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Abstract

Cancer-testis (CT) antigens are expressed in testis and malignant tumors, but rarely in non-gametogenic 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 of CT antigens for clinical vaccination trials.

We report the expression pattern of CT genes in purified MM cells (MMC) of 64 patients with newly-diagnosed MM, 12 patients with MGUS, in normal plasma cell and B cell samples and in 20 MMC lines. Out of 46 CT genes interrogated by the Affymetrix HG-U133 Set arrays, 35 are expressed in 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. MMC of 98% of the patients expressed 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 to circumvent tumor escape mechanisms – is obtained in MMC of 67% of the patients. Providing confirmation of the immunogenicity of the products of these 10 CT genes, gene expression profiling could be useful to identify which CT antigens could be used to vaccinate a given patient.

Introduction

Multiple myeloma (MM) is a B cell neoplasia characterized by the accumulation of malignant plasma cells in the bone marrow. Although complete remission could be achieved in about 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 two to three year event-free survival (1). There is ample evidence that a small fraction of MM cells (MMC) escape chemotherapy and remain present even despite a "complete remission" (2). Remaining MMC are promising targets for active or passive immunotherapy, as (i) MMC can be lysed by cytotoxic T cells *in vitro* (3-6) and (ii) 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 immunoglobulin (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 interest 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 antigens have been identified (15-17). These antigens may be shared with normal cells, as MART-1, gp100 or tyrosinase. Another category, the cancer testis (CT) antigens, 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 including 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 pre-existing immune tolerance. Thus, the testis-restricted

CT antigens are being used as targets in several vaccination trials (23). In particular, NY-ESO-1 and CT antigens 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 Scanlan *et al*'s definition (19), about one fourth of CT genes are tissue restricted, *i.e.* expressed in two or fewer non-gametogenic tissues, one fourth is differentially expressed, *i.e.* expressed in three to six non-gametogenic tissues, but at a low level compared to 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 reported the presence of functional CD8 T lymphocytes against NY-ESO-1, LAGE-1, MAGE-A1,-A2, -A3 and -A4 in the peripheral blood of MM patients. Their frequency was three-fold increased compared to healthy donors, supporting an immunogenicity of CT antigens 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. 47/64 patients and 64/64 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, 7 IgAλ MM, 24 IgGκ MM, 10 IgGλ MM, 6 Bence-Jones κ MM, 3 Bence-Jones λ MM, and 2 non-secreting MM. In the second series, 4 patients were of stage IA, 11 of stage IIA, 45 of stage IIIA and 4 of stage IIIB. Nine patients had IgAκ MM, 3 IgAλ MM, 20 IgGκ MM, 19 IgGλ MM, 7 Bence-Jones κ MM, 4 Bence-Jones λ MM, and 2 non-secreting 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 ATTC (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 cell (BMPC) and primary MMC were purified using anti-CD138 MACS microbeads. Briefly, mononuclear cells were collected by centrifugation on 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 (Fascscan, Becton Dickinson). This sorting procedure generally insures a high plasma cell purity (≥ 95%). Sorted MMC were discarded if the purity was below 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 complementary RNA (cRNA) and microarray hybridization

Microarray experiments were performed in the Institute of Research in Biotherapy at the Montpellier University Hospital (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) in accordance with the manufacturer's instructions. Biotinylated complementary RNA (cRNA) was amplified with a double in-vitro transcription, according to the Affymetrix small sample labeling protocol vII (Affymetrix). The biotinylated cRNA was fragmented and hybridized to the HG-U133A and HG-U133B or to HG-U133 Plus 2.0 GeneChip oligonucleotide arrays according to 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 under 1.

Gene expression profiling analysis

In the Affymetrix HG-U133 arrays, a gene is probed by eleven 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 analysed with our bioinformatic platform (RAGE, http://rage.montp.inserm.fr/). CT gene expression was assessed in 64 primary MMC samples, 20 HMCL samples, 8 plasma cells samples from patients with MGUS, 7 BMPC samples, 7 MB cell samples, 7 PPC samples and 5 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 4 plasma cell samples

from patients with MGUS using the HG-U133 Plus 2.0 arrays. Hierarchical clustering was performed with the Cluster and Treeview softwares from Eisen (37). We also used the gene expression profiling (GEP) from 72 human normal tissues samples available from Hogenech's group 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 and normalized to *GAPDH* for each sample, and compared with the values obtained for a positive control (*i.e.* the testis sample number 3 used in microarray hybridization) using the following formula $100/2^{\delta\delta Ct}$ where $\delta\delta Ct = \delta Ct$ unknown – δCt positive control.

Immunocytochemistry

The expression of CT proteins was assessed by immunocytochemistry on cytospin smears of 9 HMCL and of tumor samples from 3 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 minutes 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™ DAB 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 slides were submitted to secondary staining with the automaton, using a mouse anti-Goat IgG (Fc specific) mAb (Interchim) (5 µg/ml). 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.

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, BMPC) or in MMC of patients at different Durie-Salmon stages were compared with a chi-square 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 softwares from Eisen (37).

Results

Expression of known CT genes in patients with MM, in patients with MGUS, in HMCL and in normal cells according to the Present/Absent call provided by Affymetrix HG-U133 Set arrays.

