Myeloid-derived suppressor cells infiltration in non-small-cell lung cancer tumor and MAGE-A4 and NY-ESO-1 expression

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Abstract. Cancer/testis antigens melanoma-associated antigen 4 (MAGE-A4) and New York esophageal squamous cell carcinoma-1 (NY-ESO-1) are of clinical interest as biomarkers and present valuable targets for immunotherapy; however, they are poor prognostic markers in non-small cell lung cancer (NSCLC). In addition, myeloid derived suppressor cells (MDSCs) are recognized as a key element in tumor escape and progression. The aim of the present study was to investigate the diagnostic and prognostic value of MAGE-A4 and NY-ESO-1, and their association with MDSCs in NSCLC samples. The expression levels of MAGE-A4 and NY-ESO-1, and the infiltration of MDSCs ($CD33^+$), were analyzed by immunohistochemistry of 67 tissue samples from patients with NSCLC. Overall, 58.33% of the NSCLC squamous cell carcinoma tissues and 94.7% of adenocarcinoma tissues were positive for MAGE-A4. NY-ESO-1 expression was observed in 52.78% of the squamous cell carcinoma tissues and 80% of the adenocarcinoma tissues. In primary adenocarcinoma tumor tissues, MAGE-A4 and NY-ESO-1 demonstrated a higher intensity of expression compared with the squamous cell carcinoma tissues. A total of 33 (91.7%) squamous cell carcinoma and 19 (95.0%) adenocarcinoma specimens were positive for CD33. The expression of MAGE-A4 and NY-ESO-1 antigens and infiltration of MDSCs was associated with poor prognosis of patients with NSCLC. Further studies investigating the association between these findings and underlying molecular mechanisms are required.

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Introduction

As the leading cause of cancer-associated death worldwide in 2018, lung cancer causes significant challenges for cancer researchers (1). Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for ~85% of all cases (2). The long-term prognosis of patients with lung cancer is poor and the overall 5-year survival rate has been reported to be as low as 18% in USA according to the American Cancer Society (3).

Recent evidence of the clinical efficacy of immunotherapeutic approaches, including chimeric antigen receptor, T-cell therapy, immune checkpoint blockade and vaccine therapy (4-6), for lung cancer suggests that immunotherapy will become the next major therapeutic advance for this disease. Vaccines include antigen specific therapies, which induce specific antitumor immunity against relevant tumor-associated antigens. The cancer/testis (CT) family of antigens, including melanoma-associated antigen 4 (MAGE-A4) and New York esophageal squamous cell carcinoma-1 (NY-ESO-1), has been a focus of previous studies (7,8) due to their potential as immunotherapeutic targets (9,10). Furthermore, experimental studies have shown the ability of CT antigens to elicit a specific cellular response, including cytotoxic T lymphocytes (CTLs), and humoral immune responses (9,11-14). The presence of high numbers of CTLs in the tumors of patients with NSCLC is associated with enhanced survival (15-17); however, contrasting results demonstrated that high expressions of CT antigens were associated with poor survival in patients with lung cancer (18,19). A possible reason for this may be the accumulation of myeloid derived suppressor cells (MDSCs) in peripheral lymphoid organs and tumor tissues (20,21).

MDSCs represent a heterogeneous population of immature myeloid cells that can strongly inhibit anti-tumor activities of T and NK cells and stimulate regulatory T cells (Treg), leading to tumor progression. Furthermore, MDSCs can contribute to patient resistance to immunotherapy (22,23). Several studies have examined the association between the MAGE-A4 and NY-ESO-1 expression levels and survival (18,24-29), as well as the association between the MDSCs infiltrated in the tumor microenvironment and the survival of patients with different types of lung cancer (30-33). However, the prognosis of the association between the MAGE-A4 or NY-ESO-1 and MDSCs

has yet to be confirmed. The present study aimed to disclose the association between the expression levels of MAGE-A4 or NY-ESO-1 and MDSCs infiltration in patients with NSCLC.

Materials and methods

NSCLC tissue selection. A total of 67 cases of NSCLC were retrieved from the archives of the Zibo Central Hospital between February 2010 and March 2015. The patients' records included clinical data, preoperative examination results, details of surgical operations, histopathological findings and Tumor Node Metastasis (TNM) staging (34). The preoperative assessments included magnetic resonance imaging of the brain, bronchoscopy and bone scintigraphy. None of the patients underwent radiation or chemotherapy before surgery. The present study was approved by The Ethics Committee of Zibo Central Hospital (Zibo, China) in accordance with the Declaration of Helsinki and all procedures were approved by the Institutional Review Board of Zibo Central Hospital (Zibo, China). Tissue samples, including lung cancer specimens and normal tissues adjacent to tumor, were collected after informed consent was provided by all patients. The histopathological subtype, stage and grade of the tumors were determined by four pathologists according to the guidelines of the World Health Organization Classification of Lung Tumors (The 2015 World Health Organization Classification of Lung Tumors) (35). For survival analysis, follow-up was also performed. Patient survival was calculated as the time between surgery and mortality. Patients who were still alive at the time of data collection were censored in the statistical analysis (Table I).

