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**Expression of cancer/testis (CT) antigens MAGE-A1, MAGE-A3, MAGE-A4, CT-7, and NY-ESO-1 in malignant gammopathies is heterogeneous and correlates with site, stage and risk status of disease**

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## Abstract

Cancer/testis (CT) antigens are expressed in several malignant tumors, but not in normal tissues except for testicular germ cells. The expression of CT antigenic proteins in malignant gammopathies has not been characterized. We examined the expression of a panel of CT antigenic proteins in 29 patients with malignant gammopathies by immunohistochemistry using the following monoclonal antibodies (mAbs): mAb MA454 to MAGE-A1, mAb M3H67 to MAGE-A3, mAb 57B to MAGE-A4, mAb CT7-33 to CT7/MAGE-C1 and mAb ES121 to NY-ESO-1. We could detect at least one CT antigen in tumors from 27 of 29 patients. The expression pattern of MAGE-A1, -A3, -A4 and NY-ESO-1 is heterogeneous in most cases, revealing staining in <25% of the tumor cells. Monoclonal antibodies CT7-33 and M3H67 show the highest incidence of immunoreactivity. Importantly, CT-7 can also be detected on the surface of some myeloma cells by flow cytometry, and in one plasmacytoma case by immunohistochemistry. Expression of CT antigens is greater in patients with stage III extramedullary plasmacytoma or high-risk myeloma relative to other cohorts. These data suggest that CT antigens may have important biological implications in malignant gammopathies and that CT-7 may be a suitable target for T cell-based and possibly antibody-mediated immunotherapy of myeloma.

## Introduction

Cancer/testis antigens are expressed in various malignant tumors, but not in normal adult tissues, except for testicular germ cells and occasionally placenta (1, 2). CT antigens are immunogenic and elicit cellular and humoral immune responses in a subset of tumor patients (1, 3). Of the more than 20 CT genes and gene families now identified, the two best-studied systems are the MAGE family and NY-ESO-1 (2). Another CT antigen, CT-7, was identified by cDNA expression cloning using sera from a melanoma patient (4). CT-7 was independently isolated by another group and termed MAGE-C1 (5). Due to their tumor-restricted expression pattern and their immunogenicity, CT antigens are under active evaluation for immunotherapy of cancer (6).

Multiple myeloma (MM) is an incurable tumor characterized by multifocal clonal expansion of malignant plasma cells in the bone marrow. In patients with plasmacytomas, such expansions are clinically unifocal and restricted to the bone (medullary plasmacytomas) or extramedullary tissues (extramedullary plasmacytomas). Due to the modest efficacy of current therapies, there is significant interest in approaches harnessing anti-tumor immunity in myeloma. In prior studies, we and others have documented the presence of RNA transcripts encoding members of the MAGE gene family, as well as NY-ESO-1 and CT-7/MAGE-C1 in myeloma tumor cells and cell lines (7, 8, 9, 10). However, the expression of these antigens at the protein level in MM and plasmacytomas has not been analyzed. Consequently, the present study analyzes the expression of MAGE-A1, -A3, -A4, CT-7 (MAGE-C1), and NY-ESO-1 by immunohistochemistry in a panel of MMs and plasmacytomas.

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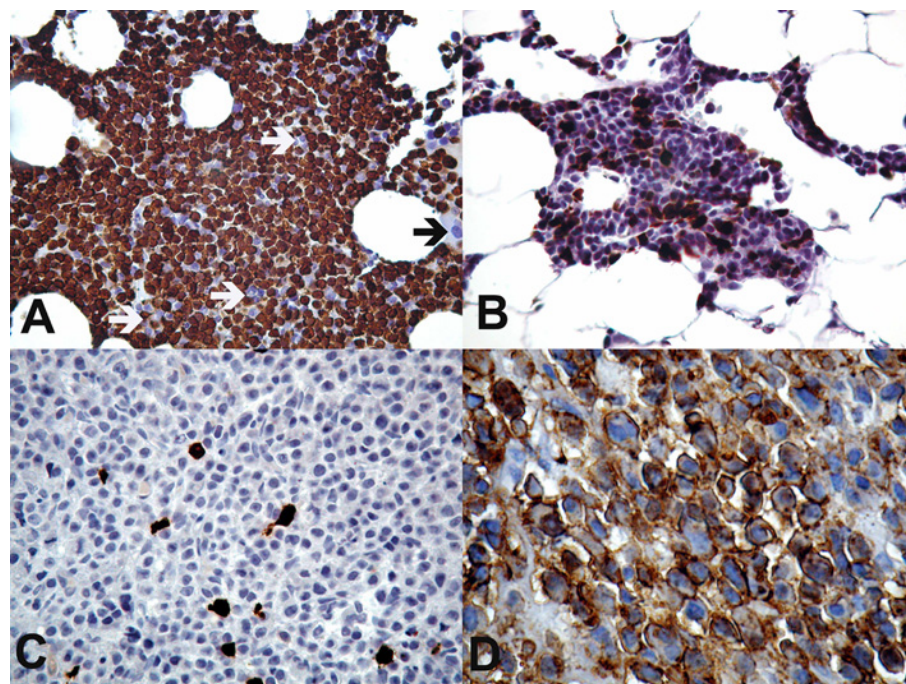
## Results

