

NY-ESO-1/LAGE-1 coexpression with MAGE-A cancer/testis antigens: A tissue microarray study

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The characterization of the expression pattern of different families of cancer/testis (C/T) antigens in different tumors, at the protein level, might be of relevance in the development of multiantigen vaccine preparations for active specific immunotherapy. We have used tissue microarray (TMA) technology to explore in large numbers of tumor specimens the expression of NY-ESO-1/LAGE-1 C/T antigens and its correlation with MAGE-A expression by using D8.38 and 57B monoclonal antibodies (MAb). The epitopes recognized by these reagents in C/T antigens were identified by molecular mapping by using a bacterial expression system. Out of 2,052 samples, 119 (5.8%) scored positive upon staining with D8.38 NY-ESO-1/LAGE-1-specific MAb. Expression in >10% of cases was detectable in melanoma and basalioma (31.6 and 18.2%, respectively), large cell carcinomas and adenocarcinomas of the lung (17.8 and 10.5%, respectively), stomach adenocarcinomas of the intestinal type (13.2%), pT2-4 bladder TCC (18.2%), nonseminomatous carcinomas of the testis (10.4%) and liposarcomas (15.4%). Simultaneous expression of NY-ESO-1/LAGE-1 and MAGE-A C/T antigens was then addressed in a TMA where 101/845 and 73/845 samples (12 and 8.6%, respectively) showed evidence of MAGE-A or NY-ESO-1/LAGE-1 specific staining, respectively. In 35/845 specimens (4.1%) concomitant expression of MAGE-A and NY-ESO-1/LAGE-1 was observed ($p = 0.0002$). Discrepancies in the expression of NY-ESO-1/LAGE-1 and MAGE-A were conspicuously detectable in squamous cell carcinomas of the skin (MAGE-A positive but NY-ESO-1/LAGE-1 negative) and in liposarcomas (NY-ESO-1/LAGE-1 positive, but MAGE-A negative). Taken together, these data suggest novel areas of application of C/T antigens targeted active specific immunotherapy possibly based on multiantigen vaccine preparations.

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Key words: cancer/testis antigens; NY-ESO-1/Lage-1; MAGE-A; epitope mapping

Cancer/testis (C/T) antigens are a group of proteins characterized by a peculiar expression profile, mostly limited to male germinal cells and cancer cells of diverse histological origin.¹

Among C/T antigens, NY-ESO-1 appears to play an important role since it is probably the most immunogenic member of the group and it is also capable to induce humoral responses in addition to specific cytotoxic T lymphocytes (CTL) and CD4+ T cells.^{2,3} Remarkably, most recently a highly homologous determinant, LAGE-1, has been reported to be able to induce specific regulatory CD25+/CD4+ in melanoma tumor infiltrating lymphocytes (TIL).⁴

Immunohistochemical detection of NY-ESO-1 has been made possible by the generation of monoclonal antibodies (MAb) capable of recognizing the target protein in paraffin embedded tissues, as reported by us and others.^{5,6} However, the fine specificity of these reagents at the molecular level was not clarified, the number of tissues tested was relatively limited and the issue of the coexpression together with other C/T antigen families was not addressed. Here, we mapped epitopes recognized by reagents used to identify C/T antigens in clinical samples and explored NY-ESO-1/LAGE-1 expression by taking advantage of tissue microarrays (TMA), a technique allowing the fast assessment of the expression of determinants recognized by specific sero-

logical reagents on large numbers of tissues. Furthermore, we comparatively evaluated the expression of NY-ESO-1/LAGE-1 as associated with MAGE-A C/T antigen expression.