Immunohistochemistry (IHC). IHC was performed according to the following protocol. Briefly, 0.9% saline washed-surgical resections were cut into 4 mm thickness and fixed with 10% formaldehyde overnight at room temperature. After dehydration with increasing concentration of ethanol (50, 70, 90 and 100%) and cleaning with xylene, tissues were embedded with paraffin (36). Consecutive sections from paraffin-embedded tissue blocks were cut into 4-μm sections, deparaffinized and rehydrated with xylene and descending ethanol (100, 90, 70 and 50%). Endogenous peroxidase activity was blocked by a 10-min incubation with 0.3% hydrogen peroxide in methanol at room temperature. Epitope retrieval was performed using 1 mM EDTA buffer (pH 8.0) in a microwave for 15 min, followed by cooling for 20 min at room temperature. Sections were washed with PBST and blocked with 10% normal goat serum (cat. no. G9023; Sigma-Aldrich; Merck KGaA) for 30 min at room temperature. The expression of NY-ESO-1, MAGE-A4 and CD33 in lung cancer and normal tissues was detected by incubation with primary monoclonal antibodies against NY-ESO-1 (1:200; cat. no. 35-6200; Thermo Fisher Scientific, Inc.), MAGE-A4 (1:150; cat. no. ab139297; Abcam) and CD33 (1:100; cat. no. 303402; BioLegend, Inc.) at 4˚C overnight. Sections were washed three times with PBST and were incubated with goat anti-mouse secondary antibody, HRP (1:2,000; cat. no. 62-6520; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Sections were washed three times with PBST and were incubated with 5% 3,3-diaminobenzidine for 10 min at room temperature until brown colors developed. Slides were counterstained with hematoxylin and mounted on glass coverslips. Slides were viewed under bright field using an upright microscope BX63 (Olympus Corporation), and representative areas were photographed using a CCD camera and processed using Olympus Image Analysis software (Olympus Stream 1.9; Olympus Corporation). The levels of MAGE-A4, NY-ESO-1 and CD33 expression were determined using a semi-quantitative four-grade scoring system (+, 5-25%; ++, 25-49%; +++, 50-75%; ++++, >75% of cells stained). Focal staining of single cells or small clusters (<5% total) was considered as negative staining. There was no significant difference between the expression levels of MAGE-A4, NY-ESO-1 and CD33 in patients with NSCLC using the four-grade scoring system. Patients were therefore classified into positive and negative groups. The positive group included tissues stained from + to ++++. Individual core counts from five replicates were available for most cases.

Statistical analysis. IHC data was evaluated using a χ^2 test. Pearson's correlation analysis was used to confirm the association between variables. The Kaplan-Meier method was used to estimate the probability of survival and survival differences were analyzed using a log-rank test. The receiver operating characteristic (ROC) curve was used to determine the optimal cut-off values for the sensitivity and specificity of both CT antigens and CD33 in NSCLC prognosis. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed using SPSS version 10.0 software (SPSS, Inc.).

Results

Clinicopathological parameters. The clinicopathological characteristics of the 67 patients with lung cancer included in this study are summarized in Table I. The average age of patients was 66 years (age range, 38-79 years). Patients included 51 men (76.12%) and 16 women (23.88%). Pathologically, 36 (53.73%), 20 (29.85%), 11 (16.42%) patients were diagnosed with squamous cell carcinoma (SCC), adenocarcinoma, and other pathologies than adenocarcinoma or SCC, including composite large-cell neuroendocrine carcinoma and squamous cell carcinoma, composite large-cell carcinoma and squamous cell carcinoma, composite clear cell carcinoma and squamous cell carcinoma, large cell (undifferentiated) carcinoma and adenosquamous carcinoma. The pathological stages were at Ia, Ib, IIa, IIb and IIIa in 11, 38, 12, 5 and 1 patients, respectively. Clinical follow-up was available for all cases. The median follow-up period was 48 months (range, 6-89 months).

MAGE-A4, NY-ESO-1 and CD33 are upregulated in NSCLC tissues according to IHC. Expression levels of MAGE-A4 and NY-ESO-1 in NSCLC specimens (specially focusing on squamous cell carcinoma and adenocarcinoma) were analyzed using IHC staining. To evaluate the immune suppressor cells infiltrating the tumor microenvironment, CD33 was detected using IHC. Furthermore, no staining of normal tissue adjacent to the positively stained tumors was detected (Fig. 1). The semi-quantitative results of immunohistochemical staining with MAGE-A4, NY-ESO-1 and CD33 monoclonal antibodies are shown in Tables II-V.

Table I. Clinicopathological parameter of patients with primary non-small cell lung cancer.