Twenty-nine cases with histologically proven malignant gammopathies (13 myelomas, 12 medullary plasmacytomas, and 4 extramedullary plasmacytomas) were retrieved. The results of immunohistochemical staining with the mAbs MA454, M3H67, 57B, CT7-33 and ES121 are listed in Table 1. Overall, reactivity with at least one of the mAbs was detected in 27 of 29 patients tested (Table 1 and Figure 1). Immunoreactivity with mAbs CT7-33 (79%) and M3H67 (48%) was higher than with MA454 (24%), 57B (21%) and ES121 (27%;  $P<0.01$ ). The staining pattern was predominantly heterogeneous for mAbs MA454, 57B, and ES121, for the most part staining <25% of the tumor cells. Representative staining showing focal immunoreactivity (Figure 1C), or expression in 25-50% tumor cells (Figure 1B) is shown. CT7-33 and M3H67 showed a homogeneous immunoreactivity pattern (Figure 1A), corresponding to a +++ - ++++ staining in our grading system in 11/24 and 6/14 positive cases respectively (Table 1). However even in these tumors, clearly immunonegative tumor cells can be observed adjacent to strongly positive cells (Figure 1A). Immunoreactivity was predominantly cytoplasmic for mAbs ES121 and MA454, and cytoplasmic as well as nuclear for mAbs CT7-33 and 57B (Figure 1, panels A-C). Interestingly, one plasmacytoma case showed a membranous staining pattern of tumor cells with mAb CT7-33 in some areas (Figure 1D). Immunoreactivity of CT7-33 correlated with M3H67 ( $r=0.53$ ,  $P<0.01$ ) and other MAGE markers, but not with ES121 ( $r=0.05$ ,  $P=0.8$ ). Expression of CT-7 was further examined by flow cytometry in myeloma cell lines, and was detected in cag cells, but not arp cells, correlating with prior RT-PCR data (7) (Figure 2). Interestingly, CT-7 expression was also seen in cells not treated by permeabilization, which was interpreted as cell surface staining.

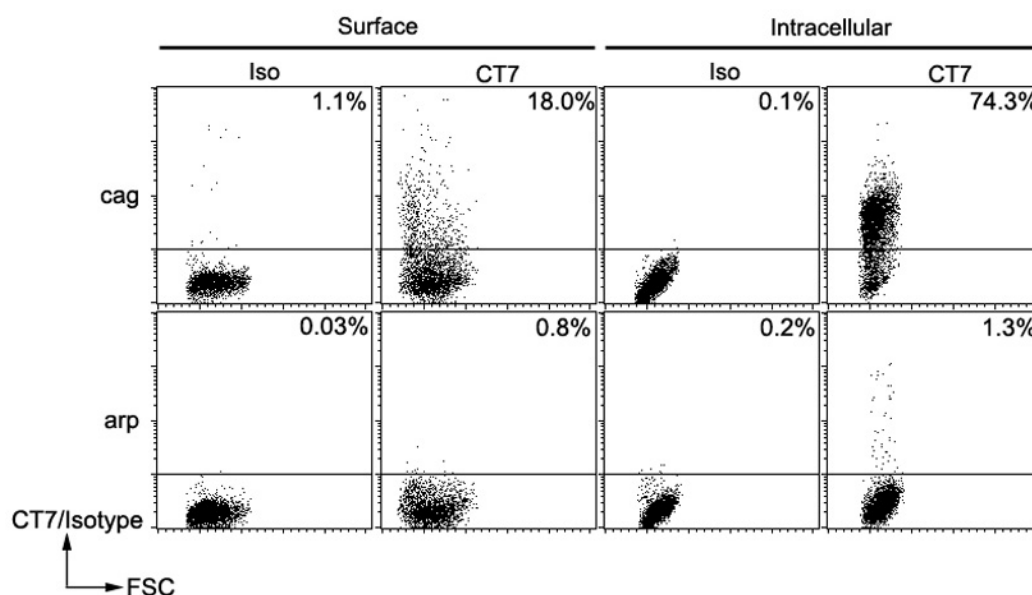
**Table 1. Patient characteristics and immunophenotyping of malignant gammopathies for expression of MAGE-A1, MAGE-A3, MAGE-A4, CT-7, and NY-ESO-1 on tumor cells<sup>a</sup>. (next page)**

