Cancer/Testis Genes in Multiple Myeloma: Expression Patterns and Prognosis Value Determined by Microarray Analysis[1]

Maud Condomines,*†‡ Dirk Hose,§ Pierre Raynaud,*‡ Michael Hundemer,§ John De Vos,*†‡ Marion Baudard,¶ Thomas Moehler,§ Véronique Pantesco,*† Marion Moos,§ Jean-François Schved,*‡ Jean-François Rossi,‡¶ Thierry Rème,*† Hartmut Goldschmidt,§‖ and Bernard Klein[2]*†‡

Cancer-testis (CT) Ags are expressed in testis and malignant tumors but rarely in nongametogenic tissues. Due to this pattern, they represent attractive targets for cancer vaccination approaches. The aims of the present study are: 1) to assess the expression of CT genes on a pangenomic base in multiple myeloma (MM); 2) to assess the prognosis value of CT gene expression; and 3) to provide selection strategies for CT Ags in clinical vaccination trials. We report the expression pattern of CT genes in purified MM cells (MMC) of 64 patients with newly diagnosed MM and12 patients with monoclonal gammopathy of unknown significance, in normal plasma cell and B cell samples, and in 20 MMC lines. Of the 46 CT genes interrogated by the Affymetrix HG-U133 set arrays, 35 are expressed in the MMC of at least one patient. Of these, 25 are located on chromosome X. The expression of six CT genes is associated with a shorter event-free survival. The MMC of 98% of the patients express at least one CT gene, 86% at least two, and 70% at least three CT genes. By using a set of 10 CT genes including *KM-HN-1*, *MAGE-C1*, *MAGE-A3/6/12*, *MAGE-A5*, *MORC*, *DDX43*, *SPACA3*, *SSX-4*, *GAGE-1–8*, and *MAGE-C2*, a combination of at least three CT genes—desirable for circumventing tumor escape mechanisms—is obtained in the MMC of 67% of the patients. Provided that the immunogenicity of the products of these 10 CT genes is confirmed, gene expression profiling could be useful in identifying which CT Ags could be used to vaccinate a given patient. *The Journal of Immunology,* **2007, 178: 3307–3315.**

Multiple myeloma (MM)[3] is a B cell neoplasia characterized by the accumulation of malignant plasma cells in the bone marrow. Although complete remission could be achieved in ~25–50% of newly diagnosed patients treated with high-dose melphalan and autologous peripheral blood stem cell transplantation (ABSCT), almost all patients will relapse with a median of 2 to 3 years of event-free survival (EFS) (1). There is ample evidence that a small fraction of MM cells (MMC) escape chemotherapy and remain present even despite a "complete remission" (2). The remaining MMC are promising targets for active or passive immunotherapy because: 1) MMC can be lysed by CTL in vitro (3–6); and 2) allograft strategies have shown that donor T cells can efficiently lyse MMC in some patients in vivo, provided that an efficient immune response is mounted (7–9). The clonal Ig produced by MMC seemed to be an ideal and specific target, but several vaccination trials using it failed to prove a clinical benefit for patients with MM (10–12), contrary to its promise in patients with B cell lymphoma (13). An explanation suggested by murine models (14) could be a deletion of the T cell repertoire related to the tumor Ig due to the large amounts of circulating tumor Ig.

In melanoma and epithelial cancers, several tumor-associated Ags have been identified (15–17). These Ags may be shared with normal cells, as MART-1, gp100, or tyrosinase. Another category, the cancer-testis (CT) Ags, is expressed by testis cells and malignant cells mainly from patients with melanoma, small cell lung cancer, hepatocellular carcinoma, or bladder cancer (18). To date, 47 CT gene families that include 93 genes have been described (19–22). Strictly testis-restricted genes that are expressed only by male germ cells and malignant cells, represent about one-half of CT genes (19). These testis-restricted genes, in particular genes belonging to the MAGE, GAGE, and SSX families, are mainly located on chromosome X. Because of their restricted expression to germ cells and malignant tissues, there should be no deletion of a high-affinity T cell repertoire and theoretically a low risk of preexisting immune tolerance. Thus, the testis-restricted CT Ags are being used as targets in several vaccination trials (23). In particular, NY-ESO-1 and CT Ags belonging to the MAGE and GAGE families have been shown to elicit spontaneous cellular and/or humoral immune responses in cancer patients (24–28). According to the definition of Scanlan et al. (19), about one-fourth of CT genes are tissue restricted, i.e., expressed in two or fewer nongametogenic tissues, one-fourth are differentially expressed, i.e.,

*Institute of Research in Biotherapy. Centre Hospitalier Universitaire Montpellier, Montpellier, France; †Institut National de la Santé et de la Recherche Médicale, Unité 475, Montpellier, France; ‡Université Montpellier, Unité de Formation et de Recherche Médecine, Montpellier, France; §Medizinische Klinik und Poliklinik V, Universitätsklinikum Heidelberg, Heidelberg, Germany; ¶Department of Hematology and Clinical Oncology, Centre Hospitalier Universitaire Montpellier, Montpellier, France; and ‖National Center of Tumour Diseases Heidelberg, Heidelberg, Germany

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[2] Address correspondence and reprint requests to Dr. Bernard Klein, Institut National de la Santé et de la Recherche Médicale U475, 99 Rue Puech Villa, Montpellier, France. E-mail address: klein@montp.inserm.fr

[3] Abbreviations used in this paper: MM, multiple myeloma; ABSCT, autologous blood stem cell transplantation; BMPC, bone marrow plasma cell; CT, cancer-testis; CT-X, a CT gene located on chromosome X; EFS, event-free survival; GEP, gene expressing profiling; HDC, high-dose chemotherapy; HMCL, human myeloma cell line; MB, peripheral blood memory B cell; MGUS, monoclonal gammopathy of unknown significance; MMC, multiple myeloma cell; PPC, polyclonal plasmablast.

Table I. *Frequencies of expression for 35 CT genes evaluated by microarray analysis in multiple myeloma cells and normal counterparts*[a]

