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Longitudinale Untersuchung der Expression von Cancer/Testis-Antigenen im Knochenmark von Patienten mit Multiplem Myelom

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II

Abstract

BACKGROUND AND OBJECTIVES: Reliable data on the persistence of the expression of tumor antigens over time and consequent analyses of its impact on the clinical course of malignancies are crucial for their evaluation as clinical parameters and immunotherapeutic targets. This study represents the first longitudinal investigation of Cancer-Testis (CT) antigen expression in Multiple Myeloma (MM) patients and its relevance for therapeutic, diagnostic, and predictive clinical use.

DESIGN AND METHODS: 330 bone marrow (BM) samples from 129 MM patients and 40 samples from healthy BM donors were screened for the expression of 4 CT antigens (MAGE-C1/CT7, MAGE-C2/CT10, MAGE-A3, and SSX-2) by conventional RT-PCR. Selected patient samples were analyzed by real-time PCR and Western Blot. Finally, statistical analyses of experimental and clinical data were carried out.

RESULTS: CT antigens were frequently and persistently expressed, indicating that down-regulation of these immunogenic targets does not represent a common tumor escape mechanism in patients with multiple myeloma. 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 further suggest a potential role for CT antigens as independent tumor markers. Interestingly, antigen MAGE-C1/CT7 was found to represent an extraordinarily frequent coexpression partner, indicating a potential role as a gatekeeper gene. In addition, MAGE-C1/CT7 represented an indicator of early relapse and dramatically reduced survival in patients following allogeneic stem cell transplantation (alloSCT).

CONCLUSIONS: Analyzing a large number of patients with MM, this study established essential parameters endorsing the use of CT antigens as immunotherapeutic target structures and for the first time suggested the use of the mRNA expression of distinct CT antigens as diagnostic and predictive markers.

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Abbreviations

AA	Amino acids
alloSCT	Allogeneic stem cell transplantation
autoSCT	Autologous stem cell transplantation
BM	Bone marrow
cDNA	Complementary deoxyribonucleic acid
CCD	Charge-coupled device
CD	Cluster of differentiation
CT	Cancer-Testis
CR	Complete remission
EBMT	European Group for Bone and Marrow Transplant
Ig	Immunoglobulin
IMWG	International Myeloma Working Group
ISS	International Staging System
GvHD	Graft-versus-Host Disease
GvM/GvL	Graft-versus-Myeloma/Graft-versus-Leukemia
HLA	Human Leukocyte Antigen
MGUS	Monoclonal gammopathy of undetermined significance
MHD	MAGE homology domain
MM	Multiple Myeloma
MNC	Mononuclear cells
MRD	Minimal residual disease
PD	Progressive disease
PR	Partial remission
RT-PCR	Reverse transcriptase polymerase chain reaction
SEREX	Serological analysis of recombinant tumor cDNA expression libraries
RNA	Ribonucleic acid

1   Introduction and Aims

Despite the introduction of novel treatment regimen (Katzel, Hari et al. 2007), Multiple Myeloma (MM) remains an essentially incurable disease. This is caused by either the reinfusion of malignant cells during autologous stem cell transplantation (autoSCT) and/or a subset of persisting chemotherapy-resistant cells eventually leading to clinical relapse, as observed in over 90% of patients with MM (Barlogie, Tricot et al. 2006). Therefore, the development of novel treatment options is essential and one of the most promising approaches in this setting represents targeted immunotherapy.

Cancer testis (CT) antigens are a diverse group of tumor antigens of which more than 240 unique members have been identified during the past two decades (Simpson, Caballero et al. 2005). CT antigens have been studied intensively regarding their expression pattern and their immunogenicity in cancer patients. Their tumor-restricted expression as well as their ability to induce spontaneous antibody and T cell responses theoretically renders CT antigens important therapeutic targets. On the other hand, CT antigens might also be used as diagnostic and/or prognostic markers. Curiously, these aspects have not been comprehensively investigated before.

Although numerous studies have demonstrated a common expression of CT antigens in a large variety of human tumor types on the RNA as well as on the protein level (Meklat, Li et al. 2007), there has not been a single study analyzing the expression of CT antigens in a human cancer over time. This is surprising since reliable data on the persistence of tumor-related CT antigen expression are a prerequisite for the evaluation of their clinical usefulness, especially considering data suggesting that immunoselection might lead to downregulation or loss of CT antigen expression in cancer patients (Jager, Stockert et al. 1999; Knuth, Jager et al. 2000).

The laboratory for tumor immunology at the II. Medical Department of the University Medical Center Hamburg-Eppendorf has recently demonstrated that CT antigens are commonly expressed and capable of inducing antibody- and T cell-mediated immunity in MM patients (Atanackovic, Arfsten et al. 2007). This finding may be of clinical relevance since MM has been considered a disease, which is, at least to a certain extent, controlled by the adaptive immune system. This is especially supported by the fact that the therapeutic efficacy of allogeneic stem cell transplantation 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 even following clinical relapse (Mielcarek and Storb 2003; Schetelig, Kiani et al. 2005). Importantly, the

finding that immune responses against CT antigens are induced by allogeneic stem cell transplantation (Atanackovic, Arfsten et al. 2007) suggests that this class of tumor antigens might indeed contain natural targets for donor-derived allo-immune or even spontaneous anti-myeloma immune responses.

The main goals of this study were the determination of the stability of Cancer-Testis antigen expression over the course of a malignant disease and their evaluation as diagnostic and predictive markers following alloSCT in patients with MM. To this end, I analyzed the expression of four CT antigens (MAGE-C1/CT7, MAGE-C2/CT10, MAGE-A3, SSX-2) commonly expressed in MM, in 330 bone marrow (BM) samples from 129 MM patients by qualitative and quantitative RT-PCR and correlated the resulting data with the clinical course of the disease. Findings derived from this study strongly support a role for CT antigens as diagnostic and predictive markers following alloSCT as well as immunotherapeutic targets in MM.

The results obtained in this study have been published under the title "Longitudinal Analysis and Prognostic Effect of Cancer-Testis Antigen Expression in Multiple Myeloma" in Clinical Cancer Research (current Impact Factor 6.5) on February 2nd 2009.

Tumor immunology

1.1.1 History

The notion that the human immune system might be able to detect and eradicate cancer cells has been disputed extensively in the last century. As early as 1909, Paul Ehrlich proposed that the immune system might be involved in the detection of malignant cells (Ehrlich 1909), an idea that was later expanded on by Burnet's and Thomas' hypothesis of "immunosurveillance", stating that the immune system not only observes but is in fact capable of eliminating tumor cells (Burnet 1957). These now widely accepted concepts became particularly unpopular during the 60s and 70s due to studies that were unable to show a significant difference in the incidence of tumors between immunocompetent and immunodeficient mice (Rygaard and Povlsen 1974).

Later on, these results were contradicted by findings of multiple groups describing an influence of the immune system on the development and the progression of malignant diseases (DUNN, BRUCE ET AL. 2002) with T cell-mediated immunity playing an important role (SMYTH, THIA ET AL. 2000; SHANKARAN, IKEDA ET AL. 2001). Accordingly, it was found that tumor infiltration by T cells and their respective proliferation rate constitute a positive prognostic factor in renal cell (NAKANO, SATO ET AL. 2001), ovarial (ZHANG, CONEJO-GARCIA ET AL. 2003; SATO, OLSON ET AL. 2005), uterine (KONDRATIEV, SABO ET AL. 2004) and colon carcinoma (ROPPONEN, ESKELINEN ET AL. 1997; NAITO, SAITO ET AL. 1998), as well as hematologic malignancies such as Non-Hodgkin lymphoma (Ansell, Stenson et al. 2001; Xu, Kroft et al. 2001).

After proposing a potential clinical impact of tumor-specific immune responses, the field of tumor immunology began the systematic search for the corresponding cellular and molecular target structures. Soon, the development of novel methodological approaches enabled the determination of a vast amount of previously unknown tumor-associated antigens (Rosenberg 2001).

1.1.2 Tumor antigens

Tumor antigens are defined by their expression pattern, as well as their ability to induce spontaneous immune responses in cancer patients. Specific immune responses can be detected in the form of significant antibody titers or the presence of antigen specific immune effector cells. In fact, these immune reactions led to the discovery and the characterization of many of the respective targets.

The systematic evaluation of antigen-specific humoral immunity was enabled by the introduction of a novel strategy to determine autoreactivity via serological analysis of recombinant cDNA expression libraries (SEREX) (Chen YT 2000). In a SEREX analysis cDNA derived from the patient's tumor is expressed by a bacteriophage system in order to generate clones displaying individual proteins on their surface. These clones are then exposed to the host's serum. In case of confirmed reactivity the respective clone is sequenced and results are compared with public gene libraries. A significant number of Cancer-Testis antigens as well as numerous overexpressed tumor-associated antigens were identified via this approach. In contrast to T cells, antibodies recognize tumor antigens in an HLA-independent manner, predominantly binding directly to small linear peptide sequences. While this observation initially suggested a relative abundance of anti-tumor antibodies compared to cellular immune responses, many antigens identified by antibodies in the sera of cancer patients, have been confirmed using T cell-based expression cloning, such as in the case of the MAGE tumor antigens.

At the same time, the development of novel immunological methods to detect and quantify antigen-specific CD4+ and CD8+ T cells allowed the discovery of numerous target structures of cellular immune responses. A relative bias towards epitopes recognized by CD8+ T cells might be related to the preferred processing pathway of the predominantly intracellular tumor antigens. While CD8+ cytotoxic T lymphocytes recognize antigenic peptides presented by HLA class I proteins, CD4+ helper T cells are activated by the HLA class II complex. Generally, internalized membranous or soluble proteins are presented using the HLA class II complex following degradation by vesicular proteases (Cresswell 1994). Conversely, endogenously generated proteins are preferably processed by the proteasome and the endoplasmic reticulum and subsequently presented via the HLA I complex (Lehner and Cresswell 1996). Following pathway specific processing tumor peptides associate with HLA molecules within the intracellular compartment and are then translocated to the cell surface. The resulting immunogenicity of these complexes is finally determined by the compatibility and affinity between the antigenic fragment and the HLA proteins (Sette, Vitiello et

al. 1994; van der Burg, Visseren et al. 1996). This at least in part explains why, while CD4+ T cells play an important role in the promotion of anti-tumor immunity, especially characterization of CD8+ cytotoxic T lymphocytes has proven to be a valuable tool in discerning antigenic peptide sequences.

The multitude of tumor antigens described by these techniques was classified according to their origin, function, and expression pattern in comparison to healthy tissues (Wang and Rosenberg 1999).

1.1.2.1 Overexpressed antigens

The use of the term "overexpressed antigens" in the literature can be considered ambiguous as it might refer to an increased expression in tumor cells in comparison to healthy cells but is also used to describe an entire tissue's expression rather than an individual cell's. The latter implies that a higher proportion of cells expressing this antigen might be responsible for the observed effect. Both perspectives are accepted in current research and often only evaluation of the underlying methodology will reveal the nature of the described findings (e.g. normalized quantitative RT-PCR of distinct populations is commonly used to determine cellular expression levels). Apart from nomenclature, the distinction of both interpretations is essential considering that the former would be more likely to be associated with specific immune responses.

Prominent genes that are commonly classified as overexpressed antigens are HER-2/neu and WT1. Both genes are currently used as therapeutic targets, showing encouraging clinical results (Bernhard, Salazar et al. 2002; Keilholz, Letsch et al. 2009).

1.1.2.2 Mutated antigens

While overexpressed antigens can be detected in healthy tissues, mutated antigens are by definition strictly associated with the malignant cells. In this case, the aberrant expression product is the result of a somatic mutation causing increased immunogenicity through either overexpression or novel protein structure. Most mutated antigens are the result of point mutations, fused translocations, as in the BCR/ABL gene, or mutations leading to frame shift and therefore irrelevant peptide products. In an immunotherapeutic setting, mutated antigens ideally code for a gene product facilitating the malignant phenotype, as the tumor cells would be less likely to undergo immunoselection leading to a relative loss of expression of the mutated gene. Furthermore, such genes would be more likely to be shared between tumors and pa-

tients. Prominent members of this group of tumor antigens include CDK4, K-Ras, and N-Ras.

1.1.2.3 Differentiation antigens

T cell responses against surface markers of mature melanocytes in healthy individuals first raised the question whether efficacious immune responses are able to target peptides that are associated with a subpopulation of differentiated healthy cells (Anichini, Maccalli et al. 1993). Although such considerations initially suggested the risk of undesired anti-self immune reactions, patients showing natural cellular immune responses against these obvious autoantigens evidenced no clinical signs of autoimmunity, indicating that immunotherapeutic approaches involving this group of antigens should be safe (Slingluff, Petroni et al. 2003; Berger, Haendle et al. 2004). Tyrosinase, an enzyme crucial for the synthesis of melanin, and MART-1/Melan-A, which is used as a diagnostic marker of melanoma, represent some of the members of this antigen family.

1.1.2.4 Cancer-Testis antigens

CT antigens are a group of genes normally expressed only in germ line tissues, but also showing aberrant expression in a large number of human cancers. Approximately 240 unique genes have been assigned CT antigen status and have been further clustered into gene families according to sequence similarity and origin (Almeida, Sakabe et al. 2009). As approximately 50% of CT antigens are located on the X chromosome, a general distinction between CT-X and non-X CT antigens, with the latter showing a distribution throughout the entire genome, has been suggested.

Interestingly, functional data regarding CT antigens remain sparse, but strictly phase-specific expression, especially of CT-X antigens, within germ line tissues during gametogenesis suggests a possible functional involvement in the associated cellular processes. Indeed, for some genes roles in transcriptional and translational regulation (Tureci, Sahin et al. 1996; Tureci, Sahin et al. 2002; Kalejs 2005) as well as control of chromosome pairing during meiosis (Tureci O 1998) have been suggested. Recent studies further demonstrated involvement in the protection from apoptosis (Cilensek, Yehiely et al. 2002), promotion of the malignant phenotype, and induction of resistance to chemotherapeutic drugs (Duan, Duan et al. 2003).

Another reason for the exploration of CT antigens as immunotherapeutic target structures represents their extraordinary immunogenicity in cancer patients. In fact,

most CT antigens have been discovered due to their high immunogenic potential and the resulting immune responses in tumor patients showing increased expression of these genes.