Diagnosis	Patient No.	Age	Stage	Cytogenetics	B2M (mg/l)	Albumin (g/dl)	LDH (U/l)	MAGE-A1 (Mab MA454)	MAGE-A3 (Mab M3H67)	MAGE-A4 (Mab 57B)	CT-7 (Mab CT7-33)	NY-ESO-1 (Mab ES121)
Myeloma	1	70	III	normal	9.4	4.1	ND	1+	3+	2+	4+	-
	2	77	III	t(13:14, 11q14)	1.3	3.7	ND	focal	focal	-	-	-
	3	41	II	normal	1.8	4.9	140	-	-	-	1+	-
	4	71	III	trisomy 11	6.7	3.1	177	-	2+	-	4+	-
	5	63	III	13q-, trisomy 11	5.2	4.1	104	-	-	-	focal	-
	6	57	III	13q-	NMA	5.4	179	-	1+	-	2+	-
	7	73	III	trisomy 11	4.9	3.6	ND	-	-	-	1+	-
	8	53	II	ND	NMA	4.2	130	-	-	-	-	-
	9	70	III	normal	7.5	2.8	107	-	2+	-	3+	1+
	10	73	I	normal	1.4	3.6	95	-	-	-	1+	-
	11	57	III	normal	7.8	2.7	ND	-	-	-	-	-
	12	42	III	NA	3.5	4	196	4+	2+	-	focal	focal
	13	79	IIIB	normal	4.7	4.3	268	-	-	-	-	1+
Extramedullary plasmacytoma	14	67		trisomy 11	ND	4	122	2+	4+	4+	4+	focal
	15	52		normal	NMA	4.5	140	1+	4+	focal	4+	-
	16	68		ND	1.3	ND	ND	-	focal	-	-	-
	17	64		ND	2.2	3.9	ND	2+	3+	2+	3+	focal
Plasmacytoma	18	58		ND	ND	3.5	ND	focal	3+	focal	4+	focal
	19	65		ND	NMA	4.3	184	-	-	-	focal	-
	20	72		normal	2.1	4.5	ND	-	focal	-	1+	-
	21	55		NA	5.7	4	99	-	-	-	-	focal
	22	56		ND	NMA	4.5	ND	-	-	focal	4+	-
	23	44		ND	ND	4.6	120	-	-	-	focal	-
	24	83		NA	10.2	4.8	151	-	-	-	focal	-
	25	53		ND	3.7	ND	ND	-	-	-	1+	-
	26	71		ND	3.5	ND	ND	-	-	-	3+	focal
	27	67		ND	8.1	ND	ND	-	3+	-	4+	-
	28	44		normal	4	ND	ND	-	focal	-	focal	-
	29	71		ND	ND	3.7	88	-	-	-	4+	-
Total number of patients with CT antigen positive tumor cells (% positive)					7 (24%)			14 (48%)			23 (79%)	
Total number of patients with CT antigen positivity in >50% tumor cells (% positive)					1 (3%)			6 (21%)			11 (38%)	
											8 (27%)	
											0 (0%)	

<sup>a</sup>Grading for expression of CT antigens on tumor cells: focal, <5%; 1+, 5-24%; 2+, 25-49%; 3+, 50-74%; 4+, 75-100%. Abbreviations: NA, not analyzable; ND, not done; NMA, no measurable amount; B2M, serum beta-2-microglobulin; LDH, serum lactate dehydrogenase.



