Cancer testis antigens in newly diagnosed and relapse multiple myeloma: prognostic markers and potential targets for immunotherapy

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ABSTRACT

Background
In multiple myeloma, expression of cancer testis antigens may provide prognostic markers and potential targets for immunotherapy. Expression at relapse has not yet been evaluated for a large panel of cancer testis antigens which can be classified by varying expression in normal tissue: restricted to testis, expressed in testis and brain and not restricted but selectively expressed in testis.

Design and Methods
Evaluation of cancer testis antigen expression was made in newly diagnosed multiple myeloma cases (HOVON-65/GMMG-HD4 trial; n=320) and in relapse cases (APEX, SUMMIT, CREST trials; n=264). Presence of expression using Affymetrix GeneChips was determined for 123 cancer testis antigens. Of these 87 had a frequency of more than 5% in the newly diagnosed and relapsed patients, and were evaluated in detail.

Results
Tissue restriction was known for 58 out of 87 cancer testis antigens. A significantly lower frequency of presence calls in the relapsed compared to newly diagnosed cases was found for 3 out of 13 testis restricted genes, 2 out of 7 testis/brain restricted genes, and 17 out of 38 testis selective genes. MAGEC1, MAGEB2 and SSX1 were the most frequent testis-restricted cancer testis antigens in both data sets. Multivariate analysis demonstrated that presence of MAGEA6 and CDCA1 were clearly associated with shorter progression free survival, and presence of MAGEA9 with shorter overall survival in the set of newly diagnosed cases. In the set of relapse cases, presence of CTAG2 was associated with shorter progression free survival and presence of SSX1 with shorter overall survival.

Conclusions
Relapsed multiple myeloma reveals extensive cancer testis antigen expression. Cancer testis antigens are confirmed as useful prognostic markers in newly diagnosed multiple myeloma patients and in relapsed multiple myeloma patients.

The HOVON-65/GMMG-HD4 trial is registered under Dutch trial register n. NTR-213. CREST, SUMMIT and APEX trials were registered under ns. M34100-024, M34100-025 and NCT00049478/NCT00048230, respectively.

Key words: multiple myeloma, relapse, testis antigen expression, prognosis, immunotherapy.

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The online version of this article has a Supplementary Appendix.

Introduction

Improvements in the treatment of multiple myeloma (MM) have resulted in significantly improved survival. However, this is still limited to 4-5 years.[1-3] For younger MM patients, treatment consisting of high-dose chemotherapy with autologous stem-cell transplant (SCT) is available, often including novel agents both pre-transplant (induction treatment) and post transplant (maintenance treatment)[4]. Residual disease after treatment is an important issue, for which specific therapeutic approaches, such as immunotherapy, may be of value.[5-7] Immunotherapy of cancer types such as melanoma and non-small lung cancer has demonstrated the clinical relevance of this treatment approach.[8] Optimized peptide and DNA vaccination protocols demonstrate ongoing improvements in immunotherapeutic intervention.[9,10] A critical requirement for immunotherapy is that tumor associated antigens (TAAs) are expressed in tumor cells when disease re-emerges after therapy. To this end, we have evaluated the gene expression of an important family of TAAs, the cancer testis antigens (CTAs) in relapse samples, and we have compared this to expression in newly diagnosed MM cases. CTA expression after treatment has been shown for a limited number of CTAs, including PASD1, CTAG1B and MAGEC1/CT7.[11-15] In addition, in MM, expression of CTA genes has been shown to be strongly correlated to clinical outcome, i.e. presence of CTA expression has been linked to shorter survival.[16] Similarly, in other tumor types, CTA expression has been linked to prognosis.[17,18] Prognostic implications of CTAs post therapy have not been systematically evaluated. Three expression patterns have been defined for CTAs: expression restricted to testis, restricted to testis and brain, and expression in other tissues but strong expression in testis (testis-selective)[19]. Evaluation of CTAs in relation to tissue restriction is highly relevant to the likelihood of side-effects of immunotherapy. Here, the expression of 123 CTAs, spanning these categories, was further evaluated in relapse cases and in newly diagnosed MM.[20-22]

Design and Methods

Patients

Bone marrow aspirates of newly diagnosed MM patients included in the HOVON-65/GMMG-HD4 trial were processed as previously described.[22] This trial is a large multicenter, prospective, randomized phase III trial, evaluating bortezomib as front-line treatment (EudraCT n. 2004-000944-26; registered at www.trialregister.nl as NTR213)[20]. Gene expression profiles of the APEX, SUMMIT and CREST trials have been collectively described.[21] These multicenter trials, which were carried out in the US, Canada, Europe and Israel, evaluated bortezomib in relapsed MM. Number of prior therapies in these trials ranged from 1 to 14, with a median of 3. Plasma cells were obtained from bone marrow aspirates as described.[21] Informed consent was obtained for all cases included in this study, in accordance with the Declaration of Helsinki. The approval of local ethics committees was obtained.