Parameter	n	%
Total	67[a]	
Age, years		
>60	51	76.12
≤60	16	23.88
Median (range)	66 (38-79)	
Sex		
Male	51	76.12
Female	16	23.88
Histology		
Squamous cell carcinoma	36	53.73
Adenocarcinoma	20	29.85
Other	11	16.42
pT status		
pT1a	19	28.36
pT1b	34	50.75
pT2a	8	11.94
pT2b	5	7.46
pT3	1	1.49
Unknown		
pN status		
pN0	51	76.12
pN1	16	23.88
Systemic metastasis (before operation)		
No metastasis	67	100
Stage		
Ia	11	16.42
Ib	38	56.72
IIa	12	17.91
IIb	5	7.46
IIIa	1	1.49
Grade		
Well-differentiated	8	11.94
Moderately differentiated	13	19.40
Poorly differentiated	46	68.66
Unknown		
Median follow-up (range), months	48 (6-89)	

[a]One case missed their follow-up.

In terms of MAGE-A4, 71.4% of NSCLC tissues demonstrated positive staining. According to the histological types, the positive expression rates of MAGE-A4 in patients with squamous cell carcinoma and adenocarcinoma were 58.33 and 94.7% (P=0.004), respectively (Table II). Regarding NY-ESO-1, NSCLC tissues demonstrated relatively low positive staining (62.5%). Considering the histological types, the positive expression rates of NY-ESO-1 were 52.78% in squamous cell carcinoma samples and 80% in adenocarcinoma samples (P=0.04; Table II). The MAGE-A4 and NY-ESO-1 double expression rate was 70% in adenocarcinoma tissues, which was significantly higher compared with the expression rate in squamous carcinoma (38.89%; P=0.026; Table III). In each histological type, tumor differentiation was associated with the expression of MAGE-A4 and NY-ESO-1 antigens. As shown in Table II, 92.90% of NSCLC tissues were positive for CD33 expression. The positive expression rates of CD33 in squamous cell carcinoma and adenocarcinoma were 91.7 and 95.0% (P=0.6; Table II).

According to sex, the MAGE-A4-positive expression rate was significantly higher in women (84.6%) compared with men (65.1%; P<0.01; Table IV). NY-ESO-1 also demonstrated a greater expression in females compared with males (69.2 vs. 53.5%; P<0.05; Table IV). As for CD33, there was a similar expression rate in women (84.6%) and men (90.1%). The expression of both CT antigens and CD33 in NSCLCs was not correlated with patients age, TNM-pT and stage (data not shown).

Association between MDSC infiltration and combined patterns of CT antigens expression. A total of four combined expressions of MAGE-A4 and NY-ESO-1 were evaluated to determine the association with MDSC infiltration (Table III). Combined positive expression for CD33, NY-ESO-1 and MAGE-A4 was significantly higher in adenocarcinoma tissues (55.00%) compared with squamous carcinoma tissues (36.11%; P=0.171). The double expression rates of CD33/MAGE-A4, CD33/NY-ESO-1 and MAGE-A4/NY-ESO-1 were higher in squamous cell carcinoma compared with adenocarcinoma tissues (Table III). A χ^2 test was used to analyze the association between MAGE-A4 and NY-ESO-1 expression and between CT antigens and CD33 expression. The group with positive MAGE-A4 or NY-ESO-1 staining demonstrated a higher number of infiltrating MDSCs compared with the groups with negative MAGE-A4 or NY-ESO-1 expression (P<0.005; Table V). In addition, the expression levels of both CT antigens in NSCLC were correlated (Pearson's r=0.411; P=0.002; Table V).

The association between the CT antigens expression and CD33 was analyzed using a χ^2 test. There was no significant association between CT antigens expression and MDSC infiltration, although CD33 was expressed more frequently in CT antigens patients with positive expression compared with patients with negative expression (Tables III and V).

Prognostic value of MAGE-A4, NY-ESO-1 and CD33 expression. By using log-rank test, CD33 expression was significantly associated with survival rate (P=0.03; Fig. 2A). MAGE-A4 and NY-ESO-1 expression levels were identified to be significantly associated with prognosis in terms of survival rate (Fig. 2B and C; P=0.005 and P=0.001, respectively). Subsequently, tissues were classified based on pathological type to examine the prognostic value of CT antigens and MDSC infiltration. By contrast, no association was identified between histology type and survival rate in patients positive in both CT antigen expression and CD33 expression (Fig. 2D-F), although there was a higher expression of CT antigens in adenocarcinoma compared with patients with squamous cell cancer (Table II; MAGE-A4, 58.33% positive expression in squamous cell cancer and 94.7% in

Table II. MAGE-A4, NY-ESO-1 and CD33 expression intensity in non-small cell lung cancer specimens.