Genes belonging to the MAGE family currently represent the most thoroughly investigated CT antigens and some of the most promising anti-tumor targets. Most members of the MAGE family have been initially detected during screenings of T cell responses against autologous tumor cells from melanoma patients (Kocher, Schultz-Thater et al. 1995; Chen, Gure et al. 1998; Lucas, De Smet et al. 1998) and several ongoing clinical studies investigate full-length recombinant MAGE protein or partial MAGE peptides as anti-tumor vaccines in melanoma and other solid cancers (Health 2000). All genes belonging to the MAGE family share the very specific, highly conserved MAGE Homology Domain (MHD) (Barker and Salehi 2002). While there are few data regarding the functional role of this approximately 200aa spanning sequence, it is likely to facilitate homologous interactions between MHD-containing proteins. Direct interactions of MAGE genes with other CT antigens, such as NY-ESO-1 (Cho, Caballero et al. 2006), and unrelated genes, e.g. gankyrin (Nagao, Higashitsuji et al. 2003), have also been described.

In this study, I investigated the expression of three MAGE antigens located on the X chromosome that had been found to be commonly expressed in MM, namely MAGE-C1/CT7, MAGE-C2/CT10, and MAGE-A3 (Pellat-Deceunynck, Mellerin et al. 2000; De Vos, Thykjaer et al. 2002; Jungbluth, Ely et al. 2005; Jungbluth, Ely et al. 2005; Taylor, Reiman et al. 2005; Atanackovic, Arfsten et al. 2007; Atanackovic, Arfsten et al. 2007; Condomines, Hose et al. 2007; Condomines, Hose et al. 2007; Tinguely, Jenni et al. 2008). MAGE-C1/CT7 and MAGE-C2/CT10 are about 800 amino acids longer than other MAGE proteins, contain a large number of unique short repetitive sequences in front of their respective MHD (Lucas, De Smet et al. 1998), and show an extraordinary mutual sequence identity.

The fourth gene investigated in this study was SSX-2. Genes belonging to the SSX family had first been described in patients with synovial sarcoma, who frequently showed antibody responses against these antigens (Gure, Wei et al. 2002).

Expression of CT antigens in human malignancies is highly heterogeneous. While solid cancers, such as breast cancer, lung cancer, and melanoma have been found to frequently express members of this gene family, a particularly rare expression was observed in hematologic malignancies, with the exception of Multiple Myeloma. Importantly, tumors that express at least one CT antigen are likely to express others and these co-expression patterns appear to be non-random (Sahin, Tureci et al.

1998; Tajima, Obata et al. 2003). This is for example illustrated by the finding that MAGE-A3 was expressed in almost all lung cancers that were found positive for at least one CT antigen (Tajima, Obata et al. 2003). This first suggested that CT-X antigens are coordinately activated as part of an expression program rather than individually. And indeed, it was found that one of the mechanisms behind the activation of such programs is the hypomethylation of CpG islands within the CT genes' respective promoter regions, a common occurrence in malignant cells (Weber, Salgaller et al. 1994; De Smet, De Backer et al. 1996).

Overall, CT antigens are commonly and specifically expressed in many malignancies, are able to induce spontaneous immune responses, play functional roles in healthy and malignant cells, and possibly provide target redundancy due to a frequent co-expression. In order to comprehensively evaluate their immunotherapeutic potential it was now necessary to determine the stability of their expression in candidate malignancies and the optimal clinical conditions for such approaches.

1.1.3 Tumor immunotherapy

As previously illustrated, cancer cells provide unique gene expression profiles, that commonly characterize the respective tumor. Immunohistochemistry already exploits this phenomenon for diagnostic purposes. The human immune system, too, is able to recognize structures associated with the malignant cells as indicated by tumor-specific antibody and T cell responses.

It has been known for several years that the presence of such tumor-specific T cell responses in cancer patients represents a favorable prognostic factor (ROPPONEN, ESKELINEN ET AL. 1997; NAITO, SAITO ET AL. 1998; NAKANO, SATO ET AL. 2001; ZHANG, CONEJO-GARCIA ET AL. 2003; KONDRATIEV, SABO ET AL. 2004; SATO, OLSON ET AL. 2005) and a multitude of studies have confirmed the anti-tumor activity of these T cells in vitro and in vivo (Koebel, Vermi et al. 2007). How this effect might be used efficiently in a targeted therapeutic setting is the subject of ongoing research.

1.1.3.1 Immunotherapy using undefined antigens

There are several options available in order to generate or enhance anti-tumor immune responses in patients. Using tumor cell lysates for vaccination or adoptive transfer of in-vitro-primed autologous T cells represents an elegant approach due to its self-provided redundancy, as a multitude of specific structures are targeted simul-

taneously. Furthermore, this approach does not require cumbersome characterization of the eventual targets and allows immediate personalized clinical application. On the other hand, the potential induction of autoimmunity and priming of the immune system against inappropriate target structures, such as non-processed intracellular proteins, represent major drawbacks of this approach. In addition, first clinical studies showed a surprisingly poor efficacy in cancer patients (Sosman, Unger et al. 2002; Nemunaitis, Sterman et al. 2004).

In contrast, another form of immunotherapy using undefined target structures, allogeneic stem cell transplantation (alloSCT), shows remarkable clinical success rates frequently leading to remission and even cure in patients with hematological malignancies (Corradini, Cavo et al. 2003; Bruno, Rotta et al. 2007). The immunotherapeutic aspects of alloSCT that play an important role in its curative potential have been termed the Graft-versus-Myeloma (GvM) effect (Aschan, Lonnqvist et al. 1996; Tricot, Vesole et al. 1996; Verdonck, Lokhorst et al. 1996).

1.1.3.1.1 Graft-versus-Myeloma/Leukemia effect

The Graft-versus-Myeloma effect was first hinted at by the observation that clinical remission of patients occurred more frequently following allogeneic rather than autologous stem cell transplantation (Cavo, Terragna et al. 2000; Martinelli, Terragna et al. 2000; Willems, Verhagen et al. 2000). Therefore, bone marrow reconstitution did not appear to be the sole mechanism of therapeutic action of the allografted cells. Further investigating this phenomenon, studies found that donor lymphocyte infusions (DLI) alone were able to lead to long-term remission even after clinical relapse following alloSCT in patients with MM (Kolb, Schattenberg et al. 1995; Collins, Shpilberg et al. 1997), as well as chronic and acute myeloid leukemia (Badros, Barlogie et al. 2002; Einsele, Schafer et al. 2003; Maloney, Molina et al. 2003). Furthermore, it has been shown that transfusion of T cell-depleted donor lymphocytes (Goldman, Gale et al. 1988; Horowitz, Gale et al. 1990; Marmont, Horowitz et al. 1991) and immunosuppression (Odom, August et al. 1978; Higano, Brixey et al. 1990) are associated with an increased rate of relapses. Graft-versus-Host-Disease (GvHD) was more frequently observed in patients treated with DLI (Kolb, Schattenberg et al. 1995) and this immunologic side effect were abrogated using CD8+ T cell-depleted DLI, while therapeutic efficacy was maintained (Alyea, Soiffer et al. 1998). This finding indicated that CD4+ T cells might represent the major effector cells mediating the GvM/GvL effect.

1.1.3.2 Antigen-specific immunotherapy

As the manifestation of Ehrlich's "magic bullet" theorem antigen-specific immunotherapy represents one of the most promising cancer treatment options to date. Following the identification of appropriate target structures, either in form of processed antigenic protein or surface antigens, the conditioning of immune effector cells against tumor specific proteins can be achieved by various mechanisms.

1.1.3.2.1 Vaccination

Vaccination using either full-length tumor protein or partial peptide sequences containing identified epitopes to be recognized by the respective effector cells is a straightforward method to induce a coordinated immune response against specific antigens. Such vaccines are usually enhanced using potent adjuvants, such as unspecific dendritic cell activators Bacillus Calmette-Guerin (Alexandroff, Jackson et al. 1999) or cytidine-phosphate-guanosine (CpG)-containing oligonucleotides (Brunner, Seiderer et al. 2000), and used in combination with other target proteins in order to prevent immunoselection and to achieve a broader anti-tumor activity. Although vaccination using full-length or partial tumor antigen carries the advantage of a more integrated immune response over highly focused approaches such as ex vivo expansion of preexisting immune responses, this aspect also harbors potential drawbacks. For example, it has been recently found that vaccination using partial MAGE-A3 peptide containing CD4+ epitopes in lung cancer patients leads to the induction of not only effector T helper cells, that are needed for the maintenance of cytotoxic CD8+ T cell activity, but also a significant number of CD25+FoxP3+ regulatory T cells (Francois, Ottaviani et al. 2009). These induced suppressor cells showed strong inhibitory activity and might be in part responsible for the lack of early clinical success of some of the current clinical vaccination studies (Rosenberg, Yang et al. 2004).

1.1.3.2.2 T cell based adoptive immunotherapy

Although technically demanding, adoptive cellular immunotherapy represents a promising approach in patients with spontaneous immune responses against tumor antigens or following tumor-specific vaccination. In brief, effector cells are initially acquired either in the form of whole blood or tumor-infiltrating lymphocytes from the respective patient. *Ex vivo* expansion of these cells can then be achieved via different methods, for example using unspecific activating cytokines or antibodies, e.g.

against the T cell receptor and CD3. In order to increase target specificity antigen-presenting cells (APC) can be pulsed using full-length or partial antigenic protein, and can subsequently be used to stimulate the expansion of autologous effector cells reactive against the respective antigen. This further allows the isolation and elimination of undesired subsets, such as immune suppressor cells, which may otherwise hamper the potential therapeutic efficiency.

1.1.3.3 Clinical studies

In the last decade multiple phase I and/or II clinical trials of active immunotherapies showed promising results leading to the recent initiation of several phase III studies. Unfortunately, all but one of these trials failed to achieve significant improvements regarding their respective clinical endpoints (Finke, Wentworth et al. 2007). As an antigen-specific immunotherapy of prostate cancer Sipuleucel-T, autologous APC loaded with the recombinant prostate cancer antigen prostatic acid phosphatase, represents the only exception showing a significant improvement in overall survival (Small, Schellhammer et al. 2006) and currently awaits FDA approval.

Although initially discouraging the majority of these findings are likely to be caused by methodological and conceptual problems (Finke, Wentworth et al. 2007). It has been shown that early immunotherapeutic treatment followed by conventional modes of therapy such as radiation and chemotherapy significantly improves survival compared with each modality alone (Demaria, Bhardwaj et al. 2005). In contrast, most of the discontinued phase III trials included highly heterogeneous patient collectives often in late stages of their disease. Furthermore, many of the failed studies investigated melanoma as it had been suggested to be subject to a significant immunologic control, but considering its generally rapid progression and high heterogeneity this disease might not be an ideal candidate for immunotherapeutic approaches in these early stages. In order to overcome these obstacles, we require detailed information on the dynamic of expression patterns of potential target structures. It has further become evident that comprehensive characterization of candidate malignancies, as well as the determination of the optimal clinical settings for immunotherapeutic approaches are essential.

Multiple Myeloma

1.1.4 Definition and epidemiology

Multiple myeloma originates from the malignant expansion of clonal plasma cells or plasmocytoid cells in the bone marrow. The excessive production of "paraprotein" by the malignant clone leads to the accumulation of complete or partial monoclonal antibodies in the serum and urine of patients (Goldschmidt 2002). This unique pathophysiology of MM was probably first described in 1845 and eventually linked to plasma cells (Clamp 1967). The introduction of methods such as electrophoresis and immunoelectrophoresis finally allowed the characterization of the observed abundance of protein as monoclonal antibodies (Longsworth LG 1939).

Contrary to prevalent misconceptions in the professional and non-professional environment, MM is not a rare disease. In fact, MM represents the second most common hematologic malignancy with approximately 3/100,000 new cases per year. Showing no significant changes in the overall incidence over the last 50 years, ethnicity appears to influence the probability of developing MM. Especially African Americans and Pacific Islanders show an increased incidence, while Asians generally show comparably low rates of this disease (Durie 2004).

Importantly, while MM is not exclusively a disease of the elderly, over 20% of patients are over the age of 70 (Kyle 1975). This demographic obviously influences the applicability of more aggressive therapeutic approaches and their outcome.

1.1.5 Etiology

The etiology of MM remains uncertain, although several contributing factors have been identified. Especially exposure to ionizing radiation (Stewart 1982), pesticides, herbicides, and dioxin represent potential pathogenic factors (Riedel and Pottern 1992). Furthermore, associations with certain viral diseases, such as HIV (Dezube, Aboulafia et al. 2004) and human herpesvirus-8 (Berenson and Vescio 1999), are discussed as causal factors in MM. Other possibilities, such as heritable genetic traits have been suggested and are currently under investigation (Chang 2005).

In 1998, Hallek proposed a linear model for the development of MM from the healthy plasma cell (Hallek, Bergsagel et al. 1998). In this model, plasma cells are initially immortalized but not transformed during the development of monoclonal gammopathy of undetermined significance (MGUS). In this stage, the plasma cells do not accumulate or cause significant end-organ damage. As the disease progresses, prolif-

erative capacity increases and the malignant plasma cells enrich within the bone marrow microenvironment. These cells start causing local bone damage through the secretion of various osteoclast-activating cytokines, such as RANK ligand, TNFα, and IL-1β. Finally, in a small subgroup of patients the previously contained MM cells are able to leave the bone marrow, proliferate even more rapidly, and ultimately lead to local and systemic organ damage.

1.1.6 Clinical manifestations

The most common specific presenting symptoms of patients with MM are bone pain, hypercalcemia, renal insufficiency, and anemia (Kyle 2004). Bone pain and hypercalcemia are usually caused by local osteoclast-activation, while renal insufficiency is in turn caused by hypercalcemia and tubular damage from light chain excretion by the malignant plasma cells. Anemia results from the production of various inhibiting cytokines by the malignant cells and bone marrow failure caused by a displacement of normal erythropoiesis through the excessive plasma cell expansion (Pezzutto 2007).

In the clinical setting proteinuria can be observed in close to 90% of patients with MM, while skeletal abnormalities as demonstrated by roentgenography are seen in approximately 80%. Immunoelectrophoresis represents one of the most specific tools in the detection of MM and is found positive in 90% of patients with confirmed MM (Kyle 1975).

1.1.7 Diagnostic criteria

In order to provide reliable diagnostic guidelines two sets of almost identical criteria have been formulated for the diagnosis of MM by the International Myeloma Working Group and the Mayo Clinic (2003). These guidelines also include criteria for related plasma cell disorders, such as MGUS, in order to allow for a more standardized determination of differential diagnoses.

In brief, diagnosis of MM requires a bone marrow infiltration with clonal plasma cells higher than 10% and the presence of urinary or serum monoclonal antibodies as determined by immunoelectrophoresis. Additionally, formal diagnosis of MM requires the presence of end-organ damage, either in the form of hypercalcemia, renal insufficiency, anemia, or bone lesions, as determined by conventional radiography. In contrast, MGUS can be differentiated from MM by a normal overall immunoglobulin

level, less than 10% bone marrow plasma cells, and the absence of end-organ damage despite the presence of monoclonal immunoglobulin.