**Figure 1. Expression patterns of CT antigens in malignant gammopathies, exemplified by immunohistochemical staining with mAb CT7-33.** (A) Homogeneous staining of MM by mAb CT7-33 with only occasional scattered immunonegative myeloma cells (arrow) (patient # 4; obj. 20x). (B) Heterogeneous CT7-33 immunoreactivity in a MM case with an overall staining of 25-50% tumor cells (patient # 6; obj. 20x). (C) Focal immunoreactivity of mAb CT7-33 with only single positive myeloma cells (patient # 12; obj. 20x). (D) Membranous CT7-33 immunoreactivity in a plasmacytoma case (patient # 26; obj. 60x).



**Figure 2. Detection of cell surface and intracellular CT-7 by flow cytometry.** FACS plots showing cell surface and intracellular staining for CT-7 using mAb CT7-33 in cag (CT-7 positive by RT-PCR) and arp cells (CT-7 negative).



Next, we explored the correlation of the expression of CT antigens with clinical presentation and major prognostic factors. For this, we pooled the data for percent expression of individual antigens in each patient to generate a CT score, which allows a more global assessment of CT antigen expression at the protein level. Patients with extramedullary plasmacytomas had greater expression of CT antigens compared to patients with medullary plasmacytomas or MM (mean CT score of 2.4 versus 0.8 and 0.9 respectively,  $P < 0.01$ ). Among MM patients, expression of CT antigens was higher in patients with stage III disease versus lower stage disease (mean CT score of 1.1 versus 0.1,  $P = 0.02$ ). Patients with high serum beta-2-microglobulin ( $> 5.5$  mg/l), which constitutes high-risk disease, also had higher expression of CT antigens than those with lower risk disease (mean CT score of 1.5 versus 0.5,  $P = 0.06$ ).

## Discussion

In myeloma and plasmacytoma, CT antigen expression has previously been characterized only at the mRNA level (7, 8, 9, 10). However, for these antigens there are discrepancies between the mRNA and protein levels determined by expression analyses, and for immunotherapy, knowledge of the actual antigen presence is essential (2). Our data demonstrate that CT antigens can be detected across the entire spectrum of malignant gammopathies, from medullary plasmacytomas to MM and extramedullary plasmacytomas. We also show that the pattern of expression of CT antigens is heterogeneous for MAGE-A1, -A4 and NY-ESO-1, with only a small proportion of cases displaying strong expression, while CT-7 and MAGE-A3 appear to be homogeneously expressed in almost half of the positive cases. The basis for the different expression patterns of CT antigens, also seen in other cancers, is not known, but has important implications for immune targeting of these antigens (2). A heterogeneous protein expression pattern of CT antigens has been observed with these reagents in many other tumor types, such as carcinomas and occasionally in sarcomas (2).

Due to the high homology of several CT antigens, cross-reactivities of anti-CT reagents cannot be fully excluded. This is illustrated by mAb 57B, which was initially thought to be MAGE-A3-specific, was subsequently considered a poly-MAGE reagent, but was eventually found to be predominantly reactive with MAGE-A4 (2). However, no cross-reactivity of these reagents to any protein outside their gene family or to any non-CT antigen has been found. Nevertheless, the fine specificity of these reagents should be regarded with caution. Our study indicates that in the present panel, CT-7 is the most dominant CT antigen in malignant gammopathies at the protein level. The immunogenicity of this antigen in myeloma should therefore be of interest. CT antigens are intracellular proteins and give rise to MHC-restricted epitopes. Surprisingly, flow cytometry of cell lines supports the notion that CT-7 can also be expressed on the cell surface of some myeloma cells. This is corroborated by the membranous immunostaining observed in one of our plasmacytoma cases. CT antigens are usually intracellular (2), as confirmed here. Detection of CT-7 on the cell surface needs further study and may relate to its somewhat unusual structure among CT antigens (2). This may however have important implications for tumor-specific targeting of myeloma using monoclonal antibodies. As seen previously in other neoplasms, expression of CT-7 correlated highly with that of other MAGE family members, such as MAGE-A3, but not of NY-ESO-1 (11), supporting the possibility that the expression of some but not all CT antigens may be co-regulated, possibly related to genome-wide demethylation in cancer.

