Longitudinal Analysis and Prognostic Effect of Cancer-Testis Antigen Expression in Multiple Myeloma

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Abstract

Purpose: Reliable data on the persistence of tumor expression of cancer-testis (CT) antigens over time and consequent analyses of the effect of CT antigen expression on the clinical course of malignancies are crucial for their evaluation as diagnostic markers and immunotherapeutic targets.

Experimental Design: Applying conventional reverse transcription-PCR, real-time PCR, and Western blot, we did the first longitudinal study of CT antigen expression in multiple myeloma analyzing 330 bone marrow samples from 129 patients for the expression of four CT antigens (*MAGE-C1*/*CT7, MAGE-C2*/*CT10, MAGE-A3,* and *SSX-2*).

Results: CT antigens were frequently and surprisingly persistently expressed, indicating that down-regulation of these immunogenic targets does not represent a common tumor escape mechanism in myeloma. We observed strong correlations of CT antigen expression levels with the clinical course of myeloma patients as indicated by the number of bone marrow – residing plasma cells and peripheral paraprotein levels, suggesting a role for CT antigens as independent tumor markers. Investigating the prognostic value of CT antigen expression in myeloma patients after allogeneic stem cell transplantation, we found that expression of genes, such as *MAGE-C1*, represents an important indicator of early relapse and dramatically reduced survival.

Conclusions: Our findings suggest that CT antigens might promote the progression of multiple myeloma and especially *MAGE-C1*/*CT7*, which seems to play the role of a "gatekeeper" gene for other CT antigens, might characterize a more malignant phenotype. Importantly, our study also strongly supports the usefulness of CT antigens as diagnostic and prognostic markers as well as therapeutic targets in myeloma.

Cancer-testis (CT) antigens are a diverse group of genes of which more than 40 families have been identified during the past 15 years (1). CT antigens have been considered promising targets for immunotherapy of human malignancies based on their tumor-restricted expression and on their immunogenicity in cancer patients. Both of these characteristics could render CT antigens important diagnostic and prognostic markers; however, thus far, this aspect of the biology of CT antigens has not intensively been explored.

Although an impressive number of studies have shown expression of CT antigens in a large variety of human tumor types on the RNA as well as on the protein level (2), there has not been a single study analyzing the expression of CT antigens in a human cancer over time. This seems surprising because reliable data on the persistence of tumor-related CT antigen expression are a prerequisite for the evaluation of these tumor-specific proteins as diagnostic markers and immunotherapeutic targets, especially considering data suggesting that immunoselection might lead to down-regulation or loss of CT antigen expression in cancer patients (3, 4).

We have recently shown that CT antigens are commonly expressed and are capable of inducing antibody-mediated and T-cell – mediated immunity in multiple myeloma (MM) patients (5). This finding may be of clinical relevance because MM has been considered a disease that is, at least to a certain extent, controlled by the adaptive immune system. The latter view is supported by the fact that the therapeutic effect of allogeneic stem cell transplantation (alloSCT) is partly mediated by immune effects exerted by donor-derived T cells and that donor T cells infused into MM patients are capable of inducing remission in case of relapse (6, 7). Importantly, our finding that immune responses against CT antigens are induced by alloSCT (5) suggests that this class of tumor antigens might indeed represent natural targets for donor-derived alloimmune or even spontaneous antimyeloma immune responses.

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Translational Relevance

Cancer-testis (CT) antigens have been proposed as immunotherapeutic targets and diagnostic markers in patients with multiple myeloma (MM) for several years. Surprisingly, despite the recent emergence of clinical trials involving this attractive group of tumor antigens, almost no data on the persistence of their expression and correlation with the clinical course are available. In this study, we investigated a collective of 129 patients with MM over the course of their disease, repeatedly analyzing the expression of four of the most promising CT antigens. We show for the first time that in these frequently expressed antigens, down-regulation is not a common tumor escape mechanism. This finding confirms their usefulness in a therapeutic setting in patients with MM. Additionally, our observation of a dramatically shortened time to relapse in patients after allogeneic stem cell transplantation who expressed *MAGE-C1/CT7* as well as the close relationship of CT antigen expression with conventional clinicopathologic parameters in MM supports their applicability as prognostic and diagnostic markers.

High-dose chemotherapy alone supported by autologous stem cell transplantation (autoSCT) has been the standard treatment of MM patients up to the age of 65 years (8); however, minimal residual disease persists in the majority of patients and will eventually lead to a fatal relapse (9, 10). CT antigens, which are specifically expressed by malignant plasma cells, represent potential markers for minimal residual disease and could also be used to target myeloma cells remaining in the patients' bone marrow (BM) after standard therapy. Undoubtedly, the usefulness of CT antigens as targets for the antigen-specific immunotherapy of MM would further be strengthened if an immediate effect of CT antigen expression on the occurrence of relapse or progression of the disease could be shown.

In our current study, we did the first longitudinal analysis of CT antigen expression in human cancer. To this end, we repeatedly analyzed expression of four CT antigens (*MAGE-C1/CT7*, *MAGE-C2/CT10*, *MAGE-A3*, and *SSX-2*) in 330 BM samples from 129 MM patients, correlating the resulting data with the clinical course of the disease. Findings derived from our study strongly support a role for CT antigens as diagnostic and prognostic markers as well as therapeutic targets in MM.

Materials and Methods

Patients and healthy stem cell donors. A total of 129 consecutive consenting MM patients (11) and 40 healthy stem cell donors were included in the study. All patients were admitted for treatment or diagnostic purposes to the University Medical Center Hamburg-Eppendorf. The study protocol had received approval by the local ethics committee (OB-038/06).

BM samples and myeloma cell lines. Three hundred thirty BM samples from MM patients were obtained during routine diagnostic procedures done from January 2004 until March 2007. From 61 patients, multiple samples were available (median, 4; range, 2-10 samples) with an average time between the first and the last sample of 14.6 mo (range, 1-35 mo). Samples were acquired at different times during follow-up, with a median time after therapy of 24 mo. Whole BM samples obtained from consented healthy donors were part of BM donations for alloSCT or were collected from blood donors, respectively. Mononuclear cells were isolated by density gradient centrifugation and were washed twice with PBS. Mononuclear cells and cells from myeloma cell line U266 were lysed using RLT Buffer (Qiagen) or protein lysis buffer containing Protease Inhibitor Cocktail (Sigma-Aldrich) and were stored at -80°C until needed.

