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Original Article

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

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Abstract: The purpose of this study was to determine the expression of the tumor associated antigens (TAAs) MAGE-A3 and PRAME and the occurrence of tyrosine-kinase inhibitor (TKI)-sensitive *EGFR* mutations in tumors from a Chinese non-small cell lung cancer (NSCLC) population including only adenocarcinomas (ACs) and squamous cell carcinomas (SCCs). Correlations between TAA expression and clinico-pathological characteristics and between occurrence of *EGFR* mutations and TAA expression were also investigated. Quantitative reverse transcription polymerase chain reaction assays with antigen-specific primers were used to detect the TAAs on 191 fresh-frozen archival tumor tissues. The *EGFR* mutation status was determined by sequencing. Of 96 AC tumors, 15.6% expressed MAGE-A3, 31.3% expressed PRAME and 37.5% expressed at least one of these TAAs. TKI-sensitive *EGFR* mutations were found in 40.6% overall, in 66.7% of those expressing MAGE-A3 and in 35.8% of those not expressing MAGE-A3. Occurrence of TKI-sensitive *EGFR* mutations was unrelated to PRAME expression. Of 95 SCC tumors, 31.6% expressed MAGE-A3, 70.5% expressed PRAME and 72.6% at least one of these TAAs. TKI-sensitive *EGFR* mutations occurred in 2.1%. No correlation was found between expression of any of the TAAs and tumor stage or the patients' age. We conclude that in this Chinese NSCLC population, each TAA was expressed about twice as often in SCC tumors as in AC tumors. Forty-one percent of the AC tumors harbored TKI-sensitive *EGFR* mutations in contrast to only 2% of the SCCs. Antigen-specific immunotherapy targeting MAGE-A3 or/and PRAME could focus on SCC tumors, which often express these TAAs.

Keywords: Non-small cell lung cancer, MAGE-A3 antigen, PRAME antigen, *EGFR* mutation, TKI-sensitive

Introduction

Worldwide, approximately 1.2 million patients die of lung cancer each year, making it the leading cause of cancer-related death [1]. Non-small cell lung cancer (NSCLC) is the most common type accounting for 80 to 85% of all lung tumors [2]. In more than 60% of the incident cases, NSCLC is diagnosed as locally advanced or metastatic disease and is generally incurable [3].

For earlier stages (I-IIIA), surgery is the standard of care, but the rate of recurrence is high and most completely resected patients die of recurrent disease within five years of resection (from 33% in stage I to 77% in stage IIIA) [4]. Moreover, many resected patients are not candidates for the current standard adjuvant treatment (four cycles of platinum-based doublet chemotherapy) due to poor performance status, co-morbidities and/or complications following lung resection. Compared to surgery only, adjuvant chemotherapy provides a limited absolute survival benefit of just 5.4% [5] and may lead to complications such as cardiovascular complications and an increase in cardiovascular related mortality [5].

To achieve better outcomes for lung cancer patients, which have improved only modestly over the last 30+ years (in the US, five-year sur-

vival increased from 12% to 17% between 1975 and 2008) [6], diagnosis at an earlier stage must be facilitated. In particular, there is an important and widely recognized need for developing more effective and less toxic adjuvant and neoadjuvant treatments to eradicate the micrometastases believed to be the cause of the high rate of recurrence after resection [3].

One possible treatment option is antigen-specific immunotherapy targeting tumor-associated antigens (TAAs). Although lung cancer is generally not considered immunogenic and relatively few studies have documented host immune infiltrates in lung tumors [7-11], more recent studies of immunotherapy in lung cancer show that responses, although infrequent, are long-lasting [12], TAA-specific immunotherapy is an attractive option because several TAAs are frequently expressed in NSCLC tumors [13-16].

TAA-specific immunotherapy aims at mobilizing the patient's immune system to recognize the tumor cells as non-self and to elicit robust and durable immune responses, both humoral and cellular, which will eventually eradicate the tumor and prevent its recurrence. Among the immunogenic TAAs identified and partly characterized over the last 20 years are MAGE-A3 (melanoma-associated antigen A3) and PRAME (preferentially expressed antigen of melanoma), which have both been detected in a variety of cancer types [17, 18].

