Cancer Immunity (1 February 2008) Vol. 8, p. 2
Submitted: 5 September 2007. Accepted: 8 October 2007.

CANCER
IMMUNITY
Article

Prognostic impact of cancer/testis antigen expression in advanced stage multiple myeloma patients

Valéria C. C. Andrade[1], André L. Vettore[2,3], Roberta S. Felix[1], Manuella S. S. Almeida[1], Fabrício de Carvalho[1], José Salvador R. de Oliveira[1], Maria de Lourdes Lopes Ferrari Chauffaille[1], Adagmar Andriolo[4], Otavia L. Caballero[5], Marco Antonio Zago[6] and Gisele W. B. Colleoni[1]

[1]*Discipline of Hematology and Hemotherapy, Universidade Federal de São Paulo, UNIFESP, São Paulo, Brazil*
[2]*Ludwig Institute for Cancer Research, São Paulo Branch, Brazil*
[3]*Universidade Federal de São Paulo, UNIFESP, Diadema, Brazil*
[4]*Laboratory Medicine Department, Universidade Federal de São Paulo, UNIFESP, São Paulo, Brazil*
[5]*Ludwig Institute for Cancer Research, New York Branch, USA*
[6]*Faculdade de Medicina de Ribeirão Preto/USP, Brazil*

Communicated by: LJ Old

This study aims to analyze the expression of 14 cancer/testis (CT) antigens in multiple myeloma (MM) to identify possible prognostic markers and therapeutic targets. The expression of *MAGEA1*, *MAGEA2*, *MAGEA3/6*, *MAGEA4*, *MAGEA10*, *MAGEA12*, *BAGE1*, *MAGEC1/CT7*, the *GAGE* family, *LAGE-1*, *PRAME*, *NY-ESO-1*, *SPA17* and *SSX1* was studied by RT-PCR in 15 normal tissues, a pool of 10 normal bone marrow samples, 3 normal tonsils and bone marrow aspirates from 6 normal donors, 3 monoclonal gammopathies of undetermined significance (MGUS), 5 solitary plasmacytomas, 39 MM samples (95% advanced stage) and the MM cell line U266. *MAGEC1/CT7* was expressed in bone marrow aspirates from one MGUS and one plasmacytoma. The frequencies at which CT antigen were found to be expressed in MM patients were *MAGEC1/CT7* 77%, *LAGE-1* 49%, *MAGEA3/6* 41%, *MAGEA2* 36%, *GAGE* family 33%, *NY-ESO-1* 33%, *BAGE-1* 28%, *MAGEA1* 26%, *PRAME* 23%, *SSX-1* 26%, *MAGEA12* 20.5%, *MAGEA4* 0%, and *MAGEA10* 0%. Cox's regression model showed that *GAGE* family expression and having >6 CT antigens expressed were independent prognostic factors when all patients were analyzed. However, *MAGEC1/CT7* expression was the only independent prognostic factor when non-transplanted patients where analyzed. Based on our findings, MAGEC1/CT7, MAGEA3/6 and LAGE-1 are good candidates for immunotherapy, since together they cover 85% of our MM cases. Furthermore, expression of the *GAGE* family, >6 CT antigens and *MAGEC1/CT7* seem to have impact on MM prognosis.

Keywords: human, multiple myeloma, CT antigens, mRNA, tissue distribution, prognosis

Introduction

Multiple myeloma (MM) is a malignancy characterized by the proliferation of a clonal plasma cell, with a resultant increase of a single immunoglobulin and its fragments in the serum and urine, osteolytic lesions, anemia and hypercalcemia (1). High-dose chemotherapy followed by autologous stem cell transplant increases complete response rates and overall survival (OS). On the other hand, an allogeneic stem cell transplant represents a chance of cure for selected cases but also results in high mortality and morbidity (2). Currently, immunomodulatory drugs, such as thalidomide and its analogs (lenalidomide), and proteasome inhibitors (bortezomib) have been used as single or combined agents for relapsed/refractory cases and have even been used as first line therapy (3). However, despite of all these new therapeutic alternatives, MM remains an incurable disease, with a median survival of 3 years (1). Therefore, insights into the biology of aberrant plasma cell differentiation in monoclonal gammopathy of undetermined significance (MGUS) and MM offer the potential for new therapeutic approaches (4). In this context, tumor vaccines are very attractive therapeutic strategies because they may induce death of tumor cells resistant to current chemotherapy protocols. In addition, immune memory mechanisms induced by some vaccines can elicit long-term tumor immunosurveillance and could be helpful to prevent disease relapse (5).

Cancer/testis (CT) antigens are genes expressed almost exclusively in the normal human germ line and in malignancies. They have become the most extensively studied antigen group in the field of tumor immunology (6, 7). CT antigens were originally described in patients with malignant melanoma due to their ability to elicit cytotoxic T cells and humoral responses (8). Because CT antigens show restricted normal tissue expression and are highly immunogenic, they are especially attractive targets for immunotherapeutic approaches in cancer patients (5, 9).

In a study comparing genes overexpressed in malignant plasmablasts versus polyclonal plasmablasts generated from peripheral blood B cells, five of the eight most expressed genes were the CT antigens *GAGE-6*, *MAGEA1*, *MAGEA2*, *MAGEA3*, and *SSX2* (10).

