ORIGINAL ARTICLE

Case-Control Study: Smoking History Affects the Production of Tumor Antigen–Specific Antibodies NY-ESO-1 in Patients with Lung Cancer in Comparison with Cancer Disease–Free Group

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ABSTRACT

Introduction: Lung cancer is the leading cause of cancer mortality worldwide; therefore, understanding the biological or clinical role of tumor-associated antigens and autoantibodies is of eminent interest for designing antitumor immunotherapeutic strategies.

Methods: Here we prospectively analyzed the serum frequencies of New York esophageal squamous cell carcinoma 1 (NY-ESO-1), human epidermal growth factor 2/neu, and melanoma-associated antigen A4 (MAGE-A4) antibodies and expression of the corresponding antigens in tumors of 121 patients with NSCLC undergoing an operation without prior neoadjuvant chemotherapy and compared them with those in 57 control age-matched patients with no history of a malignant disease.

Results: We found that only antibodies specific for NY-ESO-1 (19.8% [n = 24 of 121]) were significantly increased in the group of patients with NSCLC compared with in the controls. NY-ESO-1 seropositivity was significantly positively associated with an active smoking history in patients with NSCLC but not in smokers from the control group. In tumors, the frequency of NY-ESO-1 mRNA expression was 6.3% (in four of 64 patients), the frequency of human epidermal growth factor 2/neu (HER 2/neu) expression was 11.9% (five of 42), and the frequency of MAGE-A4 expression was 35.1% (20 of 57). MAGE-A4 expression in tumors correlated with smoking status and male sex in patients with NSCLC. Patients with squamous cell carcinoma displayed higher expression of NY-ESO-1 and MAGE-A4 in tumors than did patients with adenocarcinoma. On the other hand, 94.7% of nonsmoking patients in our study had adenocarcinoma (of whom 73.7% were women).

Conclusion: These results confirm the reported high immunogenicity of NY-ESO-1 and suggest that a smoking-induced chronic inflammatory state might potentiate the development of NY-ESO-1–specific immune responses. Moreover, smoking might contribute to the expression of other cancer/testis antigens such as MAGE-A4 at early stages of NSCLC development.

Keywords: NY-ESO-1; Smoking; NSCLC; Tumor antigens; MAGE-A4 autoantibodies

Introduction

Lung cancer is the leading cause of mortality among malignant diseases, with a 5-year survival rate of 5% to 15%. Worldwide, it remains the most common type of

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malignancy in men, with the highest incidence in central and Eastern Europe (53.5 in 100,000).[1] With the number of new lung cancer cases rising every year by 3%, smoking of tobacco products is the main risk factor responsible for up to 90% of new carcinomas diagnosed. The link between smoking and lung cancer was shown in 1950,[2] and since then the role of smoking in carcinogenesis has been well established.[3] Cigarette smoke contains a multitude of toxic compounds[4] that can directly activate oncogenes or cause epigenetic changes to the genome. Smoking further contributes to tumor-promoting inflammation, impairs mucociliary clearance, or hampers the immune system.[5] Most cases of lung cancer (80%–85%) are NSCLC, which encompasses three histological subtypes: adenocarcinoma (AC), squamous cell carcinoma (SCC), and large cell carcinoma (5%). The ratio of the different histological subtypes of NSCLC has changed over the years from squamous cell carcinoma (which now accounts for approximately 30% of cases) to adenocarcinoma now being the most prevalent histological subtype (50% of cases). The remaining 15% to 20% of lung cancer cases are attributed to small cell carcinoma. An estimated 10% to 25% of lung cancers worldwide occur in never-smokers (i.e., individuals who have smoked fewer than 100 cigarettes in their lifetime).[6] Lung cancer in never-smokers encompasses mainly adenocarcinoma and occurs with higher frequency in women than in men (in Asia > in North America > in Europe). However, the mortality rates in women and men are similar. SCLC is very rare in never-smokers. The important determining factors for non–smoking-associated lung cancer include hereditary risks, secondhand smoking, air pollution, ionizing radiation, preexisting lung disease, diet, and hormonal changes.[6,7]