MAGE-A3 is a human gene that encodes the MAGE-A3 protein (or antigen), whose biological function has not yet been elucidated. The *MAGE-A3* gene is silent in all normal adult human tissues except the testis [19]. Furthermore, the cells that express the *MAGE-A3* gene in testis, the spermatogonia, do not bear molecules from the major histocompatibility complex on their surface and therefore do not present any MAGE-A3 epitopes [20]. Thus, the MAGE-A3 antigen is considered to be virtually tumor-specific.

The function of the PRAME protein, encoded by *PRAME* and overexpressed in a variety of cancers [18], remains incompletely characterized but has been suggested to be associated with repression of retinoic acid receptor signaling [21] and thereby involved in cell-death regulation and the cell cycle. PRAME-specific cytotoxic T lymphocytes, which can lyse human cell lines expressing PRAME, have been found in melanoma patients [22].

In advanced NSCLC, the presence of activating *EGFR* mutations is a strong predictor of response to *EGFR*-TKIs [23]; patients with such mutations show response rates to first-line *EGFR*-TKI therapy of 60-75% [24, 25]. While responses rates of 10-20% have been reported in unselected patients [26]. Following negative results obtained from an early trial of adjuvant EGFR-TKI therapy in unselected NSCLC patients [4], at least three large trials of adjuvant *EGFR*-TKI therapy have been initiated in patients with the activating *EGFR* mutations predicting sensitivity to *EGFR*-TKIs [4]. Despite conflicting evidence, these *EGFR* mutations are also widely believed to be prognostic markers for the survival of patients with early stage NSCLC, who undergo resection [27, 28].

The retrospective observational study reported here aimed to assess the expression of MAGE-A3 and PRAME in tumors from a cohort of Chinese NSCLC patients, their *EGFR* mutation status and the association between *EGFR* mutant type or wild type (WT) and TAA expression. Clarifying these relationships would be helpful in identifying the patient subsets most likely to respond to the new options for adjuvant therapy considered here.

Materials and methods

This single-center, retrospective observational study was carried out at the Guangdong General Hospital in Guangzhou, China. All data and tissue specimens were taken from those already stored in the tumor bank at the investigational site. Many patients may no longer be alive, or no longer be in contact with the investigational site. Patients were not subjected to any study-specific treatments or other procedures and no questionnaires were addressed directly to them. For these reasons, it was not required that patients should give their informed consent before inclusion in the study. Strict anonymity of all study data was established and maintained. Prior to initiation, the study and its protocol were accepted by the local ethics committee that did not raise any concern related to the absence of informed consent in view of the

appropriate protocol rules applied to maintain confidentiality.

Specimen collection

Fresh frozen tumor tissue specimens from NSCLC patients with disease stage IB to IIIA were included in the study, provided the tumor belonged to one of the histological types of interest, AC and SCC. We did not intend to collect a sample of tumors representative of the actual distribution of histological types among Chinese NSCLC patients.

The corresponding data collected on patient characteristics included age, gender, smoking status and tumor stage. A smoker was defined as a patient who had smoked more than 100 cigarettes during his/her lifetime and a non-smoker as one who had smoked less than this or not at all.

The tumors' mRNA expression of MAGE-A3 and PRAME was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using TaqMan methods and the ABI PRISM 7900 Sequence Detection System. These experiments were performed by the study site laboratory according to protocols as described by Gruselle and colleagues [29]. *EGFR* mutations were analyzed by gene sequencing (Sanger method) at the study site laboratory.

RNA extraction and RNA purification method

Total RNA was extracted from the tumor tissue specimens by the Trizol RNA extraction method (Invitrogen). Genomic DNA (gDNA) was removed by DNase 1 Amplification Grade (Invitrogen) using an RNeasy kit (Qiagen) according to the instructions of the manufacturer. Total RNA was eluted in RNase-free water and RNA quality was measured using a 2100 Agilent Bioanalyzer (Agilent Technologies). DNase treated total RNA (1 µg) was converted to cDNA using random priming. Synthesis was performed in 20-µL volumes containing 1 × first strand buffer, 0.5 mM of each dNTP, 10 mM of dithiothreitol, 20 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