Reverse transcription-PCR and real-time PCR. PCR experiments were done according to previously published protocols (5, 12). Reaction mixtures for reverse transcription-PCR (RT-PCR) included transcript-specific oligonucleotides for *MAGE-C1/CT7* (forward, 5′-ACATCCTCACCCTCAGGAGGG-3′; reverse, 5′-GACGAGGATCGTCTCAGGTCAGC-3′), *MAGE-C2/CT10* (forward, 5′-CGGATCGAAGGCATTTGTGAG-3′; reverse, 5′-GTGAACTCACGGGCTCTCTTGAG-3′), *MAGE-A3* (forward, 5′-GAAGCCGGCCCAGGCTCG-3′; reverse, 5′-GGAGTCCTCATAGGATTGGCT-3′), *SSX-2* (forward, 5′-GTGCTCAAATACCAGAGAAGATC-3′; reverse, 5′-TTTTGGGTCCAGATCTCTCGTG-3′), or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH;* forward, 5′-TGATGACATCAAGAAGGTGG-3′; reverse, 5′-TTTCTTACTCCTTGGAGGCC-3′). Real-time PCR was done with transcript-specific oligonucleotides for *GAPDH* (5′-TGATGACATCAAGAAGGTGG-3′; 5′-TTTCTTACTCCTTGGAGGCC-3′) and commercially designed primers for *MAGE-C1/CT7* (Qiagen) using target-specific programs for *MAGE-C1/CT7* (95°C for 10 s, 60°C for 30 s, 72°C for 20 s) and *GAPDH* (95°C for 15 s, 61°C for 5 s, 72°C for 26 s). To assess primer specificity, PCR products were analyzed repeatedly by sequence analysis.

Western blot. Lysates prepared from total BM or from myeloma cell line U266, which was used as a positive control, were denaturated for 10 min at 70°C. Samples of lysates containing 30 μg total protein were resolved on 4% to 12% Bis-Tris SDS-PAGE gels (Invitrogen) under reducing conditions. Proteins were blotted on Hybond-ECL nitrocellulose membranes (Amersham Biosciences), blocked overnight at 4°C with Top-Block (Fluka), and incubated with 1 μg of a monoclonal primary antibody (Ludwig Institute for Cancer Research, New York, NY) for 4 h at room temperature. Secondary horseradish peroxidase–labeled anti-mouse monoclonal antibody (R&D Systems) was applied for 1 h at room temperature. Specific binding was visualized by chemiluminescence (ECL Western Blotting Analysis System, Amersham Biosciences). Appropriate blocking experiments were done for all antigens, with the exception of *MAGE-C1*, where recombinant full-length protein was not available, to confirm specificity of the bands detected.

Evaluation of clinical responses. Remission status was evaluated for all 309 BM samples from previously treated patients based on a modification of the criteria specified by the European Group for Blood and Marrow Transplantation (13). Criteria were modified to account for the limited data set available for each sample in this retrospective assessment. Complete remission was defined as BM plasma cell counts below 10%, negative serum immunofixation, physiologic levels of the patients' respective paraprotein, and lack of progressive bone or kidney pathologies. Partial remission was defined as BM plasma cell counts below 10% and one of the following criteria: positive or ambivalent serum immunofixation, moderately elevated levels of the patients' respective paraprotein (up to 150% of physiologic levels), and lack of progressive bone or kidney pathologies. "Progressive disease" was assigned to patients with one of the following criteria: BM plasma cell counts above 10%, highly elevated levels of the patients' respective paraprotein (>150% of physiologic levels), or progressive bone or kidney pathologies.

Time to relapse was defined as the time between alloSCT and clinical relapse as specified by the International Myeloma Working Group (14). Overall survival was defined as the time between alloSCT and death immediately related to MM as determined by the attending physician. Cases were censored due to death not immediately related to MM, including therapeutic complications ($n = 4$) or loss to follow-up.

Statistical analysis. Analysis of covariance was used to assess correlations between BM plasma cell counts, paraprotein levels, and *MAGE-C1/CT7* levels determined by quantitative RT-PCR. Correlations between clinicopathologic variables and CT antigen expression were assessed using Pearson's χ^2 test. Log-rank test and Cox regression analysis were done for evaluation of survival and relapse in MM patients. Results were considered significant if $P < 0.05$.

Results

Patient characteristics. Analyzing the clinicopathologic characteristics of all 129 patients (Table 1), we observed a male predominance, the typical patient was around 56 years old, and IgGκ represented the most common idiotype. Whereas 16% of the patients were included immediately after initial diagnosis, most patients had already received therapy before this study was initiated. The only clinicopathologic variable correlating with the expression of a minimum of one of the four CT antigens (*MAGE-C1/CT7*, *MAGE-A3*, *MAGE-C2/CT10*, and *SSX-2*), which we and others have previously shown to rank among the most commonly found CT antigens in MM (5, 15–17), was age at the time of inclusion (Table 1A). However, we found a significant association of the expression of at least one of the CT antigens with serum albumin levels, serum hemoglobin levels, and BM plasma cell infiltration when we analyzed all 330 samples available from our patients with MM (Table 1B). Interestingly, when we correlated clinicopathologic variables with the number of CT antigens simultaneously expressed in the same patient group, we also observed significantly higher numbers of CT antigens to be expressed in patients with serum hemoglobin levels below 13 g/dL ($P < 0.05$), elevated BM plasma cell counts ($P < 0.001$), and age above 60 at the time of inclusion ($P < 0.05$).

CT antigens are frequently expressed in MM and expression is related to stage of the disease and BM plasma cell infiltration. When we evaluated the overall frequency of CT antigen expression in BM samples derived from our patients, we found that 38% of all newly diagnosed patients and 29% of newly diagnosed patients with stage I/II disease expressed at least one CT antigen on the RNA level. In newly diagnosed patients with stage III disease, however, we observed CT antigen expression in close to 60% of cases (differences between groups are not significant; Fig. 1A).