1.1.8 Treatment options, therapy-resistance, and response criteria

High-dose induction chemotherapy followed by autologous stem cell transplantation (autoSCT) represents the therapy of choice for all patients under 65 years and patients over 65 years in good general condition (Ludwig 2005). In addition, several approaches relying on chemotherapy alone are available for the treatment of relapsed patients or individuals who are ineligible for autoSCT. Conventional protocols include the combined application of vincristin, adriamycin, and dexamethasone (VAD), or melphalan and prednisolon (MP) (Group 1998; Kyle 2008). These options have been enhanced or even replaced by the introduction of novel proteasome inhibitor bortezomib and thalidomide or its analoga, such as lenalidomide (Dimopoulos 2003; Dicato 2006). Introduction of these therapeutic approaches and clinical protocols aiding in the determination of optimal individual treatment strategies have significantly increased disease-free and overall survival (Anderson 2003; Richardson, Sonneveld et al. 2005; Lacy M 2006). Furthermore, the occurrence of severe adverse effects has been decreased significantly. Unfortunately, despite these remarkable advancements, almost all patients will eventually relapse (Bensinger 2009).

This observation has caused the investigation of distinct subpopulations of chemotherapy-resistant myeloma cells that might be responsible for the eventual outgrowth of the malignant cells and the associated clinical relapse. Recently, Matsui et al. have found that a small group of cells within the monoclonal plasma cell compartment of patients with MM show increased drug efflux and strong intracellular drug detoxification potential (Matsui, Wang et al. 2008). This population, lacking expression of the mature plasma cell surface marker CD138, was furthermore giving rise to clonogenic growth in vitro and was able to induce MM in NOD/SCID mice, while the bulk of CD138+ cells lacked this ability. These findings indicate that a stem cell-like subpopulation of MM cells might indeed be the cause for the eventual clinical relapse and therefore represent an important target of MM-specific therapeutic approaches.

High-dose induction chemotherapy followed by allogeneic stem cell transplantation might represent such a specific treatment, as it currently represents the only curative option in MM (Corradini, Cavo et al. 2003; Bruno, Rotta et al. 2007). Long-term survival or even cure in patients treated with this modality has been attributed to the

previously mentioned Graft-versus-Meloma effect. Unfortunately, despite the introduction of non-ablative induction regimen (Badros, Barlogie et al. 2002; Einsele, Schafer et al. 2003; Maloney, Molina et al. 2003), treatment-related mortality remains one of the major problems with alloSCT (Gahrton, Tura et al. 1995; Bjorkstrand, Ljungman et al. 1996; Alyea, Weller et al. 2003; Kroger, Einsele et al. 2003). Furthermore, the lack of HLA-matched family donors especially in old patients represents a major obstacle for a patient collective with a median age at diagnosis of 61 years.

The most widely used assessment of treatment response in MM has been developed by the European Group for Bone and Marrow Transplantation (EBMT) (Blade, Samson et al. 1998). The EBMT classification focuses on typical MM disease parameters, such as idiotype levels, plasma cell infiltration and end-organ damage (Table 2). In 2006 this system was replaced by a classification from the International Myeloma Working Group (IMWG), with the goal to unify assessment systems for ease of scientific comparability (Durie, Harousseau et al. 2006).

1.1.9 Staging and prognosis

Two systems are currently available for the staging and determination of median survival of patients with MM, although the more recent International Staging System (ISS) (Greipp 2005) has mostly replaced Durie-Salmon staging (Durie 1975) which had been developed over 35 years ago (Table 1). Some of the shortcomings of the Durie-Salmon system were its reliance on clinical parameters that are subject to a high interobserver variability, such as the number of osteolytic lesions, and its counter-intuitive complexity. In order to overcome these problems, ISS staging aimed to improve objectivity and simplicity by using two highly standardized parameters that can be determined in virtually any clinical laboratory, serum β2 microglobulin and serum albumin level. Both staging systems allow the classification of MM patients into three distinct groups and thereby provide the means for an estimation of median survival.

As knowledge about the molecular basis of MM increased other prognostic parameters emerged. These factors include additional serum parameters, such as elevated LDH (Hus, Dmoszynska et al. 2004), and especially the detection of cytogenetic abnormalities, such as the deletion of chromosome 13 (Fonseca, Barlogie et al. 2004) and translocations involving chromosome 14 (Fonseca, Blood et al. 2003). While these parameters are not included in current staging systems, some are already

used as prognostic factors, either individually or as part of independent staging systems (Facon, Avet-Loiseau et al. 2001).

As most samples analyzed in this study had been acquired prior to the formulation of ISS staging or even newer prognostic factors, most statistical evaluations involving disease staging were performed using the older Durie-Salmon classification.

2   Methods and material

Material and equipment

2.1.1 Patients and healthy donors

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

2.1.2 Bone marrow samples and myeloma cell line U266

330 BM samples from MM patients were obtained during routine diagnostic procedures performed between January 2004 and 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 months [range: 1-35 months]. Samples were acquired at different times during follow-up, with a median time after therapy of 24 months. Whole BM samples obtained from consented healthy donors were part of BM donations for alloSCT or were collected from blood donors, respectively. Myeloma cell line U266 was obtained from the DSMZ and was cultured according to the DSMZ's instructions.

2.1.3 Study design

This study followed a retrospective case-control pattern. Bone marrow samples were acquired from patients and healthy individuals and subsequently analyzed by RT-PCR, as well as exemplary Western Blots for their expression of four CT antigens and a housekeeping gene to determine the quality of the samples. All samples from patients who had at least once expressed one CT antigen were further analyzed using quantitative RT-PCR for longitudinal correlation with quantitative disease parameters. Clinical data were provided by the submitting department. After acquisition of the required experimental data, statistical analyses were carried out.

Methods

2.1.4 Determination of remission status

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 (EBMT) (Blade, Samson et al. 1998). Criteria were modified to account for the limited dataset available for each sample in this retrospective assessment. Complete remission (CR) was defined as BM plasma cell counts below 10%, negative serum immunofixation, physiological levels of the patients' respective paraprotein, and lack of progressive bone or kidney pathologies. Partial remission (PR) 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 physiological levels) and lack of progressive bone or kidney pathologies. "Progressive disease" (PD) was assigned to patients with one of the following criteria: BM plasma cell counts above 10%, highly elevated levels of the patients' respective paraprotein (more than 150% of physiological 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 (Durie, Harousseau et al. 2006). 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 treatment-related mortality (N=4) or loss to follow-up.

2.1.5 Separation of mononuclear cells

In a first step, the mononuclear cell (MNC) fraction was isolated from whole BM samples using Ficoll-Paque (Table 3). Due to its high specific weight, Ficoll-Paque allows separation of low-density MNC from erythrocytes and granulocytes. After separation and lysis of remaining erythrocytes, MNC were washed twice with PBS. MNC and cells from myeloma cell line U266 were lysed using RLT Buffer (Qiagen) for subsequent RT-PCR analysis or protein lysis buffer containing a Protease Inhibitor Cocktail (Sigma) for Western Blot and were stored at -80°C until needed.

2.1.6 Isolation of total RNA and cDNA synthesis

In order to determine the mRNA expression status of the investigated CT antigens, total RNA was extracted from all samples using the RNeasy Mini Kit (Table 3). RNA isolation was performed as recommended by the kit manufacturer. In brief, cells were disrupted using a QiaShredder column and 70% ethanol. Next, whole RNA was allowed to bind to a silica membrane and subsequently washed multiple times to remove DNA and protein residues. Finally, the RNA was eluted using RNAse-free water. Due to RNA's inherent instability caused by enzymatic digestion and degradation, cDNA was generated immediately after RNA isolation using 2μg RNA in a 20ul reverse transcriptase (RT) reaction, containing 1.5μl AMV RT and random primers as unspecific transcription initiation sites. Reverse transcription was run at 42 °C for 45 min followed by heat inactivation of the enzyme at 95 °C for 5 min. cDNA was stored at -20°C until needed.

2.1.7 Qualitative RT-PCR

In a process called Polymerase Chain Reaction (PCR) heat-stable DNA polymerases are used for the in vitro amplification of individual DNA sequences. These enzymes are able to synthesize double-stranded from single-stranded DNA, given appropriate initiation sites. These initiation sites are usually two short oligonucleotides containing sequences homologous to the respective ends of the desired transcript. During repeated melt-annealing cycles, PCR leads to the exponential amplification of the target sequence, which can be visualized, e.g. using gel electrophoresis and fluorescent DNA dyes.

For PCR analysis of the expression of single CT antigens, 4 μl first-strand cDNA (equivalent to 0.1 μg RNA) was amplified after preparation of 25 μl PCR reaction mixtures containing transcript-specific oligonucleotides (10 pMol), 2 U AmpliTaq Gold (Perkin Elmer, Weiterstadt, Germany), 10 nMol of each dNTP (dATP, dTTP, dCTP, dGTP), and 1.67 mM $MgCl_2$ (Table 3). PCR primer sequences and conditions used for the analysis are given in Table 4. Following 35 PCR cycles, products were separated on 1.5% agarose gels, stained with ethidium bromide, visualized with UV light, recorded using a CCD camera, and assessed for expected size. CDNA quality was tested by RT-PCR measuring expression of housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Negative controls without cDNA and cDNA derived from human testis RNA (Ambion) as a positive control were integrated into all PCR reactions.

2.1.8 Quantitative RT-PCR

Quantification of DNA amplification was achieved using the Lightcycler system (Roche) with SYBR green, a fluorescent dye with the ability to bind double stranded DNA. Primer sequences for target genes used in real-time PCR are given in Table 4. A master mix of the following components was prepared at the final concentrations indicated: 4.0 mM $MgCl_2$, 400 nM forward and reverse primers, 200 nM dNTP (Invitrogen), 1% DMF, BSA at 250 µg/ml, SYBR Green I (Sigma) diluted 1:20000, and 1 Unit FastStart taq polymerase (Roche Diagnostics) in a total volume of 20 µl (Table 3). After an initial denaturation at 95°C for 10 minutes, PCR reactions were cycled 40 times. Target-specific programs are given in Table 4. Fluorescence intensity was measured at the end of each elongation phase. A melting curve analysis was carried out immediately after amplification in order to determine specificity of the PCR reaction. A standard curve prepared from the PCR product cloned into pCR2.1 using the TA cloning kit (Invitrogen) was prepared to determine absolute copy numbers of target transcripts in cDNA samples.

2.1.9 Sequencing

Determining correct target amplifications, all PCR products were analyzed exemplarily in testis cDNA and in at least two patient samples using BigDye-terminator sequencing. In brief, terminator nucleotides are coupled to unique fluorescent labels, allowing the distinction between bases at each termination point according to the distance traveled during capillary electrophoresis. Following regular PCR amplification, the respective product was gel-purified and 6µl of the purified sample subsequently subjected to the sequencing reaction using the commercially available BigDyeKit (2.5% reaction buffer, dNTP, fluorescence-labeled terminator deoxynucleotides, and Taq polymerase) and 2.5µl 10pM forward and reverse primers in separate reactions (Table 3). Following the sequencing reaction the DNA was precipitated using sodium acetate and ethanol followed by centrifugation for 30min at 4°C and 13,000rpm. Finally, the pellet was dried using a vacuum centrifuge. Dye-terminated sequences were analyzed in an ABI Prism 3100 Genetic Analyzer (AppliedBiosystems).

2.1.10 Western Blot

Western blot analysis allows separation of proteins according to different properties. The most common approach represents sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) facilitating the migration of proteins in an electric field following denaturation. Differently sized proteins travel at different speeds and thus allow distinction by molecular weight after transfer to a nitrocellulose membrane and appropriate visualization.

Lysed BM cells and cells from myeloma cell line U266, which was used as a positive control, were denatured for 10 minutes at 70°C. Samples of lysates containing 30µg total protein were resolved on 4-12% 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 primary monoclonal antibody for 4h at room temperature (Table 3). Antibodies against CT antigens were provided by the New York branch of the Ludwig Institute for Cancer Research. Anti-β-actin antibody was obtained commercially (Santa Cruz Biotechnology). Secondary HRP-labeled anti-mouse monoclonal antibody (R&D Systems) was applied for 1h at room temperature. Specific binding was visualized by chemiluminescence (ECL Western Blotting Analysis System, Amersham Biosciences).

2.1.11 Statistics

All statistical analyses were carried out using SPSS 14 (SPSS Inc., IL, USA). Pearson's correlation was used to analyze correlations between BM plasma cell counts, paraprotein levels, and MAGE-C1/CT7-levels determined by quantitative RT-PCR. Correlations between clinicopathological parameters and CT antigen expression were assessed using Pearson's Chi-square test. Log-Rank test and Cox-regression analysis were performed for the evaluation of survival and relapse in MM patients. Results were considered significant if $p < 0.05$. Figures were created using Microsoft Excel 2003 (Microsoft Deutschland GmbH, Unterschleißheim, Germany), SigmaPlot (Systat Software Inc., San Jose, CA, USA), and Adobe Photoshop (Adobe Systems GmbH, Munich, Germany).

3   Results

Baseline analysis of CT antigen expression

3.1.1 Patient characteristics

Analyzing the clinicopathological characteristics of all 129 MM patients (Table 5A), the patient collective showed a male predominance, the typical patient was approximately 56 years old and IgG kappa represented the most common idiotype. While 16% of the patients were included immediately after initial diagnosis, most patients had already received therapy before this study was initiated. The only patient-associated clinico-pathological parameter correlating with the expression of a minimum of one CT antigen in at least one of the patient's samples was age at the time of inclusion (Table 5A).

When analyzing all 330 samples including repeated samples from the same patients, I further observed significant correlations of CT antigen expression with serum albumin levels, serum hemoglobin levels, remission status, type of therapy, and BM plasma cell infiltration (Table 5B). Interestingly, correlating clinicopathological parameters with the number of simultaneously expressed CT antigens, I observed significantly higher numbers of CT antigens expressed in patients with serum hemoglobin levels below 13g/dl ($p < 0.05$), elevated BM plasma cell counts ($p < 0.001$) and age above 60 at the time of inclusion ($p < 0.05$).

3.1.2 CT antigen expression and conventional disease parameters

Evaluating the overall frequency of CT antigen expression in BM samples obtained from all newly diagnosed MM patients (N=21), I found that 38% of patients expressed at least one CT antigen on the RNA level. Among newly diagnosed patients with stage I/II disease 29% were CT antigen-positive while the rate of subjects expressing CT antigens increased to close to 60% among patients with stage III disease (differences between groups were not significant, Figure 1A). Importantly, the control group showed no expression of CT antigens.

The observation of a correlation between the level of BM plasma cell infiltration and the detection of CT antigens indicated that this factor might represent a potential confounding variable in subsequent analyses. Therefore, I next restricted my analysis to patients with a significant tumor load, as defined by a BM plasma cell infiltra-

tion higher than 10%, applying qualitative RT-PCR (Figure 1B). Remarkably, 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%. As expected when I analyzed all 330 samples, higher BM plasma cell infiltration was not only associated with high individual expression frequencies but also a higher number of simultaneously expressed CT antigens (Figure 1C).