RNA isolation and microarray processing

For HOVON-65/GMMG-HD4, RNA from samples with a plasma cell purity of more than 80% was extracted using the RNeasy kit (Qiagen). After a double *in vitro* transcription reaction, biotinylated cRNA was hybridized to Affymetrix GeneChip HG U133 plus 2.0 arrays. A total of 320 cases passed quality controls and were included (GSE19784;[22]). Similarly, amplification, hybridization (Affymetrix HG U133 A/B) and quality control were applied to the APEX/SUMMIT/CREST samples.[21] Based on the quality control Normalized Unscaled Standard Errors (NUSE) measurement obtained using the AffyPLM package (Bioconductor), the replicate chip with the lowest NUSE value was used for the analysis. The APEX/SUMMIT/CREST dataset provided 264 cases with gene expression profiles in which a gene expression based myeloma purity score was used to exclude samples with an apparently low purity (GSE9782).[21]

Preprocessing and gene selection

The raw data files (CEL-files) were analyzed using the mas5 calls algorithm available in the affy package (Bioconductor).[23] This resulted in a presence call for a specific probe set for a specific patient, or an absence call. Here the frequency of presence calls per probe set for both the newly diagnosed and relapse patient sets was reported. The CTA list (n=253) was obtained from the current version of the CT database, a CTA classification website created by the Ludwig Institute for Cancer Research and the Laboratório Nacional de Computação Científica.[8,24] Using Affymetrix annotation in combination with the online gene compendium GeneCards V3, 48 genes did not have corresponding probe sets, with the gene symbol present (n=37) or absent (n=11) in GeneCards (*Online Supplementary Table S1*)[25]. Based on the CT database, 12 genes were excluded from further analysis, either due to being splicing variants of other genes (which are reported here) or due to overlapping and essentially being other genes (LAGE-1b, XAGE-3b, CT16.2, CTAGE-2, MMA1b (splicing variants), GAGE3, CT47B1,SPANXE, BAGE2, BAGE3, BAGE4, BAGE5). This resulted in 193 genes out of 253 genes being represented by probe sets on either the U133A and B chip (n=173) or presented on the U133 Plus 2.0 chip (n=193; 20 present only on U133Plus2.0)[25]. Based on normal testis expression (GSE1133) and the CT database, probe sets/genes not expressed in normal testis were excluded, resulting in the exclusion of 24 genes (U133 A and B) and 22 overlapping genes for the U133Plus 2.0 chip (*Online Supplementary Table S1*; GSE1133). Due to the genetic proximity of a number of genes, 26 genes on U133AB and 29 genes on Plus 2.0 were represented by probe sets corresponding to closely related CTA genes. Examples include the GAGE cluster of genes of which 16 genes are represented by 2 probe sets (*Online Supplementary Table S1*). For genes with more than one probe set, the probe set with the highest percentage of present calls was used. For 5 genes with discrepancies between the two types of chips, i.e. positive difference between probe sets in one platform and a negative difference between probe sets in another platform, arbitrarily the probe set with the highest presence call on the U133AB chip was used (*Online Supplementary Table S2*). Finally, 142 probe sets (171 genes) on U133Plus2.0 and 123 probe sets (149 genes) on U133AB were evaluated. To avoid reporting identical findings (i.e. different genes but same probe sets), the results will be presented per probe set with the most prominent gene given (*Online Supplementary Table S1*). Therefore, the set of 123 probe sets was used to represent 123 genes for comparison in presence frequency between newly diagnosed and relapse cases, and an overlapping set of 142 genes were reported on for the newly diagnosed patients (i.e. 142-123 resulting in 19 genes exclusively reported for newly diagnosed patients). To set a filter for general prevalence of CTAs, a 5% presence call frequency cut off was used for the populations tested. Genes with a presence call frequency of below 5% are reported in the *Online Supplementary Tables*. Eighty-seven CTA genes had a presence call frequency of more than 5% and were discussed in the main body of this report. It should be noted that a presence call for any given

gene using the mas5 algorithm represents a robust level of quantifiable mRNA.