Importantly, the control group showed no expression of CT antigens, which is in accordance with our previous studies investigating $CD138^+$ plasma cells and $CD34^+$ progenitor cells isolated from the peripheral blood or BM of healthy donors (5). Furthermore, these results are in line with immunohistochemical

Table 1. Patient and sample characteristics and correlation with CT antigen expression

A. Patient characteristics

Characteristics	No. patients per group	Percentage of patients expressing a minimum of one CT antigen	
Total	129	52.7	
Age at time of inclusion (y)			$P < 0.05$
≤60	79	44.3	
>60	50	66.0	
Sex	129		$P = 0.4$
Heavy chain isotype	129		$P = 0.7$
Light chain isotype	129		$P = 0.7$
Initial stage (Durie-Salmon)	129		$P = 0.1$
Deletion 13q14	48		$P = 0.27$

B. Sample characteristics

Characteristics	No. samples per group	Percentage of samples expressing a minimum of one CT antigen	
Total	330	34.2	
Percentage of BM-infiltrating plasma cells			$P < 0.001$
0-5%	174	19.5	
5-10%	74	23.0	
11-100%	82	75.6	
Serum albumin (g/dL)			$P < 0.001$
<3.5	24	75	
≥3.5	286	29.7	
Hemoglobin (g/dL)			$P < 0.001$
≤13.0	215	40.9	
>13.0	104	18.3	
Serum β_2-microgloubulin (mg/L)	110		$P = 0.5$
Serum lactate dehydrogenase (units/L)	320		$P = 0.1$

NOTE: A total of 129 patients with MM were classified according to clinical features of their disease. Information on the initial stage ($n = 119$) and deletion 13q14 ($n = 48$) were available for fewer patients. Three hundred thirty samples were classified according to sample-specific clinical criteria. Information on remission status ($n = 309$), serum β_2-microglobulin ($n = 110$), hemoglobin ($n = 319$), serum albumin ($n = 310$), as well as serum lactate dehydrogenase levels ($n = 320$) were available for fewer samples. *P* values show correlations between percentages of patients or samples expressing at least one CT antigen (*MAGE-C1, MAGE-C2, MAGE-A3,* or *SSX-2*) as determined by qualitative RT-PCR and single clinicopathologic characteristics.

analyses linking CT antigen expression specifically to the malignant myeloma cells within BM samples (16).

Next, we analyzed all samples from patients with a significant number of plasma cells, defined by a BM plasma cell infiltration >10%, applying qualitative RT-PCR (Fig. 1B). Remarkably, we found that close to 80% of these samples expressed at least one of the four CT antigens, with *MAGE-C1/CT7* being expressed in 65%, *MAGE-A3* in 52%, and *MAGE-C2/CT10* in 43% of cases. In contrast, *SSX-2* was only expressed in 12% of all BM samples with a BM plasma cell infiltration of at least 10%. Interestingly, when we analyzed all 330 samples, we found that higher BM plasma cell infiltration was not only associated with high individual expression frequencies but also a higher number of simultaneously expressed CT antigens (Fig. 1C).

Confirming CT antigen expression on the protein level, we *did* Western blot analyses for *MAGE-C1/CT7*, *MAGE-C2/CT10*, and *MAGE-A3* on BM samples of a randomly selected group of 10 MM patients and 10 healthy donors of whom lysates of total BM were available to us. We found that two patient samples expressed CT antigens as indicated by RT-PCR. Western blot analysis, however, revealed protein expression of *MAGE-C1/CT7* and *MAGE-C2/CT10* in all 10 patient samples, whereas the control group expressed none of the evaluated CT antigens on the protein or on the RNA level ($P < 0.001$). In addition, six patient samples showed expression of *MAGE-A3* protein ($P < 0.01$; Fig. 2). This finding suggests that RNA expression of CT antigens in MM directly translates into protein expression of the given antigen and also raises the possibility of noticeably higher expression rates of CT antigens in MM than previously indicated by the commonly done analysis of RNA expression using conventional RT-PCR.

The obvious discrepancy between CT antigen expression as indicated by RT-PCR versus Western blot might simply be based on a lower sensitivity of the former; however, reliable detection of CT antigen mRNA in BM containing as little as 1% malignant plasma cells might argue against this explanation. Differences in the specificity of both methods are, to our mind, also excluded by reliable results in the routinely *done* molecular sequencing of PCR products and protein blocking experiments done as part of the Western blot analyses. We, therefore, believe that the comparably weak mRNA expression of CT antigens, a phenomenon known from previous studies, and a relatively strong and stable protein expression with a low turnover rate are more likely to explain our findings.

MAGE-C1 ***might provide a gatekeeper function for other CT antigens.*** Specific CT genes, such as *MAGE-A3*, have been suggested to do gatekeeper functions in a way that expression of the gatekeeper gene is mandatory for other CT genes to be expressed in the same tumor. Thus far, it has been unclear whether such a gatekeeper CT gene exists in MM.

Investigating coexpression patterns of the four CT antigens examined in this study, we did not detect a significant influence of the presence of *MAGE-A3*, *MAGE-C2/CT10*, or *SSX-2* on the expression of the remaining antigens (Fig. 3A). In contrast, the