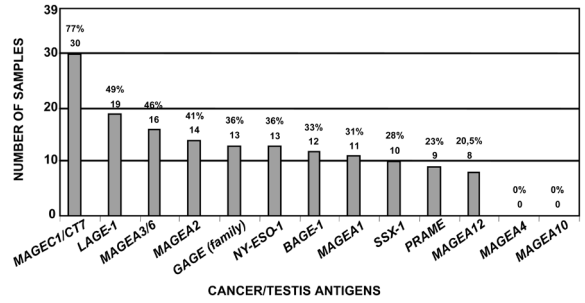
Recently, *MAGEC1/CT7* and *MAGEA3/6* were found to be frequently expressed in advanced stage MM patients (11). Higher levels of MAGEC1/CT7 and MAGEA3/6 proteins were also found to correlate with an elevated plasma cell proliferation index. These findings suggest a possible pathogenic role for such proteins in MM and also show that they could be attractive targets for immunotherapy in this disease.

This study aims to analyze the global expression of an extensive panel of CT antigens in normal tissues and bone marrow aspirates from MGUS, plasmacytomas and MM patients to detect possible prognostic markers and immunotherapeutic targets for this incurable disease.

Table 1
Primer sequences, references and RT-PCR conditions used in this study.

Gene	Primer Sequence	Product Size (bp)	Reference	RT-PCR Cycle
GAGE	5'-TATGCGGCCCGAGCAGTT-3' (F) 5'-CCTGCCCATCAGGACCATC-3' (R)	209	This study	Denaturation 94°C, 2 minutes 94°C, 45 seconds 60°C, 45 seconds | 30 cycles 72°C, 1 minute Elongation 72°C, 7 minutes
MAGEA4	5'-GCGAGCTGCTCTGTCTGAC-3 (F) 5'-AAGGACTCTGCGTCAGGC-3' (R)	523	This study	
PRAME	5'-CTGTACTCATTTCCAGAGCCAGA-3' (F) 5'-TATTGAGAGGGTTTCCAAGGGGTT-3' (R)	561	20	
SSX-1	5'-CTAAAGCATCAGAGAAGAGAAGC-3' (F) 5'-AGATCTCTTATTAATCTTCTCAGAAA-3' (R)	422	27	Denaturation 94°C, 2 minutes 94°C, 45 seconds 50°C, 45 seconds | 35 cycles 72°C, 1 minute Elongation 72°C, 7 minutes
MAGEA2	5'-TCAGGGAGTTGATGACCTTGT-3' (F) 5'-AGTGCCTCGGGTCCTACTT-3' (R)	159/257	This study	Denaturation 94°C, 2 minutes 94°C, 45 seconds 56°C, 45 seconds | 35 cycles 72°C, 1 minute Elongation 72°C, 7 minutes
MAGEA10	5'-GGAACCCCTCTTTTCTACAGA-3' (F) 5'-TCCTCTGGGGTGCTTGGTATTA-3' (R)	410/500	28	
BAGE-1	5'-TGGCTCGTCTCACTCTGG-3' (F) 5'-TCCTGTTGAGCTGCCGTCT-3' (R)	275	This study	
MAGEA3/6	5'-GAAGCCGGCCCAGGCTCG-3' (F) 5'-GGAGTCCTCATAGGATTGGCT-3' (R)	423	11	Denaturation 94°C, 2 minutes 94°C, 45 seconds 60°C, 45 seconds | 35 cycles 72°C, 1 minute Elongation 72°C, 7 minutes
MAGEA12	5'-ATTCTCGCCCTGAGCAACGG-3' (F) 5'-GATGCCTCCAACACACTCAGCT-3' (R)	835	This study	
NY-ESO-1	5'-CCCCACCGCTTCCCGTG-3' (F) 5'-CTGGCCACTCGTGCTGGGA -3' (R)	275	20	
SPA17	5'-GGCAGTTCTTACCAAGAAGAT-3' (F) 5'-GGAGGTAAAACCAGTGTCCTC-3' (R)	494	30	
MAGEC1/CT7	5'-GACGAGGATCGTCTCAGGTCAGC-3' (F) 5'-ACATCCTCACCCTCAGGAGGG-3' (R)	632	11	Denaturation 94°C, 2 minutes 94°C, 45 seconds 63°C, 45 seconds | 35 cycles 72°C, 1 minute Elongation 72°C, 7 minutes
MAGEA1	5'-GGCCGAAGGAACCTGACCCAG-3' (F) 5'-AGGGCCTCTTGTTGGGCCTCAA-3' (R)	246	This study	Denaturation 94°C, 2 minutes 94°C, 45 seconds 63°C, 45 seconds | 35 cycles 72°C, 1 minute Elongation 72°C, 7 minutes
LAGE-1	5'-CTGCGCAGGATGGAAGGTGCCCC-3' (F) 5'-GCGCCTCTGCCCTGAGGGAGC-3' (R)	332/561	29	
β-actin	5'-AAATCTGGCACCACACCTTC-3' (F) 5'-AGCACTGTGTTGGCGTACAG-3' (R)	646	31	Denaturation 94°C, 2 minutes 94°C, 45 seconds 65°C, 45 seconds | 30 cycles 72°C, 1 minute Elongation 72°C, 7 minutes

Figure 1



Number and percentage of MM patients with positive expression of the 13 CT antigens analyzed.

Results

We used RT-PCR to analyze the expression of 14 CT antigens (*MAGEA1*, *MAGEA2*, *MAGEA3/6*, *MAGEA4*, *MAGEA10*, *MAGEA12*, *BAGE-1*, *MAGEC1/CT7*, *GAGE* family, *LAGE-1*, *NY-ESO-1*, *PRAME*, *SPA17*, *SSX1*; Table 1) in 15 normal tissues, a pool of 10 normal bone marrow samples, 3 normal tonsils, bone marrow aspirates from 6 normal donors, 3 MGUS, 5 solitary plasmacytomas, 39 multiple myeloma samples and the MM cell line U266. Baseline characteristics of patients with MGUS, plasmacytoma and multiple myeloma are described in Table 2.