	MAGE-A4						NY-ESO-1						CD33					
	Total n	Extent of staining[a]					Total n	Extent of staining[a]					Total n	Extent of staining[a]				
Histology	(% positive)	-	+	++	+++	++++	(% positive)	-	+	++	+++	++++	(% positive)	-	+	++	+++	++++
Squamous cell carcinoma	36 (58.33)	15	7	5	5	4	36 (52.78)	17	8	3	4	4	36 (91.70)	3	11	16	6	0
Adenocarcinoma	20 (94.70)[a]	1	3	1	6	9	20 (80.00)[b]	4	0	2	11	3	20 (95.00)[c]	1	1	8	7	3
Total n (% positive)	40 (71.40)						35 (62.50)						52 (92.90)					

-, negative; +, 5-25%; ++, 25-49%; +++, 50-75%; ++++, >75% of the tumor cells stained. [a]P=0.004, [b]P=0.04 and [c]P=0.6. MAGE-A4, melanoma-associated antigen 4; NY-ESO-1, New York esophageal squamous cell carcinoma-1.

adenocarcinoma; NY-ESO-1, 52.78% positive expression in squamous cell cancer and 80% in adenocarcinoma). These results demonstrated that a poor prognosis was associated with positive CT antigen and CD33 expression in patients with squamous cell cancer and adenocarcinoma.

Prognostic value of MDSCs in patients with MAGE-A4 or NY-ESO-1-positive expression. In order to evaluate the association between MDSCs and prognosis in patients positively expressing CT antigens, the associated between the CT antigens expression and the infiltration of MDSCs into the tumor site was analyzed (Fig. 3). In tissues positive for MAGE-A4, there was a significant difference between CD33-positive expression and CD33-negative expression in terms of survival rate (P=0.013; Fig. 3A). Compared with CD33-positive expression, the survival rate of patients with negative CD33-negative staining was improved, whereas there was no significant difference survival rate between in NY-ESO-1-positive and NY-ESO-1-negative cases (P=0.054; Fig. 3B). For further analysis of the sensitivity and specificity of both CT antigens and CD33 in NSCLC prognosis, a receiver operating characteristic (ROC) curve was used to analyze the present results. The area under the ROC curve was 0.60, 0.626 and 0.721 for expression of MAGE-A4, NY-ESO-1 and CD33, respectively (Fig. 4).

Discussion

The evidence of clinical efficacy for immunotherapeutic approaches for lung cancer suggests that immunotherapy will become the next major therapeutic advance for this disease (5,37,38). NSCLC has historically been considered as a nonimmunogenic disease (2). Previous data has shown that much of this lack of immune responsiveness to lung cancer is due to high expression of CT antigens, which are expressed in the normal testis and placenta, but may also be expressed in tumor tissues (39-44). Thus, it is essential to determine the association between expression levels of CT antigens and prognosis of patients with lung cancer. In the present study, the expression of MAGE-A4 and NY-ESO-1 was analyzed by immunohistochemistry of 67 tissue samples from patients with NSCLC, and the survival of these patients was assessed. Patients with high expression of both CT antigens exhibited a poor prognosis, which was consistent with previous studies (19,43,45). This may be due to the immunosuppressive microenvironment of the tumor (19). Regardless of the fact that MAGE and NY-ESO-1 can trigger a strong immune reaction by stimulating lymphocyte migration into the tumor microenvironment, these T cells do not readily translate to tumor cell killing *in vivo* (9,19).

Tumor infiltrating lymphocytes are important factors in the antitumor immune response, which are associated with cancer incidence, tumor growth, response to therapy and the prognosis of the disease (46). Intensive infiltration of CTLs into the tumor nest is associated with good patient prognosis in several tumor types (47-51). Tumor-specific CTLs recognizing MAGE-A4 and NY-ESO-1 have been reported and several CTL epitopes within MAGE and NY-ESO-1 proteins have been identified (9,52,53). However, the present study did demonstrate an association between high expression of

Table III. Combined positive expression of cancer/testis antigens MAGE-A4 and NY-ESO-1 and CD33.

Histology	n	CD33/MAGE-A4/ NY-ESO-1 n (%)	CD33/MAGE-A4 n (%)	CD33/NY-ESO-1 n (%)	MAGE-A4/NY-ESO-1 n (%)
Squamous cell carcinoma	36	13 (36.11)	18 (50)	17 (47.22)	14 (38.89)
Adenocarcinoma	20	11 (55)	15 (75)	11 (55)	14 (70)
P-value	0.171	0.068	0.5778	0.026	

MAGE-A4, melanoma-associated antigen 4; NY-ESO-1, New York esophageal squamous cell carcinoma-1.

Table IV. CT antigens, CD33 expression and clinicopathologic parameters in NSCLC.

		MAGE-A4		NY-ESO-1		CD33	
Sex	n	Negative n (%)	Positive n (%)	Negative n (%)	Positive n (%)	Negative n (%)	Positive n (%)
Male	43	15 (34.9)	28 (65.1)	20 (46.5)	23 (53.5)	4 (9.1)	39 (90.1)
Female	13	2 (15.4)	11 (84.6)	4 (30.8)	9 (69.2)	2 (15.4)	11 (84.6)
P-value			<0.01		<0.05		

MAGE-A4, melanoma-associated antigen 4; NY-ESO-1, New York esophageal squamous cell carcinoma-1.

