7 | 0.78     |
| Mutated         | 7  | 24.1   | 10.3–43.5 | 9  | 21.4  | 10.3–36.8 |          |

AC, adenocarcinoma; SCC, squamous cell carcinoma; WT, wild type; N, number of specimens; n, number of specimens in a given category; %, n/number of specimens with available results × 100; CI, confidence interval; P-values, Fisher's exact test.

and exon 19 deletions, detected in 35.3% and 19.2%, respectively.

EGFR mutations were detected in 20.5% of the SCC tumors and TKI-sensitive mutations in 11.0% of tumors; the most common mutations detected were L858R and T790M, each harbored in 9.4% of the SCC tumors.

EGFR mutation status according to antigen expression

Except for the three AC tumors expressing MAGE-A3, which were all EGFR mutants, no association between EGFR mutations and TAA expression was found, regardless of the tumor histology (Table 4).
A. SCC patients according to MAGE-A3 expression

There was no association of MAGE-A3 or PRAME expression status and patient OS. Representative KM curves and HR statistics are presented in Figure 1.

HR for MAGE-A3-positive vs MAGEA3-negative: 0.68 (95% CI: 0.25 - 1.88; p = 0.46)

B. AC patients according to PRAME expression

HR for PRAME-positive vs PRAME-negative: 0.67 (95% CI: 0.27 - 1.66; p = 0.39)

Figure 1 Representative Kaplan-Meier curves for OS of the patients according to their tumor histology and its expression of MAGE-A3 or PRAME. (a) SCC patients according to MAGE-A3 expression. (b) AC patients according to PRAME expression.

Overall survival according to antigen expression status

There was no association of MAGE-A3 or PRAME expression status and patient OS. Representative KM curves and HR statistics are presented in Figure 1.

Detection of polymorphisms

Polymorphisms of the MAGEA3 gene

The presence of SNPs in MAGEA3 was examined using gDNA extracted from 200 tumor specimens (without stratification between AC and SCC tumors). As problems with the quality of the raw sequences were identified in
In 22 of the 177 specimens (12.4%), at least one SNP was identified in the *MAGEA3* coding sequence. Only one of these specimens carried an SNP in the *MAGEA3* amplicon region of the PCR assay (located at the 3’ extremity of the forward primer). The tumor specimen concerned was tested negative for *MAGE-A3* expression, suggesting that SNPs in this region might influence *MAGE-A3* expression.

**Polymorphisms of the PRAME gene**

Sequencing analyses were performed on 205 tumor specimens to detect the presence of 24 known SNPs of *PRAME*. Three *PRAME* SNPs were observed with a high frequency: rs2266988 (87.8%), rs61745687 (100%) and rs34866162 (100%). Three other SNPs were present in 8–10% of the patients (rs17497547, rs17497512 and rs34101420). The remaining SNPs were not detected or detected in less than 3% of the patients.

**DISCUSSION**

The rates of expression of *MAGE-A3* and *PRAME* in the tumors of this cohort of Taiwanese NSCLC patients were generally similar to the rates reported by two recent East Asian studies27,28; however, in this study, only three (3.3%) Taiwanese AC patients had tumors expressing *MAGE-A3* compared to 15–20% in these previous studies. The data collected in this study do not provide any clue to explain why so few AC tumors expressed *MAGE-A3*. Thongprasert et al.26 showed that tumor histology (AC vs SCC) and smoking status were independent factors significantly associated with expression of both *MAGE-A3* and *PRAME* with higher rates of TAA expression in SCC tumors and in tumors from (ex-)smokers.26 Within each histology, we did not find any association between the smoking status and the TAA expression. Both TAAs were much more frequently expressed in SCC tumors and similar differences in TAA expression between AC and SCC tumors have been frequently reported.26–28

SNPs can serve as genetic factors with implications for a patient’s response to treatment and the types and severity of experienced side effects.29 The results of a previous study in NSCLC patients showed that specific SNPs in cells of East Asian populations result in a lower *EGFR* protein expression.30 In another studies, specific gene SNPs were indicated as the potential cause of intrinsic TKI resistance in NSCLC tumors harboring TKI-sensitive *EGFR* mutations.29–31 Of the 177 specimens sequenced in this study, only one had an SNP that could have impacted the detection of *MAGE-A3* expression. Thus, this factor alone cannot explain the low *MAGE-A3* expression rate among AC tumors.

Regardless of tumor histology, no association was found between patients’ TAA expression and OS time. In contrast, a previous study reported that *MAGE-A3* expression in NSCLC AC tumors was a prognostic marker indicating lower OS.22 However, because of the very low rate of *MAGE-A3* expression in ACs, we could not investigate such association in our study.

*EGFR* mutations were detected in 62.8% of the AC tumors, midway in the range (40–79%) of *EGFR* mutation rates in NSCLC AC tumors previously reported in several Asian studies.27–29 This further confirms the ethnic differences established for AC tumors from NSCLC patients, with four large European studies reporting *EGFR* mutation rates ranging from 10% to 21%.30–32 The reasons for this ethnic difference have not been conclusively identified.

