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Immunohystochemical Expression of Cancer/Testis Antigens (MAGE-A3/4, NY-ESO-1) in Non-Small Cell Lung Cancer: The Relationship with Clinical-Pathological Features

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A B S T R A C T

The aim of this study was to explore the expression of cancer/testis tumor associated antigens (C/T TAAs) MAGE-A 3/4 and NY-ESO-1 in lung squamous cell carcinoma and adenocarcinoma, and to evaluate their association with the standard clinical-pathological features of surgically treated lung cancer patients. The study included 80 patients with non-small cell lung cancer (40 adenocarcinomas, 40 squamous cell carcinomas) who had undergone surgery in the period between 2002 and 2005. The MAGE-A3/4 and NY-ESO-1 antigen expression was analyzed immunohistochemically (IHC). The results showed MAGE-A3/4 and NY-ESO-1 positive staining in 65.1% and 23.3% of squamous cell carcinomas and 18.9% and 10.8% of adenocarcinomas, respectively. A statistically higher MAGE-A3/4 expression was observed in planocellular bronchial carcinoma ($p<0.001$), while no difference was found in the expression of NY-ESO-1 in adenocarcinoma and planocellular carcinoma ($p=0.144$). A significant association was found between the MAGE-A3/4 expression and presence of tumor necrosis in squamous cell cancer specimens ($p=0.001$), but not in adenocarcinoma ($p=0.033$). A statistically significant association was noted between the NY-ESO-1 expression and positive hilar and mediastinal lymph nodes in adenocarcinoma ($p=0.025$) whereas it was not the case in squamous cell carcinoma. Non-small cell lung cancer frequently expresses cancer/testis tumor associated antigens. Our results demonstrate that the MAGE-A3/4 and NY-ESO-1 expression was significant associated with prognostic factors of poor outcome of disease (presence of tumor necrosis and lymph node metastasis). As C/T antigens are important for inducing a specific immune reaction in lung cancer patients, there is an intention to form a subgroup of patients in the future, whose treatment would be enhanced by specific immunotherapy based on the observed scientific results.

Key words: *non small cell lung carcinoma, cancer/testis tumor associated antigens, immunohistochemistry*

Introduction

Lung cancer is the most frequent cause of cancer death and its incidence has been steadily increasing during recent decades [1]. The number of new cases is increasing in rate of about 3% annually [2]. Despite the advances in early detection of lung cancer, the overall 5-year survival still remains disappointing. Cigarette smoking remains the major risk factor on the incidence of this cancer. Today we acknowledge that tobacco smoking is the principal cause of lung cancer, accounting for 90% of lung cancer deaths in men and approximately 60% in woman[3].

Adenocarcinoma is the most frequent histological type (50%) and squamous cell carcinoma, previously the most common, accounts for approximately one third of non-small cell lung cancer (NSCLC)[4]. Small cell carcinoma accounts for 15–20% of lung cancers.

Surgery remains the initial treatment for patients with early stage non-small cell cancer, but additional therapy is necessary because of high rates of distant and local disease recurrence after surgical resection[5]. The re-

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sults of large randomized studies have demonstrated that adjuvant chemotherapy prolongs overall survival by approximately 5% at 5 years in patients with early-stage non-small cell lung carcinoma[6]. Radiotherapy constitutes one of the main treatment modalities in lung cancer and is indicated in around three-quarters of all lung cancer cases[7].

Cancer/testis tumor associated antigens (C/T TAA-s) present one of the tumor antigen groups, characterized by outstanding immunogenicity, which is why there are numerous ongoing clinical research studies examining vaccines based on these antigens. They are normally expressed in gametes[8] and trophoblasts[9], but only exceptionally in other healthy tissues. Cancer-testis antigens were recognized as a group of attractive targets for cancer immunotherapy because of their expression in numerous human neoplasms, among which there is lung cancer[10–15]. Their potential relevance as tumor markers has also been underlined. The genes, which code the melanoma antigens MAGE (Melanoma Antigen E) and NY--ESO-1 (New York Oesophageal Squamous Cell Cancer), are expressed in numerous malignant neoplasms, including lung cancer[16–19.]