We used the list of CT genes available on http://www.cancerimmunity.org/CTdatabase (19), updated with three recently-reported CT genes, *i.e. CT45* (22), *SLLP1*/*SPACA3* (20) and *KM-HN-1* (21). Out of 93 CT genes belonging to 47 families, 64 could be interrogated by 104 probesets with the Affymetrix HG-U133A or GNFH1 arrays. For a given gene, if several probesets were available, the probeset 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 non-gametogenic tissues (excluding testis, placenta and ovary samples) and therefore should be considered as "ubiquitously expressed," according to Scanlan *et al*'s definition (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 3/5 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 out of 64 MMC samples. Fig. 1*A* shows their "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 less than 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 testis. The median of the MMC/testis ratios is 0.7 and that of the HMCL/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 ten CT genes that were the more frequently expressed in MMC. *MAGE-C1* and *KM-HN-1* were the two most often expressed ones (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 more than 20% of the patients (Table I). *CTAG1B*/*NY-ESO-1,* known to encode for a highly immunogenic antigen, 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 1/12 samples: *SPANXB, CT45, GAGE1-8, SPACA3, DDX43/HAGE, MORC, MAGE-C1* and *KM-HN-1*.

Seven CT genes were expressed in some normal B cells, plasmablasts or normal bone marrow plasma cells, including the following three expressed in more than 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 hematopoietic 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 above 5%. HMCL express CT-X more frequently than primary MMC (median 50% versus 11%, $P < .001$) and with a higher expression level (median ratio 2 versus 0.8, $P < .001$). 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 ($P \leq .05$).

Validation of CT gene expression by immunochemistry

For 4 CT gene families (MAGE-A, SSX, GAGE-7, and NY-ESO1) whose antibody 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 4 monoclonal or polyclonal Abs efficiently labeled testis tissue and the HMCL with a "present" Affymetrix detection call (Fig. 4A). The HMCL with an "absent" Affymetrix detection call were not labelled (Fig. 4A). GAGE-7, MAGE-A and SSX protein expression were also evidenced on primary MMC from patients (Fig.4B). Of note, CT protein expression in primary MMC was heterogeneous, since strongly stained MMC close to negative MMC could be observed in the tumor sample of the same patient (Fig.4B).

Focus on regularly co-expressed CT genes

MMC from only one out of 64 patients (2%) expressed none of the 35 CT genes ("present" Affymetrix call). MMC from 8/64 patients (12%) expressed only one CT gene, MMC from 10/64 patients (16%) only two CT genes and MMC from 45/64 patients (70%) at least three CT genes. Fig. 5*A* shows the percentage of patient's 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 recommended to use several different CT antigens in a vaccine design. In order 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 antigen 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 to obtain a combination (actually 15 combinations) of at least three CT genes that are co-expressed in 67% of the patients. The remaining patients expressed one or two genes from this list.

Prognostic value of CT gene expression

Forty-seven out the 64 patients were treated with HDC and ABSCT with a careful clinical follow-up. In order 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 two-year follow-up. The prognostic value of each CT gene was evaluated comparing the event-free survival (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 beta 2-microglobulin level ≥ 3.5 mg/L (P = .04 and P = .03, respectively) and the *MAGE-A6* positive group contained significantly more patients with a serum albumin level < 35 g/L (P = .04). Among the *CTAG1B* or *CTAG2* positive patients, 90% had a chromosome 13 deletion compared to 46% in *CTAG1B* or *CTAG2* negative patients (P = .008). Considering 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 Affymetrix call (Fig. 7*A*). Patients in the CT-X^{high} cluster had a shorter EFS (median 16 months) compared to patients in the CT-X^{low} cluster (median 32 months, P = .003) (Fig. 7*B*). Eighty-nine percent of the patients included in the CT-X^{high} group had a stage III disease compared to 71% of the patients in the CT-X^{low} group (P = .004). Also, the CT-X^{high} group contained 51% of the patients with a beta 2-microglobulin level ≥ 3.5 mg/L, whereas the CT-X^{low} group contained 33% (P = .06). Finally, 55% of the patients included in the CT-X^{high} group had a gain of chromosome band 1q21 compared to 27% in the CT-X^{low} group (P = .008). No differences in hemoglobin level, albumin level, bone lesions, kappa light-chain isotype, age, C-reactive protein (CRP), lactate deshydrogenase (LDH), chromosome 13q deletion, chromosome 17p deletion, 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: (i) testis-restricted (expression in testis and tumor samples only), (ii) "tissue restricted" (mRNA detected in 2 or fewer non-gametogenic tissues), (iii) "differentially expressed" (mRNA detected in three to six non-gametogenic tissues), and (iv) "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 probeset signal is defined by the hybridization to 11 matched and mismatched oligonucleotides, making it possible a statistical assignment of a probeset as "present" or "absent". This Affymetrix call enables us to use together data from different Affymetrix microarrays, avoiding the normalization problem. Probesets for 64 out 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*, *TSP50*), probesets were likely inefficient because no present call were found in all testes samples (10 from Su *et al* (38) and five from our current data) as well as in the other tissues. An explanation is that the Affymetrix probesets 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 ten an "ubiquitous" expression pattern. From the latter, seven (*BAGE/CT2*, *MAGEB2/CT3.2*, *SYCP1/CT8*, *NA88A pseudogene/CT18*, *CTAGE-1/CT21*, *AF15Q14/CT29*, *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 1/64 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,* the *MAGE-A, GAGE, CTAG* and *SSX* gene families are expressed by MMC (20, 29, 31, 32, 41-44). The CT antigen *CTAG1B/NY-ESO-1*, known to be highly immunogenic (27, 45, 46), shows the highest median signal in MMC compared to 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 this 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* and *SPANXC* in 6% and *CRISP2* in 5%. For 6 CT genes – *MAGE-C1, KM-HN-1, MAGE-A3, MORC, DDX43, SSX1* – the Affymetrix values were confirmed by real time RT-PCR. Affymetrix values were also confirmed by protein labelling for 4 CT gene families - NY-ESO-1, MAGE-A, SSX and GAGE-7 - whose Abs to gene products were available. The HMCL with a "present" Affymetrix call were stained positively with the respective anti-CT antigen 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 antigens in MMC (32, 41). It would be necessary to further extend this protein validation for each of the 35 CT genes expressed in MMC, when the specific antibodies will become available.