For 94 genes out of 142 genes in newly diagnosed cases determined on the U133Plus2.0 chip, expression in normal tissue has been determined. Twenty-one were classified as testis-restricted, 64 as testis-selective, and 9 as testis/brain restricted. For the 123 genes on the U133AB chip, 17 were testis-restricted, 58 testis-selective, and 9 testis/brain restricted.[19]

Presence of CTA expression in normal plasma cells was evaluated using the GSE6477 data set.[26] Presence calls were determined as described above using the mas5 algorithm. Due to the U133A chip used in this data set, the number of genes evaluated was restricted to 82 (*Online Supplementary Table S6*).

Statistical analysis

Differences in frequency of presence calls were evaluated with Fisher's Exact test with Benjamini-Hochberg correction.[27] Survival analysis was performed in newly diagnosed cases (HOVON-65/GMMG-HD4 trial) as well as in relapse samples, restricted to samples belonging to the APEX trial (n=156). Presence of CTA genes was analyzed in relation to progression free survival and overall survival. In the HOVON-65/GMMG-HD4 trial, to prevent bias caused by patients receiving allogeneic stem cell transplantation, these patients were censored where appropriate. Clinical follow-up data was available for 229 HOVON-65/GMMG-HD4 cases. Kaplan-Meier analysis was applied with the log rank test. To correct for multiple testing, Benjamini-Hochberg correction was performed with a false discovery rate of 5%.[27] Genes significantly associated with progression free survival and overall survival were further analyzed by Cox's regression analysis by backward elimination. For the HOVON-65/GMMG-HD4 study, ISS stage and cytogenetic covariates were included (1q gain, 17p loss and translocations t(4;14), t(11;14) and t(14/20)/t(14;16)). For the APEX study, ISS stage was combined with TC classification which was used as a substitute for cytogenetic covariates. TSPY1 was excluded for survival analysis, since it is only expressed in males. To avoid analyzing too many small groups, genes with an overall presence frequency of more than 5% and less than 95% were evaluated by survival analysis (n=84, HOVON-65/GMMG-HD4; n=61, APEX study; *Online Supplementary Table S1*).

Correlation analysis

To evaluate the correlation between CTAs, cluster analysis was performed using 57 genes with known tissue restriction, presence frequency of more than 5% (Table 1A and B; *Online Supplementary Table S1*), and excluding TSPY1 (located on Y). Cluster 3.0 and TreeView software by Eisen *et al.* was used; this is available through the BRB-analysis tool.[28] After median centering of the genes, the clustering was performed using the uncentered Pearson's correlation combined with the complete linkage option. Clusters were characterized by determining the differential expression of genes within a specific cluster *versus* all other clusters using the ClassComparison tool ($P<1*10^{-7}$).

Results

Expression of CTAs in MM patients

Expression of CTA genes was evaluated in MM at diagnosis (n=320) and at relapse (n=264). Newly diagnosed cases were taken from the HOVON-65/GMMG-HD4 trial and relapse cases were taken from the combined APEX/SUMMIT/CREST trials. Patients' characteristics are provided in the *Online Supplementary Table S3*. A significant difference in age distribution was found as a result of the HOVON-65/GMMG-HD4 inclusion criteria which stipulate participation only of patients under the age of 65 years, whereas there was no age restriction for the APEX/SUMMIT/CREST trials. In addition, and in agreement with a more advanced disease state, thrombocytopenia was more frequent in relapse cases compared to newly diagnosed cases.

Based on the CT database (see *Design and Methods* for details), 123 CTA genes were available for evaluation in both newly diagnosed and relapse patients. For 87 of these 123 CTAs, a frequency of more than 5% was found in one of the study populations (Table 1, *Online Supplementary Table S4*). The genes with low presence frequency are presented in the *Online Supplementary Table S5*.