Figure 1: Correlation of CT antigen expression with conventional disease parameters
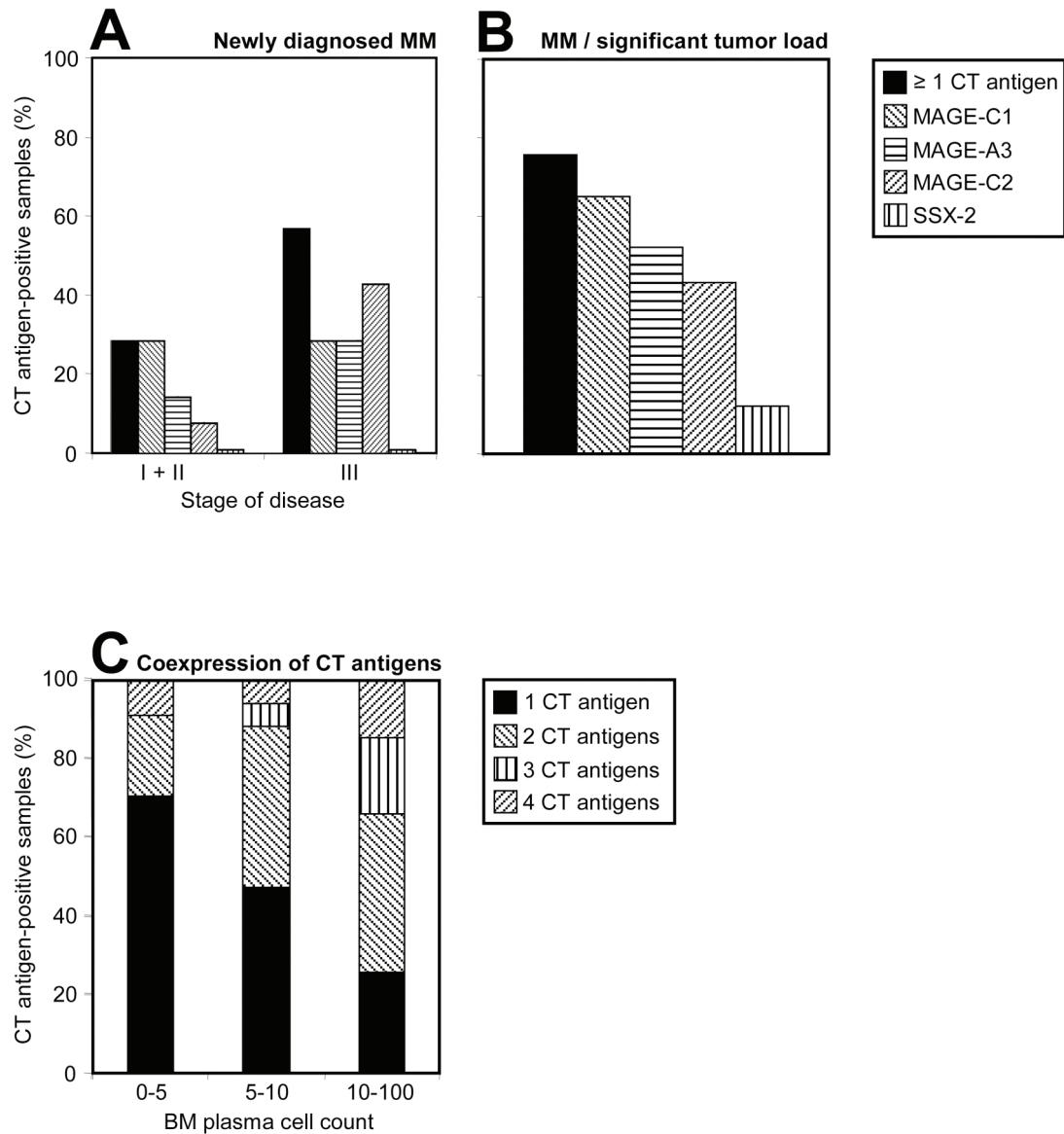
21 samples from newly diagnosed patients and 309 samples from 108 previously treated patients were analyzed for the expression of 4 CT antigens and housekeeping gene GAPDH by RT-PCR.

(A) Bars represent percentages of CT antigen-expressing samples in newly diagnosed patients (n=21) per stage.

(B) Bars represent percentages of samples (n=91) expressing CT antigens obtained from patients (n=71) with significant BM plasma cell infiltration >10%.

(C) Bars represent percentages of all CT antigen-positive samples (n=113) simultaneously expressing the respective number of antigens, depending on BM plasma cell infiltration.

3.1.3 Comparison of mRNA and protein expression levels

In order to confirm CT antigen expression on the protein level, I performed Western Blot analyses for MAGE-C1/CT7, MAGE-C2/CT10, and MAGE-A3 on BM samples from a randomly selected group of 10 MM patients and 10 healthy donors from whom lysates of total bone marrow were available. I found that two patient samples expressed all three 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 while the control group expressed none of the evaluated CT antigens on the protein or on the RNA level. In addition, 6 patients' samples showed expression of MAGE-A3 protein (Figure 2).

Figure 2: CT antigen expression on the mRNA and on the protein level
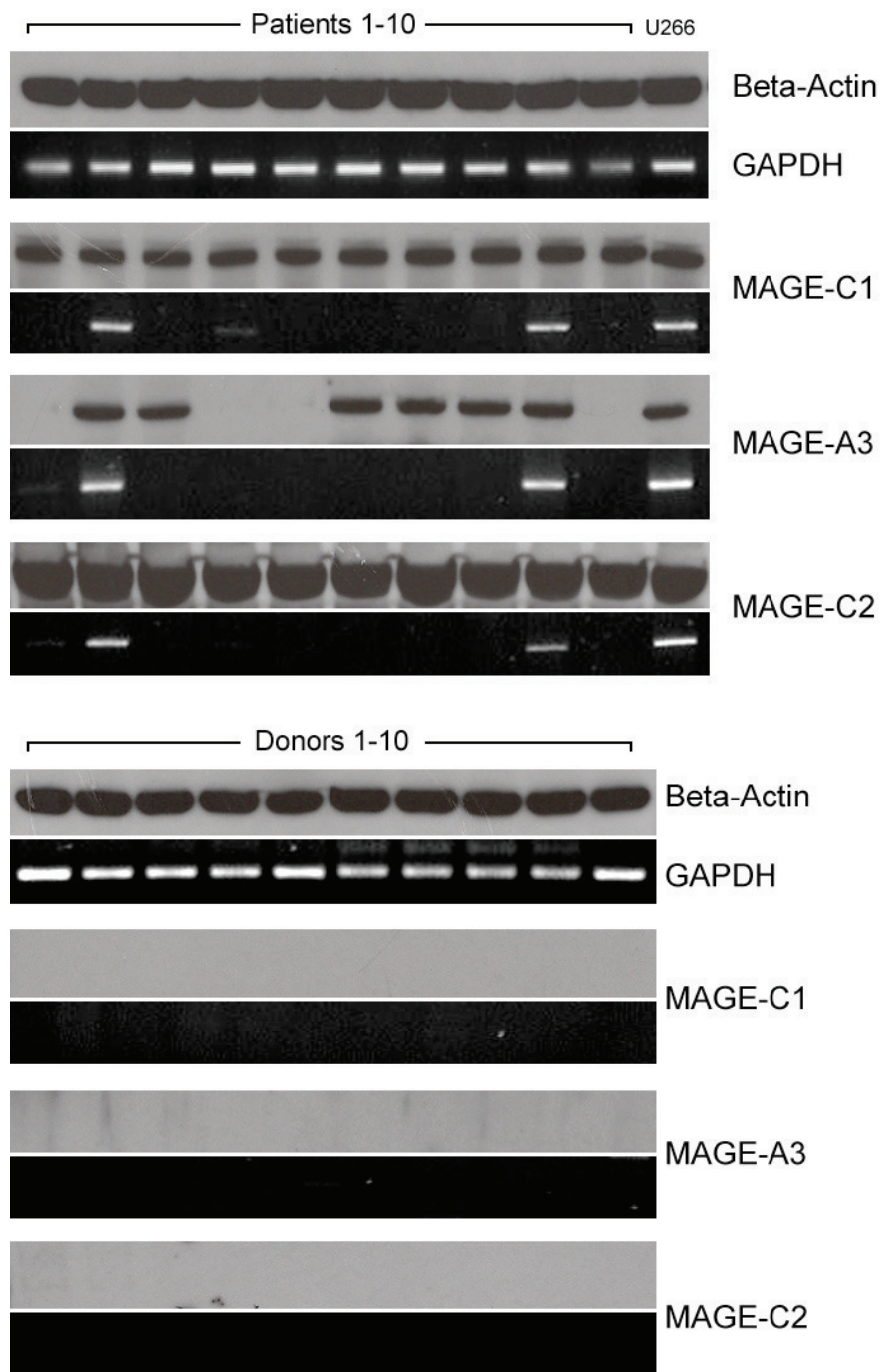
10 BM samples from MM patients and cells from myeloma cell line U266 were analyzed for the expression of MAGE-C1/CT7, MAGE-A3, and MAGE-C2/CT10, by qualitative RT-PCR (lower rows) and Western Blot (upper rows).

3.1.4 Coexpression patterns of CT antigens

Some CT genes have been suggested to perform a so called "gatekeeper" function. In theory, the presence of these genes would be required for the expression of other CT genes within the same tumor. So far, data regarding potential "gatekeeper" properties of individual CT antigens in MM remain inconclusive.

Investigating coexpression patterns of the 4 CT antigens examined in this study, I did not observe a significant influence of the presence of MAGE-A3, MAGE-C2/CT10, or SSX-2 on the expression of the remaining antigens (Figure 3). In contrast, the detection of MAGE-C1/CT7 strongly predicted concomitant expression of the remaining antigens, even of MAGE-A3 which had previously been suggested to represent a gatekeeper CT gene in solid tumors (Gure, Chua et al. 2005). These findings suggest that MAGE-C1/CT7 might provide a "gatekeeper" function for the expression of other CT antigens in MM.

Figure 3: Coexpression analysis of CT antigens in MM
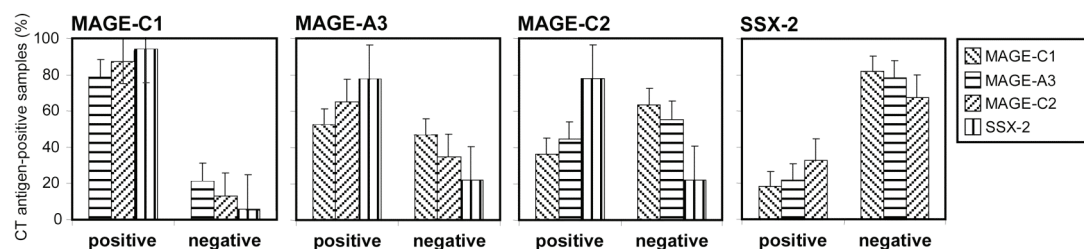
Analysis of 330 samples from 129 MM patients for the expression of 4 CT antigens suggested that MAGE-C1/CT7 might provide a "gatekeeper" function for other CT antigens. Graphs show samples positive or negative for the CT antigen indicated in the title and bars represent percentages of samples expressing the given CT antigens, flags indicate margin of error at 90% confidence.

3.1.5 Association of CT antigen expression 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, I compared the three generally available types of MM-specific therapy – conventional chemotherapy, autologous stem cell transplantation (autoSCT), and allogeneic stem cell transplantation (alloSCT) - regarding effects on the frequency of CT antigen expression in the BM of all patients who had expressed CT antigens at least once.

In a first step, I compared samples from previously treated patients in different states of clinical remission. Remarkably, I 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 non-responders to therapy or who showed progressive disease expressed CT antigen mRNA in 90% of cases (Figure 4A).

Since I had observed a highly significant ($p < 0.001$) correlation between remission status and therapeutic modality (data not shown), I also analyzed CT antigen expression depending on the mode of therapy. I found that after chemotherapy alone, 100% of all patients still expressed at least one CT antigen, while autoSCT significantly reduced expression to 77% (Figure 4B). The strongest reduction, however, was achieved in patients post alloSCT whose BM was found positive for CT antigen expression in only 40%.

Interestingly, expression of the antigens reacted differently to the individual modes of therapy. MAGE-A3 and SSX-2 expression was strongly reduced after autoSCT without alloSCT further diminishing the number of BM samples showing an expression of these CT antigens. In contrast, despite a strong reduction in MAGE-C1/CT7 and MAGE-C2/CT10 expression following autoSCT, alloSCT was able to even further decrease BM-related expression of these CT antigens.

Figure 4: Correlation of CT antigen expression with response to treatment and mode of therapy
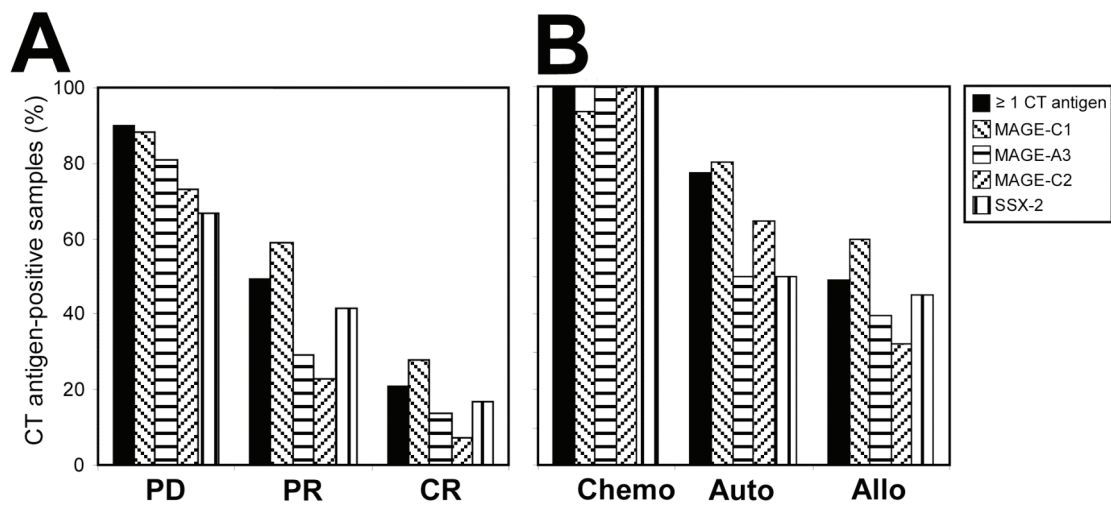
180 samples from 68 previously treated patients who had expressed the respective CT antigen at least once were analyzed for the expression of MAGE-C1/CT7 (N=137), MAGE-A3 (N=133), MAGE-C2/CT10 (N=81), SSX-2 (N=33) and housekeeping gene GAPDH by RT-PCR. Remission status was evaluated for all samples individually (A) and if more than one therapy had been applied the latest was used for the definition of treatment status (median time after therapy: 21 months) (B).