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 antigen 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 (here 0-1/11 patients with stage I MM), 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 antibody will be available. In the same view, *KM-HN1* was also expressed in purified plasma cells from 83% of the patients with MGUS, *MAGE-C1* in 33%, *DDX43* in 33%, *SPACA3, MORC, GAGE1-8, CT45* and *SPANXB* in 8%. Patients with MGUS continuously transform into MM with an annual rate of approximately 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 plasma cell antigens present (49).

It is interesting to note that CT gene expression is associated with shorter EFS, significantly in the case of six out of the 35 CT genes, all located on chromosome X, and in a "cluster" of patients driven by 25 CT-X. No difference in overall survival was found, but the median follow-up of this 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 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 the 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 of vaccination strategies that might allow eradicating residual MMC. Vaccination could be initiated directly after HDC in order to use the "lymphodepletion window" associated with the effect of HDC and 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 antigens was stronger when they were injected 14 days after HDC rather than three months (57).

We provide here a strategy for how to choose three CT genes that are frequently co-expressed. To this end, antigen cocktails are envisioned to be superior to single antigen in order to avoid tumor selection and escape. We show that a combination of at least three antigens could be designed in 67% of the patients using only ten CT genes. However, additional studies are required before such information could be helpful in the designing of vaccination strategies. In particular, the 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 antibodies to the gene product in the serum of patients with MM. According to the information available from http://www.cancerimmunity.org/ CTdatabase, T cell reactivity has been shown in other cancers for 7 out of these 10 CT gene products: KM-HN-1, MAGE-C1, MAGEA3/6, SSX4, SPACA3, MAGE-C2, and GAGE1-8. For 3 of these 10 CT genes, immunogenicity was also shown in patients with MM: MAGE-C1 specific CD8 T cell detected in patients' bone marrow (58), MAGE-A3 specific CD8 T cell detected in patients' blood (28) and SPACA3 Abs found in patient's sera (20). In an allogenic transplantation setting, Atanackovic *et al* recently shown that at least 4 CT antigens expressed by MMC were immunogenic *in vivo*. Indeed in 9/35 patients, allograft resulted in the emergence of Abs anti-SSX2, MAGE-A3 and/or NY-ESO-1 in recipients' sera (59). In the same

study, humoral response against CT antigens was detected in only 1 patient's serum out 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 demonstrated presently. Once this immunological work done, associating these CT genes peptides with other tumor-associated antigen peptides reported in MM, *e.g.* MUC-1 or HM-1.24 (61, 62), one can envision to cover almost all patients in a population with a set of three to five individually selected immunogenic peptides.

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

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

Probe set	Gene name	Chromosomal Location	MMC (%) (n=64)	Ratio MMC/ testis	HMCL (%) (n=20)	Ratio HMCL/ 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

Data are the frequencies of "present" Affymetrix call for 35 CT genes in purified myeloma cells (MMC), human myeloma cell lines (HMCL), purified plasma cells from patients with MGUS, normal bone marrow plasma cells (BMPC), normal plasmablasts (PPC) and normal memory B cells (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.

Legends to figures

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 probeset 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 testes samples with a present call.

Each symbol represents one CT gene.

FIGURE 2: Gene expression of 10 CT genes measured by pan genomic Affymetrix HG-U133 Set arrays.

Histograms show the expression level of 10 CT genes in five testis samples, seven normal memory B cell (MB) samples, seven normal polyclonal plasmablastic cell (PPC) samples, seven normal BM mature plasma cells (BMPC) samples, eight purified plasma cell samples from patients with monoclonal gammopathy with undetermined significance (MGUS), 64 MMC samples from patients with multiple myeloma (MMC) ordered in stages (I, II, 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). Empty histograms indicate an "absent" Affymetrix call and filled histograms a "present" Affymetrix call.

FIGURE 3: Validation of microarrays 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 and normalized with GAPDH. The testis sample number 3 was used as a positive control and represented by the grey 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.

FIGURE 4: Immunocytochemical detection of CT gene products in HMCL and primary MMC

A. Acetone fixed cytospin preparations of HMCL were immunostained with CT antigen-specific Abs. Antibody 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 (x 400) and (a) in XG-7 cells (x 1000) and (b) localized in the nucleus as well as in the cytoplasm of XG-19 cells (x 1000). (c) and (d), no immunoreactivity was detected in the LP1 and XG-14 HMCL with an absent Affymetrix call (x 1000). A nuclear expression of SSX was observed in testis (x 300), (e) in XG-19 cells and (f) XG-1 cells (x 1000) and no labelling of (g) LP1 cells and (h) XG-14 cells (x 630). GAGE-7 is detected in nucleus and cytoplasm of testis cells (x 400) and in the cytoplasm of (i) XG-6 and (j) XG-12 (x 1000) cells. It was not detected in (k) XG-13 cells and (l) XG-19 cells (x 1000). A cytoplasmic expression of NY-ESO-1 was observed in some testis cells (x 400) and in (m) XG-7 cells (x 630) and a cytoplasmic and nuclear one in (n) U266 cells (x 1000) and was lacking in (o) XG-1 cells and (p) XG-14 cells (x 1000).