In the present study, expression of CT antigens was higher in patients with extra-medullary disease, higher Durie-Salmon stage, and high-risk disease. These data suggest that CT antigens may be linked to clonal evolution of plasma cell tumors. Indeed, a recent study comparing gene expression profiles in normal versus tumor plasmablasts found that CT antigens were among the most upregulated genes in tumor cells, and the expression of some CT antigens (e.g. SSX-1) was higher in plasma cell leukemia (12). At present, the function of most of the CT genes is unknown, but studies suggesting their roles in cell cycle progression and apoptosis are emerging (13).

In conclusion, our study documents the expression of several CT antigens in myeloma and plasmacytoma at the level of the antigenic protein. The expression of several of the CT antigens tested is heterogeneous. However, tumor cells in a significant proportion of cases demonstrate homogeneous expression of CT-7, suggesting that this may be a useful target for immuno-therapy of myeloma and plasmacytoma. The surface expression of CT-7 merits further investigation, and might provide a novel opportunity for antibody-based therapeutics in some patients. Several groups have already initiated clinical studies targeting CT antigens (particularly MAGE-A3 and NY-ESO-1) in myeloma, based on the presence of RNA transcripts for these antigens in tumor cells. Our data suggest that the level of antigenic protein in these tumors may be variable and may affect the results of such approaches. Identification of additional tumor-specific antigens with homogeneous expression in tumor cells is warranted to develop widely applicable poly-specific myeloma vaccines.

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## Abbreviations

CT, cancer/testis; MM, multiple myeloma

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## Materials and methods

### Patients and tissue samples

Formalin-fixed paraffin-embedded samples of bone marrow biopsies or surgical tissues were obtained from the archives of the Department of Pathology at the Memorial Sloan-Kettering Cancer Center. Clinical information was obtained by retrospective chart review.

### Immunohistochemical analysis

The following monoclonal antibodies were used for the detection of the CT antigens: mAb MA454 to MAGE-A1 ([14](#)), mAb M3H67 to MAGE-A3/6 ([15](#)), mAb 57B to MAGE-A4 ([16](#), [17](#)), mAb CT7-33 to CT7/MAGE-C1 ([18](#)), and mAb ES121 to NY-ESO-1 ([19](#)). Immunohistochemistry was performed as described previously ([19](#)). Briefly,

primary antibodies were applied overnight at 4°C. A biotinylated horse anti-mouse secondary antibody (1:200; Vector, Burlingame, CA) was used to detect the primary antibody, followed by an avidin-biotin system (ABC-elite kit, Vector). 3,3-diaminobenzidine tetrahydrochloride (Biogenex, San Ramon, CA) served as chromogen. A heat-based antigen-retrieval method (AGR) was applied to all slides. DAKO hipH-solution and EDTA (1 mmol, pH 8.0) were used as the AGR solution for mAbs ES121 and MA454 respectively, and citrate (10 mmol, pH 6.0) was used for mAbs CT7-33 and 57B. The following antibody concentrations were used: MA454 1.0 µg/ml, M3H67 1.0 µg/ml, 57B 0.25 µg/ml, ES121 2.5 µg/ml, CT7-33 0.5 µg/ml. Testis with intact spermatogenesis served as a control tissue. Appropriate negative controls omitting the primary reagent were included for each case. The extent of tumor staining was estimated on the basis of tumor cells stained and graded as follows: Focal, approximately <5%; +, 5-25%; ++, >25-50%; +++, >50-75%; and +++++, >75%.

### Flow cytometric detection of CT antigens

Monoclonal antibody CT7-33 was also utilized for the detection of CT-7 in tumor cell lines cag and arp by flow cytometry. Cells were stained with mAb CT7-33 (dilution 1:100; 1 µg/ml) for 30 minutes on ice, followed by PE-conjugated goat anti-mouse IgG secondary antibody. For permeabilization, cells were preincubated with 1% saponin in FACS buffer (PBS with 1% fetal calf serum, 1% pooled human serum, and 0.2% sodium azide). Flow cytometry was performed using FACSCalibur (Becton Dickinson) and analyzed with CellQuest software.

### Statistical analysis

To correlate the antigen expression data with the clinical or biological features of the tumor, we first transformed CT antigen grading as noted above to a CT antigen score over a scale of 0-5. For some comparisons, antigen scores for individual antigens in each patient were pooled to allow a more global assessment of CT expression. Expression of CT antigens in various groups was compared using the Kruskal-Wallis test, or with the Mann-Whitney test when only two groups were compared. Correlation between different antigens was tested using Spearman's rank correlation.

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