Different normal tissue RNA samples were analyzed for the expression of the 14 CT antigens. The *SPA17* gene was found to be expressed in all seven normal bone marrow samples and all normal tissues tested. Sequencing of the *SPA17* RT-PCR products from normal testis, normal mammary gland, normal bone marrow and normal plasma cells confirmed amplification of this gene. Thus, *SPA17* was excluded from further analyses. The results of the analysis of the expression of the other 13 CT antigens in normal tissues are reported in more detail in Table 3.

The frequency of CT antigen expression in MM patients is shown in Figure 1. *MAGEC1/CT7*, *MAGEA3/6* and *LAGE-1* together were expressed in 85% of our MM patients. At least one CT antigen was expressed in 92.3% of MM cases. Of the 16 cases with a lower frequency of CT antigen expression (0, 1 or 2 CT antigens expressed), 3 (7.6%) were negative for all CT antigens tested (cases 42, 59, and 130). Nine of the remaining 13 cases (approx. 70%) were positive for *MAGEC1/CT7* expression. We did not find an association between age, MM isotype or International Staging System (ISS) (12) stage and the percentage of CT antigens expressed in the MM cases studied.

To evaluate possible prognostic factors among the variables studied, we performed univariate analyses including all patients. The following parameters gave a negative prognostic impact on overall survival: (i) a β2-microglobulin level of 5.5 mg/L or more (β2-microglobulin <5.5 mg/L: n = 15, median OS = 40 months;

Table 2
Baseline characteristics of patients with MGUS, plasmacytoma and multiple myeloma.

Characteristic	MGUS	Solitary Plasmacytomas	Multiple Myeloma
Number of patients	3	5	39
Range of ages (yr)	62-72	48-71	37-80
Sex			
Male	3	3	26
Female	0	2	13
Isotype			
IgA	-	-	11
IgG	2	1	21
N/A	1	4	7
Light chain isotype			
Kappa	1	3	25
Lambda	1	1	13
N/A	1	1	1
Durie-Salmon stage			
I	-	-	0
II	-	-	2
III	-	-	37
ISS			
1	-	-	3
2	-	-	13
3	-	-	20
N/A	-	-	3
DNA ploidy (*n* = 23)			
Hypodiploid	-	-	6
Diploid	-	-	0
Hyperdiploid	-	-	15
Pseudodiploid	-	-	1
Normal	-	-	1
N/A	-	-	16
Cytogenetic analyses (*n* = 23)			
Del 13	-	-	7
Normal	-	-	16
N/A	-	-	16

β2-microglobulin 5.5 mg/L or more: $n = 22$, median OS = 11 months; log-rank, $P = 0.0343$, data not shown), (ii) the number of CT antigens expressed (6 CT antigens or less: $n = 29$, median OS = 25 months; >6 CT antigens: $n = 10$, median OS = 4 months; log-rank, $P = 0.0062$), and (iii) *GAGE* family expression (*GAGE* family negative: $n = 26$, median OS = 40 months; *GAGE* family positive: $n = 13$, median OS = 13 months; log-rank, $P = 0.0250$) (Figure 2).

Subsequently, Durie and Salmon stage, ISS stage, β2-microglobulin level, albumin level, the number of CT antigens expressed, expression of *MAGEC1/CT7*, *GAGE* family, *LAGE-1*, *SSX1*, and del 13 were included in the Cox regression model. When all patients were included in the model, *GAGE* family expression and having >6 CT antigens expressed were identified as independent prognostic factors (Table 4). However, when only non-transplanted patients were analyzed, *GAGE* family expression and the expression of >6 CT antigens lost prognostic impact but *MAGEC1/CT7* expression showed independent prognostic significance ($P = 0.0222$) confirmed by the Cox regression model (Figure 2).

Discussion

In the present study, we demonstrate high frequencies of *MAGEC1/CT7*, *LAGE-1* and *MAGEA3/6* expression in advanced stage MM patients. We also show that some CT antigens have an impact in MM prognosis.

Although the number of patients analyzed is small, we were able to show that *MAGEC1/CT7* is the most frequently expressed CT antigen (77%) in MM. Cho and collaborators (13) also demonstrated that MAGEC1/CT7 protein is highly expressed (82%) in MM by immunohistochemistry (IHC). In addition, these authors suggested that MAGEC1/CT7 expression in MM cells could elicit a T-cell immune response due to the increase in INF-γ secretion by lymphocytes when co-incubated with *MAGEC1/CT7*-transduced autologous myeloid dendritic cells and that MAGEC1/CT7-specific vaccines may boost this immunity. On the other hand, Fontecedro and colleagues (14) showed the *in vivo* immunogenicity of *MAGEC1/CT7* and the spontaneous occurrence of anti-MAGEC1/CT7 humoral responses in 22% of MM patients by Western blot and ELISA. All of the patients were patients with advanced disease. Furthermore, Goodyear and co-workers demonstrated CD8+ T cell activity recognizing several CT antigens in many myeloma patients (15).

We share with them the opinion that MAGEC1/CT7 is a good candidate for targeted therapy in MM, with the best candidates for this approach being patients expressing *MAGEC1/CT7* and with a low tumor burden, i.e. after a successful autologous stem cell transplant.