Among the SCC tumors, 20.5% were found to harbor *EGFR* mutations, 54.0% of which were *EGFR*-TKI sensitive. This may seem unusual given that *EGFR* mutations are considered to be very infrequent in well-characterized fully excised SCC specimens without any AC component.39 Two studies have reported a very low rate of *EGFR* mutations in SCC tumors.27,36 Other studies assessing *EGFR* mutations in NSCLC tumors included only AC tumors, even though other histological types were collected.35,37 The few *EGFR*-mutant SCC were found mostly in small biopsies from patients with advanced disease, in which the differential diagnosis between SCC and adenosquamous tumors is particularly difficult.40

However, several recent Asian studies reported detecting an *EGFR* mutations rates in SCC tumors ranging from 13.3% to 29.7%40–44 using different testing methods. All existing detection methods potential disadvantages and widely diverging sensitivity and there is no consensus on the test method most appropriate for clinical decision making.45–47 The analytic sensitivity of a detection method, that is the lowest concentration of tumor cells in a test specimen in which a mutation is detected with 100% precision, is an important characteristic,39 but some mutations only detectable by highly sensitive test methods may not be clinically relevant.46

The divergent findings about the frequency of *EGFR* mutations in NSCLC SCC tumors may only have
decision-making importance, if EGFR mutations are valid predictive biomarkers for these patients’ response to EGFR-TKIs. A retrospective study of 79 SCC patients found that EGFR mutations were predictive for response to EGFR-TKIs. However, a recent review reported contradicting data showing that the response rate and PFS for EGFR-mutant SCC patients are not improved upon first-line therapy with EGFR-TKIs compared to cisplatin-based doublet chemotherapy.

Irrespective of tumor histological type, we did not find any difference in the rate of TAA expression between EGFR mutants and WTs. This is in line with the results of the only identified study investigating the association between EGFR mutation status and MAGE-A3 expression in NSCLC tumors. In this previous study, MAGE-A expression was detected by immunohistochemistry and the different proteins to the MAGE-A family could not be determined.

Although we made every effort to conduct a study that reflected the frequency of expression of MAGE-A3 and PRAME and the rate of EGFR mutations in a Taiwanese NSCLC population, the study has potential limitations. The relatively small sample size coming from two different hospitals could reduce the statistical power of the subset analyses and makes difficult the generalization of the results to overall Taiwanese NSCLC population. Furthermore, in a parallel study performed in China using the same test protocol on FF tumor specimens from NSCLC patients, a valid MAGE-A3 test result was obtained for 100% of the specimens, whereas in our study only 70% of the specimens were valid. These problems were not observed for the tests of PRAME expression and EGFR mutations. The hypothesis of a lack of sufficient tumor material cannot explain the observed difference since MAGE-A3 expression was tested first. However, the problems with the MAGE-A3 testing could explain the very low rate of MAGE-A3 expression observed in AC tumors. Another potential limitation of the study is that the survival analyses were stratified only for tumor histology and TAA expression status but not for tumor stage, because the resulting subsets would become very small leading to limited statistical power of the comparison tests.

In conclusion, this study corroborates the notion that NSCLC is a very heterogeneous disease and that AC and SCC tumors have distinct molecular profiles. Development of immunotherapeutics targeting MAGE-A3 and/or PRAME might be beneficial for patients with SCC tumors, which show high rates of expression of these TAs and therefore, are likely to respond to such immunotherapeutic compounds.

AUTHORS’ CONTRIBUTIONS

(1) Conception/design/planning: SLY, SHP, SKY, PCY, BS, AdC, NFD, OG, AM, JL
(2) Acquisition/assembling data: SLY, SHP, SKY, PCY, AdC, NFD, OG, AM, JL
(3) Quality check: SLY, SHP, SKY, PCY, BS, NK, NFD, OG, AM, JL
(4) Center coordination: SLY, SHP, PCY, BS, NK, NFD, OG, AM, JL
(5) Data extraction: SLY, SHP, SKY, PCY, BS, NK, NFD, OG, AM
(6) Performing/supervising analysis: SLY, SHP, SKY, PCY, BS, NFD, OG, AM, JL
(7) Interpretation of results: SLY, SHP, PCY, BS, AdC, NFD, OG, AM, JL
(8) Provision of study materials/subjects: SLY, SHP, SKY, PCY, BS, NK, NFD, OG, AM
(9) Provision of statistical expertise: SHP, PCY, BS
(10) Collection of data: SHP, SKY, PCY, AdC, NFD, OG, AM
(11) Acquisition of funding: PCY, AM
(12) Choice/recruitment of centers/investigators: PCY, AM
(13) Administrative/technical logistic support: SLY, SHP, SKY, PCY, NK, NFD, OG
(14) Laboratory/serology testing: SHP, SKY, JL
(15) Supervision of the study research group: SLY, SHP, PCY, NK, NFD, OG, AM, JL

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:
Table S1 Primers and probes used for the RT-PCR assays
Table S2 Primers for amplification of exons 18–21 of the EGFR gene from genomic DNA
Table S3 Primers and detection probes used for detecting T790M, L858R and exon 19 deletions by MALDI-TOF MS analysis
Table S4 Sequence of MAGE-A3 primers to detect polymorphisms of MAGE-A3
Table S5 Sequence of PRAME MassARRAY (PCR primer)
Table S6 Sequence of PRAME MassARRAY (extending probes)