Probe Set	Gene Name	Chromosomal Location	MMC (%) (n = 64)	Ratio MMC/Testis	HMCL (%) (n = 20)	Ratio HMC/Testis	MGUS (%) (n = 12)	BMPC (%) (n = 7)	PPC (%) (n = 7)	MB (%) (n = 7)
206609_atU133A	*MAGEC1*	chrXq26	66	1.5	90	1.6	33	0	0	0
230900_atU133B	*KM-HN-1*	chr4q35.1	56	0.15	15	0.1	83	29	0	0
220850_atU133A	*MORC*	chr3q13	36	1.0	30	0.8	8	0	0	0
220004_atU133A	*DDX43*	chr6q12-q13	34	0.8	10	1.0	33	29	0	0
209942_x_atU133A	*MAGEA3*	chrXq28	33	1.3	75	8.1	0	0	0	0
243621_atU133B	*SPACA3*	chr17q11.2	33	0.1	55	0.15	8	0	86	43
214612_x_atU133A	*MAGEA6*	chrXq28	31	0.9	80	4.6	0	0	0	0
210467_x_atU133A	*MAGEA12*	chrXq28	25	0.6	50	4.5	0	0	0	0
214642_x_atU133A	*MAGEA5*	chrXq28	22	0.7	75	2.0	0	0	0	0
206626_x_atU133A	*SSX1*	chrXp11.23	20	0.5	60	1.2	0	0	0	0
210394_x_atU133A	*SSX4*	chrXp11.23	20	0.5	60	1.2	0	0	0	0
207739_s_atU133A	*GAGE1–8*	chrXp11.4	17	0.2	70	0.9	8	0	0	0
215733_x_atU133A	*CTAG2*	chrXq28	14	5.1	65	15.1	0	0	0	0
210546_x_atU133A	*CTAG1B*	chrXq28	13	3.8	70	9.4	0	0	0	0
220062_s_atU133A	*MAGEC2*	chrXq27	13	0.8	45	0.9	0	0	0	0
207325_x_atU133A	*MAGEA1*	chrXq28	11	3.7	75	8.6	0	0	0	0
206897_atU133A	*PAGE1*	chrXp11.23	11	2.6	60	3.0	0	0	0	0
220057_atU133A	*XAGE1*	chrXp11.22	11	1.1	50	2.5	0	0	0	0
222259_s_atU133A	*SPO11*	chr20q13	8	0.2	20	0.3	0	0	0	0
235700_atU133B	*CT45*	chrXq26.3	6	0.2	40	0.7	8	0	0	0
220217_x_atU133A	*SPANXC*	chrXq27.1	6	0.1	35	0.1	0	0	0	0
210262_atU133A	*CRISP2*	chr6p21-qter	5	0.02	20	0.02	0	0	0	0
214603_atU133A	*MAGEA2*	chrXq28	5	2.0	50	2.4	0	0	0	0
214254_atU133A	*MAGEA4*	chrXq28	5	0.2	55	2.0	0	0	0	14
207022_s_atU133A	*LDHC*	chr11p15	3	0.03	60	0.03	0	0	57	0
210437_atU133A	*MAGEA9*	chrXq28	3	0.4	50	1.4	0	0	0	0
207534_atU133A	*MAGEB1*	chrXp21.3	3	0.9	35	2.9	0	0	0	0
210497_x_atU133A	*SSX2*	chrXp11.23	3	0.6	45	0.5	0	0	0	0
207666_x_atU133A	*SSX3*	chrXp11.23	3	0.4	40	0.6	0	0	0	0
236040_atU133B	*XAGE3*	chrXp11.22	3	4.7	15	12.5	0	0	0	0
206787_atU133A	*BRDT*	chr1p22.1	2	0.1	0	NA	0	0	0	0
241224_x_atU133B	*DSCR8*	chr21q22.2	2	0.9	40	1.3	0	0	0	0
210274_atU133A	*MAGEA8*	chrXq28	2	2.0	5	0.8	0	0	0	14
220921_atU133A	*SPANXB*	chrXq27.1	2	0.7	5	0.7	8	14	0	0
221018_s_atU133A	*TDRD1*	chr10q25.3	2	0.4	5	0.5	0	0	0	0

[a] Data are the frequencies of "present" Affymetrix call for 35 CT genes in MMC, HMCL, purified plasma cells from patients with MGUS, normal BMPC, normal PPC, and normal MB. The ratios of the median microarray signals in MMC or HMCL with a present call to the median signal in testis samples with a present call are also indicated. The expression of *GAGE-1* to *GAGE-8* were assessed by one common probe set.

expressed in 3–6 nongametogenic tissues but at a low level compared with testis, and five genes initially reported as CT genes are ubiquitously expressed.

No study has assessed the expression of CT genes on a pangenomic base in MM until now. Using DNA microarrays, we and others have shown that genes belonging to the MAGE and SSX families and *NY-ESO-1* were aberrantly expressed in a certain percentage of primary MMC (29, 30). The expression of *NY-ESO-1* has been correlated with a poor prognosis and its frequency is increased in patients displaying genetic abnormalities determined by conventional cytogenetic analyses (31). CT7/MAGE-C1 and MAGE-A3/6 protein expression correlated with elevated MMC proliferation (32). A recent study (28) reported the presence of functional CD8 T lymphocytes against NY-ESO-1, LAGE-1, and MAGE-A1,-A2, -A3, and -A4 in the peripheral blood of MM patients. Their frequency was 3-fold increased compared with that in healthy donors, supporting an immunogenicity of CT Ags in MM patients (28). In addition, van Rhee et al. have shown that anti-NY-ESO-1 CD8 T cells of MM patients were able to kill primary myeloma cells (31).

The aims of the present study are: 1) to assess the expression of CT genes on a pangenomic base in MM; 2) to estimate their prognosis value; and 3) to provide appropriate selection strategies for clinical vaccination trials.

Materials and Methods

Patients and cell samples

MMC were purified from two independent series of 64 patients with newly diagnosed MM (median age, 59 years) after written informed consent was given in agreement with French or German laws. Forty-seven of 64 patients and 64 of 64 of the first and second series, respectively, were treated with high-dose chemotherapy (HDC) and ABSCT. According to Durie-Salmon classification, in the first series 11 patients were of stage IA, 11 of stage IIA, 39 of stage IIIA and 3 of stage IIIB. Twelve patients had IgAκ MM, seven IgAλ MM, 24 IgGκ MM, 10 IgGλ MM, six Bence-Jones κ MM, three Bence-Jones λ MM, and two nonsecreting MM. In the second series, four patients were of stage IA, 11 of stage IIA, 45 of stage IIIA, and four of stage IIIB. Nine patients had IgAκ MM, three IgAλ MM, 20 IgGκ MM, 19 IgGλ MM, seven Bence-Jones κ MM, four Bence-Jones λ MM, and two nonsecreting MM.

XG-1, XG-2, XG-3, XG-4, XG-5, XG-6, XG-7, XG-10, XG-11, XG-12, XG-13, XG-14, XG-16, XG-19, and XG-20 human myeloma cell lines (HMCL) were obtained and characterized in our laboratory (3, 33–35). SKMM, OPM2, LP1, U266, and RPMI8226 HMCL were purchased from American Type Culture Collection (distributed by LGC Promochem). Normal testis RNA samples were purchased from CliniSciences. Bone marrow samples were obtained from healthy donors and patients after informed consent was given in agreement with French or German laws. Normal bone marrow plasma cells (BMPC) and primary MMC were purified using anti-CD138 MACS microbeads. Briefly, mononuclear cells were collected by centrifugation on a Ficoll-Hypaque cushion, washed twice, and incubated for 30 min at 4°C with B-B4 anti-CD138 MACS microbeads (Miltenyi Biotec). Cells were then washed and purified using the autoMACS device (Miltenyi Biotec) according to the manufacturer's instructions. The purity

of plasma cells in the sorted population was determined by labeling autoMACS-sorted cells with PE-conjugated anti-CD138 mAb (Beckman Coulter) and FACS analysis (FACScan; BD Biosciences). This sorting procedure generally insures a high plasma cell purity (≥95%). Sorted MMC were discarded if the purity was >95%. For the isolation of peripheral blood memory B (MB) cells, monocytes, NK, and T cells were first removed using anti-CD14, anti-CD16, and anti-CD3 magnetic beads (Dynal), and MB cells were then positively selected using anti-CD27 MACS microbeads (Miltenyi Biotec). Polyclonal plasmablasts (PPC) were generated from purified peripheral blood $CD19^+$ B cells in vitro as described (36).