In general, the incidence and mortality of cancer remain high; therefore, there is an urgent need to detect early stages of the disease or monitor relapses after antitumor therapy. Serological detection of autoantibodies against tumor-specific or tumor-associated antigens represents an easy tool not only to identify diagnostic and prognostic markers but also to evaluate immune response to therapy in patients with cancer.[8] The most frequently analyzed humoral responses are against the antigens p53; mucin 1, cell surface associated; c-myc; New York esophageal squamous cell carcinoma 1 (NY-ESO-1); survivin; p62; human epidermal growth factor 2/neu (HER2/neu); and cyclin B1.[9] To date, however, the clinical value of these autoantibodies in malignant diseases remains open to discussion.[8,9] In lung cancer, the only commercially available autoantibody test consisting of seven antigens (p53, NY-ESO-1, cancer associated gene, GBU4-5, sex determining region Y-box 2, HuD protein and melanoma-associated antigen A4 [MAGE-A4]) has recently been validated in a case-control setting (1613 patients) and may be used as a tool complementary to cancer/testis (CT) antigens for detection of early stages of the disease.[10,11] However, although this test reached an outstanding 90% specificity, its sensitivity remains relatively low (approximately 41%).[10,11] Despite controversial results on the autoantibody prognostic or diagnostic values, understanding the characteristics and importance of tumor-specific humoral or cellular immune responses remains a prerequisite for the development of cancer detection and immunotherapeutic strategies.

In our previous unpublished study we observed that a history of active smoking correlated significantly with the frequencies of HER2/neu, NY-ESO-1, and MAGE-A4 tumor antigen–specific antibodies in the serum of patients with NSCLC (n = 32). The frequency of all three tumor-specific antibodies was higher in smokers and declined sharply in the groups of ex-smokers and nonsmokers. On the basis of these preliminary data, we designed a prospective case-control study to compare the frequencies of HER2/neu, NY-ESO-1, and MAGE-A4 tumor antigen–specific antibodies and the corresponding antigen expression in tumoral and nontumoral tissue in two independent cohorts of 121 patients with NSCLC (cohort I [n = 57] and (cohort II [n = 64]) and in an age-matched group of cancer-free patients (n = 57). We further evaluated the frequencies of autoantibodies and antigen expression in correlation with smoking history, sex, histological subtype, tumor grade, and stage of disease in patients with NSCLC.

Materials and Methods

Study Population

Peripheral blood was obtained from 57 patients undergoing an operation for NSCLC at the University Hospital Motol in Prague between October 2009 and March 2012 (cohort I). All patients with NSCLC were as far as possible individually matched by sex, age, and smoking history to a control individual with no previous history of a malignant disease, each with no sign of malignancy on a chest radiograph. These samples from control individuals were obtained from 57 patients undergoing an operation between January 2012 and May 2013. In addition to a pulmonary radiograph, a panel of tumor markers (carcinoembryonic antigen [CEA], tissue polypeptide antigen [TPA], neuron-specific enolase [NSE], SCC, and cytokeratin 19 fragment [CYFRA 21-1]) measured by enzyme-linked immunosorbent assay (ELISA) (in the case of TPA), chemiluminiscence immunoanalysis (in the case of CEA and SCC), and electrochemiluminiscence immunoanalysis (in the case of NSE and CYFRA 21-1) was used to compare the group of patients with cancer and the group of control patients. In cohort II the peripheral blood, tumor tissue, and/or

nontumoral tissue samples were obtained from 64 patients undergoing an operation for NSCLC at the University Hospital Motol in Prague between October 2012 and March 2016. All blood and tissue samples were collected with patients' written consent and the study was approved by the institutional review board of the University Hospital Motol. None of the patients enrolled in the study had received neoadjuvant chemotherapy before the operation. The clinicopathological characteristics of the cohorts of patients are summarized in Table 1. The complete set of samples for NY-ESO-1 analyses, which included serum, tumoral tissue, and nontumoral tissue samples, was obtained for only 40 patients. Reverse-transcriptase polymerase chain reaction (PCR) analyses of HER2/neu and MAGE-A4 antigen expression was performed in only 42 and 57 patient tumor samples, respectively.