Furthermore, our study showed that *MAGEC1/CT7* expression seems to occur early in disease development, because 1 of 3 MGUS and 1 of 5 plasmacytomas were positive. These findings suggest that MAGEC1/CT7 may have a biological role in the early stages of MM and may contribute to plasma cell proliferation. Dodapkar *et al.* (16), as well as Jungbluth and co-workers (11), demonstrated that this CT antigen is expressed in plasmacytoma (by IHC) and MGUS (by RT-PCR), respectively.

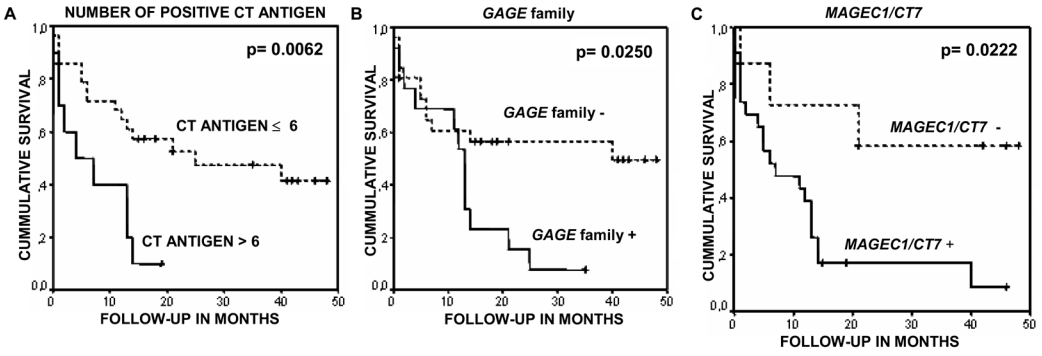
LAGE-1 was the second most frequently expressed CT antigen (49%) in our study. Tumor cells expressing LAGE-1 can be recognized by autologous cytotoxic T lymphocytes specific for *LAGE*-encoded antigenic peptides presented by various HLA class I molecules (17). *NY-ESO-1* and *LAGE-1* exhibit a high level of sequence similarity and, using single molecule fluorescence microscopy, Purbhoo and co-workers (18) demonstrated that NY-ESO1/LAGE-1-positive tumor cells present NY-ESO-1(10-50)/LAGE-1(157-165) epitopes per cell. Recently, Sun *et al.* (19) identified a new LAGE-1 peptide (LAGE-A68) which is recognized by T lymphocytes on tumor cells. In our study, the frequencies at which *LAGE-1* and *NY-ESO-1* were found to be expressed in MM patients were 49% (19/39) and 33% (13/39), respectively, in agreement with the findings of van Baren *et al.* (52% and 31% respectively; 20).

NY-ESO-1 has been shown to physically interact with MAGEC1/CT7 (21). Immunoprecipitation and immunofluorescent staining confirmed that MAGEC1/CT7 and NY-ESO-1 interact and their cytoplasmic co-localization in melanoma cells. The co-expression of these two genes was also confirmed in primary tumors, such as MM and non-small cell lung cancer (21). Among the 39 MM samples analyzed, there were 13 (33%) which were positive for *NY-ESO-1* expression and 30 (77%) which were positive for *MAGEC1/CT7* expression.

Table 3
CT antigen expression in normal tissues.

Tissue	CT Antigen Expression													
	MAGE A1	*MAGE A2*	*MAGE A3/6*	*MAGE A4*	*MAGE A10*	*MAGE A12*	*BAGE-1*	*MAGEC1 /CT7*	*GAGE family*	*LAGE-1*	*NY-ESO-1*	*PRAME*	*SPA17*	*SSX-1*
Positive controls														
Testis	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Placenta - pool of 10 samples	-	-	+	-	+	-	+	-	-	+	+	-	+	-
Placenta donor	-	-	-	-	-	-	+	-	-	-	-	-	+	-
Skeletal muscle	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Bladder	-	-	-	-	-	-	-	-	+	-	-	-	+	-
Lung	-	-	-	-	-	+	+	-	-	-	-	-	+	-
Spleen	-	-	-	-	-	-	+	+	-	-	-	-	+	-
Heart	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Brain	-	-	-	-	-	-	-	-	+	+	-	-	+	-
Thymus	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Uterus	-	-	-	-	-	+	-	-	+	-	-	-	+	-
Stomach	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Mammary gland	-	-	-	-	-	-	-	-	-	-	+	-	+	-
Pancreas	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Prostate	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Colon	-	-	-	-	-	+	-	-	-	-	-	-	+	-
Normal bone marrow (BM)														
BM - pool of 10 samples	-	-	-	-	-	-	-	-	-	-	-	-	+	-
BM 18 - donor	-	-	-	-	-	-	-	-	-	-	-	-	+	-
BM 57 - donor	-	-	-	-	-	-	-	-	-	-	-	-	+	-
BM 62 - donor	-	-	-	-	-	-	-	-	-	-	-	-	+	-
BM 138 - donor	-	-	-	-	-	-	-	-	-	-	-	-	+	-
BM 151 - donor	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Normal tonsils														
Normal plasma cell - 164	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Normal plasma cell - 166	-	-	-	-	-	-	-	-	-	-	-	-	+	-
Normal plasma cell - 168	-	-	-	-	-	-	-	-	-	-	-	-	+	-

Figure 2



Survival functions. (A) Number of positively expressed CT antigen (all patients included). (B) *GAGE* family expression (all patients included); (C) *MAGEC1/CT7* expression (only non-transplanted patients).

Simultaneous expression of *NY-ESO-1* and *MAGEC1/CT7* was observed in 26% of MM cases. This study demonstrated that the coordinated expression of CT antigens observed in many tumor types may be important for their function and for a potential role in tumorigenesis.