Material and methods

Monoclonal antibodies

Monoclonal antibodies (MAb) 57B and D8.38 were generated by using as immunogens recombinant MAGE-A3 and NY-ESO-1 proteins, respectively.^{5,7}

Epitope mapping

The FliTrx random peptide library (Invitrogen, Basel, Switzerland) is composed of 1.77×10^8 primary clones of *E. coli* with a dodecamer peptide sequence inserted within the Thioredoxin (TrxA) active site loop. The genes encoding TrxA-peptide fusion proteins are cloned into the nonessential domain of the major bacterial flagellar protein (FliC) gene under the control of a PL promoter from bacteriophage γ . When induced, the peptide sequence is expressed on the surface of the *E. coli* flagella with the N- and C-terminal ends constrained by a disulfide bond. This library was screened against the 2 different MAbs under investigation.

Two milliliters of the FliTrx library were added to IMC medium containing 100 $\mu\text{g/ml}$ ampicillin and grown by shaking at 25°C for 15 hr. The peptide library was then induced by adding approximately 1×10^{10} cells to IMC medium containing ampicillin and 100 $\mu\text{g/ml}$ tryptophan and shaken at 25°C for further 6 hr.

The target MAbs were diluted to 50 $\mu\text{g/ml}$ in sterile water and adsorbed onto 60 mm tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) by gentle agitation at 50 rpm for 1 hr. Plates were then rinsed with sterile water followed by gentle agitation for 1 hr with a blocking solution containing 100 $\mu\text{g/ml}$ ampicillin, 1% nonfat dry milk, 150 mM NaCl and 1% α -methyl mannoside.

After decanting the blocking solution, 10 ml of the induced *E. coli* culture in medium containing 1% nonfat drymilk, 150 mM NaCl and 1% α -methyl mannoside were added to culture plates and gently agitated at 50 rpm for 1 min followed by 1 hr of incubation with no agitation. The medium was then decanted and the plate gently washed 5 \times with IMC medium containing ampicillin and 1% α -methyl mannoside. Bacteria bound to the plates were eluted into 10 ml fresh culture IMC medium containing ampicillin by placing the plate on a vortex to shear the flagella and release the cells to the medium. *E. coli* were then cultured overnight and the complete procedure was repeated for 5 rounds of panning.

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TABLE II – IMMUNOSTAINING OF HEALTHY TISSUES BY NY-ESO-1/LAGE-1 SPECIFIC D8.38 MONOCLONAL ANTIBODY.

Tissues (n = 27)	n	NY-ESO-1/LAGE-1 pos ¹
Penis	4	0
Uterine cervix	4	0
Gallbladder	6	0
Esophagus	8	0
Pancreas	9	0
Oral cavity	8	0
Prostate	9	0
Colon	3	0
Gastric antrum	2	0
Gastric corpus	3	0
Ileum	3	0
Liver	2	0
Bladder	2	0
Lung	3	1
Ovary	4	0
Breast	1	0
Parathyroid	4	0
Thyroid	5	0
Parotid	5	0
Thymus	5	0
Lymph node	5	0
Brain, grey substance	5	0
Brain, white substance	5	0
Endometrium, proliferative phase	3	0
Endometrium, secretory phase	4	0
Kidney	4	0
Testis	5	4
Total	121	5 (4.2%; 95%CI [0.6%–7.8%])

¹Positivity (see Material and Methods) is defined by weak intensity staining in >66% of cells or moderate intensity staining in >34% of cells or strong intensity in any percentage of cells.

gens in TMAs were compared using the McNemar's test. The level of statistical significance was set at 0.05. All tests were 2-sided. Computations were done using SAS 8.2 (Cary, NC).

Results

Mapping of epitopes recognized by C/T antigens specific monoclonal antibodies.

We used a random peptide library method to identify epitope(s) recognized by D8.38 MAb that was generated by using recombinant NY-ESO-1 as immunogen. Plasmids from all bacterial colonies ($n=5$) specifically recognized by the D8.38 MAb displayed sequences highly homologous to a 14-mer sequence (residues 28–41) also shared by LAGE-1,¹³ a C/T antigen also encompassing identical CTL epitopes (Table Ia). A similar study was performed to clarify the fine specificity of 57B MAb, generated by using recombinant MAGE-A3 as immunogen. Indeed, immunohistochemical studies carried out in different laboratories have previously emphasized that 57B identifies multiple MAGE-A gene products¹⁴ and, in particular, that positive staining in sections from paraffin embedded tissues best correlates with MAGE-A4 gene expression.¹⁵ Sequencing of plasmids from 7 bacterial colonies indicates that this reagent recognizes an 11-mer sequence (residues 21–31) encompassed within a previously described MAGE-A3 gene fragment (Table Ib).¹⁶ Most importantly, largely homologous sequences were found to be shared by different members of the MAGE-A family including A1, -A2, -A3, -A4, -A5, -A6, -A9, -A10 and -A12.

