Clinical Cancer Research

The Pattern of Mesenchymal Stem Cell Expression Is an Independent Marker of Outcome in Multiple Myeloma

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Abstract

Purpose: Mesenchymal stem cells (MSC) are an essential component of the bone marrow microenvironment and have shown to support cancer evolution in multiple myeloma. Despite the increasing evidence that multiple myeloma MSCs differ from their healthy counterparts, little knowledge exists as to whether MSCs independently influence disease outcome. The aim of this study was to determine the importance of MSCs in disease progression and outcome in multiple myeloma.

Experimental Design: To determine the impact of MSCs on multiple myeloma outcome in an *in vivo* system, we first identified genes from cultured MSCs that were specific to MSC expression and were not or minimally expressed in plasma cells (PC) or other cells present in bone marrow aspirates. We then applied this MSC gene signature to whole bone marrow biopsies of multiple myeloma patients compared with healthy controls and determined MSC expression scores specific to multiple myeloma and predictive of outcome.

Results: We show that multiple myeloma MSC gene expression signatures can differentiate multiple myeloma from monoclonal gammopathy and smoldering multiple myeloma (SMM) as well as from healthy controls and treated multiple myeloma patients who have achieved a complete remission. We identified a prognostic gene score based on three MSC specific genes, *COL4A1*, *NPR3* and *ITGBL1*, that was able to predict progression-free survival in multiple myeloma patients and progression into multiple myeloma from SMM.

Conclusions: Our findings show that progression of multiple myeloma and of SMM into multiple myeloma does not rely solely on intrinsic PC factors, but is independently affected by the biology of the surrounding microenvironment. *Clin Cancer Res; 24(12); 2913–9. ©2018 AACR.*

Introduction

Multiple myeloma is a hematopoietic malignancy of terminally differentiated plasma cells (PC) that reside within the bone marrow. It is almost universally preceded by premalignant monoclonal gammopathy of undetermined significance (MGUS) and/or smoldering multiple myeloma (SMM) and can transform into malignant multiple myeloma over time (1, 2). Genomic analysis has so far not been able to fully explain the transition of MGUS/SMM to multiple myeloma. Recent research has shown that the bone marrow microenvironment plays a crucial role in promoting growth and disease progression, suggesting a complex interaction between the PCs and their niche that could impact clinical outcome (3–5). Mesenchymal stem cells (MSC) are a major constituent of the bone marrow microenvironment that are capable of self-renewal and can differentiate into various cell types, including fibroblasts, adipocytes, chondrocytes, and osteoblasts/osteocytes (4, 5). It is well documented that in multiple myeloma, MSCs are functionally different from normal MSCs and promote progression and drug resistance through direct cell-to-cell contact, cytokine release, and exosome secretion (6–9). The impact of the malignant plasma cells on the biology of the microenvironment and how this either predisposes to or inhibits disease progression is currently unknown. To address the lack of knowledge of the contribution of the MSCs to adverse disease behavior and clinical outcome, we investigated whether there is a distinct pattern of expression in MSC derived from cases of SMM and multiple myeloma that differentiate them from premalignant cases and whether there is a distinct pattern of expression associated with adverse clinical outcome in multiple myeloma.

Materials and Methods

Patient samples

Specimens were obtained after institutional review board approved by the University of Arkansas for Medical Sciences in accordance with the Declaration of Helsinki. All multiple myeloma samples were collected from multiple myeloma patients that had been enrolled into our Total Therapy TT2-TT5 protocols,

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Translational Relevance

Our study shows that gene expression signatures of mesenchymal stem cells (MSC) in multiple myeloma are an independent prognostic factor of outcome and can distinguish between different multiple myeloma stages, including monoclonal gammopathy of undetermined significance (MGUS), smoldering multiple myeloma (SMM), newly diagnosed multiple myeloma, and multiple myeloma in treated patients who have achieved a complete remission (CR). A specific MSC 3-gene score derived from *COL4A1*, *NPR3*, and *ITGBL1* was able to predict progression-free survival in newly diagnosed multiple myeloma patients as well as treated multiple myeloma patients who had achieved a CR and was furthermore associated with progression of MGUS/multiple myeloma patients to multiple myeloma. These results emphasize that disease evolution and progression in plasma cell (PC) dyscrasias is independently affected by the biology of the surrounding bone marrow niche. To improve treatment options and multiple myeloma outcome, therapeutic efforts should hence also be directed toward modulating MSCs within the multiple myeloma microenvironment.