Previous studies in cancer patients have shown that patients with *NY-ESO-1* antigen expression and humoral immune response to NY-ESO-1 tend to have advanced stage disease. For example, a higher frequency of *NY-ESO-1* expression in bladder cancer was correlated with muscular wall invasion (22) and an antibody response to NY-ESO-1 was correlated with advanced stage disease (23).

LAGE-1 is among the genes identified by array analysis that correlated with resistance to proteasome inhibitor bortezomib, when pre- and post-treatment myeloma samples were studied (24). Thus, if *NY-ESO-1* antigen expression was associated with advanced stages of cancer and *LAGE-1* should confer resistance to the proteasome inhibitor bortezomib, it was reasoned that the expression of these two CT antigens might contribute to a poor outcome in MM and thus represent prognostic markers for this disease. In this context, Condomines and co-workers (25) demonstrated by microarray analysis an influence of CT antigens encoded by chromosome X on MM event-free survival, particularly *CTAG1B* (*NY-ESO-1*), *CTAG2* (*LAGE-1*), *MAGEA1*, *MAGEA2*, *MAGEA3*, and *MAGEA6*.

One of the most interesting findings of this study is the frequent expression of *LAGE-1*, despite the significantly lower expression of its close homologue *NY-ESO-1* (approximately 94% homology). Due to this homology, it is possible that LAGE-1 could also elicit an immune response against NY-ESO-1. Furthermore, *LAGE-1* and *NY-ESO-1* were co-expressed in 23% of the advanced stage MM cases studied.

The MAGEA3 and MAGEA6 proteins share more than 98% sequence homology, and are frequently analyzed together. *MAGEA3/6* was the third most frequently expressed CT antigen in this study (41%). Jungbluth and group (11) demonstrated by RT-PCR that the *MAGEA-A* family was expressed in 100% of MM patients. MAGEA3/6 was expressed in 70% of stage III MM specimens by IHC. Moreover, van Baren *et al.* (20) found expression of *MAGEA3* in 29% and *MAGEA6* in 33% of MM samples (also advanced stage MM) by RT-PCR. Due to its high frequency in advanced stage MM patients, it is possible that members of the *MAGE* family participate in the biology of this fatal B cell malignancy.

Table 4

Variables identified as independent prognostic factors in all patients, and in non-transplanted patients only, by Cox's regression model.

Variable	*P* Value	Odds Ratio	Confidence Interval
All patients (*n* = 39)			
Forward stepwise	0.0153	2.9351	1.2295 - 7.0069
GAGE family expression			
Backward stepwise	0.0204	2.9645	1.1828 - 7.4303
>6 positive CT antigens			
Non-transplanted patients (*n* = 31)			
Forward stepwise	0.0520*	3.4655	0.9891 - 12.1418
MAGE-C1/CT7 expression			
Backward stepwise	0.0520*	3.4655	0.9891 - 12.1418
MAGE-C1/CT7 expression			

*Although the *P* value was only slightly greater than 0.05 and the CI was large, this result was considered to be significant.

Table 5

***MAGE-C1/CT7*, *GAGE* family and CT antigen expression in the 8 transplanted MM patients.**

Transplanted Patient Case	Situation at End of Study	Follow-up (mo)	Positive Expression		
			MAGE-C1/CT7	*GAGE* Family	>6 CT Antigens
20	dead	25	+	+	-
31	alive	54	+	-	-
58	alive	49	+	-	-
63	alive	47	+	-	-
92	alive	40	+	+	-
139	alive	23	-	-	-
145	alive	21	+	-	-
148	alive	21	+	-	-

SPA17 was the only CT antigen strongly expressed in all non-gametogenic tissues tested and this result was confirmed by automated sequencing. This finding has been described by Scanlan *et al.* (9) and, more recently, by Condomines *et al.* (25). Therefore we believe that *SPA17* does not fulfill the requirements to be classified as a CT antigen and is not a likely target for immunotherapy of any cancer type.

We attempted to correlate the International Staging System (ISS) MM stage, which is based on albumin and β2-microglobulin serum levels at diagnosis, with the number of CT antigens expressed. The hypothesis tested was that a lower number of CT antigens expressed would correlate with a lower ISS stage. In our group of MM patients, only three cases were classified as stage 1 and fewer than three CT antigens were found to be expressed in all of them. However, due to the small number of cases in this category, we were unable to demonstrate a correlation between ISS stage and the number of CT antigens expressed.

To evaluate possible prognostic factors among the variables studied, we performed OS univariate analyses including all patients. A β2-microglobulin level of 5.5 mg/L or more, expression of >6 CT antigens and *GAGE* family expression had a negative impact on OS.

Using a Cox regression model, *GAGE* family expression and expression of >6 CT antigens were found to be independent prognostic factors. The finding that a worse prognosis is associated with the expression of a larger number of CT antigens supports the hypothesis of the cumulative deleterious effect of CT antigen expression on MM disease progression. In addition, expression of *MAGEC1/CT7* showed independent prognostic significance in non-transplanted patients, suggesting that it can be a target, and also a marker, of advanced stage disease. This is now the second study demonstrating the potential prognostic relevance of CT antigen expression in MM; the other was recently published by Condomines and co-workers (25).