In agreement with these data, D8.38 or 57B recognized proteins of the expected molecular weights in lysates from a large series of cell lines of different histological origin ($n=32$), where adequate ($\geq 1\%$ of control GAPDH house keeping gene) expression of

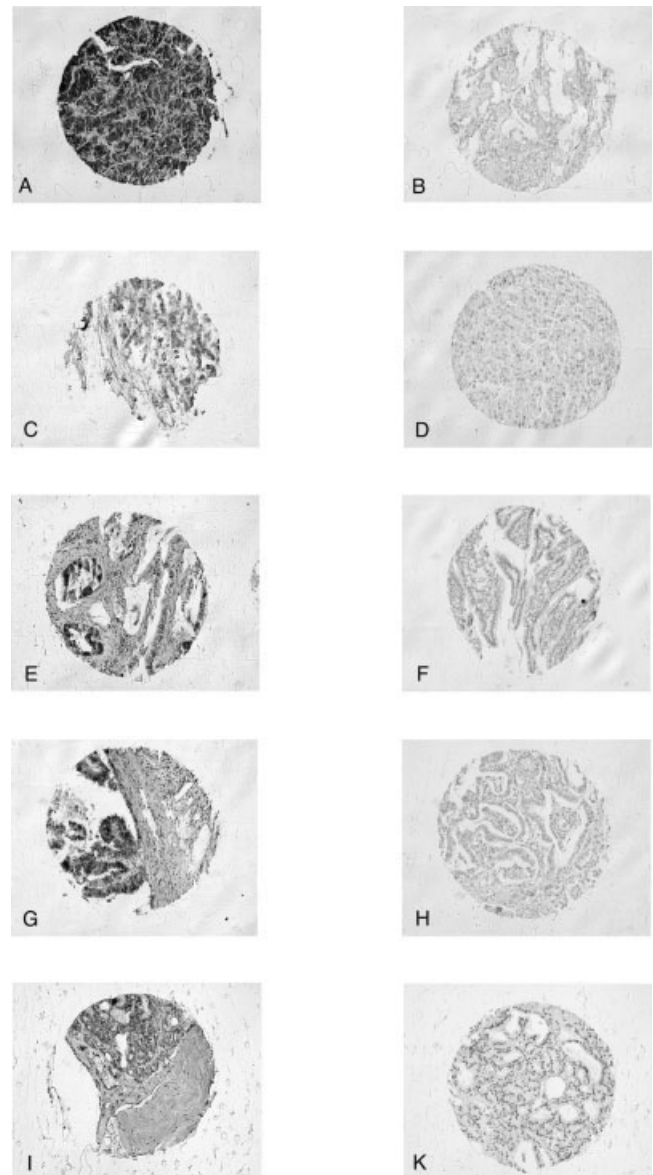


FIGURE 1 – Examples of D8.38 positive (*a,c,e,g,i*) and negative (*b,d,f,h,k*) tumors of similar histological origin: (*a,b*) Synovial sarcoma; (*c,d*) Hepatocellular carcinoma; (*e,f*) Adenocarcinoma of the small intestine; (*g,h*) Adenocarcinoma of the stomach, intestinal type; (*i-k*) Follicular carcinoma of the thyroid.

either NY-ESO-1/LAGE-1 or of 1 of the above listed MAGE-A genes was detectable by quantitative real time PCR (data not shown). In particular, specific gene products could be detected most frequently in melanoma (9/13) and in lung cancer cell lines (4/6), rarely in colorectal cancer or leukemic cell lines (1/5 and 1/4, respectively) and in none of the 3 pancreatic cancer cell lines tested.