B. Immnuostaining of primary MMC from 3 patients with anti-CT antibodies. An heterogeneous nuclear expression of MAGE (a) and SSX (b) proteins was detected in MMC from patient 1, (c) heterogeneous nuclear and cytoplasmic expression of GAGE-7 in MMC from patient 2, (d) focal expression of GAGE-7 in MMC from patient 3 (x 630). Control slides treated with the same protocols but omitting primaries Abs showed no staining (data not shown).

FIGURE 5:. Co-expression of CT genes

A. Frequency of CT gene co-expression. Histograms represent the percentage of patients whose MMC co-express the corresponding CT gene number (at least 1 to 21 CT genes). These percentages ranged from 98% of the patients expressing at least one CT gene to 2% of patients co-expressing at least 21 CT genes.

B. Combinations of three CT genes that are co-expressed in MMC of a given patient. For each combination of three CT genes, the percentage of patients whose MMC express this combination is indicated. MMC of a patient can express several combinations.

FIGURE 6: Event-free survival analysis of 111 patients with newly-diagnosed MM according to six *CT* gene expression.

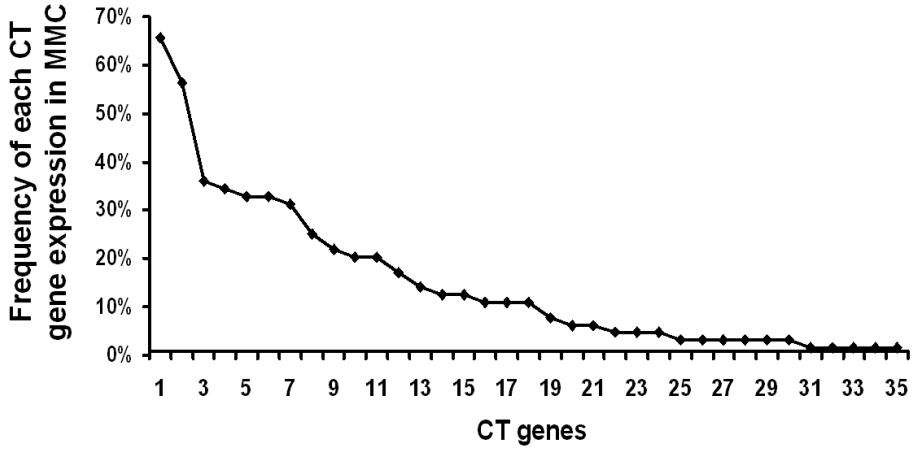
Data are the Kaplan-Meier survival curves of patients whose MMC express ("Present" call, dashed line) or did not express ("Absent" call, bold line) either *MAGE-A1*, *MAGE-A2*, *MAGE-A3*, *MAGE-A6*, *CTAG1B or CTAG2* CT gene. For each of these 6 CT genes, the log-rank test indicated that patients with 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 111 patients' MMC according to absent/present calls of the 25 CT-X genes. The gene signal of a gene was assigned 1 if it has a present call and 0 if an absent one. The signal matrix was then run with the Cluster software using log2 transformation and the Pearson correlation metrics. The clustering was then visualized with the Treeview software. Grey is for an absent call and black for a present call. Data indicate that patients co-expressing CT-X genes (about one third of the patients) cluster together.

B. Correlation of CT-X gene expression and event-free survival 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*=.003).

A


Frequency of each CT gene expression in MMC
70%
60%
50%
40%
30%
20%
10%
0%
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35
CT genes

B

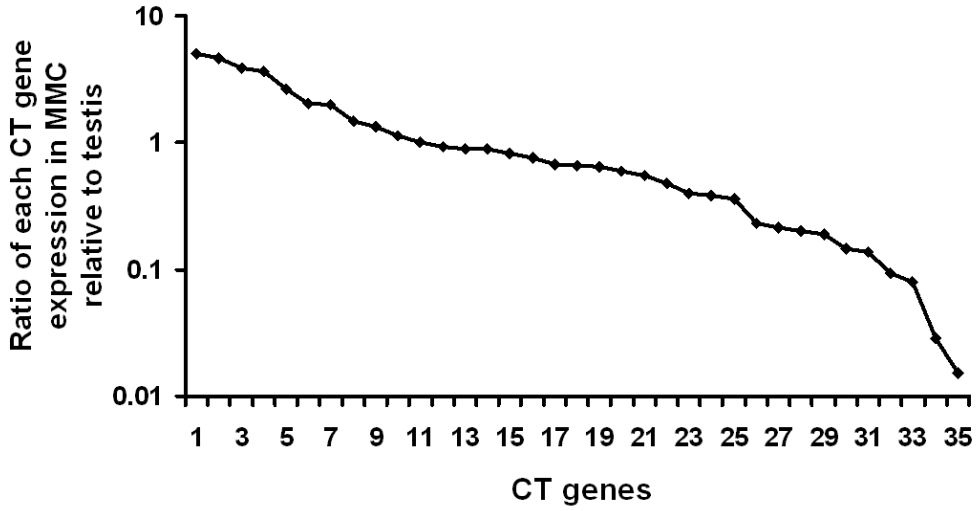

Ratio of each CT gene expression in MMC relative to testis
10
1
0.1
0.01
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35
CT genes