Preparation of cRNA and microarray hybridization

Microarray experiments were performed in Institute of Research in Biotherapy at the Montpellier University Hospital, Montpellier, France (http://irb.chu-montpellier.fr/en/laboratories_microarray.html).

RNA was extracted with the RNeasy Kit (Qiagen) or the SV Total RNA extraction kit (Promega) and TRIzol (Invitrogen Life Technologies) in accordance with the manufacturer's instructions. Biotinylated cRNA was amplified with a double in vitro transcription in accordance with the Affymetrix small sample labeling protocol VII (Affymetrix). The biotinylated cRNA was fragmented and hybridized to the HG-U133A and HG-U133B or HG-U133 Plus 2.0 GeneChip oligonucleotide arrays according to the manufacturer's instructions (Affymetrix). Fluorescence intensities were quantified and analyzed using the GCOS software (Affymetrix). Arrays were scaled to an average intensity of 100. A threshold of 1 was assigned to values <1.

Gene expression profiling analysis

In the Affymetrix HG-U133 arrays, a gene is probed by 11 pairs of perfect-match/mismatch oligonucleotides randomly spread over the chip. The signed rank MAS5 algorithm decides after scanning if the corresponding gene can be statistically declared present (P call) or absent (A call) and delivers a weighted fluorescence signal. Gene expression data were analyzed with our bioinformatics platform (RAGE; http://rage.montp.inserm.fr/). CT gene expression was assessed in 64 primary MMC samples, 20 HMCL samples, eight plasma cells samples from patients with MGUS, seven BMPC samples, seven MB cell samples, seven PPC samples, and five testis samples from healthy donors using the HG-U133 A+B set arrays. CT gene expression was assayed in a second independent series of 64 primary MMC samples from patients with newly diagnosed MM and in four plasma cell samples from patients with monoclonal gammopathy of unknown significance (MGUS) using the HG-U133 Plus 2.0 arrays. Hierarchical clustering was performed with Cluster and TreeView software from Eisen (37). We also used the gene expression profiling (GEP) from 72 human normal tissue samples available from Hogenech and coworkers on a public database (38). These GEP data were determined with Affymetrix HG-U133A and custom-designed GNFH1 arrays. The same global scaling and normalization was used for these and our data.

Real-time RT-PCR

MAGE-C1, *MAGE-A3*, *SSX1*, *MORC*, *KM-HN-1*, and *DDX43* real-time RT-PCR analysis was done as previously described (39). The Assays-on-Demand primers and probes and the TaqMan Universal Master Mix were used according to the manufacturer's instructions (Applied Biosystems). Real-time RT-PCR was performed using the ABI Prism 7000 sequence detection system (Applied Biosystems), normalized to *GAPDH* for each sample, and compared with the values obtained for a positive control (i.e., the testis sample no. 3 used in microarray hybridization) using the following formula in which Ct is cycle threshold: $100/2^{\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct$ unknown $- \Delta Ct$ positive control.

Immunocytochemistry

The expression of CT proteins was assessed by immunocytochemistry on cytospin smears of nine HMCL and of tumor samples from three patients with stage III MM. The following primary Abs were used: the mouse anti-NY-ESO-1 E978 (2.5 μg/ml), the mouse anti-MAGE-A 6C1 (4 μg/ml) mAbs (Zymed Laboratories), the mouse anti-GAGE-7 mAb clone 26 (diluted to 1/250) (BD Biosciences), and the goat polyclonal anti-SSX N-18 Ab (10 μg/ml) (Santa Cruz Biotechnology). Cytospin slides were air-dried at room temperature, fixed in glacial acetone for 10 min, and stored at −20°C until use. The thawed slides were fixed again and endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol. The slides were then incubated for 30 min in a blocking solution containing human or goat serum followed by a 15-min incubation in CAS Block™ (Zymed Laboratories). Primary and secondary staining were performed with the NEXES Ventana Medical Systems automaton using the iVIEW diaminobenzidine detection kit (Ventana Medical Systems) containing goat anti-mouse secondary Abs. Primary staining with the goat polyclonal Ab to SSX was performed manually, i.e., a 30-min incubation at room temperature and then the slides were submitted to secondary staining with the automaton using a mouse anti-Goat IgG (Fc-specific) mAb (5 μg/ml; Interchim). Sections of a formalin-fixed, paraffin-embedded normal testis were used as a positive control. Appropriate negative controls omitting the primary Ab were included for each case.

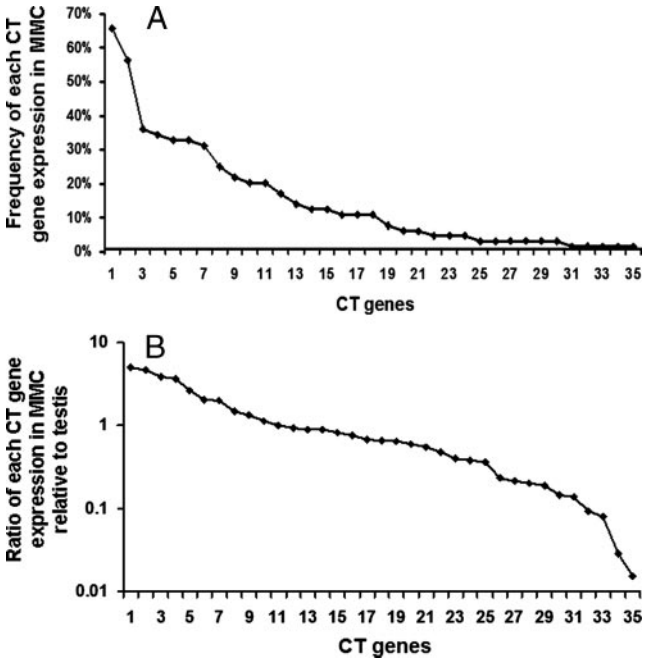


FIGURE 1. Distribution curves of 35 CT genes. *A*, The "present call" frequency curve. Frequency of expression of each CT gene was evaluated among 64 MMC samples hybridized on Affymetrix HG-U133 set arrays. The MMC sample was positive for one given CT gene if the probe set had a present call. *B*, Relative median signal level distribution curve. The curve represents, for each CT gene, the ratio of the median signal value in MMC samples with a present call to the median signal value in testis samples with a present call. Each symbol represents one CT gene.

Statistical analysis

For a given CT gene, the proportion of samples with a "present" Affymetrix call in different groups of cells (primary MMC, HMCL, MB cell, PPC, and BMPC) or in MMC of patients at different Durie-Salmon stages were compared with a χ^2 test. The correlations of gene expression signals determined with HG-U133 set arrays and real-time RT-PCR were performed with a Spearman correlation test. The prognostic values of the CT gene expression were analyzed with the Kaplan-Meier method using SPSS software. Hierarchical clustering was performed with the Cluster and TreeView software from Eisen (37).