In this study, expression of >6 CT antigens or of the *GAGE* family represent important prognostic factors when all patients were analyzed. On the other hand, expression of *MAGEC1/CT7* only represents such a factor for non-transplanted patients. It is possible that transplantation proceedings have interfered in these results. Seven out of 8 MM transplanted cases were positive for *MAGEC1/CT7* expression (Table 5). In these cases, the autologous stem cell transplant possibly overcame poor outcome related to the expression of this gene. Therefore, only after exclusion of transplanted patients did we find that *MAGEC1/CT7* expression correlated with a poor prognosis (non-transplanted, *MAGEC1/CT7*-negative: $n = 8$; median OS = not reached; non-transplanted, *MAGEC1/CT7*-positive: $n = 23$; median OS = 7 months; log rank, $P = 0.0222$).

On the other hand, only 2 out of 8 transplanted MM cases were positive for *GAGE* family expression (Table 5). Consequently, the exclusion of transplanted patients eliminated a large number of *GAGE* family-negative cases. Accordingly, transplantation proceedings (and not the absence of *GAGE*-family mRNA) justified the better prognosis seen in the *GAGE*-negative group (non-transplanted, *GAGE*-negative: $n = 20$, median OS = 7 months; non-transplanted, *GAGE*-positive: $n = 11$; median OS = 12 months; log rank, $P = 0.0894$).

Likewise, no MM transplanted patients presented expression of >6 CT antigens (Table 5). Thus, transplantation proceedings (and not the low number of CT antigens expressed) were responsible for the increased overall survival in these patients (non-transplanted, 6 CT antigens or less: $n = 21$, median OS = 13 months; non-transplanted, >6 CT antigen: $n = 10$, median OS = 4 months; log rank, $P = 0.1032$).

To date, CT antigens have been studied because they are immunogenic and are considered promising target molecules for cancer vaccines. Based on our findings, *MAGEC1/CT7*, *LAGE-1* and *MAGEA3/6* are good candidates for immunotherapy in MM, since together they are expressed in 85% of patients. Several clinical trials vaccinating with *MAGEA3* (which shows homology with *MAGEC1/CT7* and *MAGEA6*), *MAGEA12* and *NY-ESO-1* (which shows homology with *LAGE-1*) are currently open for enrollment for patients with MM (26). It will also be of interest to investigate if some CT antigens, such as the *GAGE* family or *MAGEC1/CT7*, might participate in MM pathogenesis.

Abbreviations

CT, cancer/testis; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; OS, overall survival

Acknowledgements

This work was supported by grants from FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, São Paulo, Brazil, 04/13213-3). V.C.C.A. was also supported by FAPESP (04/12855-1). The authors thank Dr. Andrew J. G. Simpson, from the Ludwig Institute for Cancer Research, for all the support and critical review of this manuscript. The authors also thank Fleury

Laboratories, São Paulo, Brazil, for the immunological characterization of MM cases.

References

1. Kyle RA, Rajkumar SV. Multiple myeloma. *N Engl J Med* 2004; **351:** 1860-1873. (PMID: 15509819)

2. Barlogie B. High-dose therapy and innovative approaches to retreatment of multiple myeloma. *Semin Hematol* 2001; **38 (2 Suppl 3):** 21-27. (PMID: 11309705)

3. Orlowski RZ. Initial therapy of multiple myeloma patients who are not candidates for stem cell transplantation. *Hematology Am Soc Hematol Educ Program* 2006; 338-347. (PMID: 17124081)

4. Huff CA, Karp JE. Should MAGE be the rage in myeloma? [comment] [Comment on Jungbluth AA, Ely S, DiLiberto M, Niesvizky R, Williamson B, Frosina D, Chen YT, Bhardwaj N, Chen-Kiang S, Old LJ, Cho HJ. In: *Blood* 2005; **106**: 167-174]. *Blood* 2005; **106:** 5.

5. Dadabayev AR, Wang Z, Zhang Y, Zhang J, Robinson WR, Lim SH. Cancer immunotherapy targeting SPA17: when should the laboratory findings be translated to the clinics? *Am J Hematol* 2005; **80:** 6-11. (PMID: 16138340)

6. Kalejs M, Erenpreisa J. Cancer/testis antigens and gametogenesis: a review and "brain-storming" session. *Cancer Cell Int* 2005; **5:** 4. (PMID: 15715909)

7. Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ. Cancer/testis antigens, gametogenesis and cancer. *Nat Rev Cancer* 2005; **5:** 615-625. (PMID: 16034368)

8. Gaugler B, Van den Eynde B, van der Bruggen P, Romero P, Gaforio JJ, De Plaen E, Lethé B, Brasseur F, Boon T. Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. *J Exp Med* 1994; **179:** 921-930. (PMID: 8113684)

9. Scanlan MJ, Simpson AJ, Old LJ. The cancer/testis genes: review, standardization, and commentary. *Cancer Immun* 2004; **4:** 1. URL: http://www.cancerimmunity.org/v4p1/031220.htm

10. Tarte K, De Vos J, Thykjaer T, Zhan F, Fiol G, Costes V, Rème T, Legouffe E, Rossi JF, Shaughnessy J Jr, Ørntoft TF, Klein B. Generation of polyclonal plasmablasts from peripheral blood B cells: a normal counterpart of malignant plasmablasts. *Blood* 2002; **100:** 1113-1122. (PMID: 12149187)

11. Jungbluth AA, Ely S, DiLiberto M, Niesvizky R, Williamson B, Frosina D, Chen YT, Bhardwaj N, Chen-Kiang S, Old LJ, Cho HJ. The cancer-testis antigens CT7 (MAGE-C1) and MAGE-A3/6 are commonly expressed in multiple myeloma and correlate with plasma-cell proliferation. *Blood* 2005; **106:** 167-174. (PMID: 15761016)

12. Greipp PR, San Miguel J, Durie BG, Crowley JJ, Barlogie B, Bladé J, Boccadoro M, Child JA, Avet-Loiseau H, Kyle RA, Lahuerta JJ, Ludwig H, Morgan G, Powles R, Shimizu K, Shustik C, Sonneveld P, Tosi P, Turesson I, Westin J. International staging system for multiple myeloma. *J Clin Oncol* 2005; **23:** 3412-3420. (PMID: 15809451)

13. Cho HJ, Farol L, Truong JT, Austin WR, Chen YT, Bhardwaj N, Niesvizky R, Old LJ, Chen-Kiang S. CT7 (MAGE-C1)-specific cellular immune responses in the bone marrow microenvironment of multiple myeloma patients [abstract]. *Blood (ASH Annual Meeting Abstracts)* 2005; **106:** abstract 356.