Multitissue array investigation of NY-ESO-1/LAGE-1 expression

The expression of NY-ESO-1/LAGE-1 was then investigated at the protein level in a multitissue array by D8.38 staining. The array under investigation included 2,052 samples from 100 different malignancies, and 121 specimens from 27 healthy tissues. Within the latter group, 4/5 control testis sections were found to

TABLE III – IMMUNOSTAINING OF TUMORAL TISSUES BY NY-ESO-1/LAGE-1 SPECIFIC D8.38 MONOCLONAL ANTIBODY

Tissues (n = 100)	n	NY-ESO-1/ LAGE-1 pos ¹	%	Tissues (n = 100)	n	NY-ESO-1/ LAGE-1 pos ¹	%
Skin tumors				Bladder, sarcomatoid cancer	7	1	—
Malignant melanoma	38	12	31.6	Bladder, squamous cell carcinoma	5	0	—
Basalioma	33	6	18.2	Bladder, adenocarcinoma	2	0	—
Merkei cell carcinoma	4	0	—	Bladder, TCC non invasive (pTa)	31	2	6.4
Kaposi sarcoma	16	1	—	Bladder, TCC (pT2-4)	33	6	18.2
Dermatofibrosarcoma protuberans	1	0	—	Renal cell carcinoma, chromophobic	13	0	—
Squamous cell carcinoma	32	3	9.4	Renal cell carcinoma, papillary	37	1	2.7
Respiratory tract tumors				Renal cell carcinoma, clear cell	48	1	2.1
Lung cancer, large cell carcinoma	45	8	17.8	Prostate cancer, hormone refractory	30	2	6.7
Lung cancer, adenocarcinoma	38	4	10.5	Prostate cancer, undifferentiated	42	0	0
Lung cancer, squamous cell carcinoma	47	3	6.4	Testis, seminoma	49	3	6.1
Lung cancer, small cell carcinoma	41	4	9.7	Testis, non-seminomatous carcinoma	46	5	10.4
Lung cancer, broncholoalveolar carcinoma	10	0	—	Testis, teratoma	6	0	—
Head and neck tumors				Testis, other tumors	8	0	—
Parotid gland, mucoepidermoid carcinoma	3	0	—	Penis, carcinoma	36	0	0
Parotid gland, acinus cell carcinoma	5	0	—	CNS tumors			
Parotid gland, malignant mixed tumor	13	1	—	Esthesioneuroblastoma	2	0	—
Parotid gland, carcinoid tumor	6	1	—	Astrocytoma	30	0	0
Larynx, carcinoma	32	3	9.4	Glioblastoma multiforme	34	0	0
Oral cavity, squamous cell carcinoma	44	1	2.3	Medulloblastoma	4	0	—
Gynecologic tumors				Oligodendroglioma	12	0	—
Breast cancer, medullary carcinoma	26	1	3.8	Craniopharyngeoma	3	0	—
Breast cancer, ductal carcinoma	44	0	0	Malignant schwannoma	8	0	—
Breast cancer, apocrine carcinoma	3	0	—	Primitive neuroectodermal tumor (PNET)	15	1	—
Breast cancer, cribriform carcinoma	5	0	—	Optic nerve glioma	1	0	—
Breast cancer, lobular carcinoma	33	0	0	Endocrine tumors			
Breast cancer, mutinous carcinoma	22	1	4.5	Adrenal gland, carcinoma	6	1	—
Breast cancer, tubular carcinoma	20	0	0	Adrenal gland, adenoma	13	0	—
Breast cancer, phylloides tumor	8	0	—	Thyroid cancer, follicular	53	4	7.5
Ovarian cancer, endometrioid	43	3	6.9	Thyroid cancer, anaplastic	5	0	—
Ovarian cancer, serous	45	4	8.9	Thyroid cancer, papillary	22	0	0
Ovarian cancer, mutinous	16	0	—	Thyroid cancer, medullary	8	0	—
Ovarian cancer, rare types	5	2	—	Blood			
Uterine cervix, squamous cell carcinoma	26	0	0	Acute myeloid leukemia	1	0	—
Uterine cervix, adenocarcinoma	2	0	—	Chronic myeloid leukemia	4	0	—
Uterine cervix, CIS, CIN III, IA1, IB1	11	0	—	Lymphoma			
Endometrium, endometrioid adenocarcinoma	37	2	5.4	Lymphoepithelial carcinoma	4	0	—
Endometrium, serous adenocarcinoma	16	0	—	Hodgkin lymphoma, mixed cellularity	14	0	—
Vagina, squamous cell carcinoma	4	0	—	Hodgkin lymphoma, nodular sclerosis	27	0	0
Vulva, squamous cell carcinoma	36	1	2.8	Non-Hodgkin lymphoma	35	2	5.7
Gastrointestinal tumors				Soft tissue tumors			
Gastrointestinal stromal tumor (GIST)	12	0	—	Synovial sarcoma	1	1	—
Anus, squamous cell carcinoma	3	1	—	Fibrosarcoma	9	5	—
Colon, adenocarcinoma	44	1	2.3	Rhabdomyosarcoma	9	2	—
Colon adenoma, severe dysplasia	30	0	0	Malignant fibrous histiocytoma	24	2	8.3
Colon adenoma, moderate dysplasia	38	0	0	Leiomyosarcoma	44	0	0
Colon adenoma, mild dysplasia	32	0	0	Liposarcoma	26	4	15.4
Esophagus, carcinoma (several types)	35	3	8.6	Leiomyoblastoma	7	0	—
Stomach, adenocarcinoma, intestinal type	38	5	13.2	Malignant mesothelioma	15	0	—
Stomach, adenocarcinoma, diffuse type	16	1	—	Alveolar sarcoma	1	0	—
Stomach, adenocarcinoma, mixed type	2	0	—	Angiosarcoma	3	0	—
Signet-ring cell carcinoma	4	0	—	Epithelioid sarcoma	2	1	—
MALT lymphoma	26	0	0	Thymus tumors			
Gallbladder, adenocarcinoma	21	0	0	Thymoma	20	0	0
Hepatocellular carcinoma	30	1	3.3	Total	2,052	119	5.8%
Pancreas, adenocarcinoma	45	0	0				95% CI
Small intestine, adenocarcinoma	6	1	—				[4.7%–6.8%]
Genitourinary tract tumors							
Bladder, small cell cancer	3	1	—				