FIGURE 1

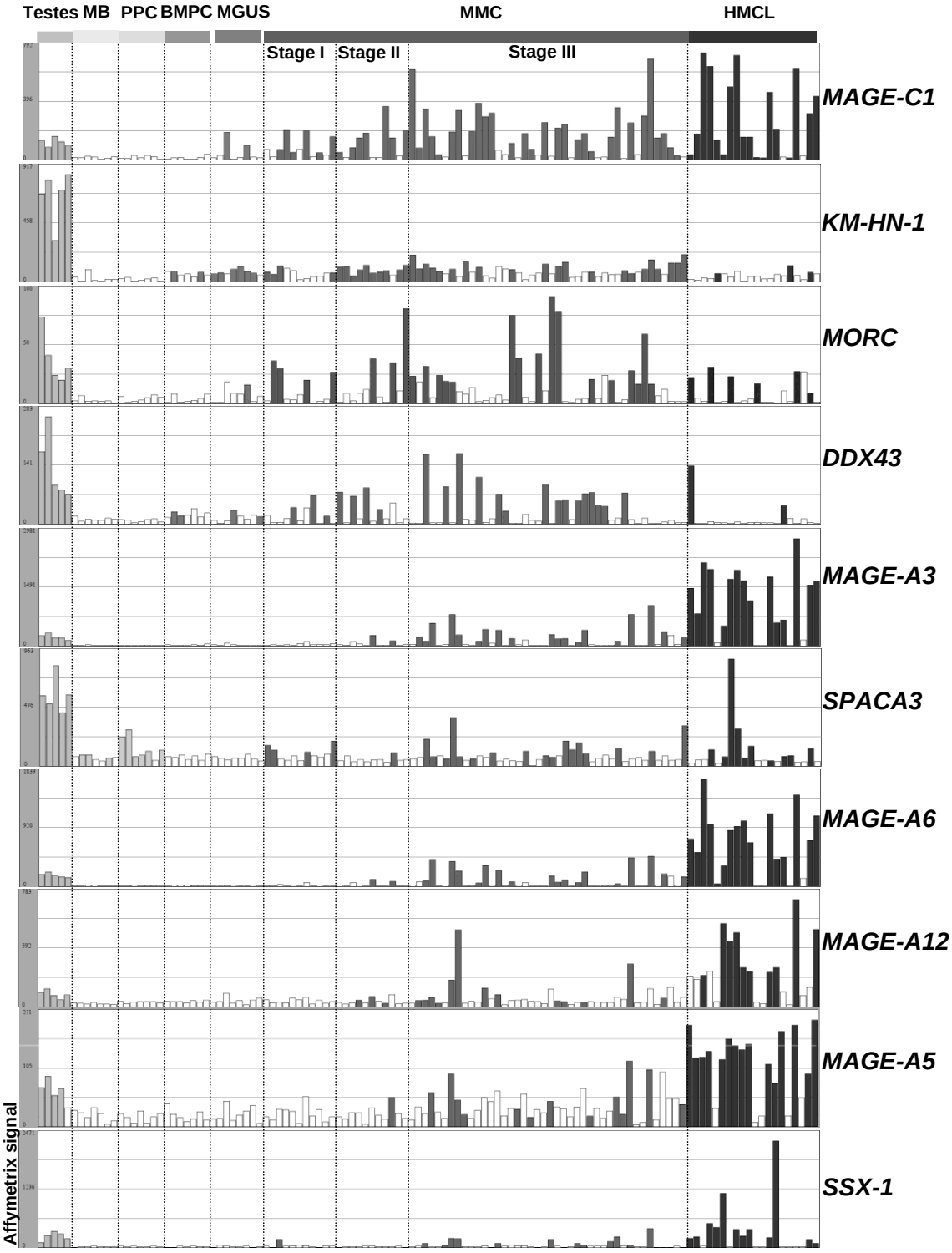

Testes
MB
PPC
BMPC
MGUS
MMC
HMCL
Stage I
Stage II
Stage III
MAGE-C1
KM-HN-1
MORC
DDX43
MAGE-A3
SPACA3
MAGE-A6
MAGE-A12
MAGE-A5
SSX-1
Affymetrix signal