MAGE-A3, *PRAME* and *β-actin* transcripts were amplified by qRT-PCR using TaqMan chemistry and 7900 ABI system (Life Technologies) 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 × TaqMan buffer, 5 mM of $MgCl_2$, 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 MGB probe. The amplification profile was 1 cycle of 2 min at 50°C, 1 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.

cDNA corresponding to 50 ng (100%) and to 2.1 ng (4.2%) UHR RNA for MAGE-A3 and β-actin were included as positive controls. For PRAME, the respective quantities were cDNA corresponding to 50 ng of UHR RNA and cDNA corresponding to 0.65 ng (1.3%) of UHR RNA. PCR mixtures, in which the RNA and DNA template were replaced with water, were used as negative controls.

Calculation of gene expression level

Antigen expression cut-off values were calculated on the basis of positive UHR RNA. The *MAGE-A3* and *PRAME* expression level of the UHR (4.2% (2.1 ng) for MAGE-A3 and 1.3% (0.65 ng) for PRAME) normalized by the *β-actin* expression level of the 100% UHR (corresponding to 50 ng of RNA) was set as the cut-off value. The gene expression cut-off value was calculated by the formula: 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 UHR RNA and b is the antigen Ct value obtained from the equivalent of 2.1 ng (4.2%) of UHR RNA.

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

Table 1. Patient and tumor characteristics

Variable	Categories	AC (N = 96)			SCC (N = 95)		
		n	%	95% CI	n	%	95% CI
Age (years)	< 45	6	6.3	2.3-13.1	4	4.2	1.2-10.4
	45-54	20	20.8	13.2-30.3	16	16.8	9.9-25.9
	55-64	30	31.3	22.2-41.5	39	41.1	31.1-51.6
	65-74	35	36.5	26.9-46.9	31	32.6	23.4-43.0
	≥ 75	5	5.2	1.7-11.7	5	5.3	1.7-11.9
Gender	Female	39	40.6	30.7-51.1	16	16.8	9.9-25.9
	Male	57	59.4	48.9-69.3	79	83.2	74.1-90.1
Tumor stage	IB	46	47.9	37.6-58.4	45	47.4	37.0-57.9
	IIA	2	2.1	0.3-7.3	8	8.4	3.7-15.9
	IIB	21	21.9	14.1-31.5	24	25.3	16.9-35.2
	IIIA	27	28.1	19.4-38.2	18	18.9	11.6-28.3
Smoking status	Non-smoker	65	67.7	57.4-76.9	36	37.9	28.1-48.4
	Smoker	31	32.3	23.1-42.6	59	62.1	51.6-71.9

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; Smoker = A patient who had smoked > 100 cigarettes during his/her lifetime; Non-smoker: A patient who had smoked ≤ 100 cigarettes during his/her lifetime (Source: ONCO RD-008 study report (17 June 2014), Table 2).

gene mRNA molecules per 1 β-actin molecule) = $2^{(c-d)}$, where c is the β-actin Ct value and d is the gene Ct value, both obtained from the equivalent of 50 ng of RNA extracted from the tumor specimen.

In addition, the following conditions had to be met for a tumor specimen to be categorized as positive for the expression of *MAGE-A3* or *PRAME*: 1) absence of PCR contamination, determined based on the two negative controls; 2) the two positive controls had to be in an appropriate range (mean ± three standard deviations); 3) the β-actin Ct value obtained for the specimen had to be < 23; if this Ct value was > 23, the quality of the RNA was considered insufficient to perform the assay; 4) absence of gDNA contamination defined as ΔCt (Ct of PCR without RT minus the Ct of PCR with RT) > 3.

EGFR mutation status

EGFR mutation status was determined by amplification of the exons 18, 19, 20 and 21, followed by direct sequencing. The primers used for amplification and probes used for identification of the mutations are presented in Table S2 in the Supplementary Material.

The tumors were examined for the following mutations: exon 19 deletions, exon 20 insertions, G719X, L858M, L858R, T790M and V819V. Of these, exon 19 deletions, L858R and G719X are categorized as TKI sensitive and exon 20 insertions and T790M as TKI resistant (just as the *EGFR* wild type, WT). The TKI sensitivity of V819V has not yet been established, so this mutation was excluded from the analyses involving TKI sensitive/resistant categorization.