qPCR Analysis of Antigen Expression

Total RNA was isolated from cell lysates using the RNeasy mini kit (Qiagen, Hilden, Germany). The RLT lysis buffer (Qiagen) contained 1% β-mercaptoethanol, and the extraction was performed in accordance with the manufacturer's protocol, including a DNA digestion step. The RNA concentration and purity was determined with a Nanodrop 2000c spectrophotometer (Fisher Thermo Scientific, Waltham, MA). Reverse trancription was performed from 1 μg of total RNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The probes were designed, synthetized, and approved by TIB Molbiol Syntheselabor GmBh (Berlin, Germany). Their sensitivity was tested previously in a study by Kloudova et al.[12] Expression of HER2/neu, NY-ESO-1, and MAGE-A4 was determined by quantitative PCR (qPCR) on a CFX96 Touch Real-Time PCR Detection System (BioRad). Each 10 μl of reaction mixture contained 5 μl of KAPA PROBE FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA), 0.5 μM each of forward and reverse primers (TIB Molbiol), 0.2 μM of TaqMan probe (TIB Molbiol), 1.5 μl of DNase-free water, and 2 μl of 10$\times$ diluted complementary DNA. Each reaction was done in duplicate. The reaction thermal protocol was as follows: 3 minutes at 95 °C followed by 45 cycles of amplification (at 95 °C for 15 seconds and 60 °C for 60 seconds). The formation of PCR products of the expected lengths was confirmed by agarose gel electrophoresis. The quantification cycle values were determined by using CFX Manager software (BioRad), and the relative levels of expression of the studied genes were calculated with GenEx software (MultiD Analyses, Göteborg, Sweden) with the cutoff at the 36th cycle. The cutoff for positive expression was set to relative expression values greater than the mean plus two SDs of the control nontumoral tissue sample.

Table 1. Clinicopathological Characteristics of Patients with NSCLC and Controls in the Study

	NSCLC (n = 121)		Controls (n = 57)	
Variable	Patients in Cohorts I/II	%	No.	%
No. patients	57/64	100/100	57	100
Age, y				
Mean	62/67		62	
Range	30-79/42-87		30-79	
Stage				
IA	13/10	22/16		
IB	11/15	19/23		
IIA	4/9	7/14		
IIB	8/7	14/11		
IIIA	15/18	26/28		
IIIB	3/1	5/2		
IV	3/4	7/6		
Histological subtype				
Squamous cell carcinoma	21/29	37/45		
Adenocarcinoma	30/35	53/55		
Others	6/0	11/0		
Differentiation				
Good (G1)	7/7	12/11		
Moderate (G2)	21/33	37/52		
Poor (G3)	20/16	35/25		
Undifferentiated	3/0	5/0		
Not specified	6/8	11/12		
Smoking history				
Smoker	30/35	53/55	29	51
Ex-smoker	18/16	32/25	19	33
Nonsmoker	9/10	16/16	9	16
Unknown	0/3	0/4	0	0

ELISA

The presence of antibodies against the three tumor antigens HER2/neu, NY-ESO-1, and MAGE-A4 in serum of patients with NSCLC and controls was detected by ELISA as adopted from Gnjatic et al.,[13] Long et al.,[14] and Stockert et al.[15] The recombinant proteins NY-ESO-1, HER-2/neu, and MAGE-A4 (Origene) were diluted in carbonate coating buffer (Invitrogen, Carlsbad, CA) to a final concentration of 1 μg/mL and coated to 96-well plates overnight at 4°C. Plates were blocked for 1 hour with Assay Buffer (Invitrogen). Human sera diluted to 1:100 and 1:200 were incubated in the antigen-coated wells for 2 hours. Plates were then incubated with secondary antibody (goat polyclonal antibody to human IgG [Abcam, Cambridge, United Kingdom]) for 1 hour. Tetramethylbenzidine substrate (Invitrogen) was added and incubated for 20 minutes. The reaction was stopped by adding Stop Solution (Invitrogen). Plates were immediately read with absorbance at 450 nm. As a positive control, the cytomegalovirus glycoprotein B protein was used.

Data Evaluation

Serum samples from 57 patients with NSCLC and 57 controls were analyzed by ELISA for the presence of NY-ESO-1, HER-2/neu, and MAGE-A4 autoantibodies. The new unthawed batch of serum samples from 57 control patients was reanalyzed for the presence of NY-ESO-1 antibodies, as were serum samples from cohort II (64 patients with NSCLC). The cutoff point of positive seroreactivity was defined as an optical density value greater than the mean plus two SDs of the controls. The patients with NSCLC and controls were divided into three groups—smokers, ex-smokers, and nonsmokers—and evaluated for seropositivity. Ex-smokers were defined as former smokers with at least a 1-year abstinence from smoking.[6] Samples from patients with NSCLC and controls for the assay were interspersed in the order in which the samples were assayed so that any batch effects would be spread over all sample types.