For 58 of the 87 genes, the tissue expression restriction in normal tissue has been evaluated previously.[19] The expression categories, i.e. restricted to testis (TR), restricted to testis and brain (TBR), and testis selective (TS) are given in Table 1 and in the *Online Supplementary Tables S1, S4 and S5*. MAGEC1, MAGEB2 and SSX1 were the most frequent TR CTAs in both data sets; present in 71%, 47% and 30% of newly diagnosed patients, respectively, and present in 61%, 28% and 30% in relapsed patients, respectively. At least one of these 3 genes was found in 266 out of 320 (83%) newly diagnosed cases, compared to 188 out of 264 (71%) relapse cases (*Online Supplementary Figure S1*). One or more of the top 3 TBR genes are found in almost all cases both in newly diagnosed cases (98%) and in relapse cases (99%) with FAM133A present in 86% and 79% of newly diagnosed and relapse cases, respectively (Table 1A). A vaccine strategy simultaneously targeting these 3 top TBR genes would be expected to be of relevance to almost all relapse MM cases. Strikingly, TS genes SPAG9, CASC5 and PBK were expressed in more than 85% of relapse cases (99.6%, 89.4% and 86.4%, respectively).

Three CTA expression categories were apparent: increased, decreased or similar frequency of expression in the relapse cohort as compared to the newly diagnosed cohort. A significantly higher frequency of presence calls in relapse cases compared to newly diagnosed cases was found for none of the TR genes, 3 out of 7 TBR genes, and 10 out of 38 TS genes (Table 1, *Online Supplementary Table S4*). The most pronounced increase in the TBR genes was noted for GAGE4 and GAGE8, with a 4-fold increase from 16.6% and 15%, respectively, in newly diagnosed MM cases to 71.2% and 61.4%, respectively, in relapse MM cases. Assessing the frequency of high expression, within the cases with a present call, GAGE4 and GAGE8 both demonstrated a much lower proportion of cases with high expression in relapse cases compared to newly diagnosed cases. For TR gene TEX14, a decreased frequency of expression is coupled to a higher frequency of high expression cases in relapse compared to newly diagnosed cases (*Online Supplementary Table S6*).

A decrease in CTA expression in relapse cases is of particular interest for immunotherapy, as decreased expression may prohibit the use of specific CTAs as vaccine targets. A significantly lower frequency of presence calls in relapse cases compared to newly diagnosed cases was found for 3 out of 13 TR genes, 2 out of 7 TBR genes, and 17 out of 38 TS genes (Table 1). For instance, TR gene MAGEB2 was found in 47% of newly diagnosed cases and in 27% of relapse cases. It is important to stress here that 75% (15 out of 20) of the most important putative

immunotherapeutic targets, i.e. TR/TBR genes, demonstrate unchanged or higher expression frequencies in relapse cases compared to newly diagnosed cases. A lower proportion, but still 55% (21 out of 38) of TS genes, are found in equal or increased frequency in relapse cases compared to newly diagnosed cases.

For the genes without known normal tissue restriction, the majority (20 out of 29) demonstrate a significantly reduced frequency in relapse cases whereas only 2 genes are significantly higher in relapse cases compared to newly diagnosed MM cases (*Online Supplementary Table S4*). SPAG4, TCC52 and RQCD1 expression is found in over 95% of both newly diagnosed and relapse cases.

Overall, 58 genes with known tissue restriction and 29 genes without known tissue restriction were evaluated in both newly diagnosed and relapse MM cases, and 45 out of 87 genes were found to remain at a comparable level or demonstrate increased presence frequency (52%). Finally, a subset of genes was not represented by the probe sets available on the U133AB platform used for relapse cases and were, therefore, only assessed in newly diagnosed cases. These genes are listed in the *Online Supplementary Table S4*. CPXCR1 (TR; 52%) and CCDC36 (TBR; 56%) are the most frequently found genes in this category in the newly diagnosed cases.

Presence of expression in normal plasma cells was analyzed (GSE6477).[26] Due to restrictions of this data set, 82 genes were evaluated here (*Online Supplementary Table S7*). A number of genes, such as SPAG4 and SPAG9, were present in all 15 normal plasma cell samples, whereas the majority (70%) were not present in any of the cases. Out of 12 testis-restricted genes shown in Table 1, MAGEB2 showed presence in 4 out of 15 normal cases and MAGEC1 and SPANXC were present in one out of 15 cases. The remaining 9 evaluated genes were not present in any of the 15 normal plasma cell cases analyzed (*Online Supplementary Table S7*).