The most studied TAAs subgroup is the MAGE-A family of C/T antigens and comprises more than 25 genes. The biologic function of TAAs in both germ lines and tumors has remained poorly understood. It has been demonstrated that MAGE-A TAAs family represses genes that are necessary for differentiation[20]. These genes have a strong immunogenic potential in humans; they induce cellular and humoral immune responses and belong to the cancer/testis (C/T) gene group[21–24]. New therapeutic strategies with limited side effects, such as immunotherapy, are constantly being investigated and have achieved promising results, and there is a continued need to develop more effective cancer immunotherapy strategies.

Materials and Methods

This is a retrospective study which included sample from a total of 80 unselected patients with adenocarcinoma and squamous cell lung carcinoma. These samples were derived from patients operated at the Department of Thoracic Surgery, Jordanovac Clinical Hospital for Pulmonary Diseases in Zagreb, Croatia, during the period from 2002 to 2005.

The patients were identified retrospectively in 2006 from pathological reports at the Departments of Pathology at the University Hospital for Tumors and the University Hospital »Sestre Milosrdnice». The patients were divided in two groups. The first group included 40 patients with adenocarcinoma, and the second group consisted of the same number of patients with squamous cell lung carcinoma.

For routine histological analysis the lung cancer tissue resected immediately after surgery was fixed in 10% buffered formalin and later embedded in paraffin. From this paraffin-embedded tumor samples 4-μm thick sections were cut and stained with hematoxylin and eosin and reviewed by the pathologist in order to establish the pathohistological diagnosis. From the same archival, formalin-fixed, paraffin-embedded tumor tissue blocks additional serial 5-μm sections were prepared for immunohistochemical staining.

The expression of MAGE-A3/4 and NY-ESO-1 in primary bronchial cancer tissues was studied by immunohistochemistry with the addition of undiluted monoclonal antibodies (mab). Expression of MAGE-A3/4 was determined by mab 57B[25,26] and of NY-ESO-1 by mab B9.8.1.1.[27].

Briefly, paraffin-embedded tumor tissue slices (2–3) were used for the immunohistochemical method. The slices were placed on silane-treated microscope glass slides (3-aminopropyltriethoxysilane, Sigma, St. Louis, Mo.). After deparaffinization, the slices were heated in an 800 W microwave oven at maximum power for 8.5 minutes, held in the 10 mmol/L citrate buffer (pH 6.0) for 5 minutes, and then rinsed with a phosphate buffer solution (PBS, pH 7.2). This was followed by spraying them with H_2O_2 to suppress endogenous peroxidase activity. After additional rinsing with PBS, the slices were incubated with a 1:10 dilution of normal rabbit serum (Dako A/S) in a wet chamber at room temperature for 20 minutes to prevent non-specific binding of immunoglobulin. This was followed by the application of monoclonal antibodies specific for MAGE-A1, MAGE-A3/4 and NY-ESO--1 in the undiluted form at room temperature for 90 minutes. Slices were then treated with biotinilized secondary antibodies (Dako-No.K0690) for 30 minutes, and this was followed by rinsing with PBS. The reaction was visualized using Kromogen-DAB (3,5-diaminobenzidine) for 5 minutes. Slices were rinsed with distilled water and Hemalaun (Dako-No.S2020) was added for 1 minute. After rinsing with water and dehydration (96% alcohol), slices were treated with xilol and fitted in Canada balsam.

The results of the immunohistochemical analysis were determined by a semi-quantitative method on at least 100 tumor cells. Primary melanoma samples were used as positive control, as they give positive reaction. If no tumor cell showed staining in the cytoplasm, the reaction was considered as negative (–). Also, when immunostaining was observed in 0–10% of cells (+, weak), it was also considered as negative in this study. The samples, in which 11–50% of the tumor cells were positive (++, moderate), and the samples, in which over 50% of the tumor cells were positive (+++, strong), were considered to be positive (Figures 1–2).

Fibroblasts and other normal cells were used as negative controls as they show no immunostaining. Intratumoral necrosis was assessed based on the criteria of presence (+) or absence (–) in the histopathology report after surgery of each patient. The differentiation of tumor cells was assessed based on accepted pathohistological indicators[28].