Results

Expression of known CT genes in patients with MM, patients with MGUS, and in HMCL and normal cells according to the present/absent call provided by Affymetrix HG-U133 set arrays

We used the list of CT genes available on the CT Gene Database (http://www.cancerimmunity.org/Ctdatabase; Ref. 19) updated with three recently reported CT genes, i.e., *CT45* (22), *SLLP1*/*SPACA3* (20), and *KM-HN-1* (21). Of 93 CT genes belonging to 47 families, 64 could be interrogated by 104 probe sets with the Affymetrix HG-U133A or GNFH1 arrays. For a given gene, if several probe sets were available, the probe set with the highest median signal value was selected. The expression of each of the 64 genes was first evaluated among 72 normal tissues or cell types. Ten CT genes had a "present call" in more than seven different

normal nongametogenic tissues (excluding testis, placenta, and ovary samples) and should therefore be considered as "ubiquitously expressed" according to the definition of Scanlan et al. (19). Furthermore, eight CT genes were not expressed ("absent call") in all samples, including the 10 testes samples. These eight genes were deleted. Among the remaining 46 CT genes with a tissue-restricted expression pattern, all had a "present call" in at least three of five testis samples, but 11 (24%) were not expressed in any of the 64 MMC-samples probed with HG-U133 set arrays. Thus, 35 CT genes, listed in Table I, had a "present call" in at least one of 64 MMC samples. Fig. 1*A* shows the "present call" frequency curve of the 35 CT genes in MMC. Of note, no CT gene was expressed in all of the 64 MMC samples and 11 CT genes were expressed in <5% of the MMC samples. The expression level of each transcript was also evaluated. Fig. 1*B* shows the distribution curve of the ratios of the median values of the 35 CT genes in MMC samples with a "present" call to that in the testis. The median of the MMC-to-testis ratios is 0.7 and that of the HMCL-to-testis ratios is 1.2 (see Table I). Fig. 2 shows the HG-U133 set array signal levels in the different groups of cells (testis, MB cells, PPC, BMPC, MGUS, MMC, and HMCL) of the 10 CT genes that were the more frequently expressed in MMC. *MAGE-C1* and *KM-HN-1* were the two most often expressed genes (66 and 56% of MMC, respectively). *MORC*, *DDX43/HAGE*, *MAGE-A3*, *MAGE-A5*, *MAGE-A6*, *MAGE-A12*, *SPACA3*, *SSX-1*, and *SSX-4* are expressed in >20% of the patients (Table I). *CTAG1B/NY-ESO-1*, known to encode for a highly immunogenic Ag, showed the highest median signal intensity relative to testes but were expressed in only 13% of patients with MM. Eleven CT genes were expressed in MMC with a median signal level at least equal to the median expression in testes samples ($R = 1$). Twenty-seven CT genes were not expressed in plasma cells from patients with MGUS and eight were expressed in at least one of 12 samples: *SPANXB*, *CT45*, *GAGE1–8*, *SPACA3*, *DDX43/HAGE*, *MORC*, *MAGE-C1*, and *KM-HN-1*.

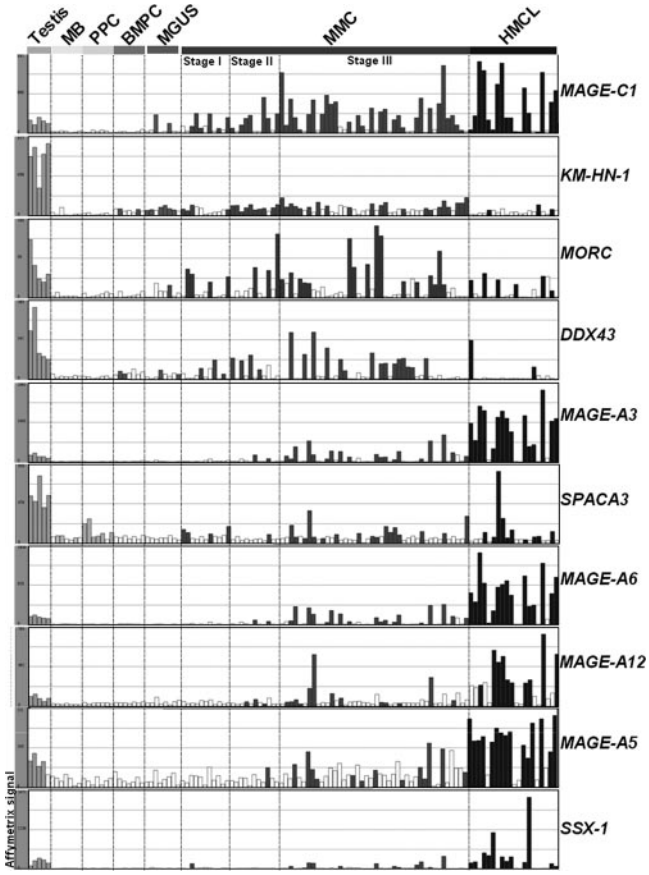


FIGURE 2. Gene expression of 10 CT genes measured by pangenomic Affymetrix HG-U133 set arrays. Histograms show the expression level of 10 CT genes in five testis samples, seven normal MB cell samples, seven normal PPC samples, seven normal BMPC samples, eight purified plasma cell samples from patients with MGUS, 64 MMC samples from patients with MM ordered in stages (I, II, and III), and 20 HMCL samples. The signal intensity for each gene is shown on the *y* axis as arbitrary units determined by the GCOS 1.2 software (Affymetrix). Open histograms indicate an "absent" Affymetrix call and filled histograms a "present" Affymetrix call.

Seven CT genes were expressed in some normal B cells, plasmablasts, or normal bone marrow plasma cells, including the following three expressed in >5% of MMC: *KM-HN1*, *SPACA3*, and *DDX43*. Analysis of their expression in the publically available GEP database of 72 normal tissues indicates that *KM-HN-1* and *SPACA3* are expressed in no normal tissues except testis and thus belong to the tissue-restricted CT category (19). *DDX43* is expressed in hemopoietic cells only (i.e., $CD14^+$ and $CD33^+$ cells). Twenty-five of the 35 CT genes are located on the chromosome X (CT-X genes), including 18 genes with a frequency in MMC of >5%. HMCL express CT-X more frequently than primary MMC (median 50 vs 11%; $p < 0.001$) and with a higher expression level (median ratio 2 vs 0.8; $p < 0.001$). This is not the case for the 10 non-CT-X genes.

Validation of CT gene microarray expression by real-time RT-PCR

Microarray data for six frequently expressed CT genes were validated by real-time RT-PCR using RNA of purified MMC from 10 patients. Data are shown in Fig. 3 and indicate that real-time RT-PCR data correlated well with microarray signals ($p \leq 0.05$).