Prognostic impact of CTA genes

CTA expression was analyzed in relation to progression free survival (PFS) and overall survival (OS) (see *Design and Methods* section). Univariate Kaplan-Meier analysis, evaluated by log rank testing, generated a set of CTA genes with prognostic value for PFS and OS for newly diagnosed cases and for relapse cases (*Online Supplementary Table S8*). Based on this analysis, both in newly diagnosed and in relapse patients, SSX1 was found to be prognostic for both PFS and OS, suggesting a universal value for this marker. Multivariate analysis indicated MAGEA6 and CDCA1 to be prognostic factors for PFS in the newly diagnosed cases, independent of cytogenetic factors and ISS. Similarly, MAGEA9 constituted an independent prognostic factor in

Table 1. Presence of CTA gene expression in newly diagnosed and relapsed MM patients. All testis-restricted, testis/brain-restricted and top 5 of testis-selective CTAs with expression presence of more than 5% in either newly diagnosed or relapse MM population are shown here. The remaining genes are given in Online Supplementary Tables S4 (>5% presence frequency) and S5 (<5% presence frequency).

Gene symbol	Probe set	Tissue restriction	Newly diagnosed		Relapse		*P* value
			n	%	n	%	Bold, significant
MAGEC1	206609_at	testis-restricted	228	71.3	160	60.6	**0.008**
MAGEB2	206218_at	testis-restricted	151	47.2	73	27.7	**<0.0001**
SSX1	206626_x_at	testis-restricted	97	30.3	78	29.5	0.9
MAGEA1	207325_x_at	testis-restricted	70	21.9	42	15.9	0.07
TSPY1	207918_s_at	testis-restricted	34	10.6	36	13.6	0.3
MAGEA2	214603_at	testis-restricted	30	9.4	22	8.3	0.8
TEX14	221035_s_at	testis-restricted	23	7.2	8	3	0.03
SSX2	210497_x_at	testis-restricted	21	6.6	17	6.4	1
PAGE2	231307_at	testis-restricted	19	5.9	6	2.3	0.04
MAGEB1	207534_at	testis-restricted	17	5.3	10	3.8	0.4
MAGEB4	207580_at	testis-restricted	17	5.3	3	1.1	**0.01**
SPANXC	220217_x_at	testis-restricted	16	5	8	3	0.3
SSX3	211670_x_at	testis-restricted	9	2.8	15	5.7	0.1
FAM133A	239481_at	testis/brain-restricted	276	86.3	209	79.2	0.03
CTNNA2	205373_at	testis/brain-restricted	194	60.6	70	26.5	**<0.0001**
CTAGE1	220957_at	testis/brain-restricted	180	56.3	242	91.7	**<0.0001**
MAGEC2	220062_s_at	testis/brain-restricted	93	29.1	25	9.5	**<0.0001**
GAGE4	208155_x_at	testis/brain-restricted	53	16.6	188	71.2	**<0.0001**
GAGE8	207086_x_at	testis/brain-restricted	48	15	162	61.4	**<0.0001**
MAGEA9	210437_at	testis/brain-restricted	35	10.9	15	5.7	0.03
SPAG9	212470_at	testis-selective	320	100	263	99.6	0.5
CTAGE5	215930_s_at	testis-selective	306	95.6	128	48.5	**<0.0001**
PBK	219148_at	testis-selective	301	94.1	228	86.4	**0.002**
ZNF165	206683_at	testis-selective	266	83.1	36	13.6	**<0.0001**
JARID1B	211202_s_at	testis-selective	264	82.5	89	33.7	**<0.0001**