Longitudinal observations

3.1.6 Persistence of CT antigen expression in patients with increased tumor load

So far, CT antigen expression has not been systematically analyzed over time in MM or in any other human cancer. When I examined repeated samples (n=31) from all MM patients, I found that when a patient was positive for a specific CT antigen at one point in time, the probability for subsequent samples with an increased tumor load, as defined by a BM plasma cell infiltration higher than 10%, to be positive for the same antigen was 98% (MAGE-C1), 92% (SSX-2), 87% (MAGE-A3) and 80% (MAGE-C2), respectively. This finding suggests a remarkably persistent expression of CT antigens in the BM-residing malignant plasma cells of patients with MM.

3.1.7 Correlation between MAGE-C1/CT7 expression levels and quantitative disease parameters

After confirming MAGE-C1/CT7 as the most frequently and persistently expressed CT antigen in this study, I investigated whether expression levels of MAGE-C1/CT7 correlated with the clinical course of the disease. To this end, I performed a longitudinal analysis of repeated BM samples from MM patients applying quantitative RT-PCR. As suggested by the high coexpression of potential "gatekeeper" gene MAGE-C1/CT7, the possibility of significant levels of MAGE-C1/CT7 in patients expressing at least one of the four antigens seemed likely. Therefore, I included all patients with at least 3 consecutive samples within a time-frame 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]) were analyzed (median follow-up: 21 [range 6-35] months).

I 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 performed during quantitative RT-PCR.

Changes in MAGE-C1/CT7 expression levels correlated with variations in the patients' BM plasma cell counts (Figure 5A). Importantly, a stronger association was found between MAGE-C1/CT7 expression and the development of the patients' paraprotein levels in the peripheral blood (Figure 5B). These results underscore the

finding that MAGE-C1/CT7 expression correlates with the clinical status of the disease, reflects the effectivity of therapeutical interventions, and might be a reliable marker for relapse and progressive disease.

Figure 5: Correlation of CT antigen expression with tumor load and paraprotein levels
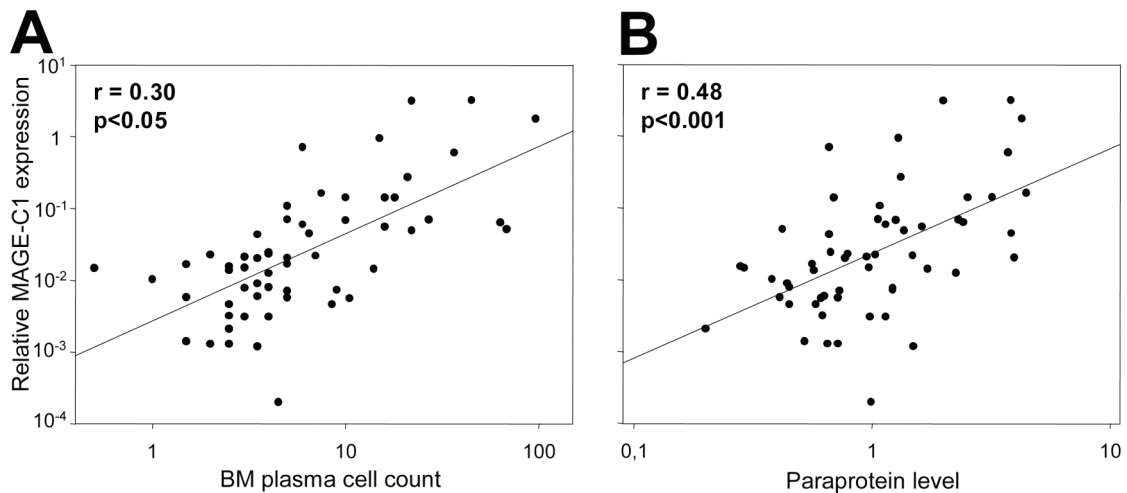
This figure illustrates the analysis of 58 samples from 11 patients who had previously expressed MAGE-C1/CT7 and from whom at least three consecutive samples within a timeframe of 12 months were available. Applying real-time PCR, MAGE-C1/CT7 levels were normalized to GAPDH levels and correlations between MAGE-C1/CT7 expression, plasma cell numbers (A), and paraprotein levels relative to the idiotype's respective upper physiological limit (B) were calculated using analysis of covariance.

3.1.8 Association of CT antigen expression with the clinical course and therapeutic interventions

To illustrate the individual consistency and reliability of CT antigen expression in patients undergoing different clinical phases of the disease all 22 patients were analyzed individually for the relationship between the clinical course of the disease including therapeutic interventions and MAGE-C1/CT7 (quantitative and qualitative RT-PCR) as well as MAGE-A3 (only qualitative RT-PCR) expression. Generally, changes in MAGE-C1/CT7-levels paralleled changes in BM plasma cell infiltration and paraprotein levels (Figure 6). Positive results from qualitative RT-PCR for MAGE-C1/CT7 were associated with higher levels measured by quantitative PCR, coinciding with increased tumor load in the BM.

The first group of patients had received alloSCT and had not relapsed in the time of observation. In these patients I found normal levels of conventional clinical response parameters and consistently low, but clearly detectable, levels of MAGE-C1/CT7 (Figure 6A). These findings suggest that low levels of MAGE-C1/CT7 expression indicate sustained remission and the persistence of minimal residual disease in the bone marrow from MM patients following alloSCT.

The second group of patients evidenced an increased tumor load at the beginning of the observation period but showed a significant response to therapeutic interventions. In these patients, decreases in conventional disease parameters paralleled a reduction of MAGE-C1/CT7 levels, as detected by real-time PCR of total bone marrow RNA (Figure 6B). Thus, serial analysis of MAGE-C1/CT7 expression represented a marker for response to therapy in MM patients but also showed that in most patients alloSCT was not able to eradicate MRD.

In contrast, the third group consisted of patients who developed increasing MAGE-C1/CT7 levels within the time of observation (Figure 6C). In two cases MAGE-C1/CT7 expression again correlated closely with clinical parameters indicating relapse of the disease. Other members of the third group showed an increase in MAGE-C1/CT7 levels at the end of the observation period, suggesting that this change in expression levels might have preceded later relapses.

Figure 6: Longitudinal analysis of MAGE-C1/CT7 expression levels and the clinical course of MM patients
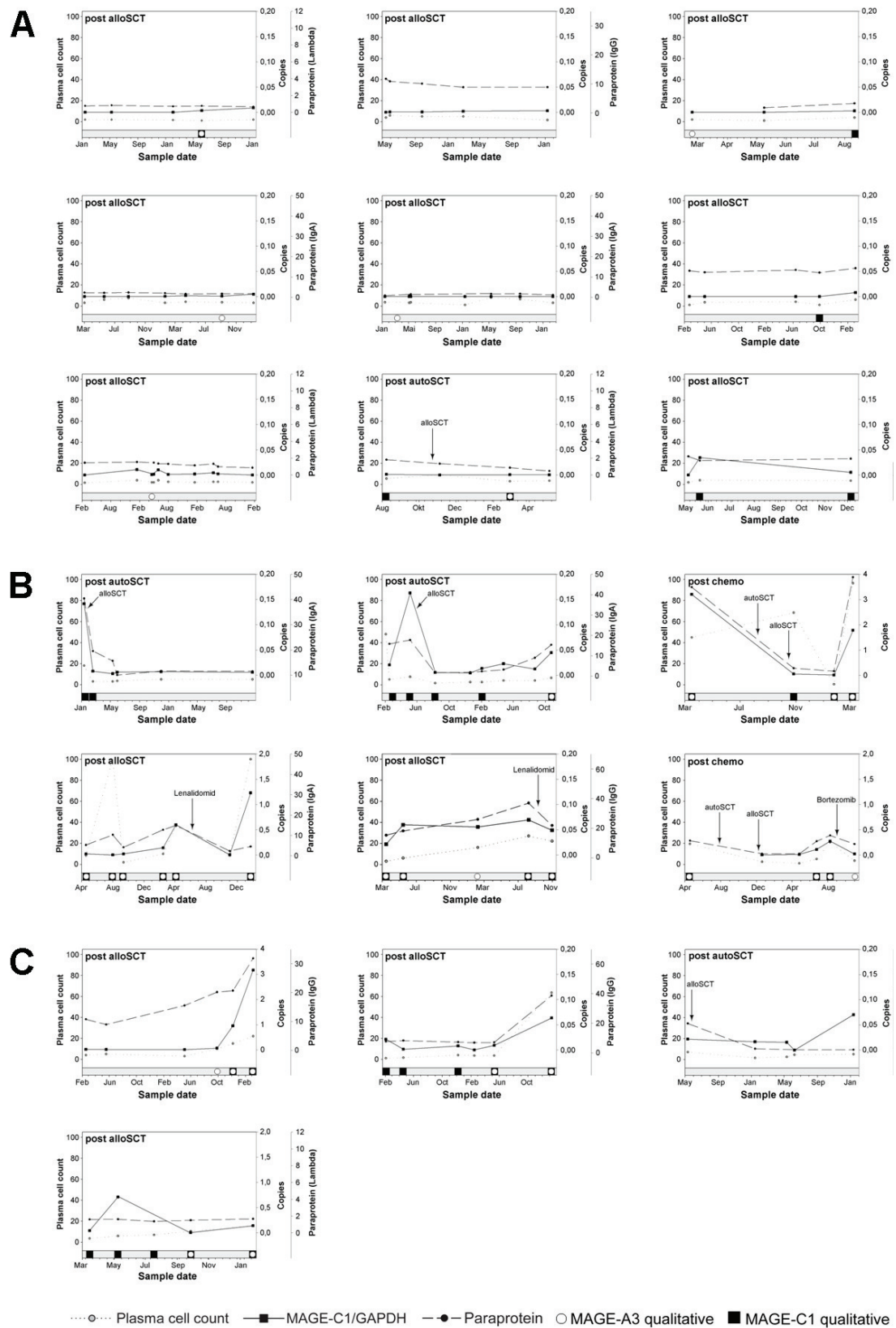
99 samples from 19 patients who had previously expressed one of the four examined CT antigens and from whom at least three consecutive samples within a time-frame of 12 months were available were analyzed for the expression of MAGE-C1/CT7 by quantitative RT-PCR. MAGE-C1/CT7 levels were normalized for the expression of hous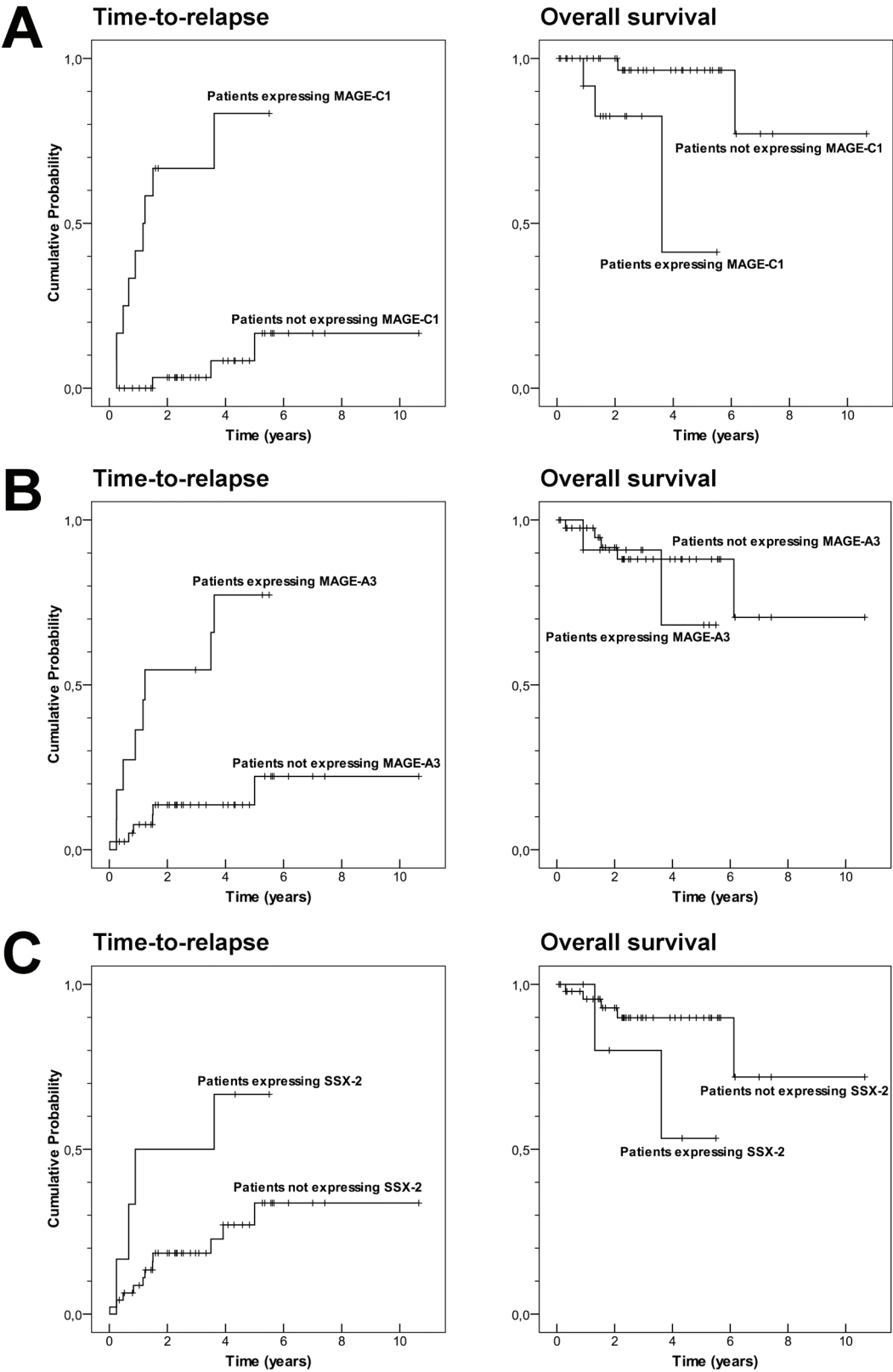ekeeping gene GAPDH. Patients were divided into three groups of patients with persistent complete remission following alloSCT (A), patients with a reduction of conventional disease parameters following therapeutic intervention (B), and patients with increasing levels of MAGE-C1/CT7 (C).