Distributions of the tested features were shown both in tables and graphically, in which descriptive statistical methods were used. The method used in the statistical

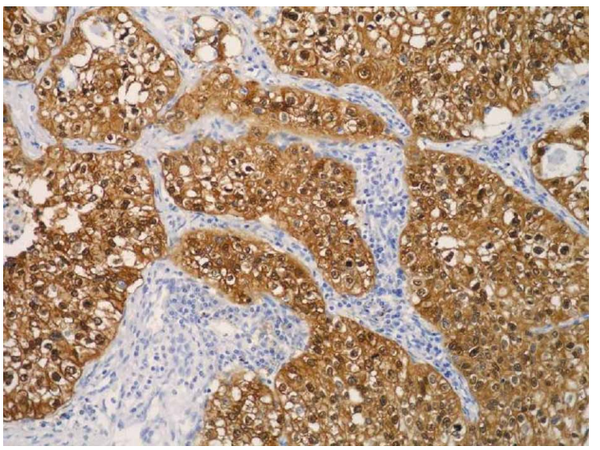

Fig. 1. MAGE-A3/4 strong positive reaction in the cytoplasm of squamous cell lung cancer.

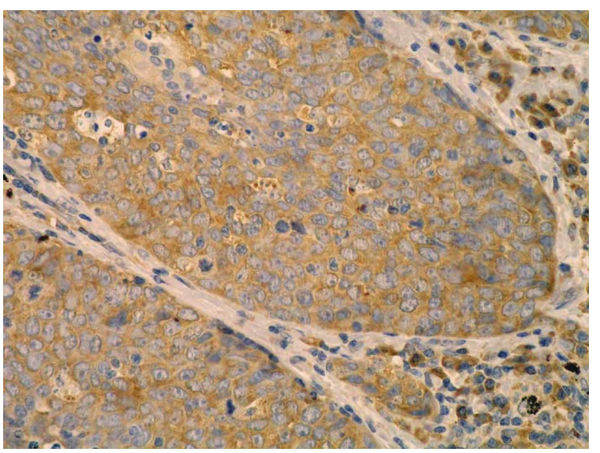

Fig. 2. NY-ESO-1 strong positive reaction in the cytoplasm of squamous cell lung cancer.

significance testing of the relationship among quantitative features was the $\chi 2$ test, that is, the Fisher's exact test when necessary, at the 0.05 significance level.

Results

This study included 64 (80%) male and 16 (20%) female patients with non-small cell bronchial cancer, with a median age of 60 years (35–88) (Table 1). Adenocarcinoma and planocellular carcinoma were equally represented in this study (40 patients in each group). The tumor was well-differentiated in 15%, moderately differentiated in 35% and poorly differentiated in 55% of patients. The largest number of patients was in stage IIIA (37.5%), followed by stage IB (26.3%), IIB (15%) and IIIB (10%). Lymph node status was negative in 47.5% of patients, positive hilar lymph nodes were found in 16.3% and positive mediastinal lymph nodes were noted in 36.3% of patients. There was no statistically significant difference between lymph node status and both tumor types (p=0,330). Tumor necrosis was found in 53.8% of patients, and there was no statistically significant difference between both tumor types (p=0,960). Squamous cell carcinoma was more frequently classified as grade III (p=0.002).

All clinical-pathological features in patients are shown in Table 1.

A possible association between the immunohistochemical expression of MAGE-A3/4 and NY-ESO-1 antigens and the clinical-pathological features of adenocarcinoma and planocellular lung carcinoma including patients' age and sex, histological tumor type, differentiation grade, tumor size, lymph node status, presence of tumor necrosis and tumor stage is presented in Table 2.

Table 2 Comparison of the expression of MAGE-A3/4 and NY-ESO-1 with regard to clinical-pathological features in patients with adenocarcinoma and squamous cell lung carcinoma

In this series, most of non-small cell lung cancer specimens expressed at least 1 C/T TAA. MAGE-A3/4 and NY-ESO-1 positive staining was observed in 65.1% and 23.3% of squamous cell carcinomas and 18.9% and 10.8% of adenocarcinomas, respectively.