14. Fontecedro AC, Knights AJ, Tinguely M, de la Rosa O, Lopes B, Moch H, Renner C, Zippelius A, Knuth A. Expression and immunogenicity of the cancer-testis antigen CT7 (MAGE-C1) in patients with multiple myeloma [abstract]. *J Clin Oncol (ASCO Annual Meeting)* 2007; **25:** 8112.

15. Goodyear O, Piper K, Khan N, Starczynski J, Mahendra P, Pratt G, Moss P. CD8+ T cells specific for cancer germline gene antigens are found in many patients with multiple myeloma, and their frequency correlates with disease burden. *Blood* 2005; **106:** 4217-4224. (PMID: 16144804)

16. Dhodapkar MV, Osman K, Teruya-Feldstein J, Filippa D, Hedvat CV, Iversen K, Kolb D, Geller MD, Hassoun H, Kewalramani T, Comenzo RL, Coplan K, Chen YT, Jungbluth AA. 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. *Cancer Immun* 2003; **3:** 9. URL: http://www.cancerimmunity.org/v3p9/030709.htm

17. Lethé B, Lucas S, Michaux L, De Smet C, Godelaine D, Serrano A, De Plaen E, Boon T. LAGE-1, a new gene with tumor specificity. *Int J Cancer* 1998; **76:** 903-908. (PMID: 9626360)

18. Purbhoo MA, Sutton DH, Brewer JE, Mullings RE, Hill ME, Mahon TM, Karbach J, Jäger E, Cameron BJ, Lissin N, Vyas P, Chen JL, Cerundolo V, Jakobsen BK. Quantifying and imaging NY-ESO-1/LAGE-1-derived epitopes on tumor cells using high affinity T cell receptors. *J Immunol* 2006; **176:** 7308-7316. (PMID: 16751374)

19. Sun Z, Lethé B, Zhang Y, Russo V, Colau D, Stroobant V, Boon T, van der Bruggen P. A new LAGE-1 peptide recognized by cytolytic T lymphocytes on HLA-A68 tumors. *Cancer Immunol Immunother* 2006; **55:** 644-652. (PMID: 16187088)

20. van Baren N, Brasseur F, Godelaine D, Hames G, Ferrant A, Lehmann F, André M, Ravoet C, Doyen C, Spagnoli GC, Bakkus M, Thielemans K, Boon T. Genes encoding tumor-specific antigens are expressed in human myeloma cells. *Blood* 1999; **94:** 1156-1164. (PMID: 10438702)

21. Cho HJ, Caballero OL, Gnjatic S, Andrade VC, Colleoni GW, Vettore AL, Outtz HH, Fortunato S, Altorki N, Ferrera CA, Chua R, Jungbluth AA, Chen YT, Old LJ, Simpson AJ. Physical interaction of two cancer-testis antigens, MAGE-C1 (CT7) and NY-ESO-1 (CT6). *Cancer Immun* 2006; **6:** 12. URL: http://www.cancerimmunity.org/v6p12/061012.htm

22. Sharma P, Gnjatic S, Jungbluth AA, Williamson B, Herr H, Stockert E, Dalbagni G, Donat SM, Reuter VE, Santiago D, Chen YT, Bajorin DF, Old LJ. Frequency of NY-ESO-1 and LAGE-1 expression in bladder cancer and evidence of a new NY-ESO-1 T-cell epitope in a patient with bladder cancer. *Cancer Immun* 2003; **3:** 19. URL: http://www.cancerimmunity.org/v3p19/031119.htm

23. Kurashige T, Noguchi Y, Saika T, Ono T, Nagata Y, Jungbluth A, Ritter G, Chen YT, Stockert E, Tsushima T, Kumon H, Old LJ, Nakayama E. NY-ESO-1 expression and immunogenicity associated with transitional cell carcinoma: correlation with tumor grade. *Cancer Res* 2001; **61:** 4671-4674. (PMID: 11406534)

24. Richardson PG, Barlogie B, Berenson JR, Singhal S, Jagannath S, Irwin D, Rajkumar V, Esseltine D, Schenkein D, Anderson KC. Prognostic factors associated with response in patients with relapsed and refractory multiple myeloma (MM) treated with bortezomib [abstract]. *Proc Am Soc Clin Oncol* 2003; **22:** 581. Abstract 2338.