¹Positivity (see Material and Methods) is defined by weak intensity staining in > 66% of tumor cells or moderate intensity staining in > 34% of tumor cells or strong intensity in any percentage of tumor cells. Percentages of positive cases are only provided when >20 samples were evaluated.

be stained by D8.38, as expected (Table II).¹⁷ Unexpectedly, however, 1 healthy lung section was also positive. We have no obvious explanation for this finding. No other healthy tissue was stained by D8.38 Mab.

A 5.8% of the tumors (119/2,052) scored positive upon staining with the NY-ESO-1/LAGE-1 specific reagent. For 48 different tumor types, representative numbers of cases (n=20) were avail-

able within the array. In 8 of these entities, evidence of NY-ESO-1/LAGE-1 expression was detectable in >10% of the cases. This group included melanoma and basalioma (31.6 and 18.2%, respectively), large cell carcinomas and adenocarcinomas of the lung (17.8 and 10.5%, respectively), stomach adenocarcinomas of the intestinal type (13.2%), pT2-4 bladder TCC (18.2%), non seminomatous carcinomas of the testis (10.4%) and liposarcomas

TABLE IV – CO-EXPRESSION OF NY-ESO-1/LAGE-1 AND MAGE-A C/T ANTIGENS IN TUMORAL TISSUES AS DETECTED BY IMMUNOSTAINING WITH D8.38 AND 57B MONOCLONAL ANTIBODIES