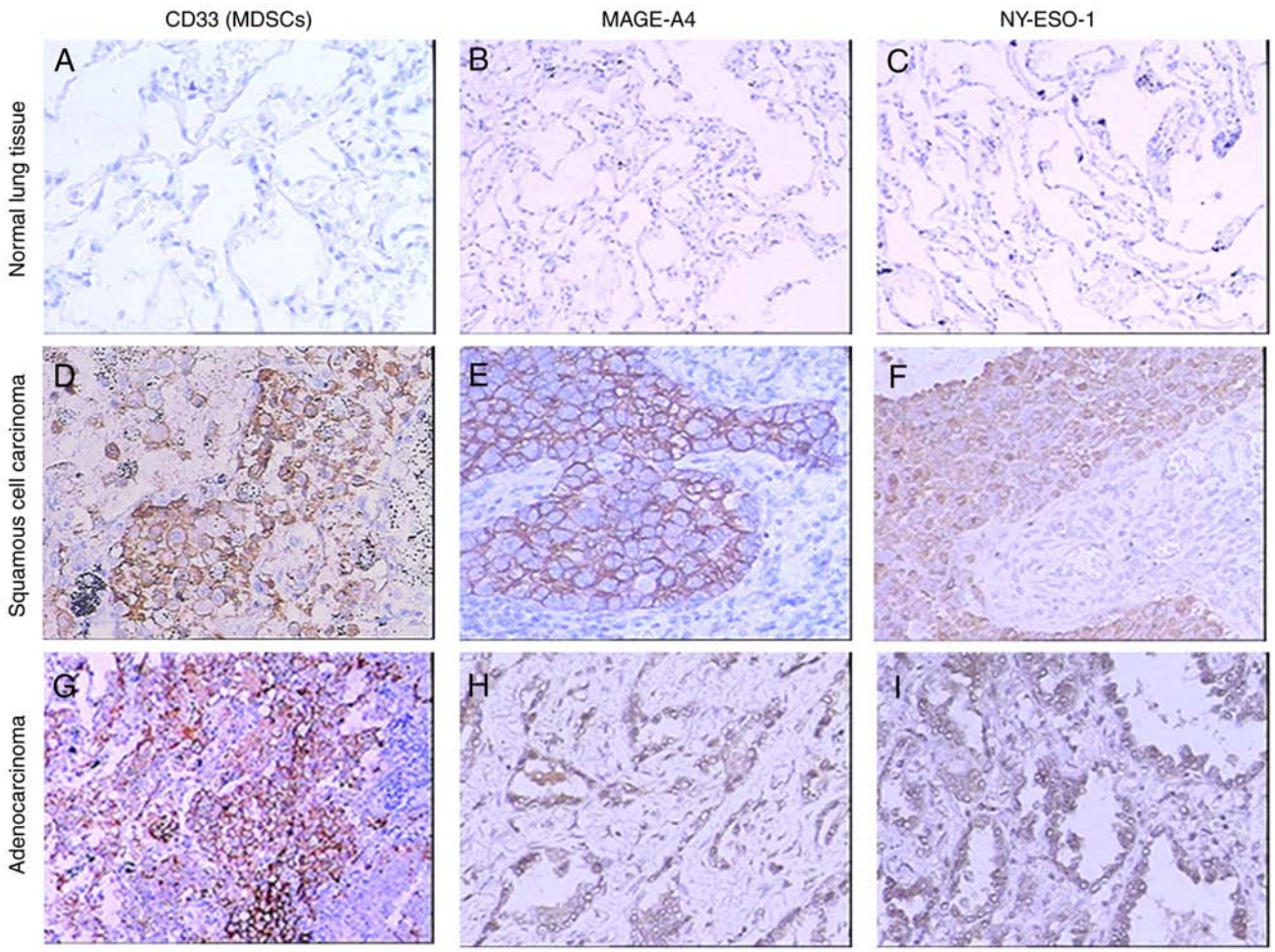

Figure 1. Immunohistochemical findings for MAGE-A4, NY-ESO-1 and CD33 in non-small cell lung cancer tissues. Specimens from the normal lung were used as a negative control. Staining of the normal lung demonstrated no reactivity for (A) CD33, (B) MAGE-A4 and (C) NY-ESO-1. Expression of (D) CD33, (E) MAGE-A4 and (F) NY-ESO-1 in squamous cell carcinoma. Expression of (G) CD33, (H) MAGE-A4 and (I) NY-ESO-1 in adenocarcinoma. Magnification, x100. MDSCs, myeloid derived suppressor cells; MAGE-A4, melanoma-associated antigen 4; NY-ESO-1, New York esophageal squamous cell carcinoma-1.

Table V. Multiple expression of CT antigens and CD33 in NSCLCs.