No statistically significant difference between male and female patients in the immunohistochemical expression of MAGE-A3/4 and NY-ESO-1 in adenocarcinoma (p=1,000, p=1.000) and squamous cell lung cancer (p=1,000,

TABLE 1
CLINICAL-PATHOLOGICAL FEATURES IN PATIENTS WITH PLANOCELLULAR CANCER (40) AND ADENOCARCINOMA (40)

Variable		n	%
Age (years; median (range))		60 (35–88)	
Sex	Male	64	80.0
	female	16	20.0
Tumour Size	< 3 cm	24	30.0
	3–6 cm	42	52.5
	> 6 cm	14	17.5
Lymph Node Status	Negative	38	47.5
	Positive hilar	13	16.3
	Positive mediastinal	29	36.3
Tumour Differentiation	Grade I	12	15.0
	Grade II	28	35.0
	Grade III	40	50.0
Tumour Necrosis	No necrosis	43	53.8
	necrosis	37	46.3
Tumour Stage	I A	5	6.3
	I B	21	26.3
	II A	2	2.5
	II B	12	15.0
	III A	30	37.5
	III B	8	10.0
	IV	2	2.5

p=0.280) was found. A statistically higher MAGE-A3/4 expression was observed in squamous cell lung carcinoma (p<0.001), while no difference was found in the expression of NY-ESO-1 in adenocarcinoma and squamous cell carcinoma (Table 2). Furthermore, no significant association was found between the MAGE-A3/4 antigen expression and tumor grade and positive lymph node finding in both histological tumor types. It is interesting that a significant association was found between the MAGE-A3/4 expression and presence of tumor necrosis in squamous cell cancer specimens (p=0.001), but not in adenocarcinoma (p=0.033) (Table 2).

The NY-ESO-1 expression in both adenocarcinoma and aquamous cell carcinoma showed no significant association with tumor size (p=0.798, p=0.981), differentiation grade (p=129, p=1.000) and tumor necrosis (p=1.000, p=0.437). A statistically significant association was noted between the NY-ESO-1 expression and positive hilar and mediastinal lymph nodes in adenocarcinoma (p=0.025) whereas it was not the case in squamous cell carcinoma (Table 2). No statistically significant correlation among the concurrent expression of two tumor antigens was found in either histological tumor types.

Smoking history showed no statistically significant association with a higher expression level of any of two tumor antigens, regardless of the pathohistological tumor type.

Discussion

C/T antigens are not expressed in normal tissues except for testis, ovary and placenta that express no MHC (Major Histocompatibility Complex) class I molecules, but are expressed in variable proportions of a wide range of different types of tumors, such as melanomas, cervical carcinoma, lung and breast carcinomas. Therefore, the CT antigens are promising targets for cancer immunotherapy.

Lung cancer is the most common cause of death from malignant tumors in men around the world and its incidence among women is growing rapidly. The most discouraging is the overall 15% 5-year survival rate for those diagnosed with lung cancer[2,6]. When compared to the spectrum of other malignancies, the dismal survival rate for lung cancer is approximated only by that of pancreatic cancer[1]. Cancer incidence rates in the Republic of Croatia show lung cancer ranking first in men (21%) and third in women (7%). In 2004, there were 2,119 new cases and 2,123 lung cancer deaths in men and 534 new cases and 512 lung cancer deaths in women[29].

All clinical prognostic factors in lung cancer accepted so far are still insufficient for a reliable prediction of the course of the disease. It is due to the insufficient knowledge of the real biological behavior of tumors. Determining the tumor differentiation grade and other patho-

TABLE 2
COMPARISON OF THE EXPRESSION OF MAGE-A3/4 AND NY-ESO-1 WITH REGARD TO CLINICAL-PATHOLOGICAL FEATURES IN PATIENTS WITH ADENOCARCINOMA AND SQUAMOUS CELL LUNG CARCINOMA