a detailed description of which has been published previously (10, 11). Samples from patients with SMM were derived from the S0120 protocol (12). Control samples were obtained from healthy individuals after informed consent.

MSC culture, PC selection, and identifying MSC characteristic genes

MSC cultures were prepared from whole bone marrow of healthy donors ($n = 10$) and multiple myeloma patients ($n = 20$) as previously described (13, 14). In brief, mononuclear cells (MC) were obtained from the aspirate by density gradient centrifugation (Ficoll). The cells were cultured in medium containing αMEM and 10% FBS with antibiotics. After 5 to 7 days, the cultures were washed three times to remove nonadherent cells. The remaining, isolated adherent cells were considered MSCs and phenotype has been described previously (14–16).

MM cells were enriched by CD138 immunomagnetic bead selection of mononuclear cell fractions of bone marrow aspirates (autoMACS; Miltenyi Biotec) and buffy coat samples were isolated from bone marrow aspirates by density gradient centrifugation (Ficoll). MSC genes were defined as being highly expressed in cultured MSCs ($\log_2$ expression >10), not expressed in CD138-selected multiple myeloma cells (log2 expression <9) and expressed only at low levels in hematopoietic cells ($\log_2$ expression <10), represented by buffycoat samples (Supplementary Table S1).

Processing of whole bone marrow core biopsies

To identify a MSC gene score able to predict clinical outcome within the microenvironment, whole bone marrow core biopsies were collected from newly diagnosed patients with multiple myeloma ($n = 461$), SMM ($n = 52$), MGUS ($n = 38$), multiple myeloma patients posttreatment in CR (sample was taken within the first 6 months of CR achievement, $n = 132$), and healthy donors ($n = 68$). Frozen whole bone marrow biopsies were ground in liquid nitrogen with a mortar and pestle. The tissue powder and liquid nitrogen were decanted into a cooled tube, and the liquid nitrogen was allowed to evaporate. RNA isolation and gene expression profiling (GEP) of biopsies and their matched CD138-enriched multiple myeloma cells were done as previously described (17, 18).

Gene expression analysis

Gene expression analysis of all samples was performed with the affymetrix U133Plus2.0 microarray platform (Santa Clara, CA) using methods previously described (17, 18). Risk status was determined by gene expression analysis of CD138-selected cells at diagnosis using the GEP70 signature and molecular subtypes were determined by the UAMS classification.

Statistical analysis and CIBERSORT

Differential expression analysis was conducted using *t*-test on each of MSC probe sets followed by multiple comparison adjustments using the *q* value method (19). Ingenuity pathway analysis (IPA; Qiagen) was used to detect pathways and biological functions enriched in multiple myeloma MSCs compared to healthy ones. A random forest model was developed to distinguish between normal and multiple myeloma samples. To develop a predictive model associated with survival, we first conducted univariate analysis on each probe set using Cox regression. We then selected top ranking probe sets to create a score and dichotomize at the median to predict the outcome (17). We used the previously validated CIBERSORT method to robustly estimate abundances of various bone marrow immune cell types in biopsies as previously described (20).