Tissues (n = 90)	Sample (total)	NY-ESO-1/ LAGE-1 pos ¹	MAGE-A pos ¹	NY-ESO-1/LAGE-1 and MAGE-A pos ¹
Skin tumors				
Malignant melanoma	6	2	2	0
Basalioma	16	5	8	3
Merkel cell carcinoma	1	0	0	0
Granular Cell Tumor	5	0	0	0
Glomus tumor	4	0	0	0
Squamous cell carcinoma	13	0	4	0
Respiratory tract tumors				
Lung cancer, large cell carcinoma	11	3	5	3
Lung cancer, adenocarcinoma	9	0	0	0
Lung cancer, squamous cell carcinoma	2	0	0	0
Lung cancer, small cell carcinoma	6	2	0	0
Lung carcinoid	5	0	0	0
Head and neck tumors				
Granular cell tumor	5	0	0	0
Larynx squamous cell carcinoma	7	0	3	0
Oral cavity, squamous cell carcinoma	4	1	2	1
Gynecologic tumors				
Breast cancer, medullary carcinoma	10	0	1	0
Breast cancer, ductal carcinoma	32	1	2	1
Breast cancer, apocrine carcinoma	1	0	0	0
Breast cancer, cribriform carcinoma	5	0	0	0
Breast cancer, lobular carcinoma	22	0	0	0
Breast cancer, mucinous carcinoma	7	0	0	0
Breast cancer, tubular carcinoma	4	0	0	0
Breast cancer, phylloides tumor	9	0	0	0
Ovarian cancer adenocarcinoma	2	0	0	0
Ovarian cancer, endometrioid	21	2	1	0
Ovarian cancer, serous	31	3	6	3
Ovarian cancer, mucinous	8	0	0	0
Ovarian cancer, rare types	4	0	2	0
Ovary, Brenner tumor	4	0	0	0
Uterine cervix, squamous cell carcinoma	9	0	2	0
Uterine cervix, adenocarcinoma	1	0	0	0
Uterine cervix, CIS, CIN III, 1A1, 1B1	1	0	0	0
Endometrium carcinoma	38	3	1	0
Endometrium stroma sarcoma	2	0	0	0
Uterus, mullerian tumor	5	1	3	1
Vagina, squamous cell carcinoma	3	0	1	0
Vulva, squamous cell carcinoma	31	1	3	1
Gastrointestinal tumors				
Gastrointestinal stromal tumor (GIST)	9	0	1	0
Anus, squamous cell carcinoma	2	0	0	0
Appendix, carcinoid	3	0	0	0
Colon, adenocarcinoma	29	1	1	0
Esophagus adenocarcinoma	1	0	0	0
Esophagus squamous cell carcinoma	8	0	0	0
Small intestine carcinoid	6	0	0	0
Stomach, adenocarcinoma	46	6	8	1
Stomach carcinoid	1	0	0	0
Gallbladder, adenocarcinoma	6	0	3	0
Hepatocellular carcinoma	17	0	0	0
Pancreas, adenocarcinoma	11	0	0	0
Small intestine, adenocarcinoma	5	0	0	0
Papilla vateri adenocarcinoma	3	0	0	0
Genitourinary tract tumors				
Bladder, small cell cancer	2	1	2	1
Bladder, sarcomatoid cancer	5	0	1	0
Bladder, inverted papilloma	1	0	0	0
Bladder, squamous cell carcinoma	4	0	1	0
Bladder, adenocarcinoma	3	0	1	0
Bladder, TCC non invasive (pTa)	16	1	0	0
Bladder, TCC (pT2-4)	10	1	4	0
Prostate cancer, hormone refractory	1	0	0	0
Testis, seminoma	32	6	9	4
Testis, non-seminomatous carcinoma	33	3	1	0
CNS Tumors				
Esthesioneuroblastoma	2	0	0	0
Astrocytoma	16	1	0	0
Glioblastoma multiforme	16	0	0	0
Medulloblastoma	3	0	0	0
Oligodendroglioma	9	0	0	0
Craniopharyngeoma	1	0	0	0
Malignant schwannoma	5	0	1	0
Primitive neuroectodermal tumor (PNET)	10	0	1	0