Carcinoma type	n(%)		MAGE-A3/4			NY-ESO-1		
			pos.	neg.	p	pos.	neg.	p
Adenocarcinoma	Grade I		1	10		2	11	
	Grade II		2	12	p=0.179	3	9	p=0.129
	Grade II		5	9		0	15	
	Necrosis yes		6	12	p=0.033	3	16	p=1.000
	Necrosis no		2	20		2	19	
	Lymph nodes	Hilar	0	5		2	16	
		Mediastinal	1	16	p=0.084	2	3	p=0.025
		Negative	6	12		0	17	
	Size	< 3 cm	0	11		2	9	
		3–6 cm	5	17	p=0.177	2	120	p=0.798
		> 6 cm	3	6		1	6	
Squamous Cell Carcinoma	Grade I		1	0		0	1	
	Grade II		10	4	p=0.834	4	11	p=1.000
	Grade II		15	10		5	29	
	Necrosis yes		17	2	p=0.001	6	13	p=0.473
	Necrosis no		10	1		4	31	
	Lymph nodes	Hilar	5	4		6	13	
		Mediastinal	7	5	p=0.705	2	7	p=0.231
		Negative	14	5		1	11	
	Size	< 3 cm	8	5		3	10	
		3–6 cm	5	3	p=1.000	4	16	p=0.891
		> 6 cm	27	14		2	5	

histological indicators can only partly indicate the disease prognosis and therapy selection.

In this paper, we determined the expression of MAGE--A3/4 and NY-ESO-1 antigens in a total of 80 patients, of whom 40 had adenocarcinoma and 40 patients had squamous cell carcinoma.

According to the largest number of studies, the MAGE gene expression was observed in about 30–50% of non--small cell lung cancer tissue samples[30,31], and our data concurs with the results of these studies.

In patients affected by non-small cell lung cancer, multitissue arrays identified the expression of MAGE-A as an independent negative prognostic factor[32]. Other studies confirmed an inverse correlation between MAGE--A3/4 expression and patient survival in advanced stage lung cancers[15], and MAGE-A activation probably takes place very early in the carcinogenesis of lung cancer[33,34].

The expression rate of MAGE-A3/4 did not appear to vary significant with lymph node status and tumor grade, and there was no significant correlation between the NY-ESO-1 expression and presence of tumor necrosis.

It is interesting here to see the results in our study of the statistically significant association among the intratumoral necrosis of the squamous cell carcinoma and a higher expression of MAGE-A3/4. A similar observation is found in the association between a higher incidence of positive lymph nodes and NY-ESO-1 expression. Such a correlation between necrosis and C/T TAA expression can testify to their association with more rapidly growing tumors not accompanied by adequate neovascularization, which results in the necrosis of the neoplasm tissue. A similar conclusion could apply to a higher incidence of metastases in the hilar and mediastinal lymph nodes. These observations suggest that MAGE A-3/4 and NY-ESO-1 are involved in the early steps of tumor progression.

These data suggest that evaluation of the MAGE-A3/4 protein expression is useful in the identification of groups of NSCLC characterized by severe prognosis, thus possibly providing indications for early MAGE TAAs-targeted immunotherapy[35]. To date, only one clinical study using MAGE-A3 protein as a vaccine has been reported[36]. It is shown that vaccination with the recombinant protein of CT antigens provides strong Ag-specific $CD4^+$ T cell help along with antibodies and $CD8^+$ T cell responses, and leads to integrated immunity[37].

MAGE-A3/4 and NY-ESO-1 were expressed in the great majority of samples examined. In association with the recognized characteristic of having strong antigenic properties, this makes these TAAs very attractive for trials on immunotherapy. The heterogeneous expression of these antigens suggests that future immunotherapy trials in lung cancer patients will have to adopt use of polyvalent vaccines.

In conclusion, lung cancer frequently expresses MAGE--A3/4 and NY-ESO-1. As C/T antigens are important for inducing a specific immune reaction in lung cancer patients, there is an intention to form a subgroup of patients in the future, whose treatment would be enhanced by specific immunotherapy based on the observed scientific results. More studies are necessary to define the possible role of these antigens as immunotherapy targets. The results revealing high expression levels of two researched C/T TAAs in most paraffin-based samples of planocellular and lung adenocarcinoma favor the hypothesis that these patients might benefit from active specific immunization[38], and the results shown in this paper might be useful in the development of new tumor treatment strategies (multimodal treatment or polyvalent vaccines) using C/T TAA gene products, and also in the prevention of lung cancer [39].