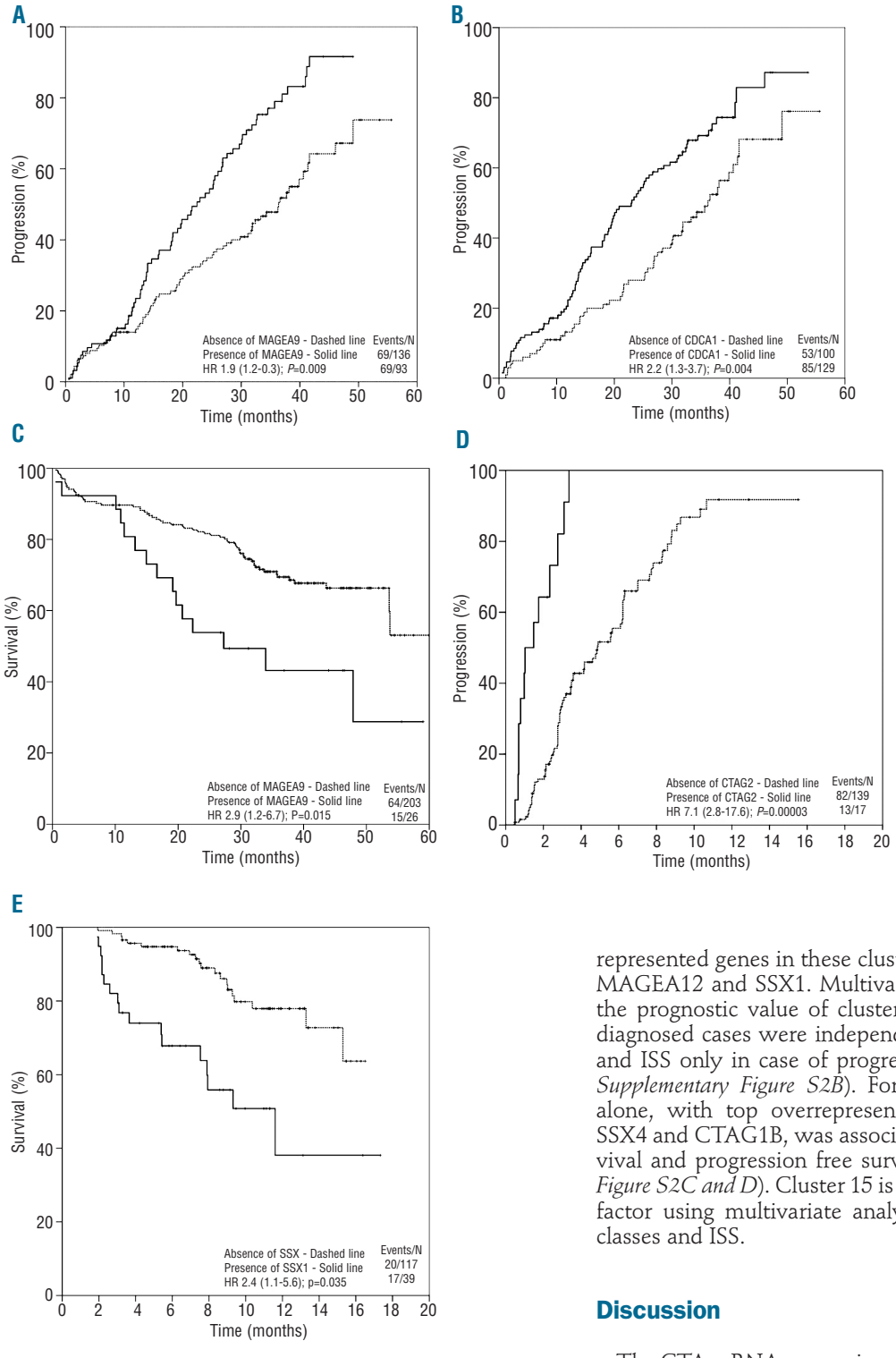

Figure 1. Survival analysis according to CTA gene expression status in the HOVON-65/GMMG-HD4 trial and in the APEX trial. Presence and absence of gene expression are indicated by solid lines and dashed lines, respectively. In HOVON-65/GMMG-HD4 presence of MAGEA6 (A) and CDCA1 (B) are prognostic for a significantly shorter PFS. Presence of CTA MAGEA9 (C) is prognostic for a significantly worse survival (overall survival, OS) in the newly diagnosed HOVON-65/GMMG-HD4 patients. In the APEX trial of relapse patients, presence of CTAG2 is prognostic for shorter PFS (D) and SSX1 for shorter OS (E). The prognostic value of all five CTAs shown is independent of ISS and cytogenetic covariates/TC class (see text). Hazard ratios (HR), 95% confidence intervals and *P* values, adjusted for ISS, are shown.

terms of overall survival (Figure 1). In the relapse cases, CTAG2 (PFS) and SSX1 (OS) were found to be independent of the TC classification, used as a substitute marker for cytogenetic markers, and ISS (Figure 1). Correlation between different CTA genes was evaluated using cluster analysis of 57 CTA genes (i.e. >5%, known tissue restriction and without TSPY1). This analysis resulted in 15 clusters (*Online Supplementary Figure S2A*). Cases belonging to clusters 5, 12 and 15 had a shorter overall survival and progression free survival in newly diagnosed cases. Top over-represented genes in these clusters combined are XAGE1, MAGEA12 and SSX1. Multivariate analysis showed that the prognostic value of clusters 5, 12 and 15 for newly diagnosed cases were independent of cytogenetic factors and ISS only in case of progression free survival (*Online Supplementary Figure S2B*). For relapse cases, cluster 15 alone, with top overrepresented genes being CTAG2, SSX4 and CTAG1B, was associated with poor overall survival and progression free survival (*Online Supplementary Figure S2C and D*). Cluster 15 is an independent prognostic factor using multivariate analysis incorporating the TC classes and ISS.