Validation of CT gene expression by immunochemistry

For four CT gene families (MAGE-A, SSX, GAGE-7, and NY-ESO1) whose Ab to gene products were available, Affymetrix microarray data were confirmed by protein labeling. We used the 6C1 mAb that recognizes several members of the MAGE-A family (A1–3, A4, A6, A10, and A12), the N-18 Ab recognizing SSX1–4, 6, and 8, and mAbs to GAGE-7 and NY-ESO-1. The four monoclonal or polyclonal Abs efficiently labeled testis tissue and the HMCL with a "present" Affymetrix detection call (Fig. 4*A*). The HMCL with an "absent" Affymetrix detection call were not labeled (Fig. 4*A*). GAGE-7, MAGE-A, and SSX protein expression were also evidenced on primary MMC from patients (Fig. 4*B*). Of note, CT protein expression in primary MMC was heterogeneous, because strongly stained MMC close to negative MMC could be observed in the tumor sample of the same patient (Fig. 4*B*).

Focus on regularly coexpressed CT genes

MMC from only one of 64 patients (2%) expressed none of the 35 CT genes ("present" Affymetrix call). MMC from eight of 64 patients (12%) expressed only one CT gene, MMC from 10 of 64 patients (16%) only two CT genes, and MMC from 45 of 64 patients (70%) at least three CT genes. Fig. 5*A* shows the percentage of patients' MMC expressing different numbers of CT genes.

Because proteins encoded by CT genes are rarely homogeneously expressed within tumors and to circumvent possible immune-escape mechanisms, it would be advisable to use several different CT Ags in a vaccine design. To focus on a limited set of CT genes, we have explored the combinations of three CT genes, making it possible to cover the MMC expressing at least three CT genes. *MAGE-A3*, *-A6*, and *-A12* were considered as a single Ag

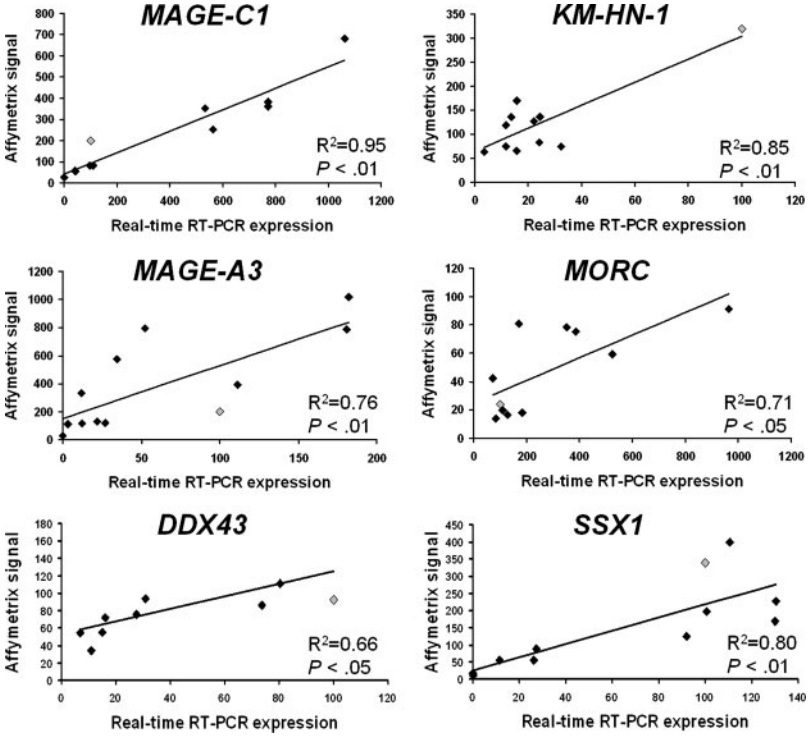


FIGURE 3. Validation of microarray data. Gene expression of *KM-HN-1*, *MAGE-C1*, *MAGE-A3*, *MORC, SSX1*, and *DDX43* were assayed with real-time RT-PCR in 10 MMC samples and normalized with GAPDH. Testis sample no. 3 was used as a positive control and is represented by the gray diamond. The coefficients of correlation and *p* values for the correlations between microarrays and real-time RT-PCR signals were determined with a Spearman test.

because they encode proteins that are cleaved similarly to generate one common HLA-A2-restricted peptide (cancerimmunity.org/peptidedatabase). The 10 CT genes depicted in Fig. 5*B* permit us to obtain a combination (actually 15 combinations) of at least three CT genes that are coexpressed in 67% of the patients. The remaining patients expressed one or two genes from this list.

Prognosis value of CT gene expression

Forty-seven out the 64 patients were treated with HDC and ABSCT with a careful clinical follow-up. To assess the prognostic value of CT gene expression in a larger series of patients, a second series of 64 consecutive newly diagnosed patients was included. The GEP of purified MMC from these patients were analyzed with HG-U133 Plus 2.0 arrays, but the use of the absent/present Affymetrix call avoids the problem of microarray normalization. Of note, the frequencies of present call for the 35 CT genes were similar in the two series of patients. These 111 patients were treated with the same HDC and ABSCT protocol and had a median 2-year follow-up. The prognostic value of each CT gene was evaluated comparing the EFS of patients with a present call to that of patients with an absent call. Significantly shorter EFS was found for six CT genes that were all encoded by chromosome X: *CTAG1B*, *CTAG2*, *MAGE-A1*, *MAGE-A2*, *MAGE-A3*, and *MAGE-A6* (Fig. 6). We investigated a correlation of CT gene expression with other conventional prognostic parameters. The *MAGE-A3*- and *MAGE-A6*-positive groups contained significantly more patients with a β2-microglobulin level $\geq$3.5 mg/L ($p = 0.04$ and $p = 0.03$, respectively), and the *MAGE-A6*-positive group contained significantly more patients with a serum albumin level $<$35 g/L ($p = 0.04$). Among the *CTAG1B*- or *CTAG2*-positive patients, 90% had a chromosome 13 deletion compared with 46% in *CTAG1B*- or *CTAG2*-negative patients ($p = 0.008$). Considering that only the 25 CT genes encoded by chromosome X, a CT-X^{high} cluster comprising MMC of one-third of the patients (35 of 111) could be defined using a binary hierarchical clustering based on the Affymetrix call (Fig. 7*A*). Patients in the CT-X^{high} cluster had a shorter EFS (median 16 mo) compared with patients in the CT-X^{low} cluster (median 32 mo; $p = 0.003$) (Fig. 7*B*). Eighty-nine percent of the patients included in the CT-X^{high} group had a stage III disease compared with 71% of the patients in the CT-X^{low} group ($p = 0.004$). Also, the CT-X^{high} group contained 51% of the patients with a β2-microglobulin level $\geq$ 3.5 mg/L, whereas the CT-X^{low} group contained 33% ($p = 0.06$). Finally, 55% of the patients included in the CT-X^{high} group had a gain of chromosome band 1q21 compared with 27% in the CT-X^{low} group ($p = 0.008$). No differences in hemoglobin level, albumin level, bone lesions, κ L chain isotype, age, C-reactive protein, lactate dehydrogenase, chromosome 13q deletion, chromosome 17p deletion, and t(11;14) or t(4;14) translocations were found between the CT-X^{high} and the CT-X^{low} groups.