Statistical Methods

Pearson's chi-square test for cross-tables was used to compare frequencies in the groups according to the levels of studied factors (the zero hypothesis being that there is no statistical dependence between the studied factors, with the hypothesis refused for p values less than 0.05, as a result of which the dependency of factors is demonstrated). Pearson's chi-square test of good fit was used to compare frequencies in individual subgroups of the control group compared with the group of patients with cancer. The two-sample t test or its modification with separate variance estimates when the assumption of homogeneity of variance has been violated was used to evaluate the dependence of the average tumor marker level (continuous variables) on the seropositivity of specific antitumor antibodies (the zero hypothesis being that the average tumor marker level is equal in both groups, with the hypothesis refused for $p < 0.05$, as a result of which a difference in average levels is demonstrated). Data analysis was performed using StatSoft STATISTICA software (version 12) (StatSoft Inc., Tulsa, OK). Relative gene expression levels were calculated by using the ΔΔCt method and normalized to the expression levels of β-actin, with the values given in log2 scale. The graphs in Figure 1 were generated in GraphPad PRISM 6 (GraphPad Software, La Jolla, CA).

Results

Clinicopathological Characteristics of Patients with NSCLC and Their Correlation with Patients' Smoking History

Clinicopathological characteristics of cohort I (serum samples [n = 57]) and cohort II (serum and tumoral and nontumoral tissue samples [n = 64]) of patients with NSCLC and control patients with no history of malignant disease are summarized in Table 1. Patients with NSCLC were distinguished from controls by a panel of tumor markers (CEA, TPA, NSE, SCC, and CYFRA 21-1) (data not shown). A statistically higher level of CYFRA 21-1 ($p = 0.008$) was detected in the NSCLC group according to a paired t test with a separate variance estimate. An average level of 4.69 ± 4.87 ng/mL in patients with NSCLC versus an average level of 1.86 ± 1.33 ng/mL in control patients was detected. No statistically significant difference between patients with NSCLC and the controls according to the average values of CEA, TPA, NSE, and SCC was found for other tumor markers.[16] No statistically significant difference in age and years of smoking abstinence was found between the group of all patients with NSCLC (n = 121) and the control cancer-free group (n = 57) (Table 1). The range of years of smoking abstinence in the group of ex-smokers (n = 34) was 2 to 40 years, with a median of 13.5 years. However, in nonsmoking patients in both cohorts, we found that significantly more women

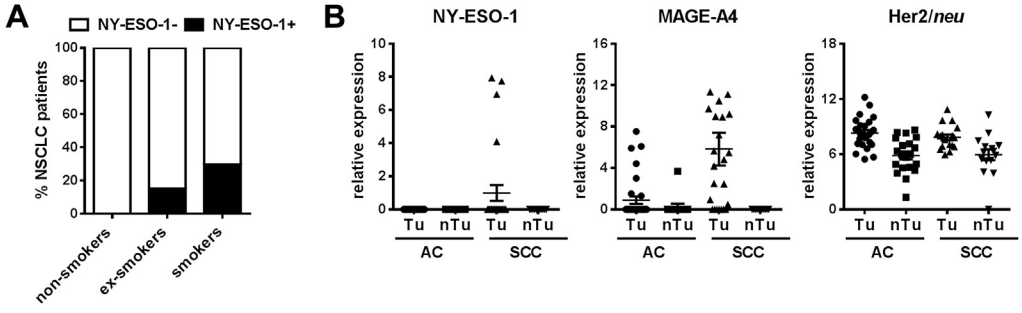

Figure 1. (*A*) Difference in frequency of New York esophageal squamous cell carcinoma 1 (NY-ESO-1) autoantibodies between the group of 121 patients with NSCLC and the controls according to smoking history. (*B*) The relative expression of human epidermal growth factor 2/neu (HER 2/neu), NY-ESO-1, and melanoma-associated antigen A4 (MAGE-A4) antigens in tumor (Tu) and nontumoral tissue (nTu) patients with NSCLC detected by quantitative polymerase chain reaction. AC, adenocarcinoma; SCC, squamous cell carcinoma.

(14 of 19 ([73.7%]) than men (five of 19 [26.3%]) have lung cancer (Table 2). The main histological subtype in nonsmoking patients was adenocarcinoma (94.7%) (Table 3).