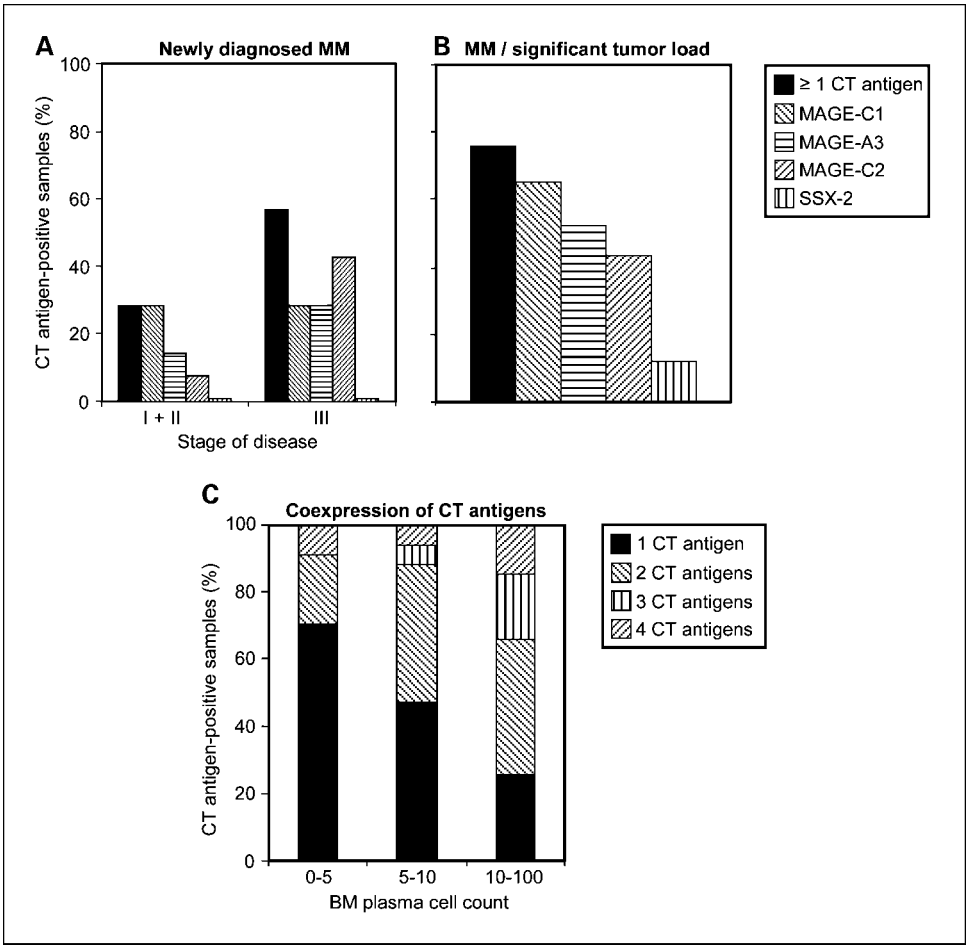

Fig. 1. CT antigens are commonly expressed in MM and expression depends on BM plasma cell infiltration and stage of disease. Twenty-one samples from newly diagnosed patients and 309 samples from 108 previously treated patients were analyzed for the expression of four CT antigens and housekeeping gene *GAPDH* by RT-PCR. Columns, percentages of CT antigen – expressing samples in newly diagnosed patients ($n = 21$) per stage (*A*) and percentages of samples ($n = 91$) expressing CT antigens acquired from patients ($n = 71$) with significant BM plasma cell infiltration >10% (*B*), respectively. *C,* columns, percentages of all CT antigen – positive samples ($n = 113$) simultaneously expressing the respective number of antigens, depending on BM plasma cell infiltration.

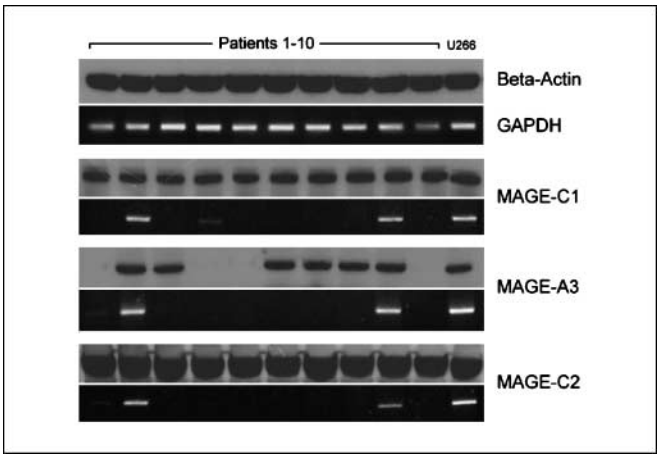

Fig. 2. CT antigens are commonly expressed on the protein level. Ten BM samples from MM patients and cells from myeloma cell line U266 were analyzed for the expression of *MAGE-C1/CT7, MAGE-A3,* and *MAGE-C2/CT10* by qualitative RT-PCR and Western blot. Expression of SSX-2 could not be analyzed on the protein level because an appropriate monoclonal antibody was not available. Due to the small number of samples, only a descriptive analysis was done.

presence of *MAGE-C1/CT7* strongly predicted concomitant expression of the remaining antigens, even of *MAGE-A3*, which has previously been indicated to represent a gatekeeper CT gene in solid tumors (18). Hence, we propose that *MAGE-C1/CT7* might provide a gatekeeper function for the expression of other CT antigens in MM.

CT antigens are persistently expressed in MM. Thus far, CT antigen expression has not systematically been analyzed over time in MM or in any other human cancer. When we examined samples of all patients (n = 17) with at least two samples showing a BM plasma cell infiltration of at least 10% and simultaneous expression of a specific CT antigen in at least one sample, the probability for subsequent samples with a significant number of plasma cells to be positive for the same antigen was 98% (*MAGE-C1/CT7*), 92% (*SSX-2*), 87% (*MAGE-A3*), and 80% (*MAGE-C2/CT10*), respectively (median time of observation, 7 months). This finding suggests a remarkably persistent expression of CT antigens in the BM-residing malignant plasma cells of patients with MM.

CT antigen expression is associated with therapeutic interventions and remission status. Therapy for MM is targeting malignant plasma cells in the BM of patients and might, therefore, be related to the number of CT antigens detected in this compartment. In addition, therapy for MM might also affect the biology of the malignant clone and could have an influence on the expression level of a given CT antigen per cell. However, possible associations between status of therapy and CT antigen expression have never been analyzed in MM. Therefore, we compared the three generally different types of MM-specific therapy—conventional chemotherapy, autoSCT, and alloSCT—regarding effects on the frequency of CT antigen expression in the BM of all patients that had expressed CT antigens at least once.

First, we compared samples of previously treated patients in different states of clinical remission. Remarkably, we found a strong correlation between remission status and CT antigen expression frequency. Of all samples from patients in partial remission, only 50% expressed at least one CT antigen and this number was even further reduced to 21% for samples from patients in complete remission. In contrast, samples from MM patients who were considered nonresponders to therapy or who showed progressive disease expressed CT antigen RNA in 90% of cases (Fig. 3B).

Because we had observed a highly significant ($P < 0.001$) correlation between remission status and the type of therapy that had been applied (data not shown), we next investigated the effect of the individual mode of therapy applied on the degree of CT antigen expression in a given patient. We found that after chemotherapy alone, 100% of all patients still expressed at least one CT antigen, whereas autoSCT significantly reduced expression to 77%. The strongest reduction, however, was achieved in patients after alloSCT whose BM was found to be positive for CT antigen expression in only 40% (Fig. 3B). Interestingly, we observed that the four CT antigens reacted differently to the individual modes of therapy. *MAGE-A3* and *SSX-2* expression was strongly reduced after autoSCT and alloSCT did not further diminish the number of BM samples showing an expression of these CT antigens. In contrast, expression of *MAGE-C1/CT7* and *MAGE-C2/CT10* was already markedly reduced after autoSCT with alloSCT further diminishing BM-related expression of these CT antigens in our MM patients. These findings suggest that chemotherapy plus alloSCT, in addition to simply reducing the total tumor load, might put other mechanisms in place specifically targeting *MAGE-C1/CT7* – expressing and *MAGE-C2/CT10* – expressing clones.