Discussion

One aim of this study was to provide an overview of the expression of known CT genes in primary MMC from newly diagnosed patients. CT genes have previously been classified by Scanlan et al. (19) into four categories according to their expression profile measured by RT-PCR in 13 somatic tissues: 1) testis-restricted (expression in testis and tumor samples only); 2) "tissue restricted" (mRNA detected in two or fewer nongametogenic tissues); 3) "differentially expressed" (mRNA detected in 3–6 nongametogenic tissues); and 4) "ubiquitously expressed". To classify accordingly, we looked for the expression of CT genes among 72 normal (including gametogenic) tissues samples whose GEP were available from Su et al. (38). An advantage of Affymetrix microarrays is that each probe set signal is defined by the hybridization to 11 matched and mismatched oligonucleotides, making possible a statistical assignment of a probe set as "present" or "absent." This Affymetrix call enables us to use together data from different Affymetrix microarrays, avoiding the normalization problem. Probe sets for 64 of the 93 reported CT genes were available on Affymetrix HG-U133A or GNFH1 arrays. For eight CT genes (*CSAGE2*, *FTHL17*, *MAGE-A10*, *MAGE-C3*, *SAGE-1*, *SSX-5*, *TFDP3*, and *TSP50*), probe sets were likely inefficient because no present call were found in any of the testes samples (10 from Su et al. (38) and five

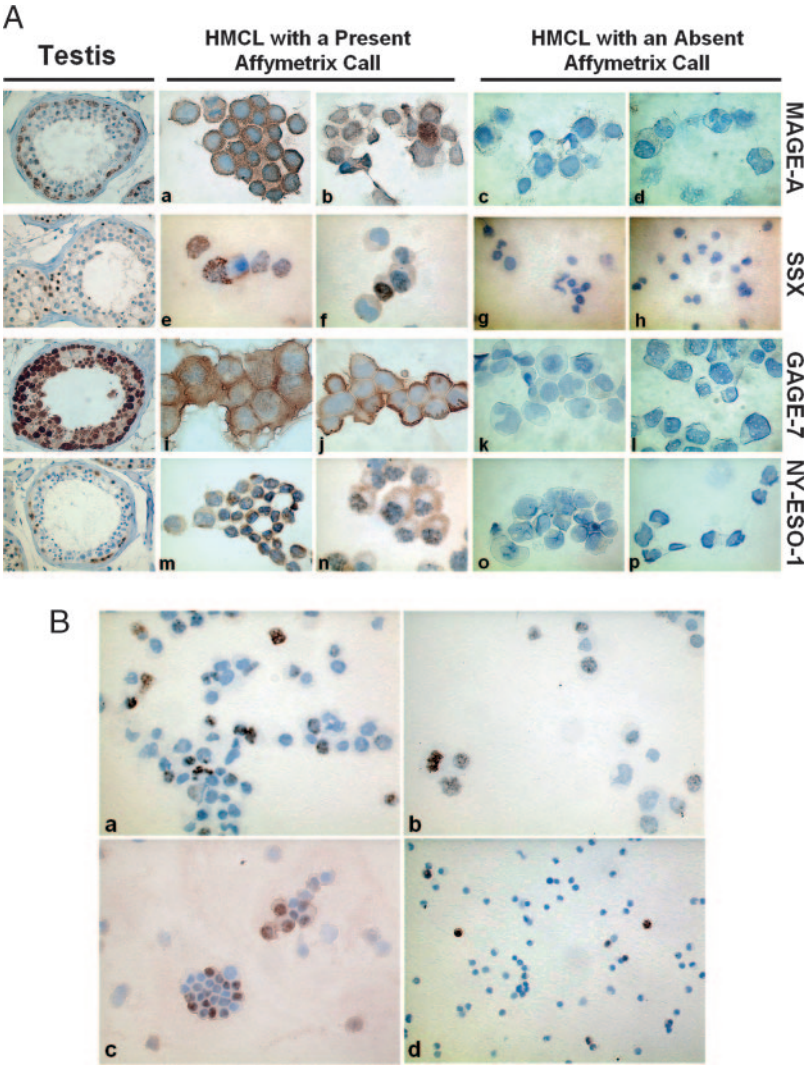


FIGURE 4. Immunocytochemical detection of CT gene products in HMCL and primary MMC. *A*, Acetone-fixed cytospin preparations of HMCL were immunostained with CT Ag-specific Abs. Ab binding is indicated by brown staining. MAGE proteins detected with the 6C1 Ab presented a cytoplasmic localization in intratubular germ cells of the testis sample (original magnification ×400) and in XG-7 cells (*a*; original magnification ×1000) and localized in the nucleus as well as in the cytoplasm of XG-19 cells (*b*; original magnification ×1000). In *c* and *d*, no immunoreactivity was detected in the LP1 and XG-14 HMCL with an absent Affymetrix call (original magnification ×1000). A nuclear expression of SSX was observed in testis (original magnification ×300) and in XG-19 cells (*e*) and XG-1 cells (*f*) (original magnification ×1000), and no labeling of LP1 cells (*g*) and XG-14 cells (*h*) (original magnification ×630). GAGE-7 is detected in the nucleus and cytoplasm of testis cells (original magnification ×400) and in the cytoplasm of XG-6 (*i*) and XG-12 (*j*) (original magnification ×1000) cells. It was not detected in XG-13 (*k*) or XG-19 cells (*l*) (original magnification ×1000). A cytoplasmic expression of NY-ESO-1 was observed in some testis cells (original magnification ×400) and in XG-7 cells (*m*; original magnification ×630) and both cytoplasmic and nuclear expressions were seen in U266 cells (*n*; original magnification ×1000) but expression of NY-ESO-1 was lacking in XG-1 (*o*) and XG-14 cells (*p*) (original magnification ×1000). *B*, Immunostaining of primary MMC from three patients with anti-CT Abs. A heterogeneous nuclear expression of MAGE (*a*) and SSX (*b*) proteins was detected in MMC from patient 1, heterogeneous nuclear and cytoplasmic expression of GAGE-7 in MMC from patient 2 (*c*), and focal expression of GAGE-7 in MMC from patient 3 (*d*) (original magnification ×630). Control slides treated with the same protocols but omitting primaries Abs showed no staining (data not shown).

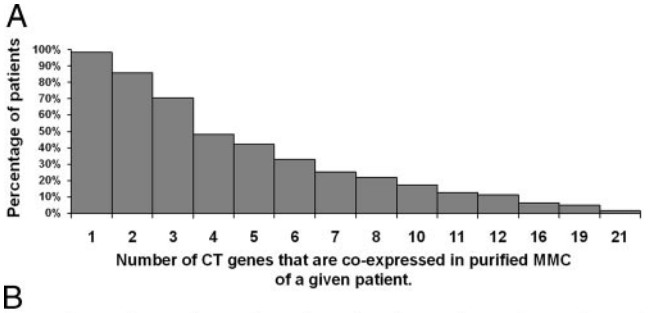
from our current data) as well as in the other tissues. An explanation is that the Affymetrix probe sets were "computer designed" and do not always work. Of the remaining 56 CT genes that could be interrogated by Affymetrix arrays, 27 showed a "testis-restricted" expression. Ten genes showed a "tissue-restricted", nine a "differentially expressed" and 10 a "ubiquitous" expression pattern. From the latter, seven (*BAGE/CT2*, *MAGEB2/CT3.2*, *SYCP1/CT8*, *NA88A* pseudogene*/CT18*, *CTAGE-1/CT21*, *AF15Q14/CT29*, and *TPTE/CT44*) have previously not been reported as "ubiquitously expressed". We removed these 10 genes from the current analysis. Thus, 46 CT genes answering the criteria defined by Scanlan et al. (19), i.e., testis restricted, tissue restricted, or differentially expressed, were retained.