Discussion

The CTA mRNA expression profile in relapse MM cases was determined and compared to the profile in newly diagnosed cases. Forty-five out of 87 CTAs demonstrated either increased or equal expression in the relapse cohort. Although 2 out of 3 of the top CTA genes with testis restricted expression demonstrate significantly reduced frequency of expression in relapse cases, the proportion of samples expressing one of the top 3 genes in relapse cases is still 71%, which compares favorably to the 83% in newly diagnosed cases. In a study by Atanackovic *et al.*,[13] the frequency of MAGEC1, MAGEA3, MAGEC2 and SSX2 expression was determined in a set of myeloma

cases: 65%, 52%, 43% and 12%, respectively. The expression presence in our study was lower for most of these genes; 71%, 38%, 29% and 7%, respectively. However, as indicated below, our results corresponded well to a previous report on CTA expression as determined using Affymetrix GeneChips in newly diagnosed cases only.[16] The difference between studies may be attributed to differences in the techniques used. CTA expression in myeloma has been evaluated in large patient sets but emphasis has so far been on newly diagnosed patients.[13,16,29,30] Compared to the study of Condomines *et al.*,[16] presence of gene expression frequency differed no more than 2-fold in 73% of overlapping genes when compared to the frequencies found in newly diagnosed patients in our study. Of these, 12 are highly correlated with a difference in frequency of no more than 1.5-fold, including MAGEC1, MAGEA5 and SSX3; 61%, 26% and 3% in our study and 66%, 22% and 3% in the study of Condomines *et al.*[16] In some cases, different probe sets belonging to the same gene may explain the difference in expression frequencies between the two studies.

Comparison of 13 CTAs in treated and untreated MM has previously been reported for a small study group.[14] In that study, CTA expression was evaluated by RT-PCR on non-purified samples[14] whereas we have reported on GeneChip data on purified plasma cells. Despite these clear differences, some similarities in CTA expression pattern were found. Overall, the expression frequency determined in our study on purified plasma cells was higher (1.2 - 1.3-fold) but the change between newly diagnosed and previously treated cases was comparable in direction in 8 out of 10 genes.

In this study, the testis-restricted antigen MAGEC1 was confirmed as an important antigen in MM. Presence of MAGEC1, also referred to as CT7, is high post therapy, and was observed in 61% of relapse cases. Expression of MAGEC1 by Q-PCR has also been reported to correlate with disease burden following therapy.[13] A small series of 10 cytospins derived from purified CD138-positive tumor cells was stained for MAGEC1 by immunohistochemistry (IHC) (*Online Supplementary Figure S3*); a clear correlation was observed for gene presence call and protein expression. In fact, MAGEC1 presence calls were found only in cases with more than 50% of the tumor cells positive for MAGEC1 by IHC. The heterogeneity of MAGEC1 expression in MM, i.e. level of MAGEC1 positive cells by IHC, has recently been reported to be correlated to survival and proliferation.[15] A linked mRNA and protein expression has now been reported for multiple CTAs where suitable antibodies for specific CTA detection are available.[12,31,32] Protein expression is naturally a requirement for immunotherapy for epitopes to be presented for CTL recognition and further studies need to assess this aspect in detail for the targeted CTAs.

SSX1, another notable testis restricted gene, was found to be expressed in equal frequencies in relapse cases compared to newly diagnosed cases. When assessed in newly diagnosed MM, co-expression of SSX1,2,4,5 was found to predict reduced survival of which SSX2 alone has been reported to yield the strongest association with reduced survival.[33] Here we find SSX1 to be the sole CTA which by univariate log rank analysis was found to be correlated to shorter overall survival and progression free survival in both independent cohorts, i.e. newly diagnosed (HOVON-65/GMMG-HD4 trial) and relapse (APEX trial) MM.