25. Condomines M, Hose D, Raynaud P, Hundemer M, De Vos J, Baudard M, Moehler T, Pantesco V, Moos M, Schved JF, Rossi JF, Rème T, Goldschmidt H, Klein B. Cancer/testis genes in multiple myeloma: expression patterns and prognosis value determined by microarray analysis. *J Immunol* 2007; **178:** 3307-3315. (PMID: 17312182)

26. Houet l, Veelken H. Active immunotherapy of multiple myeloma. *Eur J Cancer* 2006; **42:** 1653-1660. (PMID: 16797966)

27. Gure AO, Türeci O, Sahin U, Tsang S, Scanlan MJ, Jäger E, Knuth A, Pfreundschuh M, Old LJ, Chen YT. SSX: a multigene family with several members transcribed in normal testis and human cancer. *Int J Cancer* 1997; **72:** 965-971. (PMID: 9378559)

28. Rimoldi D, Salvi S, Reed D, Coulie P, Jongeneel VC, De Plaen E, Brasseur F, Rodriguez AM, Boon T, Cerottini JC. cDNA and protein characterization of human MAGE-10. *Int J Cancer* 1999; **82:** 901-907. (PMID: 10446460)

29. Wang Y, Wu XJ, Zhao AL, Yuan YH, Chen YT, Jungbluth AA, Gnjatic S, Santiago D, Ritter G, Chen WF, Old LJ, Ji JF. Cancer/testis antigen expression and autologous humoral immunity to NY-ESO-1 in gastric cancer. *Cancer Immun* 2004; **4:** 11. URL: http://www.cancerimmunity.org/v4p11/040812.htm

30. Lim SH, Wang Z, Chiriva-Internati M, Xue Y. Sperm protein 17 is a novel cancer-testis antigen in multiple myeloma. *Blood* 2001; **97:** 1508-1510. (PMID: 11222401)

31. Kikuchi S, Yamada D, Fukami T, Masuda M, Sakurai-Yageta M, Williams YN, Maruyama T, Asamura H, Matsuno Y, Onizuka M, Murakami Y. Promoter methylation of DAL-1/4.1B predicts poor prognosis in non-small cell lung cancer. *Clin Cancer Res* 2005; **11:** 2954-2961. (PMID: 15837747)

Materials and methods

Material

A panel of normal tissues (testis, placenta, skeletal muscle, bladder, lung, spleen, heart, brain, thymus, uterus, stomach, mammary gland, pancreas, prostate and colon; Clontech, Palo Alto, CA, USA), a pool of ten normal bone marrow samples from Clontech (Palo Alto, CA, USA), six normal bone marrow aspirates (from donors for allogeneic stem cell transplants) and three normal tonsils (obtained from children submitted to tonsillectomy) were used to test all the primer sets. Bone marrow aspirates from three monoclonal gammopathies of undetermined significance (MGUS), five solitary plasmacytomas, and 39 multiple myeloma samples (2 Durie and Salmon stage II and 37 stage III) were obtained between June 2002 and April 2006 from the Hospital Sao Paulo UNIFESP/EPM, Sao Paulo, Brazil. All of these patients were newly diagnosed and treatment naïve at the time of bone marrow harvest. The cases were classified according to the International Staging System (12) as 3 stage 1, 13 stage 2 and 20 stage 3 cases (Table 2). In the 39 MM patients, bone marrow biopsies showed 10% to 98% of plasma cells (median of 80%). Patients were initially treated with standard chemotherapy protocols, namely melphalan and prednisone (MP) or vincristine, doxorubicin and dexamethasone (VAD). Eight patients also underwent melphalan-based autologous stem cell transplantation. This study was approved by the institution's Ethical Review Committee and all patients provided written informed consent prior to collection of bone marrow samples. The MM cell line U266 (TIB-196, ATCC, Manassas, USA) was also analyzed.

RT-PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA was recovered from the aqueous phase by ethanol precipitation and the pellets were dissolved in 30 µL of diethyl pyrocarbonate (DEPC) treated water. First-strand cDNA synthesis, primed with oligo (dT) and 2 µg of RNA template, was reverse transcribed (Superscript II, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions and diluted 10x in water. After cDNA synthesis, the expression of β-actin was determined in all samples. The 14 CT antigens described in Table 1 were evaluated by RT-PCR and 2% agarose gel electrophoresis. Normal testis was used as template for positive controls in all RT-PCR reactions. PCR reactions were performed in 0.2 mL tubes and carried out in a total volume of 25 µL containing 2 µL of cDNA template, 1x PCR buffer, 1 mM MgCI2, 0.2 µM dNTPs, 10 pmol each of the forward and reverse primers, and 1.25 U of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA). The PCR steps were performed in an Eppendorf MasterCycler Gradient thermocycler (Hamburg, Germany) and primer sequences and cycle conditions are given in Table 1. Initial denaturation at 94°C for 2 minutes and single step elongation at 72°C for 7 minutes were used in all cycles. The *GAGE* family was analyzed using consensus primers because its members have high sequence homology. All reactions were undertaken in duplicate and amplifications of target fragments were considered positive, independent of the intensity of the bands.

Sequencing

Amplification of *SPA17* for automated sequencing was carried out with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems, Foster City, CA) using an ABI Prism 377 DNA Sequencer (Perkin Elmer, Norwalk, CT, USA).

Statistical analyses

Associations between the variables studied were tested by the Pearson chi-square test. Overall survival (OS) time was calculated from the date of diagnosis of MM until death or last follow-up. Actuarial probabilities of OS were estimated according to the Kaplan-Meier method and the curves were compared using the log-rank test. The Cox regression model was also employed to evaluate which variables could be considered independent prognostic factors on OS in this group of patients. The level of significance for all statistical tests was 5%. Statistical analysis was performed using SPSS 8.0 software.

Contact

Address correspondence to:

Gisele Colleoni, MD, PhD
UNIFESP/EPM - Rua Botucatu, 740, 3º andar
Hematologia, CEP 04023-900
Vila Clementino
São Paulo, SP
Brazil
Tel.: + 55 11 5579-1550
Fax: + 55 11 5571-8806
E-mail: gcolleoni@hemato.epm.br