Thirty-five of these 46 CT genes have a present Affymetrix call in at least one of 64 MMC. *MAGE-C1* is the most frequently expressed CT gene (66%) by MMC of newly diagnosed MM-patients as previously observed in a small group of 29 patients by Dhodapkar et al. (40). We also confirmed that *SPACA3/SLLP1, SPANXB*, and the *MAGE-A*, *GAGE*, *CTAG*, and *SSX* gene families are expressed by MMC (20, 29, 31, 32, 41–44). The CT Ag *CTAG1B/NY-ESO-1*, known to be highly immunogenic (27, 45, 46), shows the highest median signal in MMC compared to the testis with a frequency of presence of 13% in MM patients, which is lower than the frequency observed by van Rhee et al. (31).

SPA17 was proposed as a target for immunotherapy in MM (47). However, Scanlan et al. reported it to be expressed ubiquitously (19), and this was confirmed by the present study. In addition, *SPA17* was expressed in MMC of 4% of patients with MM only (results not shown).

Ten CT genes whose expression was not presently reported in MMC were identified: *KM-HN-1* in 56% of the patients, *MORC* in 36%, *DDX43* in 34%, *MAGE-C2* in 13%, *PAGE1/GAGE-9* in 11%, *XAGE-1* in 11%, *SPO11* in 8%, *CT45*, *SPANXC* in 6%, and *CRISP2* in 5%. For six CT genes—*MAGE-C1*, *KM-HN-1*, *MAGE-A3*, *MORC*, *DDX43*, and *SSX1*—the Affymetrix values were confirmed by real time RT-PCR. Affymetrix values were also confirmed by protein labeling for four CT gene families—NY-ESO-1, MAGE-A, SSX, and GAGE-7—whose Ab to gene products were available. The HMCL with a "present" Affymetrix call were stained positively with the respective anti-CT Ag Abs, unlike the HMCL with an "absent" Affymetrix call. Previous studies already showed a correlation between gene and protein expression for MAGE-A or NY-ESO-1 CT Ags in MMC (32, 41). It will be necessary to further extend this protein validation for each of the 35 CT genes expressed in MMC when the specific Abs become available.

The *KM-HN-1*, *SPACA3*, *MORC*, *DDX43*, and *SPO11* genes are also expressed by normal memory B cells, normal plasmablasts, or bone marrow plasma cells. *KM-HN-1* could be a B cell differentiation Ag as it is expressed by B or plasma cells only, unlike 72 other normal tissues. Whereas CT genes encoded by chromosome X (except *MAGE-C1*) are rarely expressed in early disease (zero to



B

(%) of patients	KM-HN-1	MAGEC1	MAGE-A3/6/12	MORC	DDX43	SSX4	SPACA3	MAGEA5	MAGEC2	GAGE 1-8
20	■	■	■							
20	■	■		■						
14	■	■			■					
13	■	■				■				
13	■		■	■						
11		■					■			■
11		■	■			■				
9		■		■			■			
9	■		■				■			
8			■	■		■				
8	■			■				■		
5	■				■		■			
5		■					■		■	
3				■	■		■			
3	■				■			■		

FIGURE 5. Coexpression of CT genes. *A*, Frequency of CT gene coexpression. Histograms represent the percentage of patients whose MMC coexpress the corresponding CT gene number (at least 1–21 CT genes). These percentages ranged from 98% of the patients expressing at least one CT gene to 2% of patients coexpressing 21 CT genes. *B*, Combinations of three CT genes that are coexpressed in MMC of a given patient. For each combination of three CT genes the percentage of patients whose MMC express this combination is indicated. The MMC of a patient can express several combinations.

one of 11 patients with stage I MM in this study), it is noteworthy that *MORC*, *KM-HN-1*, *DDX43*, and *SPACA3* are expressed in MMC from 27 to 36% of the patients with stage I disease. The function of the products of these genes in normal or cancer biology is presently unknown. As vaccination strategies could be of interest for early-stage patients, the identification of these CT genes is of importance and needs further confirmation at the protein level when the Ab will be available. In the same view, *KM-HN-1* was also expressed in purified plasma cells from 83% of the patients with MGUS, *MAGE-C1* in 33%, *DDX43* in 33%, and *SPACA3*, *MORC*, *GAGE1–8*, *CT45*, and *SPANXB* in 8%. Patients with MGUS continuously transform into MM with an annual rate of ~1% (48) and these data may suggest initiating vaccination strategies early in these patients, i.e., when there is still an efficient T cell repertoire against the plasma cell Ags (49).

It is interesting to note that CT gene expression is associated with shorter EFS, significantly in the case of six of the 35 CT genes, all located on chromosome X, and in a "cluster" of patients driven by 25 CT-X genes. No difference in overall survival was found, but the median follow-up of these patients' series was too short to conclude that point. This is in agreement with recent findings in other cancers showing that CT-X gene expression is often coordinated due to hypomethylation of their promoters (50, 51). In addition, CT-X gene expression in patients with epithelial cancers is often associated with a poor patient outcome (52–54). Our data are also in agreement with recent studies emphasizing that the MMC of patients that express *MAGE-C1*, *MAGE-A3*, and/or *NY-ESO-1* had an increased proliferative activity and are obtained mainly from patients with stage III MM (32, 40). These gene products might have a functional role in the pathogenesis of cancer leading to a bad evolution. Such a bad prognosis value was recently published for one of the six genes reported here, *NY-ESO-1/CTAG1B*, in which expression was correlated to abnormal cytogenetics (31). We also found an association between *CTAG1B* expression and chromosome 13 deletion.

The fact that ultimately all patients with MM relapse, even after HDC and ABSCT, and the concomitant observation that even in the case of a "complete remission" residual MMC remain detectable emphasize the interest in vaccination strategies that might allow the eradication of residual MMC. Vaccination could be initiated directly after HDC to use the "lymphodepletion window" associated with the effect of HDC and the concomitant endogenous production of several cytokines, as reported in a murine melanoma model and in patients with melanoma (55, 56). This strategy is further promoted by the fact that in patients with MM, the efficacy of T cells to pneumococcus Ags was stronger when they were injected 14 days after HDC rather than 3 months (57).