Results

Defining genes characteristic of MSCs and distinguishing between normal and multiple myeloma MSCs

MSC genes were defined as being highly expressed in cultured MSCs, not expressed in multiple myeloma cells and expressed only at low levels in hematopoietic cells, represented by buffycoat. We designated genes as being MSC-specific if they satisfied all of the following criteria in 90% of the samples: gene expression signal (Log_2) in MSC samples >10 and <9 in multiple myeloma plasma cells (PC) from any molecular subgroup, and <9 in buffycoat aspirate samples (representing bone marrow hematopoietic cells). The ability to identify MSCs by a distinct gene signature was essential to recognize MSC genes within cell mixture of the whole bone marrow biopsy. This analysis identified a total of 469 probe sets, satisfying the MSC criteria, representative of 345 unique genes, listed in Supplementary Table S1. We then applied these 469 MSC specific probe sets to whole bone marrow biopsies and identified MSCs based on their gene signature. Out of these 469 MSC specific probe sets, we identified 124 genes that had significant different gene expression patterns between healthy and multiple myeloma MSCs ($P < 0.05$, FDR < 0.01; Supplementary Table S2). Pathway analysis demonstrated that these 124 differentially expressed probe sets were enriched in several pathways pertinent to cancer biology ($P < 0.001$), cellular movement ($P < 0.001$), and connective tissue disorders ($P < 0.001$). Detailed results of the pathway analysis are given in Supplementary Table S3. We further performed random forest analysis (RFA) to determine 34 MSC-specific probe sets that were best able to distinguish between healthy donors and multiple myeloma patient bone

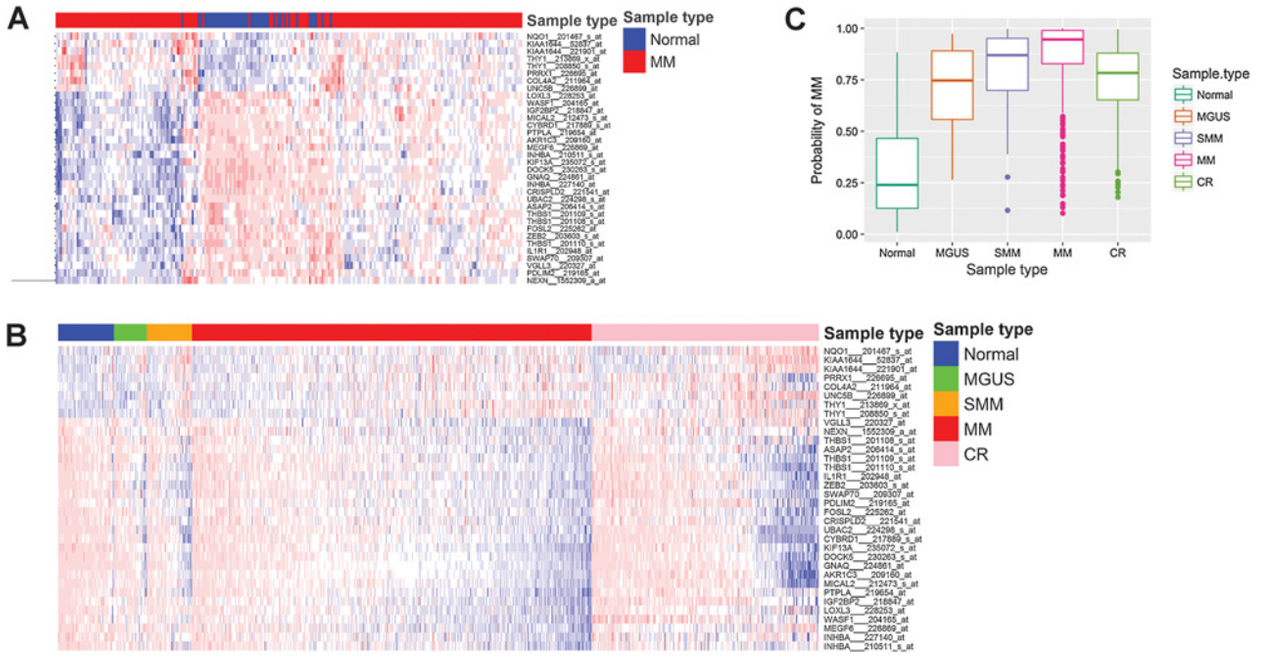

Figure 1.