3.1.9 Relationship between CT antigen expression, overall survival, and time to relapse

Based on the observation of a strong association of CT antigen expression with stage of the disease and remission status, I 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, I evaluated the course of the disease of the largest collective of patients who were in a state of PR after alloSCT (N=52; median time since alloSCT: 31 months [range 2-128 months]). While a higher risk for relapse was indeed associated with the expression of MAGE-A3 (p<0.001), SSX-2 (p=0.03), and MAGE-C2/CT10 (p<0.001) (Figure 7A-D), I observed the most distinct association for MAGE-C1/CT7 (Figure 7A). Patients who had not expressed MAGE-C1/CT7 showed a favorable course of the disease, with only 7% of patients evidencing a relapse within a median time of observation of 41 months (Figure 7A). 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 (Figure 7A). The difference in time-to-relapse between the CT antigen-positive and CT antigen-negative group was highly significant (p<0.001).

Next, I 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 post alloSCT. I indeed observed that disease-related death occurred more frequently and earlier after alloSCT in the MAGE-C1/CT7 positive group (p=0.003; Figure 7A). The same observation, albeit to a lesser extent, was made for SSX-2 (p=0.02; Figure 7C). A subsequently performed multivariate Cox regression analysis taking into account a number of relevant clinicopathological parameters confirmed MAGE-C1/CT7 as the only significant and independent predictor of relapse (Table 6) and overall survival (Table 7) in MM patients treated with alloSCT (initial stage, deletion 13q14 and isotype were rejected as covariates due to insignificant Log-rank results).

Figure 7: Relationship between CT antigen expression, overall survival, and time to relapse

D

Time-to-relapse

Cumulative Probability

Patients expressing MAGE-C2

Patients not expressing MAGE-C2

Time (years)

Overall survival

Patients expressing MAGE-C2

Patients not expressing MAGE-C2

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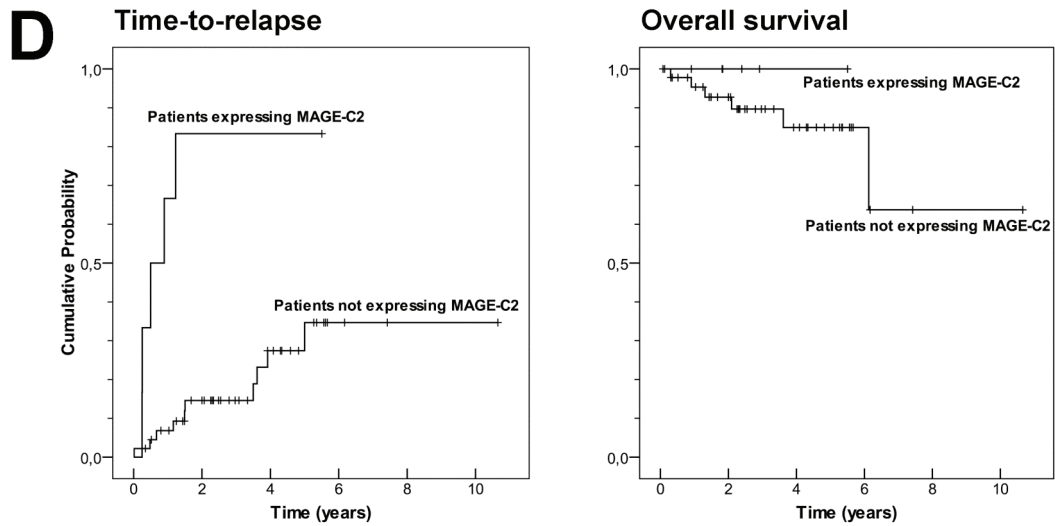
52 Patients, who had received alloSCT and from whom follow-up data were available, were analyzed for time-to-relapse, as well as overall survival and divided into groups according to expression of MAGE-C1/CT7 (A), MAGE-A3 (B), SSX-2 (C), and MAGE-C2/CT10 (D) as measured by RT-PCR. Curves represent Kaplan-Meier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

4   Discussion

General considerations

The introduction of autologous stem cell transplantation and novel chemotherapeutic agents has vastly improved the outcome of MM-specific therapy over the past two decades. However, a median survival of 4-5 years, the frequent development of therapy-resistance, and relapse in over 90% of patients with MM clearly demonstrate the need for alternative therapeutic options. Targeted immunotherapy represents a promising approach in this setting and CT antigens have been suggested as potential therapeutic targets due to their high tumor-specificity and immunogenicity in this disease. In addition, these properties would theoretically render them ideal diagnostic and prognostic tools. Despite these promising features, CT antigens had not been analyzed over time in patients and this study represents the first evaluation of longitudinal CT antigen expression and its predictive impact on MM.

Patients and baseline expression of CT antigens

In order to determine whether the included patient collective was representative for the general MM population I investigated patient characteristics and indeed observed a common distribution of conventional parameters age, sex, idiotype, and initial stage. The overall frequency of the tumor-specific expression of CT antigens in MM had been established previously (Pellat-Deceunynck, Mellerin et al. 2000; De Vos, Thykjaer et al. 2002; Gure, Wei et al. 2002; Jungbluth, Ely et al. 2005; Jungbluth, Ely et al. 2005; Taylor, Reiman et al. 2005; Atanackovic, Arfsten et al. 2007; Atanackovic, Arfsten et al. 2007; Condomines, Hose et al. 2007; Condomines, Hose et al. 2007; Tinguely, Jenni et al. 2008). Determining baseline CT antigen expression frequencies my findings closely mirrored the published results indicating a general comparability of the collective. Importantly, the control group showed no expression of CT antigens, which is in accordance with previous studies investigating CD138+ plasma cells and CD34+ progenitor cells isolated from the peripheral blood or bone marrow from healthy donors (Atanackovic, Arfsten et al. 2007).

Evaluating CT antigen expression in all patient samples I observed significant correlations with established clinical disease parameters hemoglobin, serum albumin, and BM plasma cell infiltration, as well as modality of treatment, and response to treatment. In the group of newly diagnosed patients, initial stage also correlated with CT antigen expression. Although I observed a trend of a more frequent CT antigen ex-

pression following the development of increased serum β2-microglobulin levels, this observation did not achieve statistical significance. Altogether, these findings indicate that CT antigen expression is associated with increased disease activity/progression, a finding, which is in line with previous studies investigating solid tumors (Bodey, Siegel et al. 2002; Honda, Tamura et al. 2004; Bergeron, Picard et al. 2009).

While this might simply be the result of an increased tumor load, several observations indicate a distinct biological change in the malignant cells leading to the initiation of CT antigen expression, such as the coordinated coexpression of multiple CT antigens (Sahin, Tureci et al. 1998; Tajima, Obata et al. 2003). The hypothesis of a CT antigen activating cellular program was tentatively confirmed by findings of a frequent association of CT antigen expression with hypomethylation of the genes' respective promoter regions (Sigalotti, Coral et al. 2002; Simpson, Caballero et al. 2005; Meklat, Li et al. 2007). Such hypomethylation is a frequent occurrence in many types of cancer and might therefore represent an epigenetic switch causing the malignant cells to coordinately express CT antigens. My finding of a more frequent CT antigen expression in patients older than 60 years might also be related to an association of differential methylation patterns with age (Bjornsson, Sigurdsson et al. 2008).

When I performed exemplary immunoblots against the three MAGE antigens using highly specific monoclonal antibodies I observed a surprisingly frequent protein expression. All of the 10 randomly selected patient samples evidenced protein expression of MAGE-C2/CT10 and MAGE-C1/CT7. Eight showed expression of MAGE-A3. In contrast mRNA expression indicated significant expression levels in only three patients. This finding not only suggests that mRNA expression of CT antigens in MM directly translates into protein expression of the given antigen but also raises the possibility of noticeably higher expression rates of CT antigens in MM than previously indicated by the commonly performed analysis of mRNA expression using conventional RT-PCR.

The obvious discrepancy between CT antigen expression as indicated by RT-PCR and Western Blot might theoretically 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 as determined in this study argues against this explanation. It would further be conceivable that, despite the comparably weak mRNA expression of CT antigens, a phenomenon known from previous studies (Atanackovic, Arfsten et

al. 2007), the strong protein expression is in fact the result of a low protein turnover rate.

Interaction of CT antigens

The identification of several CT antigens as transcription factors (van der Bruggen, Traversari et al. 1991; Cho, Lim et al. 2002; Wang, Han et al. 2002) and their common coexpression suggest that some members of this gene family might be able to regulate the expression of others. Such gatekeeper genes would be particularly promising targets of therapeutic approaches, due to their presumed functional relevance for the tumor and associated expression of downstream antigens as combination partners in an immunotherapeutic setting. MAGE-A3 had been previously proposed as a putative gatekeeper gene (Atanackovic, Arfsten et al. 2007).

In order to determine whether individual antigens might exert such regulatory functions, I evaluated coexpression patterns of all investigated antigens. While I did not observe an association with MAGE-A3, I found that expression of MAGE-C1/CT7 was indeed commonly associated with MAGE-C2/CT10, MAGE-A3, and SSX-2 expression. As MAGE-C1/CT7 represented the most frequently expressed antigen in this study, a common coexpression was expected. However, the magnitude of this observation suggested that additional mechanisms might be in place requiring the coexpression of MAGE-C1/CT7.

Influence of therapy and treatment response on CT antigen expression

In order to identify ideal clinical settings for future immunotherapeutic approaches and a potential use of CT antigens as response parameters I analyzed changes in their expression frequency and patterns in both settings.

Only a small minority of patients in complete remission expressed one of the four antigens. 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. These findings indicated a very close relationship between CT antigen expression and the extent of the disease. In accordance with my previous observation of a correlation between plasma cell infiltration and CT antigen expres-

sion, these findings are likely to be caused in part by a reduced number of malignant cells following therapeutic intervention.

Evaluating the impact of the three types of therapy on the expression of CT antigens, the strongest effect was observed for alloSCT. This finding might be an unspecific effect of high-dose chemotherapy and alloSCT and might be based on an increased depth of remission following this mode of therapy. However, it may also be the result of a specific elimination of CT antigen expressing myeloma cells by transplantation-induced immune mechanisms. One immediate consequence of such an active elimination of CT-antigen-positive malignant plasma cells may be the comparably strong reduction in the expression of MAGE-C1/CT7 and MAGE-C2/CT10 in the BM from MM patients following alloSCT as both antigens have previously been shown to elicit spontaneous immune responses in different solid tumors (Gure, Stockert et al. 2000; Wang, Han et al. 2002; Li, Qian et al. 2003; Ma, Germeau et al. 2004) as well as in MM (Curioni-Fontecedro, Knights et al. 2008).

Persistence of CT antigen expression

Evaluation of CT antigens as future immunotherapeutic targets and novel diagnostic markers in human cancers requires information about their expression over time, as downregulation and immunoselection represent potential mechanisms of tumor escape. Therefore, I carried out a longitudinal analysis of CT antigen expression to answer the question whether these antigens are consistently expressed and whether RT-PCR represents a sufficiently sensitive approach for their quantification.

I found that, if a patient had expressed a CT antigen at least once, the probability for recurrence of the same antigen during clinical relapse was close to 100% for some antigens, suggesting that these genes are indeed consistently expressed over the course of months and years and, most importantly, that downregulation of the investigated CT antigens is not a common mechanism of tumor escape in this disease. In addition, my findings suggest that CT genes might even be used as markers for the repeated detection of MRD present in the bone marrow of patients with MM.

Performing analyses of 19 patients' individual clinical courses, a remarkably close correlation between BM plasma cell counts, paraprotein levels and MAGE-C1/CT7 expression levels in patients with persisting remission as well as patients with complex progressions was observed. While these strong correlations retrospectively might appear trivial, reflecting the earlier relationship between disease stage and CT antigen expression, this study showed for the first time in an intraindividual approach

that independent loss or downregulation of CT antigens represents a rare exception. This finding was further substantiated by my observation of a low but distinct expression of MAGE-C1/CT7 over the course of the disease even in patients evidencing clinical remission.

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 caused by 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 the finding, that simultaneous expression of CT antigens is linked to higher BM plasma cell counts, CT antigens might specifically be activated in proliferating tumors as discussed earlier. This would further be supported by the close relationship between CT antigens and cellular functions involved in malignant transformation.

CT antigens as markers of minimal residual disease and immunotherapeutic targets

Minimal residual disease has been suggested to represent the leading cause for relapse in patients with MM. It has been associated with distinct functional properties that render a subset of cells capable of excessive proliferation and escape from conventional chemotherapeutic approaches.

A recent study showed that a small population of stem cell-like MM cells are resistant to conventional chemotherapy and were able to initiate clonogenic growth, while the bulk of myeloma cells lacked this ability (Matsui, Wang et al. 2008). In addition, studies showed that differential overexpression of anti-apoptotic proteins in MM commonly promotes the protection of MM cells especially from induced apoptosis (Spets, Stromberg et al. 2002; Bharti, Shishodia et al. 2004).

Importantly, several studies have implicated CT antigens in these functional systems. The expression of CT antigen CAGE, for example, has been associated with the proliferative capacity of cancer cell lines derived from solid tumors (Cho, Lim et al. 2002; Shim, Shim et al. 2006). In addition, recent findings by Jungbluth et al. had indicated that in MM patients, an increased expression of either MAGE-C1/CT7 or MAGE-A3 was associated with a significantly higher proportion of proliferating plasma cells within the bone marrow (Jungbluth, Ely et al. 2005), an observation which is in agreement with results obtained by Tinguely et al. (Tinguely, Jenni et al.

2008). The expression of MAGE-A genes has further been found to be associated with a chemotherapy-resistant phenotype (Monte, Simonatto et al. 2006). Altogether, these findings strongly suggest that CT antigens play an important role in functional systems associated with MRD.

While CT antigens are likely to be associated with the MRD population of MM cells, specific members of this gene family had not been identified as potential markers or target structures in this disease. My finding of a frequent expression of MAGE-C1/CT7 in the BM from patients during clinical remission, as well as its persistence over the course of the disease indicates that this gene may represent such a MRD parameter. Furthermore, my results show that determination of MAGE-C1/CT7 mRNA expression by quantitative RT-PCR is not only highly sensitive but also very specific.

Apart from these diagnostic considerations, CT antigens have been mostly pursued as immunotherapeutic targets, especially in solid tumors. Expression data from this and previous studies, however, suggest that MM might represent one of the most promising candidates for CT antigen-specific immunotherapy. This idea is further supported by the fact that MM has been found to be controlled to a large degree by the human immune system with CD4+ T cells emerging as the main effector population (Corradini, Cavo et al. 2003; Bruno, Rotta et al. 2007).

Unfortunately, the only available treatment that is able to harness this curative potential, alloSCT, is associated with a markedly increased treatment-related mortality. In addition, it is limited to a subset of patients (Gahrton, Tura et al. 1995; Bjorkstrand, Ljungman et al. 1996; Alyea, Weller et al. 2003; Kroger, Einsele et al. 2003) and the lack of HLA-matched family donors further reduces the number of patients eligible for this modality. Despite the promise of a potential cure, alloSCT therefore does not constitute a valid option for the majority of patients with MM.