Statistical methods

No formal sample size calculation based on prespecified hypotheses was performed, the study was entirely exploratory and only descriptive statistics are presented; no inferential analyses were performed. Antigen expression results are presented as the proportion (with exact 95% confidence interval (CI)) of tumors with a valid test result that are positive for the tested antigen. *EGFR* mutation results are presented in the same way. A Fischer exact test was performed to assess the association between *EGFR* mutation and expression of MAGE-A3/PRAME. For these exploratory comparisons, a *p*-value below 0.05 was used to highlight potential differences. Any potential differences must be considered with caution, however, given that no correction for multiplicity has been made and that the clinical relevance of any difference was not taken into account in the planning of these exploratory analyses.

All test results are presented for the AC and SCC cohorts separately. It must be stressed that the study did not aim to be representative for the actual distribution of histological types in the Chinese NSCLC population.

Results

In total, 191 tumor specimens (96 ACs and 95 SCCs) were collected; we obtained valid test results for MAGE-A3 and PRAME expression and for *EGFR* mutation status for all specimens. Notable differences between the two cohorts

Table 2. Rates of expression of MAGE-A3 and PRAME, in the total cohorts and in subsets determined by patient and tumor characteristics

AC (N = 96)									
Variable	Subsets	N	MAGE-A3 expression			N	PRAME expression		
			n	%	95% CI		n	%	95% CI
Total	NA	96	15	15.6	9.0--24.5	96	30	31.3	22.2-41.5
Age	26-44	6	0	0	0.0-45.9	6	1	16.7	0.4-64.1
(Years)	45-54	20	4	20.0	5.7-43.7	20	6	30.0	11.9-54.3
	55-64	30	4	13.3	3.8-30.7	30	12	40.0	22.7-59.4
	65-74	35	6	17.1	6.6-33.6	35	11	31.4	16.9-49.3
	≥ 75	5	1	20.0	0.5-71.6	5	0	0.0	0.0-52.2
Gender	Woman	39	4	10.3	2.9-24.2	39	11	28.2	15.0-44.9
	Man	57	11	19.3	10.0-31.9	57	19	33.3	21.4-47.1
Smoking	Non-smoker	65	8	12.3	5.5-22.8	65	18	27.7	17.3-40.2
status	Smoker	31	7	22.6	9.6-41.1	31	12	38.7	21.8-57.8
Stage	IB	46	6	13.0	4.9-26.3	46	15	32.6	19.5-48.0
	IIA	2	0	0.0	0.0-84.2	2	1	50.0	1.3-98.7
	IIB	21	5	23.8	8.2-47.2	21	10	47.6	25.7-70.2
	IIIA	27	4	14.8	4.2-33.7	27	4	14.8	4.2-33.7
SCC (N = 95)									
Variable	Subsets	N	MAGE-A3 expression			N	PRAME expression		
			n	%	95% CI		n	%	95% CI
Total	NA	95	30	31.6	22.4-41.9	95	67	70.5	60.3-79.4
Age	26-44	4	0	0.0	0.0-60.2	4	1	25.0	0.6-80.6
(Years)	45-54	16	6	37.5	15.2-64.6	16	11	68.8	41.3-89.0
	55-64	39	8	20.5	9.3-36.5	39	25	64.1	47.2-78.8
	65-74	31	14	45.2	27.3-64.0	31	26	83.9	66.3-94.5
	≥ 75	5	2	40.0	5.3-85.3	5	4	80.0	28.4-99.5
Gender	Woman	16	2	12.5	1.6-38.3	16	6	37.5	15.2-64.6
	Man	79	28	35.4	25.0-47.0	79	61	77.2	66.4-85.9
Smoking	Non-smoker	36	8	22.2	10.1-39.2	36	20	55.6	38.1-72.1
status	Smoker	59	22	37.3	25.0-50.9	59	47	79.7	67.2-89.0
Stage	IB	45	20	44.4	29.6-60.0	45	32	71.1	55.7-83.6
	IIA	8	2	25.0	3.2-65.1	8	6	75.0	34.9-96.8
	IIB	24	4	16.7	4.7-37.4	24	16	66.7	44.7-84.4
	IIIA	18	4	22.2	6.4-47.6	18	13	72.2	46.5-90.3

AC = Adenocarcinoma; SCC = Squamous cell carcinoma; N = number of specimens tested with a valid test result for the respective antigen; n = number of specimens in a given category with expression of the respective antigen; % = (n/N) × 100; CI = Confidence interval (Source: ONCO RD-008 study report (17 June 2014), Tables 3-7).

were that 83.2% of patients in the SCC cohort were men and 62.1% smokers, whereas the AC cohort included 59.4% men and 32.3% smokers (**Table 1**).