The 34 MSC gene score can distinguish multiple myeloma from healthy MSCs and identifies different multiple myeloma disease stages. Unsupervised clustering of the 34 MSC-specific probe sets based on random forest analysis in MSCs from normal donors and patients with multiple myeloma taken from whole bone marrow biopsies (**A**). Expression of the 34 MSC-specific probe sets based on random forest analysis (**B**) in bone marrow biopsies from normal donors and patients with MGUS, SMM, and multiple myeloma, and posttreatment patients in CR. Probability of disease stage based on the 34 MSC-specific probe set signature in bone marrow biopsy samples (**C**).

marrow biopsies (Supplementary Fig. S2); unsupervised clustering showed that healthy and multiple myeloma MSCs cluster into different groups according to the 34 gene signature (Fig. 1A). Of these 34 differentially expressed genes, only eight were upregulated [*THY1* (11q23.3), *KIAA1644* (22q13.31), *PRPX1* (1q24.2), *COL4A2* (13q34), *UNC5B* (10q22.1), and *NQO1* (16q22.1)]. Using this 34 gene model we were able to demonstrate that MSC expression differs in healthy donors and patients with MGUS, SMM, and multiple myeloma, and CR samples (Fig. 1B). Notably, the probability of multiple myeloma based on the random forest model increased from healthy controls through MGUS, SMM, and was highest in multiple myeloma patients, whereas the achievement of a CR after treatment decreased the probability of multiple myeloma to levels similar to those of MGUS patients (Fig. 1C). Taken together, these data indicate that MSC genes are differentially expressed in bone marrow of healthy donors and patients at different stages of the disease.

Development of a prognostic MSC score at baseline

To determine the MSC genes most prognostic for PFS, we tested 461 multiple myeloma patients of whom we had whole bone marrow biopsy samples at baseline, and randomly selected these patients into two-third for training and one-third as a validation set. The top 15 MSC genes that were most significantly associated with PFS are listed in Supplementary Table S4. We developed a three-gene MSC score based on the top three genes associated with PFS; *COL4A1 (13q34)*, *NPR3* (*5p13.3*), and *ITGBL1* (*13q33.1*). Higher expression of COL4A1 was associated with high hazard ratio (HR), whereas lower expression of NPR3 and ITGBL1 was associated with low HR. We examined their expression in individual samples (Fig. 2A) and the association of the three-gene MSC score with clinical parameters (Table 1).

A high three-gene MSC score was independently and significantly associated with several adverse clinical parameters, such as higher ISS stage, low hemoglobin and platelet count, elevated LDH as well as cytogenetic abnormalities and GEP 70 defined high risk multiple myeloma (Table 1). Importantly, the three-gene MSC score did not correlate with the plasma cell gene score (20) in the same whole bone marrow biopsy samples, and was hence not simply a reflection of disease bulk (Supplementary Fig. S2).

A score based on these three genes characterized one third of the test population as high risk, which correlated significantly with worse PFS in the training (Fig. 2B, $P < 0.001$) and test set (Fig. 2C, $P = 0.0077$). The three-gene MSC score further predicted progression of MGUS/SMM patients to multiple myeloma (Fig. 3A, $P = 0.002$) and had a trend towards prediction of PFS in treated multiple myeloma patients who had achieved a CR (Fig. 3B, $P = 0.12$).

We assessed the interaction between the three-gene MSC score with the GEP70 risk score, derived from plasma cells of the corresponding samples. In GEP70 defined low-risk (LR) patients, the high three-gene MSC score had a significant negative impact on PFS in the training (Fig. 4A, $P < 0.0001$) and a trend of worse PFS in the test set (Fig. 4B, $P = 0.1$). In GEP70 high-risk (HR) patients, the three-gene MSC score was not significantly associated