FIGURE 2

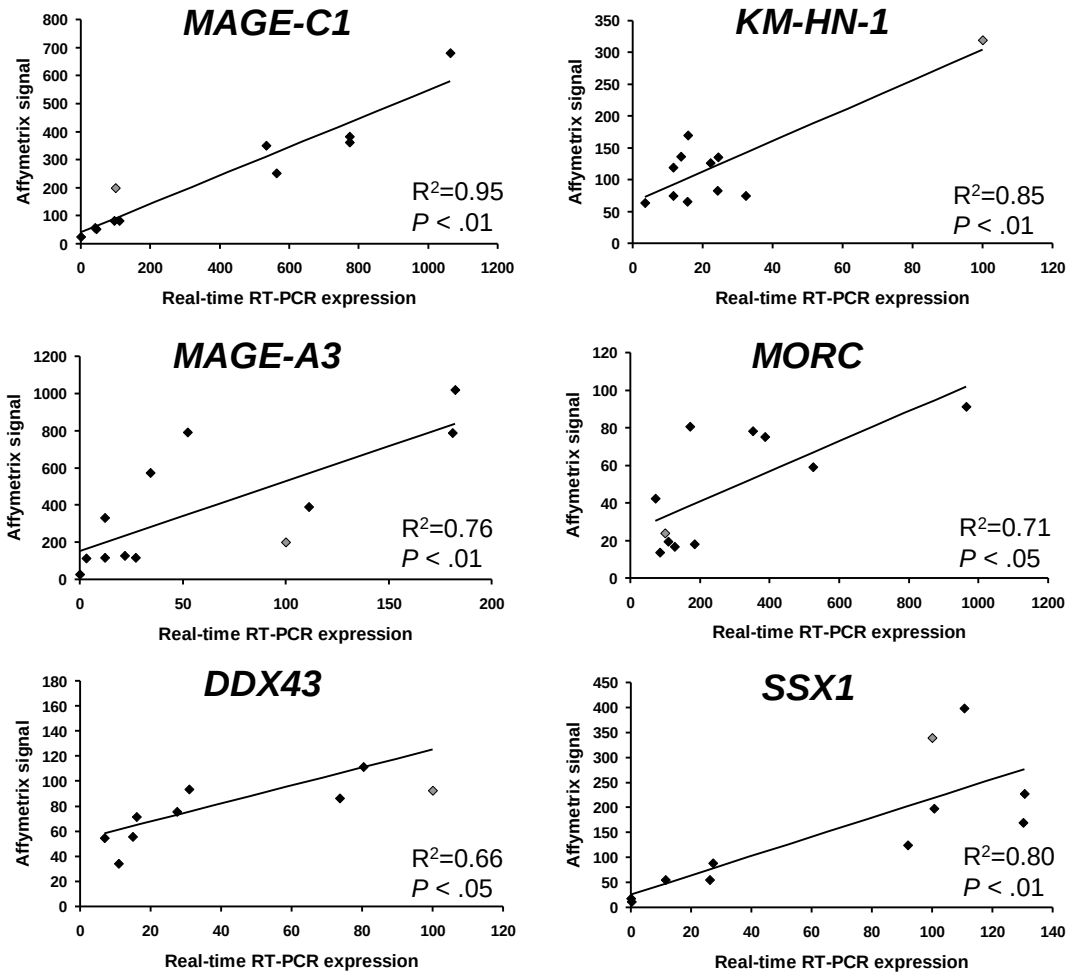
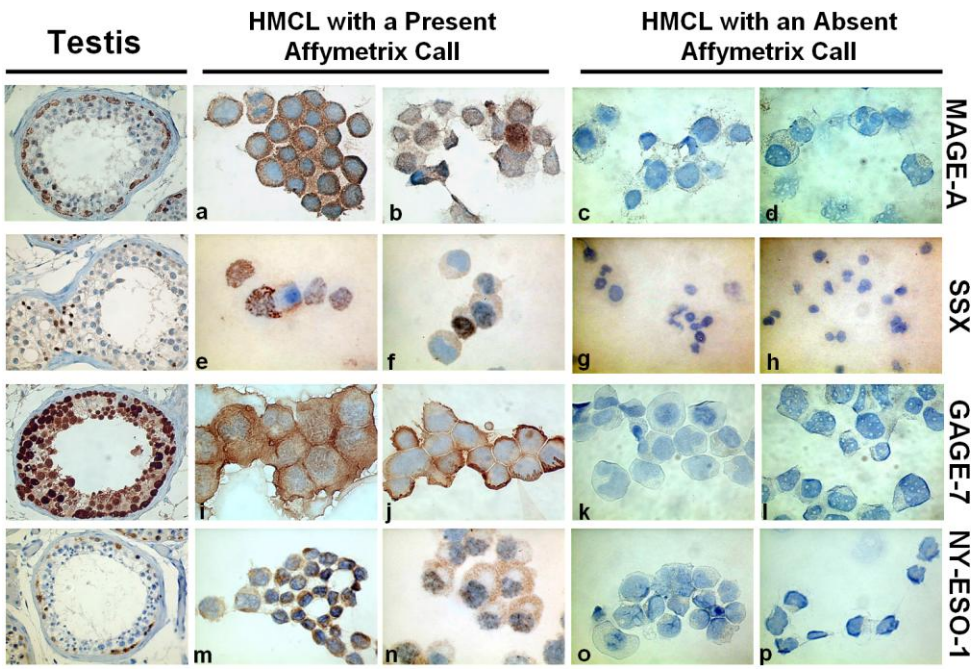

MAGE-C1
Affymetrix signal
Real-time RT-PCR expression
R2=0.95
P < .01
KM-HN-1
Affymetrix signal
Real-time RT-PCR expression
R2=0.85
P < .01
MAGE-A3
Affymetrix signal
Real-time RT-PCR expression
R2=0.76
P < .01
MORC
Affymetrix signal
Real-time RT-PCR expression
R2=0.71
P < .05
DDX43
Affymetrix signal
Real-time RT-PCR expression
R2=0.66
P < .05
SSX1
Affymetrix signal
Real-time RT-PCR expression
R2=0.80
P < .01

FIGURE 3

A


Testis
HMCL with a Present Affymetrix Call
HMCL with an Absent Affymetrix Call
MAGE-A
SSX
GAGE-7
NY-ESO-1
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p