TABLE IV – CO-EXPRESSION OF NY-ESO-1/LAGE-1 AND MAGE-A C/T ANTIGENS IN TUMORAL TISSUES AS DETECTED BY IMMUNOSTAINING D8.38 AND 57B MONOCLONAL ANTIBODIES (CONTINUED)

Tissues (n = 90)	Sample (total)	NY-ESO-1/ LAGE-1 pos ¹	MAGE-A pos ¹	NY-ESO-1/LAGE-1 and MAGE-A pos ¹
Endocrine tumors				
Adrenal gland, carcinoma	5	1	1	1
Thyroid cancer, follicular	20	9	5	5
Thyroid cancer, anaplastic	4	0	1	0
Thyroid cancer, papillary	17	2	1	1
Thyroid cancer, medullary	6	0	0	0
Blood				
Chronic myeloid leukemia	4	0	0	0
Lymphoma				
Lymphoepithelial carcinoma	2	0	0	0
Hodgkin lymphoma, mixed cellularity	13	1	0	0
Hodgkin lymphoma, nodular sclerosis	5	0	0	0
Non-Hodgkin lymphoma	6	0	0	0
Soft tissue tumors				
Synovial sarcoma	3	1	2	1
Fibrosarcoma	5	5	4	4
Rhabdomyosarcoma	5	1	1	1
Malignant fibrous histiocytoma	12	1	1	0
Liposarcoma	15	5	0	0
Leiomyoblastoma	43	0	1	0
Malignant mesenchymoma	7	1	2	1
Malignant mesothelioma	4	0	0	0
Hemangiopericytoma	4	2	2	2
Angiosarcoma	1	0	0	0
Epithelioid sarcoma	1	0	0	0
Mucoepidermoidcarcinoma	3	0	0	0
	845	73	101	35
		8.6% 95% CI [6.7%–10.5%]	12.0% 95% CI [9.8%–14.1%]	4.1% 95% CI [2.8%–5.5%]

¹Positivity (see Material and Methods) is defined by weak intensity staining in >66% of tumor cells or moderate intensity staining in >34% of tumor cells or strong intensity in any percentage of tumor cells.

(15.4%, Table III). Examples of positive stainings are reported in Figure 1.

Coexpression of NY-ESO-1/LAGE and MAGE-A C/T antigens

NY-ESO-1/LAGE-1 share a number of characteristics with MAGE-A C/T antigens.¹ Among healthy cells, both are almost exclusively expressed in germ cells at different maturation stages, whereas their expression is detectable in malignancies of different histological origin. Furthermore, both are encoded within chromosome X, albeit in regions distant from each other. Importantly, coexpression of these C/T antigens might provide a strong incentive to the design of multiantigen vaccines. Prompted by these considerations, we have stained an array including over 800 of the previously investigated tumor samples with multi-MAGE-A specific 57B antibody.

In this series, 101/845 samples (12%) showed evidence of MAGE-A specific staining, while in 73/845 samples (8.6%) NY-ESO-1/LAGE-1 gene products were detectable by immunohistochemistry; 35/845 specimens (4.1%) showed concomitant expression of MAGE-A and NY-ESO-1/LAGE-1. Thus, the expression of the 2 sets of C/T antigens was found to be significantly correlated ($p=0.00002$). In particular, in basalionomas, large cell carcinomas of the lung, serous ovarian cancers, seminomas and follicular thyroid carcinomas the C/T TAA under investigation were found to be largely coexpressed (Table IV).