Antigen expression

Of the 96 AC tumors, 15.6% expressed MAGE-A3, 31.3% expressed PRAME, 9.4% co-expressed both genes and 37.5% expressed at least one of them (**Tables 2** and **3**). Exploratory subgroup analyses of associations between the patients' age, gender, smoking status or TNM stage and TAA expression showed no obvious correlations (**Table 2**).

Of the 95 SCC tumors, 31.6% expressed MAGE-A3, 70.5% expressed PRAME, 29.5% co-expressed both antigens and 72.6% expressed at least one of them (**Tables 2** and **3**). The subset analyses (**Table 2**) suggested that the frequency of expression of both MAGE-A3 and PRAME was higher in men than in women and in smokers compared to non-smokers.

EGFR mutations

In the AC cohort, *EGFR* mutations were detected in 43.8% of the tumors. The most frequent *EGFR* mutations detected were L858R (21.9% of the tumors) and exon 19 deletions (19.8%), both of which are TKI sensitive. The overall rate of TKI-sensitive mutations was 40.6% (**Table 4**). TKI-sensitive *EGFR* mutations were detected in 66.7% (10/15) of the MAGE-A3 expressing tumors and in 35.8% (29/81) of the tumors not expressing MAGE-A3, (P = 0.04, **Table 5**). TKI-sensitive *EGFR* mutations were detected in 46.7% (14/30) of the PRAME expressing tumors and in 37.9% (25/66) of the tumors not expressing PRAME (P = 0.50, **Table 5**). The rate of *EGFR* mutations was the same in patients with tumors expressing MAGE-A3, irrespective of their PRAME expression status. For tumors not expressing MAGE-A3, the rate of *EGFR* mutations was 38.3% (23/60) for tumors not expressing

Table 3. Co-expression of MAGE-A3 and PRAME, total cohorts

Co-expression of MAGE-A3 and PRAME	AC (N = 96)			SCC (N = 95)		
	n	%	95% CI	n	%	95% CI
MAGE-A3-positive/PRAME-positive	9	9.4	4.4-17.1	28	29.5	20.6-39.7
MAGE-A3-positive/PRAME-negative	6	6.3	2.3-13.1	2	2.1	0.3-7.4
MAGE-A3-negative/PRAME-positive	21	21.9	14.1-31.5	39	41.1	31.1-51.6
MAGE-A3-negative/PRAME-negative	60	62.5	52.0-72.2	26	27.4	18.7-37.5
Positive for at least one of them	36	37.5	27.8-48.0	69	72.6	62.5-81.3

AC = Adenocarcinoma; SCC = Squamous Cell Carcinoma; N = number of specimens; n = number of specimens in a given category; % = n/Number of specimens with available results × 100; CI = Confidence interval (Source: ONCO RD-008 study report (17 June 2014), Table 13).

Table 4. EGFR mutation status overall and details on mutation types

Mutation type	Categories	AC (N = 96)			SCC (N = 95)		
		n	%	95% CI	n	%	95% CI
EGFR mutation status	Mutation (s)	42	43.8	33.6-54.3	5	5.3	1.7-11.9
	WT	54	56.3	45.7-66.4	90	94.7	88.1-98.3
Exon 19 deletions	Yes	19	19.8	12.4-29.2	1	1.1	0.0-5.7
L858R	Yes	21	21.9	14.1-31.5	0	0	0.0-3.8
L858M	Yes	0	0	0.0-3.8	0	0	0.0-3.8
G719X	Yes	1	1.0	0.0-5.7	1	1.1	0.0-5.7
V819V*	Yes	0	0	0.0-3.8	1	1.1	0.0-5.7
T790M	Yes	2	2.1	0.3-7.3	0	0	0.0-3.8
Exon 20 insertions	Yes	1	1.0	0.0-5.7	2	2.1	0.3-7.4
EGFR mutations TKI sensitivity	TKI Resistant	57	59.4	48.9-69.3	92	97.9	92.5-99.7
	TKI Sensitive	39	40.6	30.7-51.1	2	2.1	0.3-7.5
	Missing	0	-	-	1	-	-