B

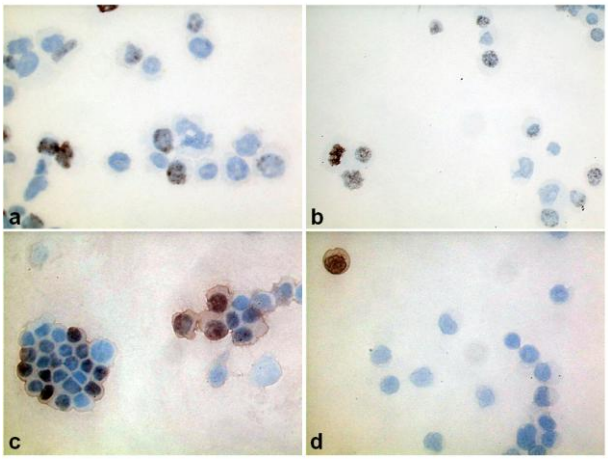
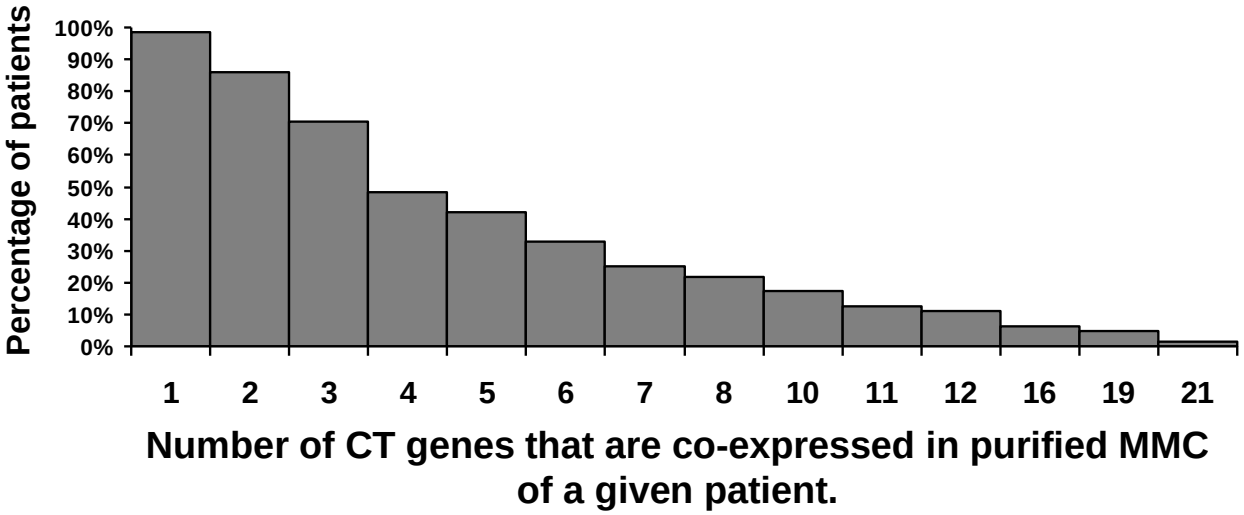

a
b
c
d

FIGURE 4

A



B

(%) of patients	KM-HN-1	MAGEC1	MAGE-A3/6/12	MORC	DDX43	SSX4	SPACA3	MAGEA5	MAGEC2	GAGE1-8
20	■	■	■							
20	■	■		■						
14	■	■			■					
13	■	■				■				
13	■		■	■						
11		■					■			■
11		■	■			■				
9		■		■			■			
9	■		■				■			
8			■	■		■				
8	■			■				■		
5	■				■		■			
5		■					■		■	
3				■	■		■			
3	■				■			■		

FIGURE 5

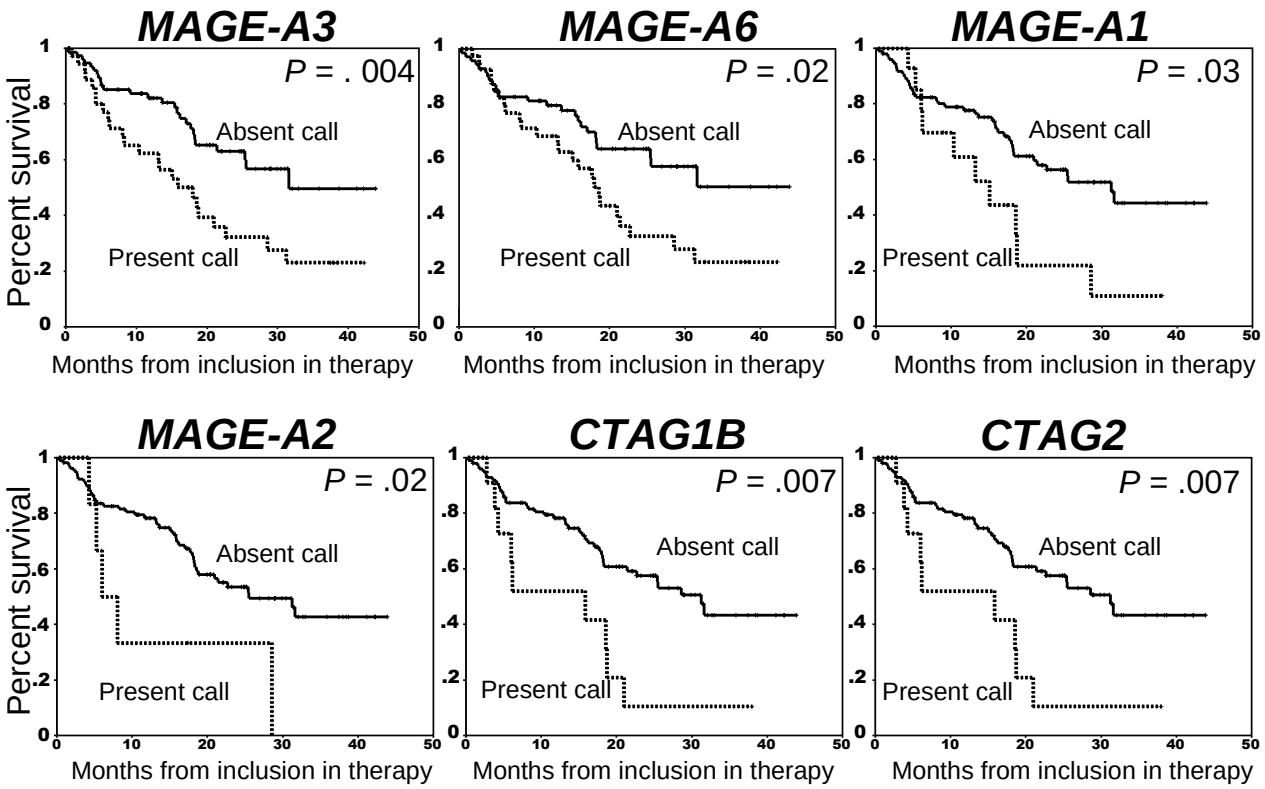
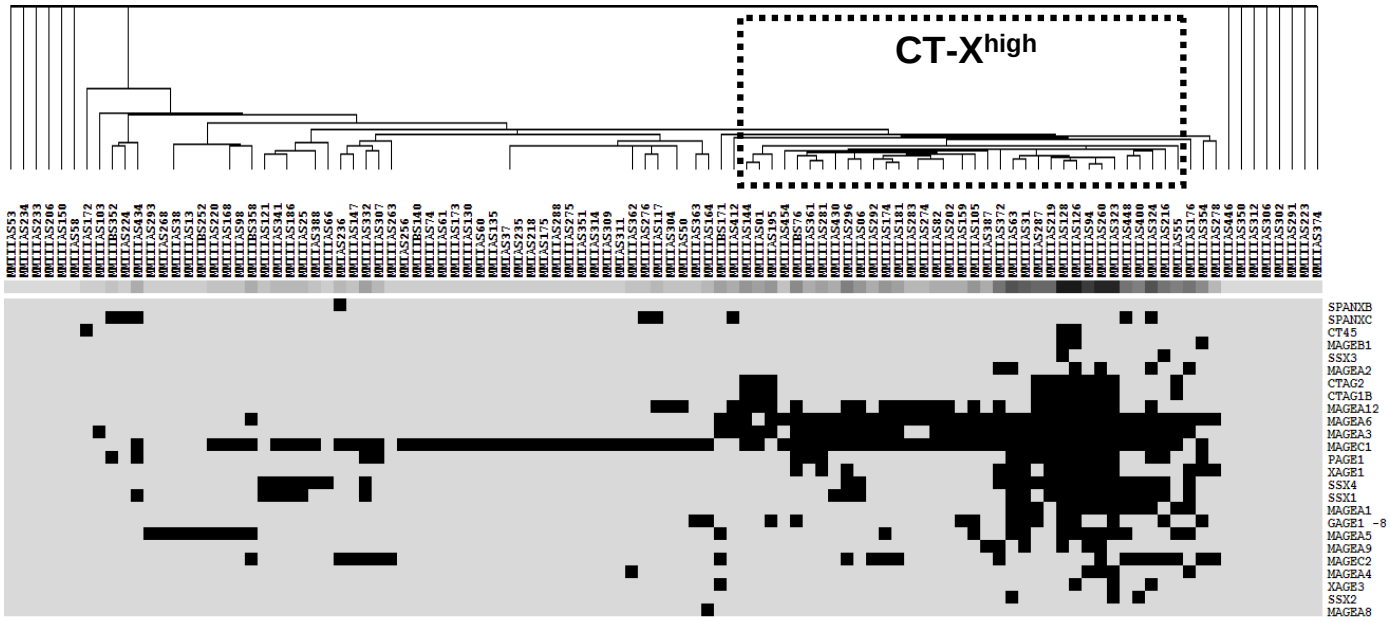