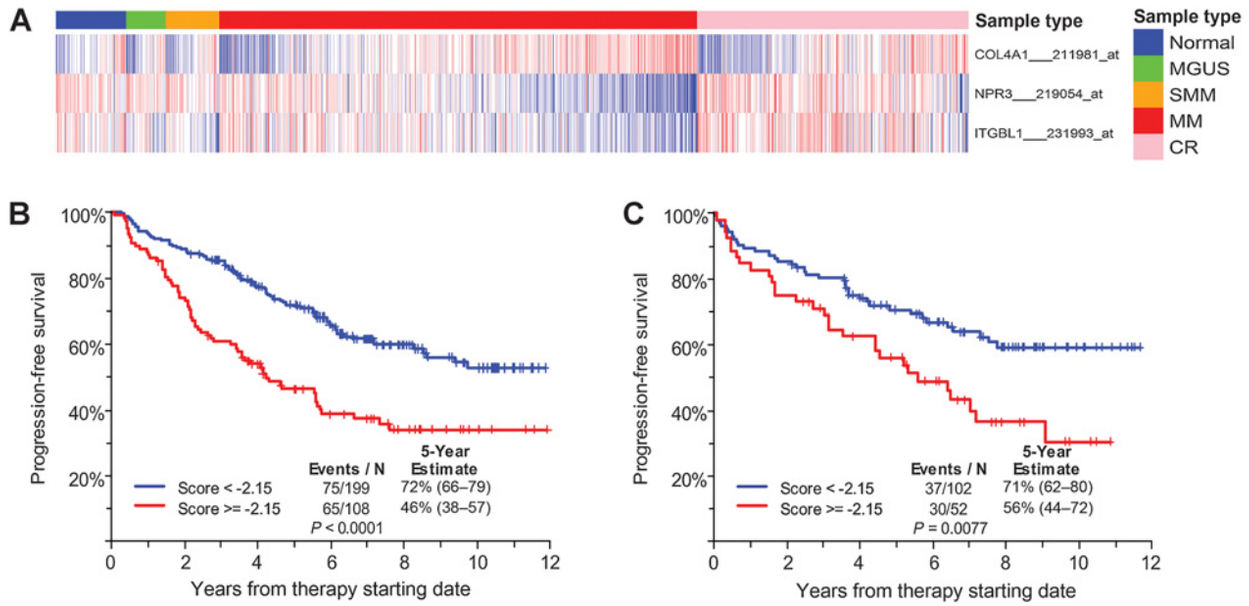

Figure 2.

Development of a prognostic MSC gene score and its association with outcome. The expression in whole bone marrow biopsy samples of the top three MSC genes associated with PFS according to disease stage are shown in **A**. Higher expression of *COL4A1* was associated with high HR, whereas lower expression of *NPR3* and *ITGBL1* was associated with lower HR (Supplementary Table S3). PFS of multiple myeloma patients in the training (**B**) and test set (**C**) shows significantly worse outcome in patients with a high 3-MSC gene score.

with outcome in either the training or test set, suggesting that the aggressive nature of HR multiple myeloma cells is the major driver of the disease (Fig. 4A and B).

Expression patterns of immune cells in the multiple myeloma microenvironment according to the three-gene MSC score

We used the CIBERSORT method to analyze whether the three-gene MSC score was associated with changes in the composition of immune cells in the multiple myeloma microenvironment (20). The results show that of the population identified by the 22 leukocyte subsets gene scores, defined by the CIBERSORT, most subsets, including monocytes, neutrophils, mast cells, macrophages, natural killer (NK) cells, and T-lymphocytes were significantly lower in bone marrow biopsies with a high three-gene MSC score (Fig. 5; Supplementary Table S5). This suggests that immune dysregulation and dysfunction within the microenvironment of these patients is present and likely further promotes disease progression and possibly also plays a role in tumor response to immune-therapies. A deconvolution analysis of MGUS/SMM patients was also performed, which showed a similar trend of changes as seen in multiple myeloma, yet the results did not achieve significance, possibly due to lower proportion of cases with high three-gene MSC gene score and lower number of total samples (Supplementary Fig. S2).