Frequency of NY-ESO-1, HER 2/neu, and MAGE-A4 Antibodies in Patients with NSCLC and Controls and Their Correlation with Patients' Smoking History

First, we evaluated the frequency of autoantibodies against NY-ESO-1, HER2/neu, and MAGE-A4 in cohort I (n = 57 patients with NSCLC) and the controls (n = 57). Only antibodies to NY-ESO-1, but not antibodies to HER2/neu and MAGE-A4, were significantly increased in 26.3% of patients with NSCLC (15 of 57) versus in 3.5% of the control group (two of 57) (Table 4). We also found that significantly higher NY-ESO-1 seropositivity was detected in patients with NSCLC with an active smoking history (Table 4). As this is the first report on the prevalence of NY-ESO-1 antibodies in patients with NSCLC with an active smoking history, we decided to validate our results in a second cohort (cohort II) (n = 64 patients with NSCLC). NY-ESO-1 autoantibodies were significantly increased in 14% of the patients with NSCLC from cohort II (nine of 64) versus in the reanalyzed samples of 1.8% of the control group (one of 57) (Table 4). In total, 19.8% of patients with NSCLC (24 of 121) tested positive for NY-ESO-1 antibodies. Because of the low number of NY-ESO-1–seropositive patients in cohort II (seven smokers, two ex-smokers, and no nonsmokers), we were not able to detect a significant correlation with an active smoking history. Statistical evaluation of NY-ESO-1–seropositive smokers from the total 121 patients with NSCLC (cohort I + cohort II), however, confirmed that the presence of NY-ESO-1 autoantibodies positively correlates with an active smoking history in patients with NSCLC. Of 65 smokers with NSCLC, 27.7% (18 of 65) were NY-ESO-1–positive in contrast to 17.6% of ex-smokers (six of 34) and 0% of nonsmokers (none of 19) (Fig. 1*A*). We did not detect any significant difference between pack-years in seven of 64 NY-ESO-1–seropositive smokers (31% ± 17.7%) and 28 of 64 NY-ESO-1–seronegative smokers (31.5% ± 14.5%). We did not detect any correlation among NY-ESO-1 seropositivity, sex, or stage of the disease in patients with NSCLC (data not shown) or AC versus SCC. Interestingly, despite the small number of patients with other histological diagnoses (three patients with large cell carcinoma and three patients with anaplastic carcinoma in cohort I), this group of patients contributed significantly (five of six) to NY-ESO-1 antibody production.

NY-ESO-1, HER2/neu, and MAGE-A4 Expression in NSCLC Tumors and Their Correlation with Smoking History, Histological Subtype, and Sex

We further analyzed NY-ESO-1, HER2/neu, and MAGE-A4 antigen expression by using qPCR from primary tumor and nontumoral tissue samples (Fig. 1*B*). We detected a statistically significantly higher expression of NY-ESO-1 in 6.3% of patients (four of 64) and MAGE-A4 in 35.1% of patients with SCC (20 of 57) than in patients with AC (Table 5). Only the expression of MAGE-A4 was strongly associated with a history of smoking and male sex (45.5% of men as opposed to 19.2% of women) (Table 5). All NY-ESO-1–positive tumors (n = 4) were from male patients. However, smoking was not found to be a confounding factor even though there were more nonsmokers among women. HER2/neu antigen was overexpressed in 11.9% of patients with NSCLC (five of 42) when compared with nontumoral tissue, and it was not correlated with any other clinicopathological parameter tested (Table 5.) We did not observe any significant differences among expression of antigens and tumor grade or stage of the disease.

Table 2. Sex and Smoking Status of Patients with NSCLC

Group of Patients with NSCLC	Smoker	Ex-smoker	Nonsmoker	Significance Level
Women				
Cohort I (n = 20)	n = 11 (55%)	n = 3 (15%)	**n = 6 (30%)**	***p* = 0.036**
Cohort II (n = 27)	n = 15 (55.6%)	n =4 (14.8%)	**n = 8 (29.6%)**	***p* = 0.02**
Total (n = 47)	n = 26 (55.3%)	n = 7 (14.9%)	**n = 14 (29.8%)**	***p* = 0.0008**
Men				
Cohort I (n = 37)	n = 19 (51.4%)	n = 15 (40.5%)	n = 12 (35.3%)	**73.7% of nonsmoking women with tumor vs. 26.3% of nonsmoking men with tumor**
Cohort II (n = 34)	n = 20 (58.8%)	n = 3 (8.1%)	n = 2 (5.9%)	
Total (n = 71)	n = 39 (54.9%)	n = 27 (38%)	n = 5 (7%)	

Note: Boldface indicates the key finding resulting from the table.