		MAGE-A4 (positive)		NY-ESO-1 (positive)			CD33 (positive)			MAGE-A4 (positive)[b]			NY-ESO-1 (positive)	
Histology	n	CD33 (negative) n (%)	CD33 (positive) n (%)	n	CD33 (negative) n (%)	CD33 (positive) n (%)	n	MAGA- 4 (negative) n (%)	NY-ESO-1 (negative) n (%)	n	NY-ESO-1 (negative) n (%)	NY-ESO-1 (positive) n (%)	n	MAGE-A4 (negative) n (%)	MAGE-A4 (positive) n (%)
Squamous cell carcinoma	21	3 (14.3)	18 (85.7)[a]	18	1 (5.6)	17 (94.4)[a]	33	15 (45.4)	16 (48.5)	21	7 (33.3)	14 (66.7)	18	4 (22.2)	14 (77.8)
Adenocarcinoma	18	3 (16.7)	15 (83.3)[a]	14	3 (21.4)	11 (78.6)[a]	17	1 (5.9)	6 (35)	18	1 (0.06)	17 (94.4)	14	0	14 (100)

[a]P<0.005. [b]Pearson's, r=0.411, P=0.002. MAGE-A4, melanoma-associated antigen 4; NY-ESO-1, New York esophageal squamous cell carcinoma-1.

MAGE-A4 or NY-ESO-1 and a poor prognosis in patients with NSCLC. One possible explanation for this apparent contradiction may be some association with suppressive immune cells, including MDSCs (54,55).

The immune system plays a paradoxical role in the response to tumors by either preventing tumor growth or by permitting tumor escape and stimulating tumor development (7). MDSCs are a group of immature immune cells, which normally are not found in the circulation but accumulate in the blood and tumor of patients with cancer (56,57). MDSCs are involved in immune evasion of tumors (21). The importance of MDSCs in cancer-related immunosuppression is evident by the inhibitory effect on T cell proliferation and function and the fact that removal of MDSCs can restore T cell effector function (58,59). Until now, the role of MDSCs in the expression of MAGE-A4 or NY-ESO-1 has remained elusive. Thus, the present study aimed to address the integrated relationship among the expression level of MAGE-A4 and NY-ESO-1, overall survival and infiltration of MDSCs into the tumor site in patients with NSCLC.

The positive frequency of MAGE-A4, NY-ESO-1 and CD33 expression in NSCLC in present study was higher than that in previous studies (28,60). Notably, MAGE-A4 and NY-ESO-1 were more highly expressed in adenocarcinoma compared with in squamous cell carcinoma tissues. These results are inconsistent with previous research (61). Intra-tumoral heterogeneity may partly explain the different extent to which certain CT antigens were re-expressed in tumors in the present study compared with previous work (62,63). In addition, discrepancies between RNA and protein expression levels are not uncommon and may contribute to the variety of expression levels reported (64); however, the association between CT antigen expression and disease development and tumor malignancy remains unclear, including NSCLC (65-69). It has been hypothesized that there is no association between the expression of CT antigens and sex (70,71); however, the present study demonstrated that a higher proportion of NSCLC tissue samples from female patients stained positive for CT antigens compared with samples from males. The genes that encode MAGE-A4 and NY-ESO-1 map to the X chromosome and are referred to as CT-X genes (62,72). This may be indicative of the association of CT antigens with the female sex and could be associated with a dominance of female patients within adenocarcinoma group showing a higher expression of CT antigens (73-76). Regarding cancer stage, CT antigen expression patterns are associated with disease stage and no expression of CT antigens has been observed in benign tissues (64). However, the present study demonstrated a weak association between tumor stage and the expression of both CT. The possibility may be not at advanced stage with these observed samples.

As for survival, the high expression of MAGE-A4 and NY-ESO-1 was a prognostic marker for a less favorable prognosis in patients with NSCLC. These results were indicative of a possible role of MAGE-A4 and NY-ESO-1 in determining greater malignant potential in types of lung cancer, although the underlying mechanism of function of these CT antigens in tumor biology has not been fully elucidated. The poor prognostic association of MAGE-A4 and NY-ESO-1 in lung cancer suggests that the development of therapy targeting both