MAGE-C1/CT7 ***levels correlate with the clinical course of MM.*** After confirming *MAGE-C1/CT7* as the most frequently and persistently expressed CT antigen in MM, we next investigated whether expression levels of *MAGE-C1/CT7* correlated with the clinical course of the disease. To this end, we did a longitudinal analysis of repeated BM samples from MM patients applying real-time PCR. As suggested by the high coexpression of gatekeeper gene *MAGE-C1/CT7*, the possibility of significant levels of *MAGE-C1/CT7* in patients expressing at least one of the four antigens seems likely. Therefore, we included all patients with at least three consecutive samples within a timeframe of 12 months who had expressed a minimum of one CT antigen. Thus, a total of 99 samples from 19 MM patients (median number of samples per patient, 5; range, 3-10 samples) were analyzed (median follow-up, 21 months; range, 6-35 months).

We found that in 64% of samples from patients who had been tested negative by qualitative RT-PCR, significant levels of *MAGE-C1/CT7* were readily detectable using real-time PCR. Possible explanations for this increase in sensitivity include the use of more efficient primer pairs and a higher number of cycles done during quantitative RT-PCR. Changes in *MAGE-C1/CT7* expression levels correlated with variations in the patients' BM plasma cell counts (Fig. 3C). Importantly, an even stronger association was found between *MAGE-C1/CT7* expression and the development of the patients' paraprotein levels in the peripheral blood (Fig. 3C). These results underscore our finding that *MAGE-C1* expression strongly correlates with the clinical status of the disease, reflects the effectivity of therapeutic interventions, and is a reliable marker for relapse and progressive disease.

To illustrate the individual consistency and reliability of CT antigen expression in patients undergoing different clinical

phases of the disease, we analyzed all 19 patients individually for the relationship between the clinical course of the disease and *MAGE-C1/CT7* (quantitative and qualitative PCR) as well as *MAGE-A3* (only qualitative PCR) expression. Generally, changes in *MAGE-C1/CT7* levels paralleled changes in BM plasma cell infiltration and paraprotein levels (Fig. 4, representative graphs shown for four patients). Positive results from qualitative PCR for *MAGE-C1/CT7* were usually associated with higher levels measured by quantitative PCR, coinciding with increased tumor load in the BM.

The first group consisted of patients who had received alloSCT and who did not relapse in the time of observation. In these patients, we found normal levels of conventional clinical response variables and consistently low levels of *MAGE-C1/CT7* (Fig. 4A). These findings show that low levels of *MAGE-C1/CT7* expression reliably indicate persisting remission in patients following alloSCT.

The second group consisted of patients who had a clinically relevant tumor load at the beginning of the observation period but who showed a significant response to therapeutic interventions. In these patients, decreases in conventional markers of disease paralleled a reduction in *MAGE-C1/CT7* levels, as detected by real-time PCR of total BM RNA (Fig. 4B). Thus, serial analysis of *MAGE-C1/CT7* expression seemed to