Targeted immunotherapy of MM represents a promising approach especially for the eradication of residual tumor cells, as described above. This study showed that MAGE-C1/CT7 is a potential candidate for such approaches due to its aforementioned expression characteristics as well as the association with a more aggressive course of the disease. Furthermore, the inherent immunogenicity of this antigen may have been demonstrated indirectly by my observation of a distinct reduction in MAGE-C1/CT7 levels following alloSCT. Ideally, these findings will be complemented by future studies investigating the functional involvement of CT antigens in the malignant cells and the therapeutic relevance of the eradication of MM cells expressing CT antigens.

CT antigens as predictive markers in patients treated with allogeneic stem cell transplantation

While the recent development of reduced intensity conditioning regimen opened the door to a wider application of alloSCT, optimization of patient stratification procedures remains a crucial task in order to increase overall and event-free survival (Bensinger 2006). Furthermore, treatment-related mortality remains a common obstacle to the broader introduction of this therapeutic modality. Novel parameters for the prediction of treatment-related adverse events as well as long-term survival are needed in order to improve the selection of patients eligible for this therapeutic option.

When evaluating the predictive value of MAGE-C1/CT7 expression in MM, I found that in patients following alloSCT, time-to-relapse as well as overall survival were dramatically decreased if the patient had evidenced expression of MAGE-C1/CT7. Confirmed by multivariate Cox regression analysis these findings indicate that MAGE-C1/CT7 is a highly significant and independent negative predictive factor in MM patients following alloSCT.

Perspective

In order to harness the therapeutic potential of MAGE-C1/CT7 multiple immunotherapeutic strategies are conceivable. Importantly, early immunotherapeutic intervention has been found to represent the most promising course of action in some solid cancers (Demaria, Bhardwaj et al. 2005). This observation might not hold true for hematologic malignancies, such as MM, as a significant reduction of the tumor bulk can generally be achieved by conventional chemotherapy and/or autoSCT. In fact, an ideal strategy of immunotherapy in patients with MM may instead represent the eradication of residual cells following conventional therapeutic strategies. This study demonstrated in a comprehensive approach that such elimination could potentially be achieved specifically targeting CT antigen-expressing MM cells.

Several ongoing studies are evaluating and optimizing current immunotherapeutic strategies, including the evaluation of DC stimulation and expansion, inhibition of regulatory immune cells, and additional unspecific immunostimulatory options. Although it is therefore impossible to predict the eventually emerging therapies some

key concepts are likely to play important roles. A straightforward procedure involving CT antigens as immunotherapeutic targets in MM represents vaccination using the synthetic full-length or partial MAGE-C1/CT7 protein. Further considering the remarkable cellular immunogenicity of this antigen, *ex vivo* expansion of MAGE-C1/CT7-specific T cells and dendritic cells following this initial vaccination might even further enhance the efficacy of this approach. Ideally, studies investigating these options will focus on a homogeneous patient collective following the performance of a standardized pretreatment regimen and include randomization according to pre-treatment response. Furthermore, this study showed that more than one CT antigen could be targeted at the same time in order to achieve broader anti-tumor responses and to prevent immunoselection that might be induced by immunotherapy causing an increased selection pressure.

Criticism

From an exclusively experimental perspective, this study did not investigate whether expression of the detected CT antigens actually occurred within the malignant cells. Since the general approach was to analyze the complete bone marrow environment of myeloma and not to focus solely on the malignant cells, I instead chose to include a large control population to rule out the possibility of aberrant CT antigen expression by healthy bone marrow cells. Using comprehensive statistical evaluation I further demonstrated that the methodological approach allowed the detection of very small quantities of CT antigen expressing cells in BM samples from patients. In addition, different groups have demonstrated that the investigated MAGE genes are in fact expressed by the malignant myeloma cells (Jungbluth, Ely et al. 2005).

Due to the retrospective character of this study it was impossible to consistently obtain some of the more recently established clinical parameters such as ISS staging or prognostically relevant genetic aberrations. This represented a particularly important consideration when performing survival analyses, but also the more basic correlations between stage of the disease, response criteria and CT antigen expression. To a degree these obstacles were overcome by using the Durie-Salmon staging system and modified EBMT response criteria that show a strong correlation with current clinical systems which had been designed for scientific comparability. Nevertheless, with the available dataset it was impossible to rule out the possibility that in the related statistical analyses clinical associations with CT antigen expression represent mere epiphenomena of hidden stratifications associated with more recent classification systems. Importantly, due to the comparably small sample size, the evaluation of larger cohorts in a prospective study design will be necessary for the confirmation of MAGE-C1/CT7 as an independent predictive parameter and its evaluation as a potential prognostic factor in newly diagnosed MM patients.

As most patients had been admitted for bone marrow transplantation in the University Medical Center Hamburg-Eppendorf the majority had already received some form of treatment prior to admission, therefore only a small population of untreated patients was included in this study. This circumstance might account for the possible misinterpretation of correlations despite statistical significance in a highly heterogeneous study population.

Importantly, some of the aforementioned shortcomings associated with this study's dataset have been alleviated in the meantime by recent confirmatory findings from

other groups suggesting that the results presented in the framework of my study in fact contain little statistical error and indeed represent clinically significant findings (Condomines, Hose et al. 2007; Perez, Herrmann et al. 2008; Tinguely, Jenni et al. 2008).

5   Conclusions

This study demonstrated that CT antigens are persistently expressed in MM and that expression levels of CT antigens, such as MAGE-C1/CT7, correlate with multiple conventional clinico-pathological parameters and the clinical course of the disease. Longitudinal analysis further showed that CT antigen expression is remarkably stable over the course of the disease and can be commonly detected in the bone marrow from patients in clinical remission. Analysis of CT antigen expression might therefore allow the monitoring of MRD. Finally the expression of MAGE-C1/CT7 represented an important predictive factor in MM patients following alloSCT.

Overall, results from this study strongly support the use of CT antigens, and especially MAGE-C1/CT7, as diagnostic and predictive factors and provide crucial information for their evaluation as immunotherapeutic target structures.

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Tables

Table 1: Durie-Salmon Staging System

Stage I:Low Tumor Mass (<0.6 x 10^{12} Myeloma Cells/m^2)

All of the following must be present:

Hemoglobin >10.5 g/dl or hematocrit >32 volume %

Serum calcium normal

Low myeloma protein production

IgG peak <5 g/dl

IgApeak <3 g/dl

Bence Jones protein <4 g/24 hour

No bone lesions

Stage II:Intermediate Tumor Mass (0.6 to 1.2 x 1012 Myeloma Cells/m2)

Fitting neither Stage I nor Stage III.

Stage III:High Tumor Mass (>1.2 x 10^{12} Myeloma Cells/m^2)

One or more of the following abnormalities must be present:

Hemoglobin <8.5 g/dl

Serum calcium >12 mg/dl

Very high myeloma protein production

IgG peak >7 g/dl

IgApeak >5 g/dl

Bence Jones protein >12 g/24 hour

>3 lytic lesions on bone survey (bone scan not acceptable)

Table 2: EBMT treatment response criteria

CRa	Absence of M-protein in both serum and urine upon immunofixation and electrophoresis, which must be maintained for a minimum of 6 weeks Absence of plasmacytomas Bone marrow aspirate containing <5% plasma cells Stable bone disease.
PRa	Greater than or equal to50% decrease in serum M-protein, maintained for >6 weeks >90% (or >200 mg) decrease in urinary M-protein, maintained for >6 weeks >50% reduction in size of plasmacytomas Stable bone disease.
MRa	25–50% decrease in serum M-protein, maintained for >6 weeks 50–90% (or >200 mg) decrease in urinary M-protein, maintained for 6 weeks 25–50% reduction in size of plasmacytomas Stable bone disease.
SD (plateau)	M-protein levels that remain stable (within 25%) of the value that was recorded before therapy, which must be maintained for >3 months Failure to achieve minimal response; no disease progression or relapse.
PD (definite increase in disease activity in patients in partial remission or disease stabilization)	>25% increase and a minimum absolute increase of 5 g/L in serum M-protein, confirmed by at least one repeated investigation >25% increase and a minimum absolute increase of 200 mg in urinary M-protein, confirmed by at least one repeated investigation >25% increase in plasma cells Increase in size of existing bone lesions, or development of new lesions Increase in size of plasmacytomas, or development of new plasmacytomas Development of cancer-attributable hypocalcemia.

Abbreviations: CR, complete response; EBMT, European Group for Blood and Marrow Transplant; MR, minimal response; PD, progressive disease; PR, partial response; SD, stable disease.

(A) Achievement of a CR, PR or MR requires that all criteria within each respective response category be met.

(B) A designation of PD only requires that at least one of the criteria within this category be met.

(C) Stable bone disease was defined as no increase in size or number of lytic bone lesions.

Table 3: Equipment and material

Cell culture		
10% FCS	Summit Biotechnology	Fort Collins ,USA
RPMI 1540, culture medium	Invitrogen	Karlsruhe, Germany
Tissue culture flasks	Sarstedt	Nümbrecht, Germany

Cell separation		
Ficoll Paque	Biochrom AG	Berlin, Germany
Phosphate-buffered saline (PBS)	Gibcoll	Paisley, GB
Centrifuge Megafuge 1.0R	Heraeus	Hanau, Germany
2-ß-Mercaptoethanol	Carl Roth GmbH	Karlsruhe, Germany
Tryptan blue solution 0,4%	Sigma-Aldrich	St. Louis, MO, USA
Microscope Telaval 31	Zeiss	Jena, Germany
RLT-Buffer	Qiagen	Hilden, Germany

RNA isolation		
RNeasy Mini kit	Qiagen	Hilden, Germany
70%-Ethanol	J.T.Baker	Deventer, Netherlands
DEPC water	Sigma	Steinheim, Germany
Microcentrifuge tubes	Eppendorf	Hamburg, Germany

cDNA synthesis		
10x Buffer	Promega	Mannheim, Germany
$MgCl^2$	Promega	Mannheim, Germany
Random Primer	Invitrogen	Karlsruhe, Germany
dNTP (dATP, dGTP, dUTP, dCTP)	Invitrogen	Karlsruhe, Germany
RNAsin	Promega	Mannheim, Germany
AMV-reverse transkriptase	Promega	Mannheim, Germany
Master cycler gradient	Eppendorf	Hamburg, Germany

RT-PCR		
$MgCl^2$	Perkin-Elmer	Waltham, MA, USA
10xPuffer	Perkin-Elmer	Waltham, MA, USA
dNTP(dATP, dGTP, dTTP, dCTP)	Invitrogen	Karlsruhe, Germany
Custom primer	MWG-Biotech	Ebersberg, Germany
AmpliTaqGold	Perkin-Elmer	Waltham, MA, USA
DEPC treated water	Sigma	Steinheim, Germany
0,2ml-Röhrchen-Kette	Sarstedt	Nümbrecht, Germany
Human testis RNA	Ambion	Austin, TX, USA
Master cycler gradient	Eppendorf	Hamburg, Germany

Gelelectrophoresis for RT-PCR		
Trisbase	Sigma	Steinheim, Germany
Aqua bidest	Th.Geyer	Berlin, Germany
Hydrochloric acid	Sigma	Steinheim, Germany
EDTA (0,5M EDTA, pH 8)	Sigma	Steinheim, Germany
DEPC treated water	Sigma	Steinheim, Germany
DNA Subcell, 1000/500	Biorad	Munich, Germany
Ethidiumbromid	Invitrogen	Karlsruhe, Germany
The Imager	Appligene	Cedex, France

Sequencing

Qiaquick Gel Extraction Kit	Qiagen	Hilden, Germany
BigDyeKit V.1.1	Applied Biosystems	Foster City CA, USA
Primer	MWG Biotech,	Ebersberg, Germany
DEPC treated water	Sigma	Steinheim, Germany
Master cycler gradient	Eppendorf	Hamburg, Germany
100%-Ethanol	J.T.Baker	Deventer, Niederlande
70%-Ethanol	J.T.Baker	Deventer, Niederlande
Centrifuge, Biofuge 13R	Heraeus Sepatech	Hanau, Germany
Speedvac, Hetovac, Intermed CT60 e	Heto	Holten, Germany
ABI Prism 3100 Genetic Analyzer	Applied Biosystems	Foster City CA, USA

Quantitative RT-PCR

Lightcycler II	Roche	Mannheim, Germany
Lightcycler Capillaries	Roche	Mannheim, Germany
FastStart Taq DNA Polymerase	Roche	Mannheim, Germany
$MgCl^2$	Perkin-Elmer	Waltham, MA, USA
10xPuffer	Perkin-Elmer	Waltham, MA, USA
dNTP(dATP, dGTP, dTTP, dCTP)	Invitrogen	Karlsruhe, Germany
Custom primer	MWG-Biotech	Ebersberg, Germany
DEPC treated water	Sigma	Steinheim, Germany

Immunoblot

Amersham Hyperprocessor	GE Healthcare	Buckinghamshire, UK
Consort EV202 power supply	Sigma-Aldrich	Steinheim, Germany
Xcell II blot chamber	Invitrogen	Karlsruhe, Germany
Xcell SureLock electrophoresis cell	Invitrogen	Karlsruhe, Germany
NuPage Bis-Tris SDS-PAGE gel	Invitrogen	Karlsruhe, Germany
NuPage transfer buffer	Invitrogen	Karlsruhe, Germany
NuPage running buffer,	Invitrogen	Karlsruhe, Germany
Methanol	J.T.Baker	Deventer, Niederlande
Sodium chloride	J.T.Baker	Deventer, Niederlande
Tris base	Sigma-Aldrich	Steinheim, Germany
Tween 20	Sigma-Aldrich	Steinheim, Germany
Amersham Hyperfilm MP	GE Healthcare	Buckinghamshire, UK
Amersham ECL Detection reagents	GE Healthcare	Buckinghamshire, UK
Protran nitrocellulose membrane	Whatman	Dassel, Germany
Gel blotting paper	Whatman	Dassel, Germany
Vibrax VXR, JK	IKA Werke	Staufen, Germany
Top-Block	Fluka	Buchs, Switzerland
Anti ACTB antibody	Santa Cruz Biotechnology	Santa Cruz, CA, USA
HRP-labeled anti-mouse IgG	R&D Systems	Minneapolis, MN, USA