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IMUNOHISTOKEMIJSKA IZRAŽENOSTI CANCER/TESTIS ANTIGENA (MAGE-A3/4, NY-ESO-1) U BOLESNIKA S RAKOM PLUĆA NEMALIH STANICA : POVEZANOST SA KLINIČKO PATOLOŠKIM POKAZATELJIMA

S A Ž E T A K

U ovom radu ispitivali smo značenje imunohistokemijske izraženosti MAGE-A3/4 i NY-ESO-1 antigena u usporedbi sa kliničko-patološkim obilježjima karcinoma pločastih stanica i adenokarcinoma pluća. Produkti MAGE (Melanoma antigen E) obitelji gena te NY-ESO-1 (New York Oesophageal Squamous Cell Cancer) spadaju u skupinu tumor-pridruženih antigena (Tumor Associated Antigens – TAAs) i imaju snažan imunogeni potencijal u ljudi. Normalno su izraženi u gametama i trofoblastima, a samo su iznimno u drugim zdravim tkivima. Izraženi su u brojnim ljudskim novotvorinama, među kojim je i rak pluća. Imunohistokemijsko određivanje C/T antigena relevantna je metoda za određivanje njihove ekspresije u raka pluća. Kako su C/T antigeni važni za izazivanje specifične imunosne reakcije u bolesnika s rakom pluća, u budućnosti će se nastojati odrediti podskupina bolesnika čije bi liječenje bilo unaprijeđeno specifičnom imunoterapijom temeljem dobivenih znanstvenih rezultata. U studiju je uključeno 80 bolesnika s rakom pluća nemalih stanica (40 adenokarcinom, 40 karcinom pločastih stanica) operiranih u razdoblju od 2002.–2005.g. Ekspresija MAGE-A3/4 antigena određivana je imunohistokemijski primjenom monklonskih protutijela 57B Mab (dr.G. Spagnoli, Research Laboratory, Basel, Switzerland), a ekspresija NY-ESO-1 monoklonskim protutijelom B9.8.1.1 mAb (dr.G. Spagnoli, Research Laboratory, Basel, Switzerland). Prisustvo ekspresije ovih C/T antigena uspoređivano je sa kliničko-patološkim obilježjima tumora. Prosječna dob bolesnika bila je 60 g. (raspon 35-88). Karcinom pločastih stanica češće je klasificiran kao gradus 3. Statistički je značajna veća imunohistokemijska izraženost MAGE-A3/4 nađena kod planocelularnog karcinoma bronha ($p<0,001$). Nije nađena povezanost između veličine tumora i izraženosti sva tri tumorska antigena. Ekspresija MAGE-A3/4 bila je statistički značajnija u karcinoma pločastih stanica ($p=0,001$), a i zamiječen je značajno veći udio tumorske nekroze kod tumora sa ekspresijom MAGE-A3/4 ($p=0,001$), ali nije nađena povezanost sa pozitivnim limfnim čvorovima. Nadalje, značajna je povezanost ekspresije NY-ESO-1 sa nalazom pozitivnih limfnih čvorova u adenokarcinoma, ali ne i u karcinoma pločastih stanica. Kako su C/T antigeni važni za izazivanje specifične imunosne reakcije u bolesnika s rakom pluća, u budućnosti će se nastojati odrediti podskupina bolesnika čije bi liječenje bilo unaprijeđeno specifičnom imunoterapijom temeljem dobivenih znanstvenih rezultata. Nalaz bi visokog postotka izraženosti sva tri istraživana C/T TAA u većini parafinskih uzoraka planocelularnog i adenokarcinoma pluća govori u prilog hipotezi da bi ti bolesnici mogli imati koristi od aktivne specifične imunizacije, a rezultati prikazani u ovom radu mogli bi biti korisni u razvoju novih tumorskih cjepiva koristeći produkte C/T TAA gena, ne samo za liječenje nego i za prevenciju raka pluća. Ova studija podržava rezultate ranijih imunohistokemijskih studija koje su pokazale da bi ekspresija MAGE-3 i NY-ESO-1 u bolesnika sa rakom pluća nemalih stanica mogla vrijedan prognostički čimbenik te pomoći u određivanju podskupine bolesnika koji bi imali koristi od imunološke terapije.