AC = Adenocarcinoma; SCC = Squamous Cell Carcinoma; WT = Wild type. TKI resistant: T790M, Exon 20 insertions + WT; TKI sensitive: Exon 19 deletions, L858R, L858M and G719X. Note: Two patients in the AC cohort with L858R mutation also had a T790M mutation and were classified as TKI resistant. *So far, the V819V silent mutation has not been demonstrated associated with resistance or sensitivity to TKI. Consequently, the patient carrying this mutation has been excluded from the analyses using the TKI resistant/sensitive dichotomy. N = number of specimens; n = number of specimens in a given category; % = n/Number of specimens with available results × 100; CI = Confidence interval (Source: ONCO RD-008 study report (17 June 2014), Table 15).

PRAME and 42.9% (9/21) for tumors expressing PRAME (**Table 6**).

In the SCC cohort, the rate of *EGFR* mutations was 5.3% (5/95) and the rate of TKI-sensitive *EGFR* mutations was 2.1% (2/95) (**Table 4**), too few to investigate any relationship with antigen expression.

Discussion

In this Chinese NSCLC population, the rate of expression of both MAGE-A3 and PRAME was about twice as high in SCC tumors as in AC tumors, whereas the rate of *EGFR* mutations was 43.8% in AC tumors and negligible in SCC tumors. With regard to MAGE-A3 expression and *EGFR* mutations, these observations are in line with several previous studies of NSCLC patients [30, 31].

The findings from our exploratory analyses for these Chinese patients contrast with those from a similar study investigating formalin-fixed paraffin-embedded (FFPE) tumor specimens from 1260 German NSCLC patients [32]. In this previous study, 29.8% of AC tumors expressed MAGE-A3, 44.1% expressed PRAME and 10.1% had *EGFR* mutations. Of the SCC tumors, 43.2% expressed MAGE-A3, 79.8% expressed PRAME, while the SCC tumors were not tested for *EGFR* mutations.

Although neither study actually documented the ethnicity of the patients included and with the proviso that they used different types of

Table 5. EGFR mutation status according to expression of MAGE-A3 or PRAME, AC patients

Categories	MAGE-A3-negative N = 81			MAGE-A3-positive N = 15				PRAME-negative N = 66			PRAME-positive N = 30			
	n	%	95% CI	n	%	95% CI	*p*-value	n	%	95% CI	n	%	95% CI	*p*-value
EGFR WT	49	60.5	49.0-71.2	5	33.3	11.8-61.6	0.087	39	59.1	46.3-71.0	15	50.0	31.3-68.7	0.51
EGFR Mutation	32	39.5	28.8-51.0	10	66.7	38.4-88.2		27	40.9	29.0-53.7	15	50.0	31.3-68.7	
TKI Resistant	52	64.2	52.8-74.6	5	33.3	11.8-61.6	0.043	41	62.1	49.3-73.8	16	53.3	34.3-71.7	0.50
TKI Sensitive	29	35.8	25.4-47.2	10	66.7	38.4-88.2		25	37.9	26.2-50.7	14	46.7	28.3-65.7	

AC = Adenocarcinoma; WT = Wild type; TKI resistant: T790M, Exon 20 insertions + WT; TKI sensitive: Exon 19 deletions, L858R, L858M and G719X; N = number of specimens; n = number of specimens in a given category; % = n/Number of specimens with available results × 100; CI = Confidence interval (Source: ONCO RD-008 study report (17 June 2014), Tables 17 and 20).