Table 4: Primer sequences and PCR cycle conditions

CT antigen	Primer sequence		PCR cycle condition	Size
MAGE-C1/CT7 *Qualitative*	F:	5'- ACA TCC TCA CCC TCA GGA GGG -3';	95°C 60s; 60°C 60s; 72°C 60s;	632bp
	R:	5'-GAC GAG GAT CGT CTC AGG TCA GC -3';		
MAGE-C1/CT7 *Quantitative*	Commercially produced (Qiagen, Hilden, Germany)		95°C 10s; 60°C 30s; 72°C 20s;	121bp
MAGE-A3	F:	5'- GAA GCC GGC CCA GGC TCG -3';	95°C 60s; 60°C 60s; 72°C 60s;	423bp
	R:	5'-GGA GTC CTC ATA GGA TTG GCT-3';		
MAGE-C2/CT10	F:	5'- CGG ATC GAA GGC ATT TGT GAG -3';	95°C 60s; 55°C 60s; 72°C 60s;	884bp
	R:	5'-GTG AAC TCA CGG GCT CTC TTG AG -3';		
SSX-2	F:	5'-GTG CTC AAA TAC CAG AGA AGA TC -3';	95°C 60s; 60°C 60s; 72°C 60s;	434bp
	R:	5'- TTT TGG GTC CAG ATC TCT CGT G -3';		
GAPDH *Qualitative*	F:	5'- TGA TGA CAT CAA GAA GGT GG -3';	95°C 45s; 60°C 45s; 72°C 45s;	246bp
	R:	5'- TTT CTT ACT CCT TGG AGG CC -3';		
GAPDH *Quantitative*	F:	5'- TGA TGA CAT CAA GAA GGT GG -3';	95°C 15s; 61°C 5s; 72°C 26s;	246bp
	R:	5'- TTT CTT ACT CCT TGG AGG CC -3';		

F, forward primer; R, reverse primer; Size, size of PCR product; bp, base pairs

Table 5: Patient and sample characteristics and correlation with CT antigen expression

(A) Patient characteristics		
Characteristics	*Number of patients per group*	*Percentage of patients expressing a minimum of one CT antigen*
Total	129	52.7
Age at time of inclusion		** p<0.05
≤60 years	79	44.3
>60 years	50	66.0
Sex		p=0.4
Male	84	54.8
Female	45	48.9
Heavy chain isotype		p=0.7
IgG	69	50.7
IgA	38	57.9
IgD	1	100.0
Light chain	21	47.6
Light chain isotype		p=0.7
Kappa	78	55.1
Lambda	42	47.6
Initial stage (Durie-Salmon)		p=0.1
I	23	39.1
II	21	42.9
III	75	61.3
Previously treated		p=0.1
Yes	108	55.6
No	21	38.1

(B) Sample characteristics

Characteristics	Number of 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	
Remission status			* p<0.05
Complete remission	109	8.3	
Partial remission	117	28.2	
Progressive disease	83	75.9	
Treatment			*** p<0.001
None	21	38.1	
Chemotherapy alone	31	67.7	
Autologous stem cell transplantation	36	47.2	
Allogeneic stem cell transplantation	242	27.7	
Serum β_2-microgloubulin (mg/l)			p=0.5
< 3.5	89	31.5	
3.5 - 5.5	10	40.0	
> 5.5	11	54.5	
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 LDH (U/l)			p=0.1
≤ 210	237	31.2	
> 210	83	41,0	

A total of 129 patients with multiple myeloma 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. 330 samples were classified according to sample-specific clinical criteria. Information on remission status (N=309), serum β2-microglobulin (N=110), hemoglobin (N=319), serum albumin (N=310), as well as serum lactate dehydrogenase levels (LDH; N=320) were available for fewer samples. P values show correlations between percentages of patients or samples expressing at least one CT antigen (MAGEC1, MAGEC2, MAGE-A3, or SSX-2) as determined by qualitative RT-PCR and single clinicopathological characteristics. Asterisks indicate significant results (*p<0.05; ***p<0.001).

Table 6: Multivariate Cox regression analysis of time-to-relapse

Covariates	B	SE	Wald	df	Sig.	Exp(B	95.0% CI for Exp(B)	
	Lower	*Upper*	*Lower*	*Upper*	*Lower*	*Upper*	*Lower*	*Upper*
MAGE-C1 expression	-4.322	1.036	17.401	1	.000	.013	.002	.101
Age (>60 years)	-.726	.643	1.274	1	.259	.484	.137	1.707
Sex	1.588	.770	4.257	1	.039	4.896	1.083	22.142
Ig idiotype	.374	.422	.787	1	.375	1.454	.636	3.325
Initial stage			2.661	3	.447			
Samples since alloSCT	-.148	.130	1.287	1	.257	.863	.668	1.114

Table 7: Multivariate Cox regression analysis of overall survival

Covariates	B	SE	Wald	df	Sig.	Exp(B	95.0% CI for Exp(B)	
	Lower	*Upper*	*Lower*	*Upper*	*Lowe*	*Upper*	*Lower*	*Upper*
MAGE-C1 expression	-3.89	1.336	8.473	1	.004	.020	.001	.281
Age (>60 years)	-1.39	1.246	1.237	1	.266	.250	.022	2.877
Sex	1.34	1.150	1.353	1	.245	3.811	.400	36.318
Ig idiotype	1.94	.914	4.492	1	.034	6.946	1.157	41.697
Initial stage			.908	3	.823			
Samples since alloSCT	-.586	.267	4.826	1	.028	.556	.330	.939

Attachments

Patient information and consent form

Patientenaufklärung und einverständniserklärung

Untersuchung von Blut und Knochenmark

(einschl. genetischer Untersuchung)

Forschungsprojekt

Untersuchung von Expressionsmuster und immunologischer

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> **Vorname, Name und Geburtsdatum des**
> **Patienten bzw. Patientenaufkleber**

1.2 Patienteninformation

Sehr geehrte Patientin, sehr geehrter Patient,

bei Ihnen besteht der Verdacht auf das Vorliegen einer Erkrankung des blutbildenden Systems, des Multiplen Myeloms, oder die Erkrankung konnte bereits zu einem früheren Zeitpunkt diagnostiziert werden.

Zur Diagnosestellung sind die Durchführung einer Knochenmarkentnahme und auch die Entnahme von peripherem Blut nötig. Auch im Rahmen einer möglicherweise durchzuführenden Behandlung werden bei Ihnen gegebenenfalls regelmäßig Blutuntersuchungen, aber auch Knochenmarkuntersuchungen durchgeführt werden. Die dabei anfallenden Untersuchungen erfolgen, um bei Ihnen rechtzeitig Komplikationen erkennen zu können, aber auch, um den Krankheitsverlauf und den Therapieerfolg beurteilen zu können. Diese Untersuchungen sind für Ihre Behandlung dringend erforderlich und gehören zum standardisierten Ablauf der Behandlung. Das nach einer solchen Untersuchung verbliebene Knochenmark bzw. Blut wird in der Regel entsorgt.

Es steht mittlerweile fest, dass das Immunsystem einen Einfluss auf die Entstehung und den Verlauf vieler Erkrankungen, einschließlich bösartiger Tumoren, hat. Wir möchten anhand der Ihnen entnommenen Knochenmarkproben untersuchen, ob das Gewebe genetische Merkmale aufweist, die vom Immunsystem als „fremd" erkannt werden. Außerdem möchten wir ermitteln, ob Ihr Immunsystem bereits auf das mögliche Vorhandensein „fremder" Strukturen aufmerksam geworden ist. Hierzu möchten wir Sie um eine zusätzliche Blutprobe (20 ml) bitten, die im Rahmen der routinemäßig entnommenen Blutproben mit gewonnen werden kann.

Um künftig bei Patienten, die an einem ähnlichen Leiden wie Sie erkrankt sind, Therapieentscheidungen noch besser treffen zu können, aber auch, um an Ihrem Material vielleicht später neue Erkenntnisse zu gewinnen, möchten wir Sie um Zustimmung bitten, dass dieses Knochenmark- und Blutmaterial asserviert, d. h. aufbewahrt, und später gesondert untersucht werden kann. Das während der Diagnostik bzw. während der Transplantation und im nachfolgenden Verlauf Ihrer Erkrankung gewonnene Material (Blut / Knochenmark) wird im Forschungslabor der II. Medizinischen Klinik des UKE in Hamburg aufbewahrt. Im Rahmen der vorliegenden Untersuchung werden Knochenmark und Blut dann im Verlauf auf das Vorliegen bestimmter Gene hin untersucht werden, die Tumoren für das menschliche Immun-

system erkennbar und damit angreifbar machen. Weiterhin sollen die Proben daraufhin untersucht werden, ob eine solche durch Antikörper- oder Immunzellen vermittelte Anti-Tumor-Antwort bereits vorliegt.

Durch diese zusätzlichen Untersuchungen entstehen für Sie keine Unannehmlichkeiten bzw. Aufwendungen, weil keine zusätzliche Knochenmark- bzw. Venenpunktion notwendig ist. Außerdem birgt die zusätzliche Entnahme von maximal 10 ml Knochenmark keinerlei spezielle Risiken. Es handelt sich bei dem hier beschriebenen Projekt um ein reines Forschungsprojekt und nicht um eine klinische Studie. Falls im Rahmen eines späteren Forschungsprojekts anhand Ihres Blutes bzw. Ihres Knochenmarks klinische Daten notwendig sind, bitten wir schon jetzt um Ihre Zustimmung, die klinischen Daten Ihres Krankheitsverlaufs zu erheben. Diese Daten werden pseudonymisiert, d. h. es erfolgt durch Ersetzen des Namens und andere Identifikationsmerkmale (z. B. Geschlecht) durch eine spezielle Kennzeichnung zu dem Zweck, die Bestimmung des Betroffenen auszuschließen oder wesentlich zu erschweren. Diese Maßnahme dient dazu, die Kenntnisnahme der klinischen Daten und der Materialdaten durch Dritte ohne Ihre Einwilligung auszuschließen. Die Untersuchungsergebnisse können in medizinischen Fachzeitschriften veröffentlicht werden, allerdings ohne Offenlegung Ihrer persönlichen Daten. Bei der Verarbeitung Ihrer personenbezogenen Daten werden die Bestimmungen des Bundesdatenschutzgesetzes eingehalten werden.

Die geplanten Untersuchungen werden ausschließlich in der II. Medizinischen Klinik des UKE durchgeführt. Eine Weitergabe an Dritte ist nur in Ausnahmefällen vorgesehen. Diese Ausnahmefälle liegen vor, wenn bestimmte wissenschaftliche Methoden nur in entsprechenden Speziallabors durchgeführt werden können. In diesem Fall werden die Daten pseudonymisiert, d. h. die personenbezogenen Daten werden so verändert, dass die Einzelangaben über persönliche und sachliche Verhältnisse nicht mehr oder nur mit einem unverhältnismäßig großen Aufwand an Zeit, Kosten, Arbeitskraft einer bestimmten oder bestimmbaren natürlichen Personen zugeordnet werden können.

Sollten andere Forschungslaboratorien oder Universitätsklinika sich an gemeinsamen Forschungsprojekten beteiligen, so werden die von uns bei Ihnen erhobenen Daten, insbesondere die klinischen Daten, ebenfalls nur in pseudonymisierter Form weitergegeben, d. h. in einem solchen Fall sind Ihre persönlichen Daten derart verändert, dass Einzelangaben über persönliche und sachliche Verhältnisse nicht mehr oder nur mit einem enormen, unverhältnismäßig großen Aufwand an Zeit,

Kosten und Arbeitskraft einer bestimmten oder bestimmbaren natürlichen Person zugeordnet werden können.

Aus diesem Grunde bitten wir Sie, diese Aufklärung durch Ihre Unterschrift zu bestätigen. Für eine ggf. sich ergebende Fragestellung, die nicht im Zusammenhang mit Ihrer derzeitigen Grunderkrankung steht, würden wir Sie gesondert kontaktieren und um Ihre Zustimmung bitten.

Über die konkreten Ergebnisse der jeweiligen Begleituntersuchungen werden Sie nicht gesondert informiert. Die Informationen aus diesen Untersuchungen werden erst bei künftig zu behandelnden Patienten, die an der gleichen Erkrankung wie Sie leiden, verwendet werden können. Selbstverständlich besteht jedoch die Möglichkeit, dass Ihnen auf Anfrage die Ergebnisse der Untersuchung mitgeteilt werden. Sollten die Daten jedoch vollständig anonymisiert werden, schließt das auch eine Information über Untersuchungsergebnisse Ihnen gegenüber aus. Die gesammelten Untersuchungsergebnisse werden getrennt von Ihrer Patientenakte aufbewahrt.

Ihre Zustimmung zur Untersuchung des Blut- bzw. Knochenmarkmaterials ist vollkommen freiwillig und kann von Ihnen jederzeit widerrufen werden. In diesem Falle erfolgt die Löschung all Ihrer gespeicherten Daten und die Vernichtung vorhandenen Blut- und Knochenmarkmaterials. Dieser Widerruf muss schriftlich erfolgen.

Ihre Zustimmung zur Untersuchung Ihres Bluts und Ihres Knochenmarks ist unabhängig von Ihrer ggf. vorliegenden Zustimmung zur Teilnahme an einer Therapiestudie.

Patientenaufklärung und -einverständniserklärung

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Vorname, Name und Geburtsdatum des Patienten bzw. Patientenaufkleber

1.2 Patienteinverständniserklärung

Ich willige ein, dass mein Blut- und/oder Knochenmarkmaterial zu Forschungszwecken verwendet wird, die ausschließlich im Zusammenhang mit meiner o. g. Erkrankung bzw. mit Erkenntnissen über die Stammzelltransplantation stehen.

Ich weiß, dass die Im Rahmen dieser Studie erhobenen Daten und persönlichen Mitteilungen der ärztlichen Schweigepflicht unterliegen und zur Verarbeitung und Auswertung nur ohne meinen Namen (pseudonymisiert) zusammengeführt werden dürfen. Ich weiß, dass die Untersuchungsergebnisse in medizinischen Fachzeitschriften veröffentlicht werden können, allerdings ohne Offenlegung meiner persönlichen Daten. Ich wurde darüber aufgeklärt, dass bei der Verarbeitung meiner personenbezogenen Daten die Bestimmungen des Bundesdatenschutzgesetzes eingehalten werden.

Ich wurde über den Inhalt der geplanten Untersuchungen detailliert aufgeklärt; mir wurden alle Fragen vollständig beantwortet. Eine Kopie der unterschriebenen Einverständniserklärung wurde mir ausgehändigt. Ich versichere, dass ich die Patien-

tenaufklärung sorgfältig gelesen und sie verstanden habe und dass ich keine weiteren Fragen habe.

Ort / Datum	(Eigenhändig unterzeichnet: Name, Datum und Unterschrift)	Patientin/Patient

Ort / Datum	(Eigenhändig unterzeichnet: Name, Datum und Unterschrift)	Aufklärende(r) Ärztin/Arzt

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Erklärung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Unterschrift: _____

Tim Cornelis Lütkens