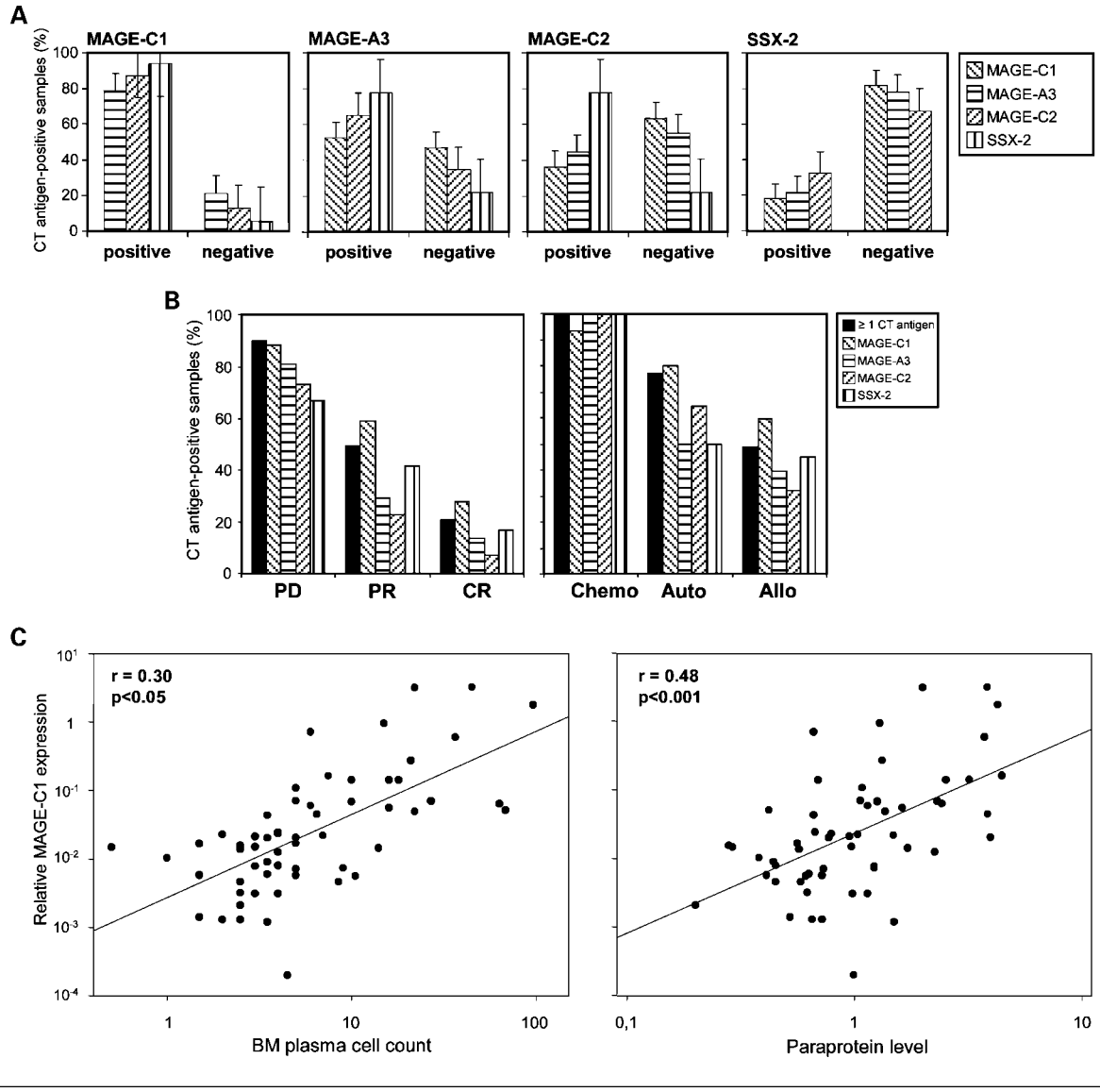

Fig. 3. CTantigen expression correlates with tumor load, remission status, and mode of therapy. Analyzing 330 samples from 129 MM patients for the expression of four CTantigens, we show that *MAGE-C1/CT7* might provide a gatekeeper function for other CTantigens. *A,* graphs show samples positive or negative for the CTantigen indicated in the title, bars represent percentages of samples expressing the given CTantigens, and flags indicate margin of error at 90% confidence. Next, we analyzed 180 samples from 68 previously treated patients who had expressed a respective CTantigen at least once for the expression of *MAGE-C1/CT7* ($n = 137$), *MAGE-A3* ($n = 133$), *MAGE-C2/CT10* ($n = 81$), *SSX-2* ($n = 33$), and housekeeping gene *GAPDH* by RT-PCR. *B,* remission status was evaluated for all samples individually, and if more than one therapy had been applied, the latest was used for the definition of treatment status (median time after therapy, 21 mo). *C,* our analysis of 58 samples from 11 patients who had previously expressed *MAGE-C1/CT7* and from whom at least three consecutive samples within a timeframe of 12 mo were available. Applying real-time PCR, *MAGE-C1/CT7* levels were normalized to *GAPDH* levels and correlations between *MAGE-C1/CT7* expression, paraprotein levels normalized to the respective upper physiologic limit of the paraprotein, and plasma cell numbers were calculated using analysis of covariance. Resulting *P* values and correlation coefficients are indicated.

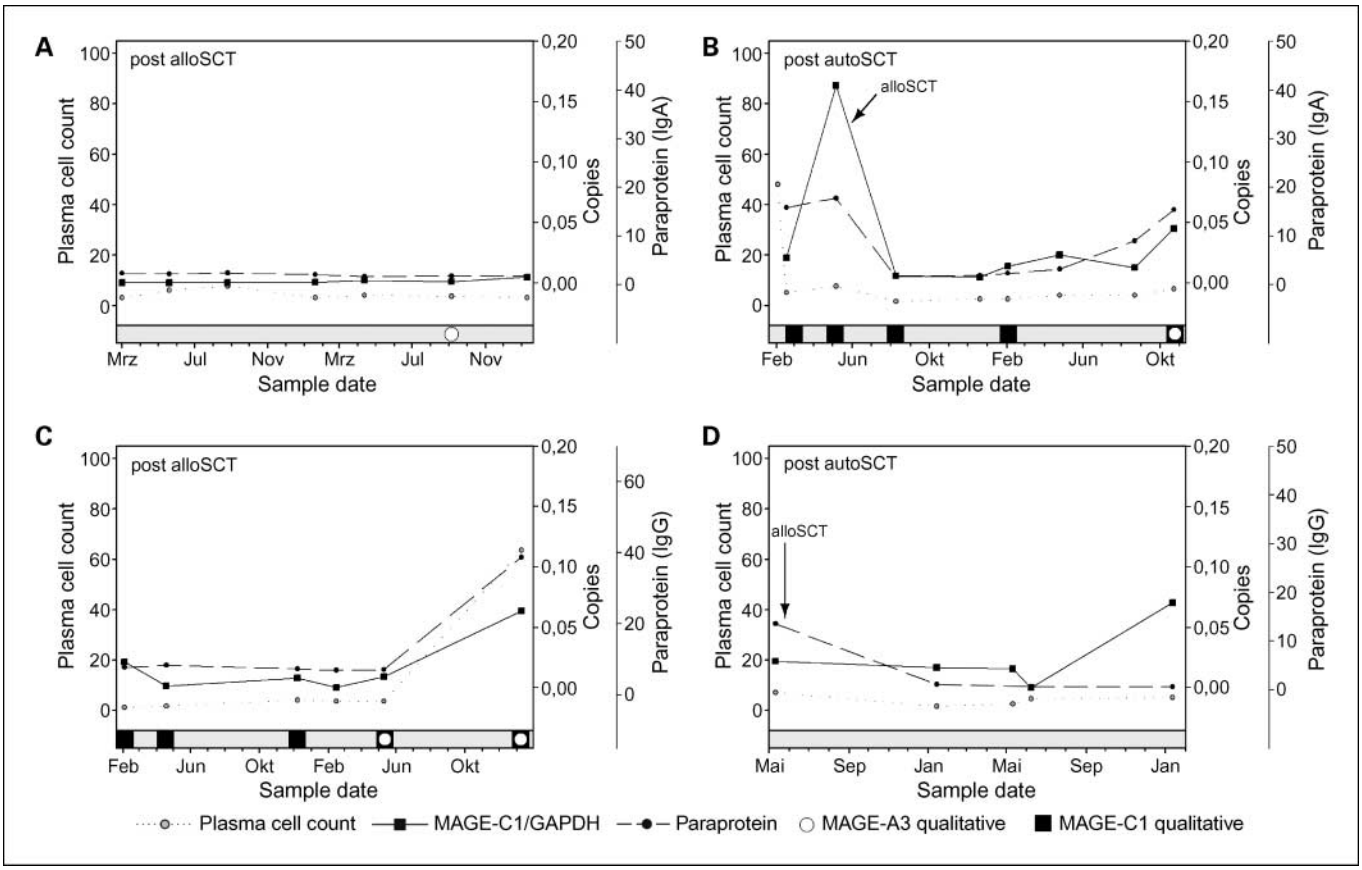

Fig. 4. *MAGE-C1/CT7* expression correlates with clinical course. Ninety-nine samples from 19 patients who previously expressed one of the four examined CT antigens and from whom at least three consecutive samples within a timeframe of 12 mo were available were analyzed for the expression of *MAGE-C1/CT7* by quantitative RT-PCR. *MAGE-C1/CT7* levels were normalized for the expression of housekeeping gene *GAPDH*. Patients were divided into four groups consisting of patients with persistent complete remission following alloSCT (*A*), patients with a reduction of conventional clinical variables following therapeutic intervention (*B*), patients with increasing paraprotein and *MAGE-C1/CT7* levels (*C*), and patients with independently increasing *MAGE-C1/CT7* levels (*D*).

represent a reliable marker for response to therapy in MM patients.

In contrast, the third group consisted of patients who developed increasing *MAGE-C1/CT7* levels within the time of observation (Fig. 4C). In two cases, *MAGE-C1* expression correlated closely with clinical variables indicating relapse of the disease. In certain members of the third group of patients, *MAGE-C1* levels increased at the end of the observation period, suggesting that this change in expression levels might precede the occurrence of relapses at later time points (Fig. 4D). In conclusion, we propose that *MAGE-C1/CT7* is a reliable and possibly earlier indicator for relapse in patients with MM.