MAGE-A3
P = . 004
Absent call
Present call
Percent survival
Months from inclusion in therapy
MAGE-A6
P = .02
Absent call
Present call
Months from inclusion in therapy
MAGE-A1
P = .03
Absent call
Present call
Months from inclusion in therapy
MAGE-A2
P = .02
Absent call
Present call
Percent survival
Months from inclusion in therapy
CTAG1B
P = .007
Absent call
Present call
Months from inclusion in therapy
CTAG2
P = .007
Absent call
Present call
Months from inclusion in therapy

FIGURE 6

A


CT-X high
SPANXB
SPANXC
CT45
MAGEB1
SSX3
MAGEA2
CTAG2
CTAG1B
MAGEA12
MAGEA6
MAGEA3
MAGEC1
PAGE1
XAGE1
SSX4
SSX1
MAGEA1
GAGE1 -8
MAGEA5
MAGEA9
MAGEC2
MAGEA4
XAGE3
SSX2
MAGEA8

B

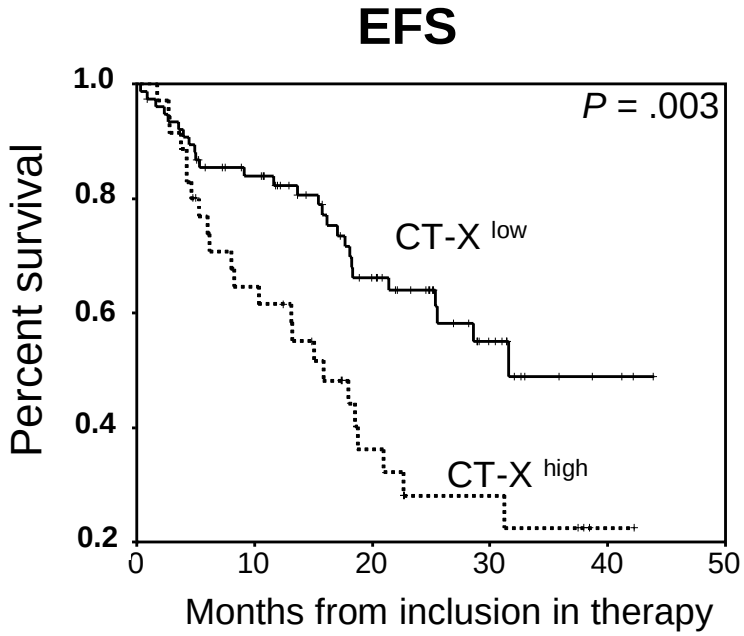

EFS
P = .003
CT-X low
CT-X high
Percent survival
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
Months from inclusion in therapy

FIGURE 7