Table 6. EGFR mutation status according to antigen co-expression, AC patients

MAGE-A3 expression	PRAME expression	N	*EGFR* mutation status	AC (Total = 96)		
				n	%	95% CI
MAGE-A3-positive	PRAME-positive	9	*EGFR* WT	3	33.3	7.5-70.1
			EGFR mutation	6	66.7	29.9-92.5
	PRAME-negative	6	*EGFR* WT	2	33.3	4.3-77.7
			EGFR mutation	4	66.7	22.3-95.7
MAGE-A3-negative	PRAME-positive	21	*EGFR* WT	12	57.1	34.0-78.2
			EGFR mutation	9	42.9	21.8-66.0
	PRAME-negative	60	*EGFR* WT	37	61.7	48.2-73.9
			EGFR mutation	23	38.3	26.1-51.8

AC = Adenocarcinoma; SCC = Squamous Cell Carcinoma; WT = Wild type; N = number of specimens in a given category of MAGE-A3 and PRAME co-expression; n = number of specimens in a given category; % = n/Number of specimens with available results × 100; CI = Confidence interval (Source: ONCO RD-008 study report (17 June 2014), Table 25).

tumor tissue and detection assays, the differences between the two studies are in-line with ethnic differences demonstrated previously. Indeed previous findings have shown much higher rates of *EGFR* mutations in AC tumors in Asian patients (40.3-78.9%) [31, 33-36]; 43.8% in the current study, than in European patients 10.1%-21.3%) [24, 32, 37, 38]. By contrast, the rates of tumors expressing MAGE-A3 or PRAME were lower in the Chinese patients, most markedly for AC tumors.

In a previous study by Thongprasert and colleagues [36] of MAGE-A3 and PRAME expression in FFPE tumor tissue specimens from 377 East Asian NSCLC patients, 20% of the AC tumors expressed MAGE-A3 and 36.4% expressed PRAME, while of the SCC tumors 36.4% expressed MAGE-A3 and 80.0% expressed PRAME. In that study, explorative univariate regression analyses with antigen expression as the dependent variable and available patient and tumor characteristics as the explanatory factors showed an identical pattern for MAGE-A3 and PRAME. They found significantly higher antigen expression rates for men than for women, for (ex)-smokers than for non-smokers, for larger than for smaller tumors and for SCC than for AC tumors. The differences in subset point estimates of antigen expression rates observed in the present study were consistent with the findings of the Thongprasert study [36], although this was more pronounced in the SCC than in the AC cohort. It should be noted, however, that in the Thongprasert study gender was not found to be an independent explanatory factor when controlled for smoking status in a multivariate logistic regression analysis [36], and so may have a confounding effect in the analysis of smoking status.

Combined, the findings of these two studies indicate that the lowest rates of MAGE-A3 or/and PRAME expressing tumors are observed in patient subsets (women, non-smokers, AC tumors) that generally have been found to have the highest rate of *EGFR* mutations [39, 40]. The highest frequencies of expression of MAGE-A3 and PRAME are thus found in subsets of NSCLC patients with low rates of *EGFR* mutation, i.e., patients who are less likely to respond to treatment with *EGFR*-TKIs.

Of the AC tumors with *EGFR* mutations, 19/42 had exon 19 deletions and 21/42 had L858R point mutation. This is in line with several previ-

ous studies reporting that these two types of mutations account for approximately 85-90% of the lung cancer specific *EGFR* mutations [24, 36, 40].

A single study investigating the relationship between *EGFR* mutation status and MAGE-A expression has been identified but the detection of antigen expression was done by immunohistochemistry and so could not distinguish between the various proteins belonging to the MAGE-A family. This study found no statistically significant difference between *EGFR* wild type (WT) and mutated tumors in the proportion of MAGE-A expressing tumors [30]. The differences observed in our cohort of AC patients were not statistically significant, neither for MAGE-A3, which was expressed by 9.3% (5/54) of the WT tumors and by 23.8% (10/42) of the EGFR mutated (P = 0.09), nor for PRAME which was expressed by 27.8% (15/54) of the WT tumors and by 35.7% (15/42) of the EGFR mutated (P = 0.50). Due to the small number of specimens tested, a firm conclusion about the relationship between *EGFR* mutation status of AC tumors and their expression of MAGE-A3 and PRAME cannot be reached without additional studies with a larger number of patients to substantiate these findings.