Expression of* MAGE-C1/CT7 *represents an important indicator of early relapse and reduced survival in patients with MM after alloSCT. Based on our observation of a strong association of CT antigen expression with stage of the disease and remission status, we examined its reliability as an indicator for relapse in patients with MM, defined by a significant increase in the patient's respective paraprotein level or BM plasma cell infiltration. To this end, we evaluated the course of the disease of our largest collective of patients who were in a state of partial remission after alloSCT (n = 52; median time since alloSCT, 31 months; range, 2-128 months). Whereas we found that a higher risk for relapse was indeed associated with the expression of *MAGE-C1/CT7* ($P < 0.001$), *MAGE-A3* ($P < 0.001$), *SSX-2* ($P = 0.03$), and *MAGE-C2* ($P < 0.001$; Fig. 5A-D), the most distinct association was observed for *MAGE-C1/CT7* (Fig. 5A). Remarkably, we found that patients who did not express *MAGE-C1/CT7* showed a favorable course of the disease, with only 7% of patients evidencing a relapse with a median time of observation of 41 months (Fig. 5A). In marked contrast, patients who showed BM expression of *MAGE-C1/CT7* following alloSCT relapsed in 75% of cases, with a median time to relapse of 14 months after alloSCT (Fig. 5A). The difference in time to relapse between the CT antigen–positive and CT antigen–negative group was highly significant ($P < 0.001$).

Importantly, when we analyzed whether the influence of *MAGE-C1/CT7* expression on the occurrence of relapse would translate into an effect on overall survival of MM patients after alloSCT, we indeed observed that disease-related death occurred more frequently and earlier after alloSCT in the *MAGE-C1/CT7*–positive group ($P = 0.003$; Fig. 5A). The same observation, albeit to a lesser extent, was made for *SSX-2* ($P = 0.02$; Fig. 5C). Subsequently done multivariate Cox regression analysis taking into account several relevant clinicopathologic variables confirmed *MAGE-C1/CT7* as the only significant and independent prognostic variable for relapse [*MAGE-C1/CT7*: $P < 0.001$ (hazard ratio, 39.2; 95% confidence interval, 8.2-186.8); sex: $P = 0.19$; age: $P = 0.47$; number of samples

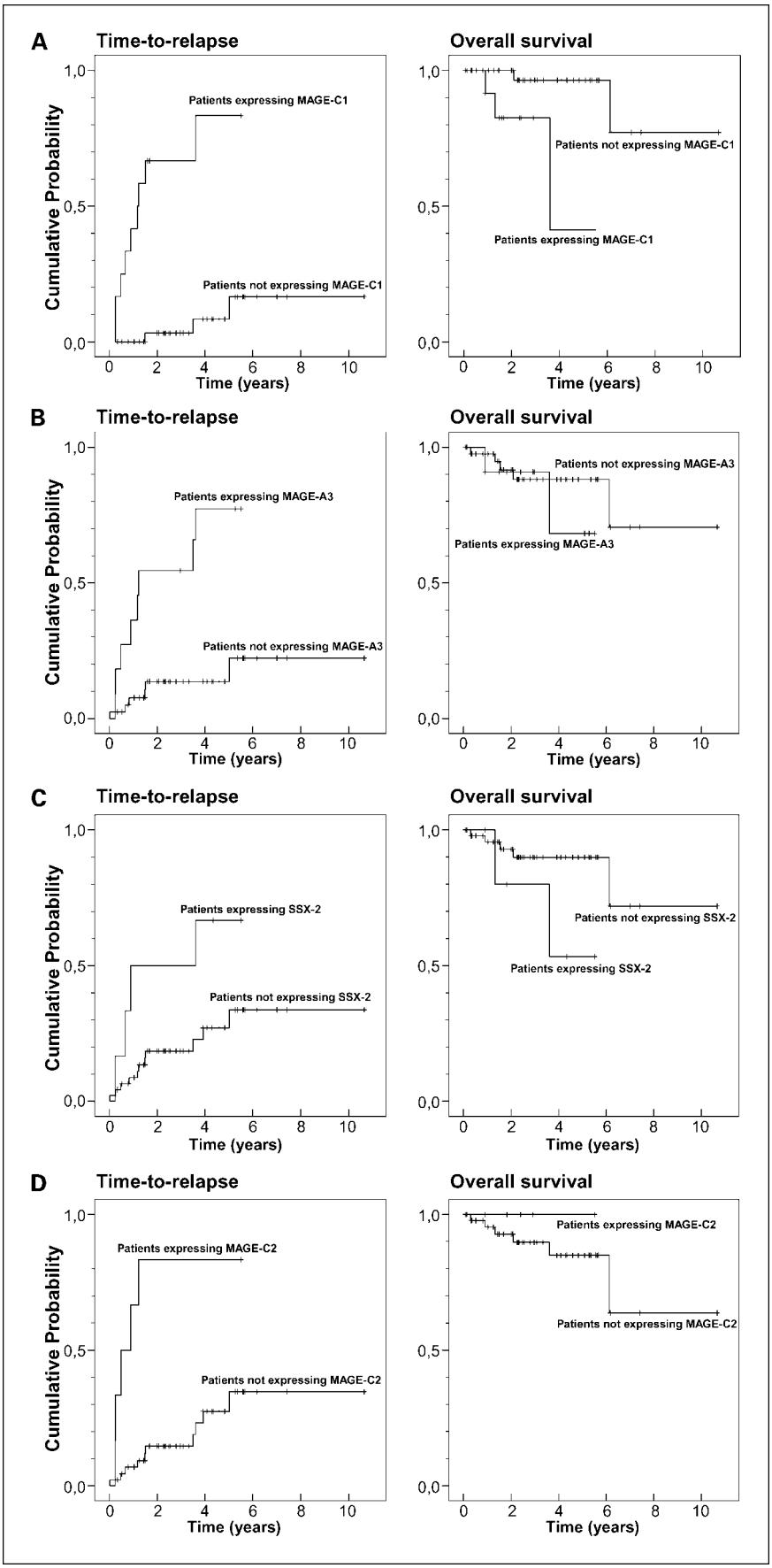

Fig. 5. *MAGE-C1/CT7* expression correlates with an increased risk for relapse following alloSCT. Fifty-two patients, who had received alloSCT and from whom follow-up data were available, were analyzed for time to relapse as well as overall survival and divided into groups according to expression of *MAGE-C1/CT7* (*A*), *MAGE-A3* (*B*), *SSX-2* (*C*), and *MAGE-C2/CT10* (*D*) as measured by RT-PCR. Curves represent Kaplan-Meier estimates of the percentages of patients experiencing a relapse or disease-related death during the time of observation.

screened since alloSCT: $P = 0.11$; initial stage, deletion 13q14, and isotype were rejected as covariates due to insignificant log-rank results] in MM patients treated with alloSCT. Unfortunately, due to the age of our samples and the associated clinical data, we were unable to include more recently established disease parameters, such as the newer International Staging System Staging or additional cytologic criteria, in the multivariate analysis.