For immunotherapy, genes with the most restricted expression pattern in normal tissue, i.e. the TR category, represent the most suitable targets.[8] Very limited expression in normal plasma cell samples was found for TR genes in this study, with presence of MAGEB2 expression in 4 out of 15 samples representing the highest value. Presence of expression in normal plasma cells indicates that a gene does not comply fully with the previously reported tissue restriction pattern, in this case TR. For CTAGE1, in the TBR category, presence of expression was found in almost all normal plasma cell samples, and its status as TBR gene may, therefore, be questioned. In addition, CTA genes with almost universal expression in MM, such as SPAG9, also demonstrated very high frequency of expression in normal plasma cells. SPAG9 is present in all but one sample in this study, and has been described to mediate JNK signaling.[34-35] For immunotherapy, expression in normal tissues raises concerns of generating autoimmune responses following vaccination. Still, some TS antigens, like MAGEA3, are targeted in current immunotherapeutic protocols.[36-37] It is of interest that not all TS antigens demonstrated expression in normal plasma cell samples.

In total, 15 out of 87 CTA genes demonstrate a reduction of 30% or more in the relapse set compared to the newly diagnosed cases. The majority of these genes are found either in the set of TS genes (7 out of 38) or in the set without known tissue restriction (7 out of 29). The most pronounced differences were found in ZNF165 (70% reduction; TS) and TMEFF1 (57% reduction; no tissue restriction known) which emphasizes the importance of monitoring the presence of antigens post therapy.

Clearly, our study does not allow for analysis of the patterns of CTA expression in the same patient longitudinally. However, our data indicate that a large number of CTAs, but importantly not all, are prevalent in relapse MM patients and offer potentially suitable targets for immunotherapy. Atanackovic *et al.* reported on the longitudinal analysis of 4 CTA genes in 17 patients during treatment, and in most cases the expression of these genes persisted from initial presentation to relapse.[13] Only MAGEC2 demonstrated a clear reduction in terms of fold-change in our study and is also the gene which was most frequently decreased in the study of Atanackovic *et al.*[13]

The relationship between CTA expression and its prognostic value is underlined by the expression of SSX1 mentioned above. Moreover, multivariate analysis identifies SSX1 as an independent prognostic factor associated with poor overall survival in relapse cases, with CTAG2 prognostic for progression free survival in this set. For newly diagnosed cases, MAGEA6 and CDCA1 were independent prognostic factors for progression free survival, and MAGEA9 is an independent risk factor in terms of overall survival. CDCA1 is a cell division protein and forms a part of protein complex associated with the centromere. The prognostic impact of this gene correlates well with the known poor prognosis of MM with high proliferation.[38] Previous studies confirm the presence call of CTAG2 and MAGEA6 to be of prognostic importance in terms of event free survival alongside the presence call of MAGEA3, MAGEA1, MAGEA2 and CTAG1B.[16] Others have demonstrated prognostic impact of MAGEC1, SSX2 and CT45.[13,16,34,39-40] A recently published signature for high-risk disease contains the genes GAGE1 and GAGE12 which further confirms the relationship between CTA genes and prognosis.[41]

Cluster analysis further confirms the correlation between different CTAs and especially those located on chromosome X. In cluster 15, part of the prognostically important set of clusters in the newly diagnosed MM cases and the most important cluster in relapse cases, the top 10 overrepresented genes are all derived from chromosome X.

In our classification of MM, we have described a new cluster in MM: the CTA group. This cluster demonstrated expression of CTA genes but without concomitant expression of proliferation genes, setting it apart from the proliferation cluster originally identified by Zhan *et al*.[22,42] Future studies will expand on the prognostic value and underlying biology of this classification. It is also important to consider CTA expression in relation to treatment using demethylating agents.[43] Despite the clear advantage of potentially derepressing genes such as p53, the effects on inducing CTA expression must be taken into account. Also drug regimens currently in use in MM may have an effect on methylation status. Indeed, bortezomib treatment has been demonstrated to have effects on gene demethylation.[44-45] In our study, however, gene expression analysis in both newly diagnosed and in relapse patients was performed prior to bortezomib treatment.

Although the function of CTA genes is generally not extensively characterized, repression of MAGEC1 and MAGEA3 has been shown to result in increased apoptosis in myeloma cell lines.[46] A functional role for CTAs in MM ties in with the finding of CTA expression post relapse in this study. Finally, CTA expression has been reported in cancer stem cells, among others in melanoma and glioma.[47,48] In MM, a putative cancer stem cell has been reported, and may be CD19+CD138-ve.[49,51] The putative importance of CTA genes in these cells is subject to future investigation. In conclusion, evaluating a large panel of CTA genes suggests that many of these antigens are relevant to tumor cells at relapse, offer putative immunotherapeutic targets, and have value as prognostic markers. Future studies are aimed at validating CTA genes as risk factors in MM progression, as well as analyzing optimal targets for immunotherapy.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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