TAA expression was investigated by qRT-PCR as mRNA expression although it may be argued that protein expression is more biologically relevant than mRNA expression [41], while analysis of protein function would be ideal from the biological point of view [42]. mRNA may for example be subject to post-transcriptional degradation or be retained in the nucleus and not translated into protein implying that mRNA expression testing would lead to an overestimation relative to the protein expression level [41]. The processing method used for archiving the tissue specimens may also lead to mRNA degradation but in the present study fresh frozen tissue was used which should lead to much less degradation than in tissues archived as formalin-fixed paraffin-embedded blocks [e.g., 43].

However, until the present no MAGE-A3-specific antibody to be used for protein expression detection by immunohistochemistry (IHC) has been identified, so IHC cannot be used to distinguish between the different members of the *MAGE-A* family, in particular *MAGE-A3* and *-A6*, which are 98% homologous [44]. Furthermore, IHC allows only a qualitative test, while the qRT-PCR assays used here lead to more precise, quantitative results (even though these are only reported in a semi-quantitative way, as positive or negative relative to a cut-off value). From a technical point of view, analyses of mRNA expression lead to more reproducible results than IHC [42].

In general, the relationships between gene amplification, mRNA levels and protein expression are not well understood and may also depend on the particular gene investigated and perhaps the tumor type. Although it is well established that there often is a lack of concordance between mRNA and protein concentration data [45], particular studies frequently report excellent concordance, for instance in breast cancer [42, 46], while the opposite may also be found, for instance in head and neck squamous cell carcinomas [41].

In conclusion, in this Chinese NSCLC population, the frequency of SCC tumors expressing MAGE-A3 and, in particular, PRAME was high, whereas very few SCC tumors had TKI-sensitive *EGFR* mutations. By contrast, forty percent of the AC tumors harbored TKI-sensitive *EGFR* mutations while their rates of MAGE-A3 or PRAME expressing tumors were about half the rates found among the SCC tumors. The highest proportions of patients who could potentially respond to immunotherapy targeted at MAGE-A3 or/and PRAME were thus found in patient subsets among whom relatively few could benefit from TKI-therapy.

Both antigens were frequently expressed in early stage tumors. This should encourage the development of antigen-specific immunotherapy for these patients, who are expected to be most able to benefit from this therapeutic approach. That the rates of antigen-positive tumors are much higher in SCC patients for whom molecularly targeted therapy is currently not available might favor selection of this large patient population for the evaluation of new therapeutic approaches including combinations of antigen-specific immunotherapy and conventional therapy.

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Disclosure of conflict of interest

None.

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Table S1. Primers and probes for MAGE-A3, PRAME and β-actin

Gene	Type	Sequence
MAGE-A3		
	TMF	GTC-GTC-GGA-AAT-TGG-CAG-TAT
	TMR	TGG-GGT-CCA-CTT-CCA-TCA-G
	TMP	5' FAM- AAA-GCT-TCC-AGT-TCC-TT--MGBNFQ 3'
PRAME		
	TMF	GAG-GCC-GCC-TGG-ATC-AG
	TMR	CGG-CAG-TTA-GTT-ATT-GAG-AGG-GTT-T
	TMP	5' FAM--TGC-TCA-GGC-ACG-TGA-T--MGBNFQ 3'
β-actin		
	TMF	CTG-GAA-CGG-TGA-AGG-TGA-CA
	TMR	CGG-CCA-CAT-TGT-GAA-CTT-TG
	TMP--reverse	5' VIC-TGC-TCG-CTC-CAA-CC--MGBNFQ 3'

Table S2. Primers for amplification of exons 18, 19, 20 and 21 of the EGFR gene from genomic DNA

Exon	Sequence
18	F: CAA ATG AGC TGG CAA GTG CCG TGT C
	R: CCA AAC ACT CAG TGA AAC AAA GAG
19	F: GCA ATA TCA GCC TTA GGT GCG GCT C
	R: CAT AGA AAG TGA ACA TTT AGG ATG TG
20	F: CCA TGA GTA CGT ATT TTG AAA CTC
	R: CAT ATC CCC ATG GCA AAC TCT TGC
21	F: ATG AAC ATG ACC CTG AAT TCG G
	R: GCT CAC CCA GAA TGT CTG GAG A

(Source: Adapted from Tsai TH, Sy KY, WU SG et al. RNA is favorable for analysing EGFR mutations in malignant pleural effusion of lung cancer. Eur Respir J 2012; 39: 677-84).