Table 3. Histological Subtype and Smoking Status of the Patients with NSCLC

Group of Patients with NSCLC		Smoker	Ex-smoker	Nonsmoker	Significance Level
Adenocarcinoma					
Cohort I	n = 30	n = 15 of 30 (50%)	n = 7 of 18 (23.3%)	n = 8 of 9 (26.6%)	$p = 0.28$
Cohort II	n = 32	n = 16 of 35 (50%)	n = 6 of 16 (18.8%)	**n = 10 of 10 (31.3%)**	**$p = 0.004$**
Total	n = 62	n = 31 of 65 (50%)	n = 13 of 34 (21%)	**n = 18 of 19 (29%)**	**$p = 0.005$**
Squamous cell carcinoma					
Cohort I	n = 21	n = 12 of 30 (57.1%)	n = 8 of 18 (38.1%)	n = 1 of 9 (4.8%)	**Adenocarcinoma in 94.7% of nonsmokers**
Cohort II	n = 29	n = 19 of 35 (65.5%)	n = 10 of 16 (34.5%)	**n = 0 of 10 (0%)**	
Total	n = 50	n = 31 of 65 (62%)	n = 18 of 34 (36%)	**n = 1 of 19 (2%)**	

Note: Boldface indicates the key finding resulting from the table.

Correlation between NY-ESO-1 mRNA Antigen Expression and the NY-ESO-1 Autoantibody Seropositivity in Patients with NSCLC

We showed that nine of 64 patients displayed high titers of anti–NY-ESO-1 autoantibodies. Four of these nine seropositive patients were not analyzed for NY-ESO-1 mRNA expression owing to the lack of primary tumor and nontumoral tissue samples. Only two of the five remaining seropositive patients displayed NY-ESO-1 mRNA expression in primary tumors. In addition, NY-ESO-1 mRNA expression by reverse-transcriptase PCR was detected in other two patients. However, their anti–NY-ESO-1 antibody titers were just below the cutoff values for ELISA positivity. In summary, we detected a positive correlation between NY-ESO-1 mRNA expression and the presence of NY-ESO-1 autoantibodies in 5% of patients (two of 40) analyzed in parallel for the presence of NY-ESO-1 autoantibodies in serum and NY-ESO-1 mRNA expression in tumors.

Discussion

In this study we compared the frequencies of NY-ESO-1, Her2/neu, and MAGE-A4 antibodies and the antigen expression in tumors of patients with NSCLC and controls with respect to smoking history. Moreover, the frequencies of these antibodies and antigen expression levels were correlated with histologic subtype, sex, and grade and stage of the disease.

We detected significantly higher frequencies of NY-ESO-1 antibodies, but not HER2/neu and MAGE-A4 antibodies, in patients with NSCLC than in the cancer-free control patients. We found that frequencies of specific

Table 4. Seropositivity of Antibodies against NY-ESO-1, HER2/*neu*, and MAGE-A4

Group of Patients with NSCLC		NSCLC	Controls	Significance Level
NY-ESO-1-positive				
Cohort I	n = 57	n = 15 of 57 (26.3%)	n = 2 of 57 (3.5%)	**$p = 0.00063$**
Cohort II	n = 64	n = 9 of 64 (14%)	n = 1 of 57 (1.8%)	**$p = 0.009$**
Total	n = 121	n = 24 of 121 (19.8%)	n = 3 of 114 (2.6%)	**$p = 0.00084$**
HER2/*neu*-positive				
Cohort I	n = 57	n = 3 of 57 (5.3%)	n = 2 of 57 (3.5%)	$p = 0.647$
MAGE-A4-positive				
Cohort I	n = 57	n = 5 of 57 (8.8%)	n = 2 of 57 (3.5%)	$p = 0.242$

Group of patients with NSCLC		Smoker	Ex-smoker	Nonsmoker	Significance level
NY-ESO-1-positive					
Cohort I	n = 57	n = 11 of 30 (36.7%)	n = 4 of 18 (22.2%)	n = 0 of 9 (0%)	**$p = 0.027$**
Cohort II	n = 64	n = 7 of 35 (20%)	n = 2 of 16 (12.5%)	n = 0 of 10 (0%)	$p = 0.27$
Total	n = 121	n = 18 of 65 (27.7%)	n = 6 of 34 (17.6%)	n = 0 of 19 (0%)	**$p = 0.028$**
HER2/*neu*-positive					
Cohort I	n = 57	n = 1 of 30 (3.3%)	n = 2 of 18 (11.1%)	n = 0 of 9 (0%)	$p = 0.518$
MAGE-A4-positive					
Cohort I	n = 57	n = 3 of 30 (10%)	n = 2 of 18 (11.1%)	n = 0 of 9 (0%)	$p = 0.780$

Note: Boldface indicates statisically significant *p* values.
NY-ESO-1, New York esophageal squamous cell carcinoma 1; HER2, human epidermal growth factor 2; MAGE-A4, melanoma-associated antigen A4.