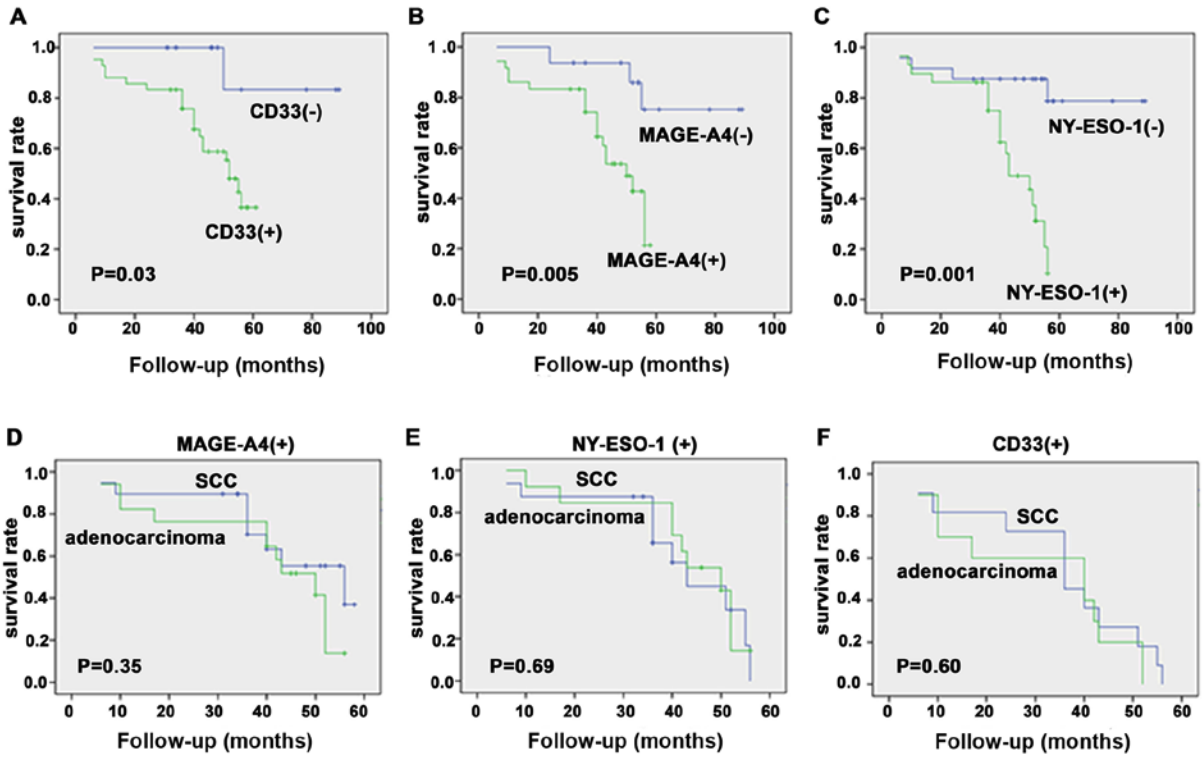

Figure 2. Survival analysis of patients with lung cancer. Patients with squamous cell cancer and adenocarcinoma were analyzed by Kaplan-Meier method. Pair-wise differences were analyzed using the log-rank test. (A) CD33 expression and survival rate. (B) MAGE-A4 expression and survival rate. (C) NY-ESO-1 expression and survival rate. (D) MAGE-A4-positive expression and survival rate for patients with SCC or adenocarcinoma. (E) NY-ESO-1-positive expression and survival rate for patients with SCC or adenocarcinoma. (F) CD33-positive expression and survival rate for patients with SCC or adenocarcinoma. SCC, squamous cell cancer; MAGE-A4, melanoma-associated antigen 4; NY-ESO-1, New York esophageal squamous cell carcinoma-1.

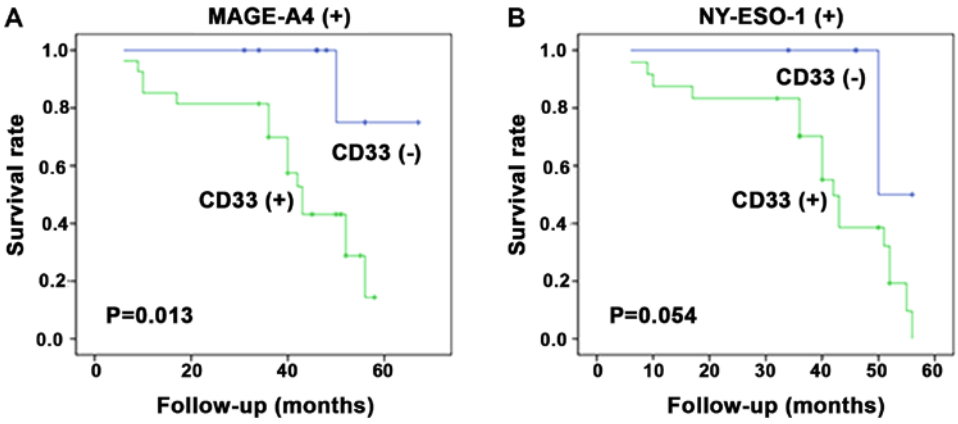

Figure 3. Survival analysis of patients with lung cancer with positive MAGE-A4 and NY-ESO staining based on positive or negative CD33 expression. Data were analyzed by Kaplan-Meier method. Pair-wise differences were analyzed using the log-rank test. (A) CD33 expression and survival rate for patients with positive expression of MAGE-A4. (B) CD33 expression and survival rate for patients with positive expression of NY-ESO-1. MAGE-A4, melanoma-associated antigen 4; NY-ESO-1, New York esophageal squamous cell carcinoma-1.

of these CT antigens may be a potential novel treatment for patients with NSCLC.