However, in defined types of cancer discrepancies in the expression of NY-ESO-1/LAGE-1 and MAGE-A C/T antigens were conspicuously detectable. For instance, 4/13 squamous cell carcinomas of the skin were MAGE-A positive but none was NY-ESO-1/LAGE-1 positive. Conversely, 5/15 liposarcomas were NY-ESO-1/LAGE-1 positive, but none was MAGE-A positive.

Discussion

C/T antigens are of particular interest in tumor immunology since they are detectable in histologically unrelated tumors and may induce both CTL and CD4+ T cell responses.^{3,18} In clinical materials, their expression has mostly been studied at the gene expression level by polymerase chain reaction (PCR), a technology providing limited information because it does not allow the quantitation of TAA positive cancer cells. These data are important for clinical studies since effective immunization targeting tumor associated antigens expressed in low percentages of tumour cells, may be of modest therapeutic relevance. Thus, a number of serological reagents have been produced and studied in different tumors.¹⁹

Clearly, the identification of target epitopes, resulting in improved specificity assessments,¹⁵ might emerge as a crucial requirement for a wider use of these reagents. Here we mapped the epitopes identified by 2 MAb generated by using recombinant MAGE-A3 (57B) and NY-ESO-1 (D8.38) as immunogens. We found that the former recognizes sequences shared by MAGE-A1-A2, -A4-A5, -A6,-A9, -A10 and -A12, whereas the latter is specific for sequences shared by both NY-ESO-1 and LAGE-1. These data, providing the first epitope mapping of C/T antigen specific MAbs, should nevertheless be interpreted cautiously because paraffin embedding of tissues, fixation and antigen retrieval might bias antigen recognition in forms intrinsic to immunohistochemical techniques but not to bacterial expression systems.

TMA represent a powerful screening tool allowing the assembling of large databases on the expression of gene products recognized by specific MAb, thereby suggesting additional investigations focusing on discrete neoplastic diseases. An obvious limitation, regarding multitumor TMA is represented by the relatively small number of cases for individual types of cancer. This

holds true in particular for the second MTA used in this work (845 samples for 90 different types of tumors). Furthermore, especially for markers characterized by focal expression, data obtained with TMA might not be fully congruent with data derived from the staining of conventional, larger size sections.

While 57B MAGE-A specific reagent has been studied on TMA12, data on NY-ESO-1/LAGE-1 expression at the protein level are limited to relatively small numbers of cases.⁶ Here we provide a large database on immunohistochemical detection of these determinants, consistent with an expression closely matching that of MAGE-A C/T antigens.

Importantly, C/T antigens have been suggested to be typically expressed in the presence of widespread DNA demethylation, as detectable in clinical specimens or in tumor cell lines²⁰ untreated or following treatment with demethylating agent 5-aza-2'-deoxycytidine.^{21,13} This notion may fit well with our findings of a highly significant association, at the protein level, of the expression of both MAGE-A and NY-ESO-1/LAGE-1 C/T antigens in tumors of different histological origin. On the other hand, detection of preferential expression of either C/T antigen family in discrete types of cancer might suggest the existence of specific DNA demethylation profiles.

Successful active specific immunotherapy of cancer might result in the immunoselection of tumor variants failing to express relevant tumor associated antigens included in vaccine preparations. Multiantigen specific immunization might help in limiting this immunoescape phenomenon. Coexpression of MAGE-A and NY-ESO-1/LAGE-1 C/T antigens has rarely been studied at the protein level.^{22,23} Our data suggest that vaccine preparations inclusive of both MAGE-A and NY-ESO-1/LAGE-1 epitopes might be advantageously used in the treatment of defined types of tumor.

On the other hand, the TMA data presented in this work may underestimate the immunological relevance of C/T antigen expression in clinical specimens. Most obviously, we cannot exclude that even minor positive reactivities do indeed reflect a gene expression sufficient to provide epitopes capable of stimulating immune responses upon HLA binding.

Taken together, these data set the stage for future investigations addressing the basic cell biology mechanisms underlying multiple C/T antigen expression in cancer and the therapeutic potential offered by multiantigen vaccine preparations in active specific immunotherapy.²⁴

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