Table 5. HER2/*neu*, NY-ESO-1, and MAGE-A4 mRNA Expression in NSCLC Tumors and Their Correlation with Smoking History, Histological Subtype, and Sex

Group of Patients with NSCLC	Group Size	Smoker	Ex-smoker	Nonsmoker	Significance Level
NY-ESO-1-positive	n = 4	n = 2 of 25 (8%)	n = 1 of 20 (5%)	n = 0 of 11 (0%)	$p = 0.61$
Her2/*neu*-positive	n = 5	n = 3 of 21 (14.3%)	n = 1 of 11 (9%)	n = 0 of 8 (0%)	$p = 0.51$
MAGE-A4 +	n = 20	n = 9 of 26 (34.6%)	n = 8 of 18 (44.4%)	n = 0 of 9 (0%)	**$p = 0.06$**
		Adenocarcinoma	Squamous cell carcinoma		Significance level
NY-ESO-1-positive	n = 4	n = 0 of 36 (0%)	n = 4 of 26 (15.4%)		**$p = 0.015$**
Her2/*neu*-positive	n = 5	n = 4 of 25 (16%)	n = 1 of 17 (5.9%)		$p = 0.32$
MAGE-A4-positive	n = 20	n = 5 of 36 (13.9%)	n = 15 of 23 (65.2%)		**$p = 0.00005$**
		Men	Women		Significance level
NY-ESO-1-positive	n = 4	n = 4 of 35 (11.4%)	n = 0 of 29 (0%)		$p = 0.07$
Her2/*neu*-positive	n = 5	n = 3 of 22 (13.6%)	n = 2 of 20 (10%)		$p = 0.71$
MAGE-A4-positive	n = 20	n = 15 of 33 (45.5%)	n = 5 of 24 (20.8%)		**$p = 0.03$**

Note: Boldface indicates statisically significant *p* values.
HER2, human epidermal growth factor 2; NY-ESO-1, New York esophageal squamous cell carcinoma 1; MAGE-A4, melanoma-associated antigen A4; mRNA, messenger RNA.

antibodies against NY-ESO-1 (19.8% [n = 24]) detected in our study in 121 patients with NSCLC were comparable with the frequencies of 20% and 23% for NY-ESO-1 that were reported in the two previous lung cancer studies.[17,18] We detected NY-ESO-1 mRNA expression in 6% (four of 64), Her2/neu in 11.9% (five of 42), and MAGE-A4 in 35% (20 of 57) of patients with NSCLC. The published frequencies for these antigens in NSCLC are similar, ranging from 2% to 32% for NY-ESO-1,[19–24] 13% to 35% for MAGE-A4,[19,20,22,23] and 3% to 10% for Her2/neu.[25,26] A positive correlation between NY-ESO-1 mRNA expression in tumors and the presence of NY-ESO-1 autoantibodies was detected in only 5% of patients (two of 40) for whom serum and tumor analyses were done in parallel. No NY-ESO-1 mRNA expression was detected in remaining three NY-ESO-1–seropositive patients with available tumor samples. It has been shown that also in high NY-ESO-1–expressing cancers such as ovarian carcinoma or melanoma 5% to 15% of patients have no detectable level of NY-ESO-1 expression in tumor cells despite NY-ESO-1 seropositivity.[15,27,28] However, because of the low number of seropositive samples analyzed in our study, it would be highly speculative to draw any conclusions.

NY-ESO-1 antibodies were found to be present at higher frequencies in patients with SCC than in those with AC,[18] but we could not confirm this observation. The same study reported more frequent detection of NY-ESO-1 autoantibodies in undifferentiated tumors (40%) compared with in SCC and AC.[18] Here in a small group of patients with large cell carcinoma and anaplastic carcinoma, we observed a significant contribution to NY-ESO-1 antibody production (in five of six) among patients with NSCLC (15 NY-ESO-1–seropositive patients in total). Whether NY-ESO-1 is preferentially expressed in NSCLC subtypes other than SCC and AC or in undifferentiated tumors needs to be determined in a larger group of patients.