Table 1. Clinical parameters related to the 3-MSC gene score show that adverse prognostic markers are significantly more frequent in patients with a high 3-MSC gene score

Factor	All patients	Score < -2.15	Score ≥ -2.15	*P*
Age $\geq$ 65 years	135/461 (29%)	95/301 (32%)	40/160 (25%)	0.138
Female	179/461 (39%)	99/301 (33%)	80/160 (50%)	<0.001
White	411/461 (89%)	273/301 (91%)	138/160 (86%)	0.150
Albumin <3.5 g/dL	174/461 (38%)	99/301 (33%)	75/160 (47%)	0.003
B2M $\geq$3.5 mg/L	242/460 (53%)	125/300 (42%)	117/160 (73%)	<0.001
B2M >5.5 mg/L	122/460 (27%)	58/300 (19%)	64/160 (40%)	<0.001
ISS stage 1	164/460 (36%)	132/300 (44%)	32/160 (20%)	<0.001
ISS stage 2	174/460 (38%)	110/300 (37%)	64/160 (40%)	0.483
ISS stage 3	122/460 (27%)	58/300 (19%)	64/160 (40%)	<0.001
Creatinine $\geq$2 mg/dL	24/461 (5%)	17/301 (6%)	7/160 (4%)	0.553
CRP $\geq$8 mg/L	296/459 (64%)	188/299 (63%)	108/160 (68%)	0.322
Hb <10 g/dL	164/461 (36%)	74/301 (25%)	90/160 (56%)	<0.001
LDH $\geq$190 U/L	100/461 (22%)	54/301 (18%)	46/160 (29%)	0.008
Platelet count $<150 \times 10^9$/L	70/461 (15%)	28/301 (9%)	42/160 (26%)	<0.001
Cytogenetic abnormalities	172/451 (38%)	90/294 (31%)	82/157 (52%)	<0.001
GEP 70 high risk	54/440 (12%)	20/283 (7%)	34/157 (22%)	<0.001

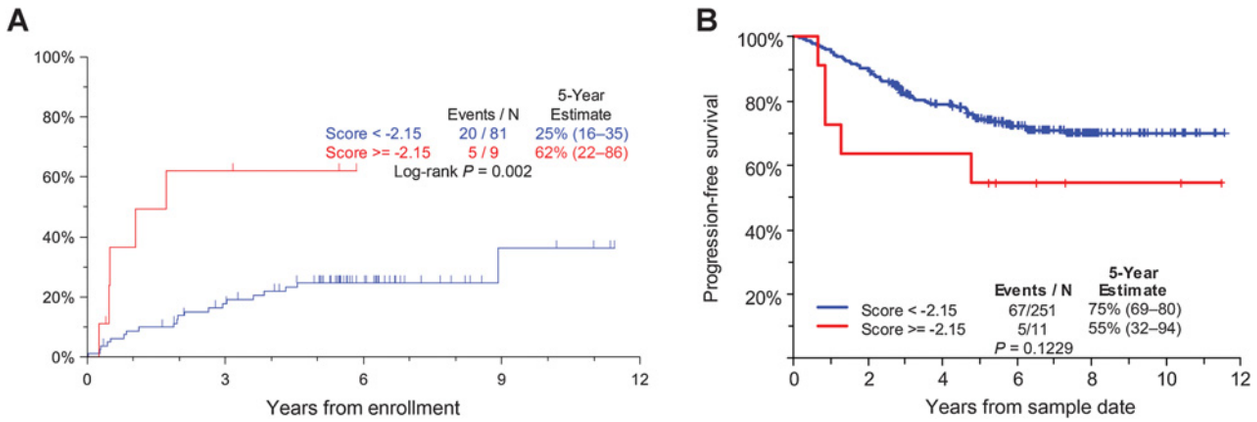

Figure 3.

High 3-gene MSCs gene score is associated with early progression of patients with premalignant disease and early relapse of multiple myeloma patients in CR. **A,** A high 3-gene MSC score was able to predict rapid progression of patients with MGUS/SMM to clinical multiple myeloma compared with patients with a low 3-gene MSC score ($P = 0.002$). **B,** A high 3-gene MSC gene score in MM CR samples (taken during the first 6 months of CR) had a trend toward worse PFS in patients ($P = 0.12$).