There was no clear association between MDSCs infiltration and MAGE-A4 or NY-ESO-1-positive expression in the present study and the results were inconsistent with our previous animal experiment (77). This may be explained by variation among species, different immunohistochemical sensitive antibodies depending on laboratory conditions and tumor cell heterogeneity. Previously, ROC curve analysis was used to assess diagnostic tests, which could also be used to assess predictive models (78-82). Limited by sample size, the ROC curves in the present study were based on patient survival without a series of cut-off points. The results indicated that MAGE-A4, NY-ESO-1 and CD33 expression were better markers of the prognosis of patients with NSCLC.

When analyzing the prognosis in different pathological types, squamous cell cancer and adenocarcinoma, there was no significant difference in survival between these two pathological types; however, MAGE-A4 and NY-ESO-1 expression had a significant difference in expression between squamous

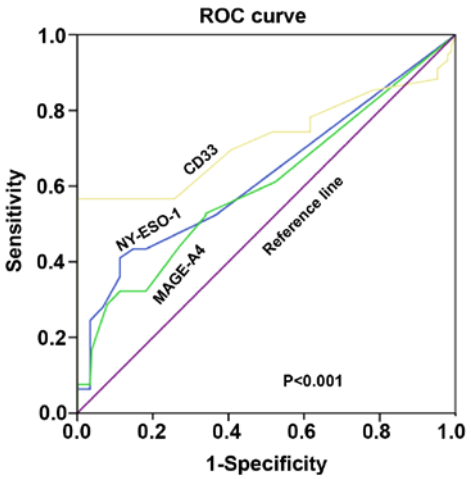

Figure 4. ROC curve of MAGE-A4, NY-ESO-1, CD33 expression and survival of patients with non-small cell lung cancer. The area under the curve for MAGE-A4, NY-ESO-1 and CD33 was 0.600, 0.626 and 0.721, respectively. ROC, receiver operating characteristic; MAGE-A4, melanoma-associated antigen 4; NY-ESO-1, New York esophageal squamous cell carcinoma-1.

cell cancer and adenocarcinoma. A possible reason for this may be that the similar expression rate of CD33 in squamous cell cancer and adenocarcinoma (83). The present results indicated that higher CD33 expression levels were correlated with poorer prognosis. Given MDSC inhibition of T cells activation in a nonspecific or antigen-specific manner (22,23), altering the dendritic cell peptide presenting ability of the major histocompatibility complex class I molecules on tumor cells (24,25), the functional analysis of $CD8^+$ T cells might help understanding how MDSC could undermine the activity of CD8+ T cells within tumor nests. (22,84).

The limitations of the present study should be noted. The sample size was small, as all data were obtained from 67 cases. Due to the heterogeneity in patients with lung cancer, survival may be affected by histology and staging. In present study, the survival of patients with squamous cell carcinoma and adenocarcinoma was analyzed. In our previous experiment, we found that tumor infiltrating MDSCs enhanced the expression of MAGE-A4 in an animal model (77). Thus, the purpose of present study was to investigate the association between the expression levels of MAGE-A4 or NY-ESO-1 and MDSCs infiltration in patients with NSCLC. Once there is positive or negative correlation between CT antigens expression and MDSCs the molecule and pathway which affects the protein expression should be investigated which may then be beneficial to cancer therapy. Given the immunogenicity of CT antigen and immune suppression of MDSC, CT antigen expression can be upregulated, which would induce anti-tumor immune response and MDSCs be downregulated, which would attenuate MDSCs-induced immunosuppression. However, the present study was limited by the sample size and no such correlation was observed, although there was a tendency to some degree between CT antigens expression and MDSC infiltrating in tumor microenvironment. In future research, considering that larger sample sizes produce more reliable results with greater precision and power (85-87), the study population should consist of long-surviving and short-surviving patients with NSCLC and the correlation between CT antigens expression and CTL infiltration or between CTL and MDSC infiltrations should be investigated.

In conclusion, the poor prognosis of patients with NSCLC with MAGE-A4 and NY-ESO-1 expressing tumors with high infiltrating MDSCs suggests that the spontaneous immune response is not sufficient against these antigens. The development of a combination therapy is required for patients with NSCLC with tumors expressing CT antigens, such as vaccinating with MAGE-A4 and NY-ESO-1 recombinant proteins or peptides. In addition, this combination therapy should combine with an inhibitor targeting CD33 to reduce the suppressive MDSCs.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GS was involved in the study design and data interpretation; drafting, revision and final approval of the manuscript. ZH performed the pathological evaluation of the specimens and participated in patients' follow-up. XL was responsible for the recruitment of the patients in the study and obtained the informed consent. XW and ZZ performed the immunohistochemistry staining. All authors had intellectual input into the study and read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Zibo Central Hospital (Zibo, China) in accordance with the Declaration of Helsinki, and all procedures were approved by the Institutional Review Board of Zibo Central Hospital (Zibo, China). Informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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