We provide here a strategy for how to choose three CT genes that are frequently coexpressed. To this end, Ag mixtures are envisioned to be superior to single Ag to avoid tumor selection and escape. We show that a combination of at least three Ags could be designed in 67% of the patients using only 10 CT genes. However, additional studies are required before such information could be helpful in the design of vaccination strategies. In particular, the

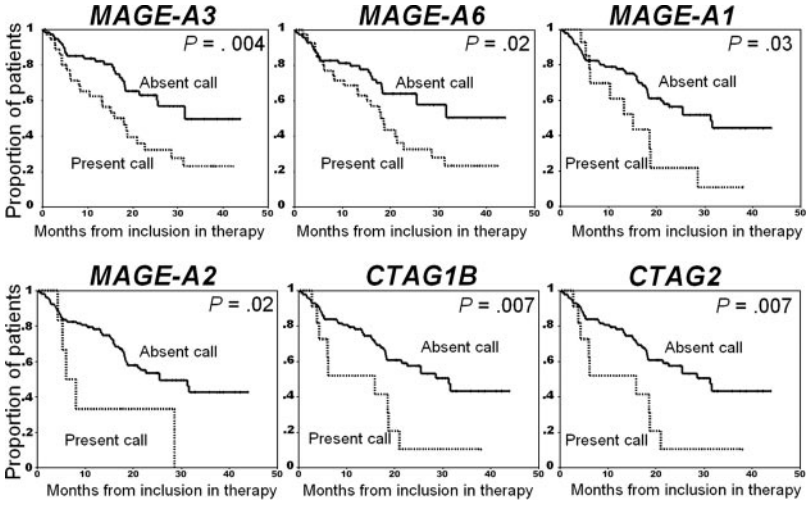


FIGURE 6. EFS analysis of 111 patients with newly diagnosed MM according to the expressions of six CT genes. Data are the Kaplan-Meier survival curves of patients whose MMC express ("present" call; dashed line) or do not express ("absent" call; bold line) the *MAGE-A1*, *MAGE-A2*, *MAGE-A3*, *MAGE-A6*, *CTAG1B*, or *CTAG2* CT gene. For each of these 6 CT genes, the log-rank test indicated that patients with a present Affymetrix call had lower EFS than patients with an absent Affymetrix call. The *p* values are indicated in each plot.

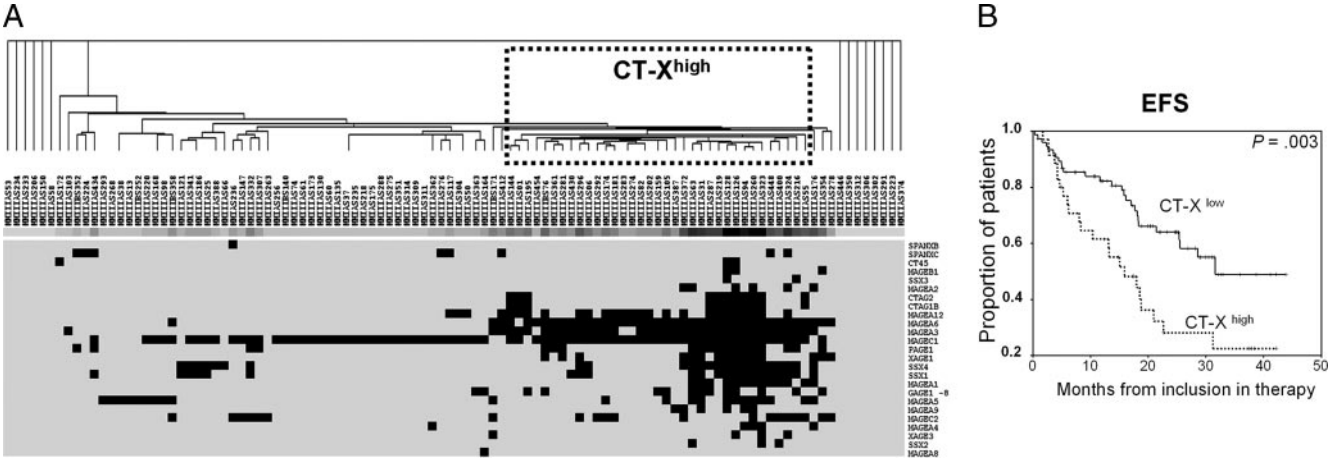


FIGURE 7. The CT gene signature. *A*, Hierarchical clustering of the MMC of 111 patients according to absent/present calls of the 25 CT-X genes. The gene signal of a gene was assigned "1" if it had a present call and "0" if it had an absent one. The signal matrix was then run with the Cluster software using log2 transformation and the Pearson correlation metrics. The clustering was visualized with the TreeView software. Gray is for an absent call and black for a present call. Data indicate that patients coexpressing CT-X genes (about one-third of the patients) cluster together. *B*, Correlation of *CT-X* gene expression and EFS in MM patients. Kaplan-Meier survival curves comparing EFS of patients included in the CT-X^{high} group (*dashed line*) with that of patients included in the CT-X^{low} group (*bold line*). The log-rank test revealed that CT-X^{high} patients had a significantly lower EFS than CT-X^{low} patients ($p = 0.003$).

immunogenicity of the gene products of each of these 10 CT genes must be proved, showing either the presence of T cells recognizing antigenic peptides and/or the presence of specific Abs to the gene product in the serum of patients with MM. According to the information available from the CT Gene Database (web site address is given in *Results*), T cell reactivity has been shown in other cancers for seven of these 10 CT gene products: KM-HN-1, MAGE-C1, MAGEA3/6, SSX4, SPACA3, MAGE-C2, and GAGE1–8. For three of these 10 CT genes, immunogenicity was also shown in patients with MM: a *MAGE-C1*-specific CD8 T cell detected in patients' bone marrow (58), a *MAGE-A3* specific CD8 T cell detected in patients' blood (28), and *SPACA3* Abs found in a patient's sera (20). In an allogenic transplantation setting, Atanackovic et al. recently showed that at least four CT Ags expressed by MMC were immunogenic in vivo. Indeed in nine of 35 patients, an allograft resulted in the emergence of anti-SSX2, anti-MAGE-A3, and/or anti-NY-ESO-1 Abs in recipients' sera (59). In the same study, a humoral response against CT Ags was detected in the serum of only one patient of 22 treated with HDC and ABSCT. It is of note that autograft leukapheresis products contain a low number of lymphocytes with an increased proportion of regulatory T cells (60). Given the current data, it is now of major interest to demonstrate that CTL directed against currently identified immunogenic peptides from KM-HN-1, SSX4, SPACA3, MAGE-C2, and GAGE1–8 can be elicited in patients with MM. In addition, we will need to identify immunogenic peptides for MORC, DDX43, and MAGE-A5 gene products for which T cell reactivity is not presently demonstrated. Once this immunological work done, i.e., by associating these CT genes peptides with other tumor-associated Ag peptides reported in MM, e.g., MUC-1 or HM-1.24 (61, 62), one can envision covering almost all patients in a population with a set of 3–5 individually selected immunogenic peptides.

In conclusion, the determination of GEP with pangenomic microarrays could be very useful in defining an optimal combination of patient-specific CT Ags that could overcome tumor escape mechanisms as well as prophylactic vaccination against Ags that will occur in late stage myeloma.

Disclosures

The authors have no financial conflict of interest.

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