The correlation of the frequency of autoantibodies and smoking history revealed a significantly higher frequency of NY-ESO-1 antibodies in those patients with NSCLC who were active smokers than in patients with NSCLC who were ex-smokers or nonsmokers when compared with the control group. In two studies a significant association of CT antigen expression with smoking history was found,[20,22] but the production of NY-ESO-1 antibodies that might be induced by smoking has not been previously described. Autoantibody production in smokers, together with antigen expression in tumors, might indicate a chronic inflammatory state in premalignant lesions that is induced by smoking and potentiates the development of tumor-specific immune responses. This would be supported by our observation that nonsmokers displayed neither antibodies nor tumor-specific expression of NY-ESO-1, MAGE-A4, or HER2/neu antigens. Smoking, however, is likely to serve as an important cofactor, rather than as the primary inducer, of other molecular changes that determine NSCLC carcinogenesis, as lung cancer does not develop in all the smokers in their lifetime. The New York esophageal squamous cell carcinoma 1 gene (*NY-ESO-1*) is located on the X chromosome together with genes coding some other CT antigens.[29] Cancer/testis X- linked genes have been shown to be regulated by demethylation of critical CpG residues within their promoter regions.[30] The expression of NY-ESO-1 could be upregulated by using demethylating agents such as decitabine.[31] Chemicals in

cigarette smoke have been shown to induce epigenetic changes in DNA[4,5]; therefore, it could be speculated that a similar mechanism might contribute to coordinated expression of CT antigens.[20] MAGE-A4 might be coordinately expressed with NY-ESO-1, as we found its strong correlation with smoking. MAGE-A4 was expressed mainly in men in our study, which has been previously reported.[22,32] Higher expression of CT antigens was observed in SCC,[20,24] and this NSCLC subtype has been connected almost exclusively with a history of smoking. In our study we observed that 94.7% of nonsmoking patients have AC, of whom 73.7% were women. This is in agreement with the previous studies on lung cancer in never-smokers.[6] Cigarette smoking is the predominant risk factor not only for lung cancer but also for chronic obstructive pulmonary disease (COPD).[3] In industrialized countries smoking accounts for 95% of COPD cases.[33] Approximately 30% of patients with COPD have been reported to die from lung cancer.[34] Unfortunately, we have no data on the incidence of COPD among our patients, but it would be of interest to determine whether the presence of NY-ESO-1 autoantibodies in patients with COPD would be predictive of the development of lung cancer. The biological role of NY-ESO-1 during carcinogenesis is not known[28,29]; however, it has been shown to interact with MAGE-C1 antigen.[35] The MAGE family members were shown to be involved in regulation of apoptosis or cell cycle progression.[36] Of note, MAGE-A4 protein was suggested to act as a tumor suppressor during early stages of carcinogenesis,[37] sensitizing cancer cells to apoptotic stimuli such as chemotherapeutic agents.[38] Interestingly, the expression of NY-ESO-1 and MAGE-A4 in tumors was shown to correlate with a poor prognosis in patients with NSCLC[20,23,39] despite NY-ESO-1 immunogenicity and a proapoptotic role of MAGE-A4. NY-ESO-1 was shown to be one of the most immunogenic tumor antigens,[28,29] as the average autoantibody frequency against single antigens in unselected patients with tumors rarely exceeds 15%.[9] High frequencies of NY-ESO-1 antibodies have also been detected in many other types of cancers together with NY-ESO-1–specific CD8-positive and CD4-positive T cells.[40] The high frequency of humoral and cell-mediated responses gave a rationale for using NY-ESO-1 as a target for cancer vaccines.[28,29]

Conclusion

In summary, we have shown here for the first time that autoantibodies against the CT antigen NY-ESO-1 correlate with smoking history in patients with NSCLC, which suggests that smoking-related chronic inflammation might induce the development of NY-ESO-1–specific immune responses. We have also observed that tumor-associated expression of MAGE-A4 correlated with a smoking history, which might suggest its role in early stages of NSCLC development. Because the tumor-associated expression of both NY-ESO-1 and MAGE-A4 were reported to be in an inverse correlation with survival in advanced stages of the disease,[20,23,39] the clinical relevance and immunotherapeutic value of our findings remain to be determined.

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