Discussion

Little is known about variations in CT antigen expression during the course of the disease in patients with MM, which seems surprising considering the emergence of immunotherapeutic approaches using CT antigens despite a previously described possibility of down-regulation or even loss of expression of target antigens in different clinical settings. Therefore, we longitudinally analyzed an extensive collection of previously treated as well as newly diagnosed patients with MM for the expression of four CT antigens. Baseline expression frequencies, measured by RT-PCR, determined *MAGE-C1/CT7* as the most frequently detected antigen, possibly performing a gatekeeper function for the other antigens examined. Importantly, 80% of patients with a significant number of plasma cells expressed at least one of the four antigens investigated. Comparing these results with conventional clinicopathologic variables, we found significant positive correlations with the patients' age at the time of inclusion, serum albumin levels, serum hemoglobin levels, as well as BM plasma cell counts. In the group of newly diagnosed patients, we also confirmed the observation that initial stage correlates with CT antigen expression (19). These data already indicated a close correlation between CT antigen expression and the clinical course of MM.

To answer the question whether these correlations could be linked to the clinical status of the individual patient at the time of analysis, we assessed response criteria for each sample and compared the resulting groups. Only a small minority of patients in complete remission were found to be CT antigen–positive. In contrast, half of the patients in partial remission were found to be CT antigen positive and expression frequencies were highest in patients with progressive disease in whom CT antigens were even more commonly found than in newly diagnosed patients. Thus, our findings indicate a very close relationship between CT antigen expression and the extent of the disease.

Evaluating the effect of the three types of therapy on the expression of CT antigens, we observed the strongest effect for alloSCT. This finding may be an unspecific effect of high-dose chemotherapy and alloSCT and may be based on an increased depth of remission following this mode of therapy. However, it might also be the result of a specific elimination of CT antigen–expressing myeloma cells by local or systematic donor-derived immune responses. One immediate consequence of such an active elimination of CT antigen–positive malignant plasma cells might be the comparably strong reduction in the expression of *MAGE-C1/CT7* and *MAGE-C2/CT10* in the BM of MM patients following alloSCT. The induction of persisting remissions by alloSCT has previously been linked to antitumor immunity exerted by donor lymphocytes (20–22). *MAGE-C2/CT10* has been shown to elicit spontaneous immune responses in different solid tumors (23–26) and we have recently shown that alloSCT induces CT antigen–specific immune responses in MM (5). Therefore, we are currently investigating whether alloSCT-induced immune responses against *MAGE-C1/CT7* and *MAGE-C2/CT10* might lead to an eradication of myeloma cells expressing these antigens from the patients' BM.

Evaluation of CT antigens as potential immunotherapeutic targets and novel diagnostic markers in human cancers requires information about their expression over time. Therefore, we did a longitudinal analysis of CT antigen expression to answer the question whether these antigens are consistently expressed. We show here that, if a patient expressed a CT antigen at least once, the probability for occurrence of the same antigen in relapse was close to 100% for antigens such as *MAGE-C1/CT7*, suggesting that CT antigens are indeed consistently expressed over the course of months and years and, most importantly, that down-regulation of CT antigens is not a common mechanism of tumor escape in this disease. Doing analyses of 19 patients' individual clinical courses, we showed remarkable correlations of BM plasma cell counts and paraprotein levels with *MAGE-C1/CT7* expression levels in patients with persisting remission as well as patients with complex progressions. Although these strong correlations retrospectively might at first glance seem trivial reflecting a correlation of disease stage and CT antigen expression, we convincingly show for the first time in an intraindividual approach that independent loss or down-regulation of CT antigens is highly unlikely and that these proteins, therefore, indeed represent valuable targets for antigen-specific immunotherapies in all clinical stages of myeloma, including minimal residual disease.

Multiple explanations for the close relationship between *MAGE-C1/CT7* expression and the clinical course of the disease are conceivable. First, the low expression level of CT antigens in the state of remission might be simply due to reduced numbers of BM plasma cells following therapy. Second, MM cells might reduce their overall transcription rate in times of lower tumor activity, which seems unlikely considering persistently high expression of housekeeping gene *GAPDH*. Third, supported by our finding, that simultaneous expression of CT antigens is linked to higher BM plasma cell counts, CT antigens might specifically be activated in proliferating tumors either individually or as part of coordinated expression programs, for example, by promoter hypomethylation that has recently been linked to the activation of several CT antigens (27, 28). We favor the latter explanation, especially considering the close relationship of CT antigens to functions involved in malignant transformation, such as regulation of transcription (24, 29–31), translation (32, 33), apoptosis (34), and promotion of the malignant phenotype through induction of resistance to chemotherapeutic drugs (35).

Finally, evaluating the prognostic value of *MAGE-C1/CT7* expression in MM, we show that in patients following alloSCT, time to relapse as well as overall survival were dramatically worse if their malignant BM plasma cells expressed *MAGE-C1/CT7*. Confirmed by multivariate Cox regression analysis, these findings indicate that *MAGE-C1/CT7* is a highly significant and independent negative prognostic factor in MM. In association with our observation of a very persistent expression in patients with recurrent disease, these data suggest a central role of CT antigens in the promotion of tumor progression. Therefore, antigen-specific eradication of CT antigen–expressing malignant

cells from the patients' BM might target an "Achilles' heel" of the malignancy and, therefore, has the potential to result in long-lasting remission or even cure in MM patients.

In conclusion, CT antigens are consistently expressed in MM and expression levels of CT antigens, such as *MAGE-C1/CT7*, correlate strongly with conventional clinicopathologic variables and the clinical course of the disease. In our sequentially analyzed patients, we did not observe down-regulation or loss of CT antigen expression. Analysis of CT antigen expression may allow for the monitoring of minimal residual disease and the expression of CT antigens represents an important prognostic factor in patients with MM. These results render CT antigens highly attractive targets for diagnostic purposes and antigen-specific immunotherapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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