Discussion

We show that *in vivo* gene expression patterns of MSCs in patients with PC dyscrasias differ significantly from their healthy counterparts and have a prognostic impact on clinical outcome. We show that the expression of MSC specific genes within the microenvironment varies substantially between disease stages, suggesting progression of the supporting bone marrow niche towards a more aggressive phenotype as the disease evolves. Importantly, this is the first study to show that the gene expression pattern of MSCs has an independent prognostic impact on survival. In contrast to previous GEP-based studies that investigated genes differentially expressed between cultured MSCs from multiple myeloma patients and healthy controls (21), this study first defined MSC-specific genes, based on high expression in MSCs but not by PCs and other cells in the bone marrow niche. This approach allowed to investigate changes in MSCs in an *in vivo* model using whole bone marrow biospies.

A MSC gene score derived from overexpression of *COL4A1*, and underexpression of *NPR3* and *ITGBL1* in multiple myeloma microenvironment, had a significant impact on PFS and was also able to reliably predict the PFS of treated multiple myeloma patients that had achieved a CR. Furthermore, a high-risk MSC gene score was associated with progression of MGUS/SMM patients to multiple myeloma. These results emphasize that

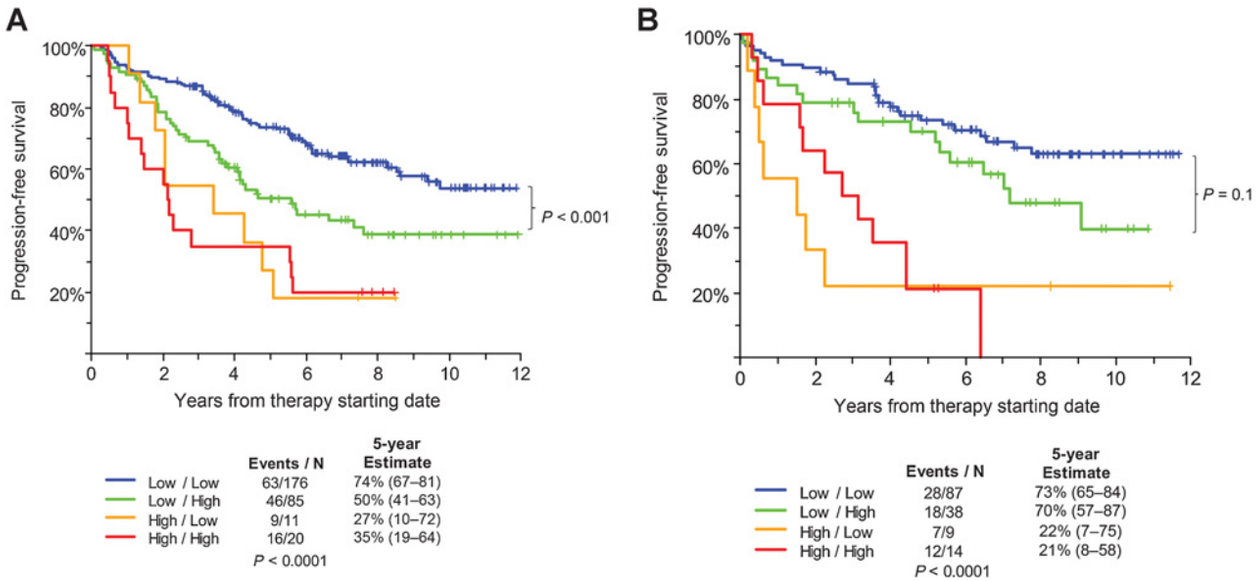

Figure 4.

The prognostic impact of the 3-gene MSC score is independent of the plasma cell–derived GEP70 signature. The 3-gene MSC score was able predict PFS in patients with GEP70-defined low-risk disease (training set $P < 0.001$, test set $P = 0.1$; **A** and **B**), but could not split the curves in patients with GEP70-defined high-risk disease (**A** and **B**).

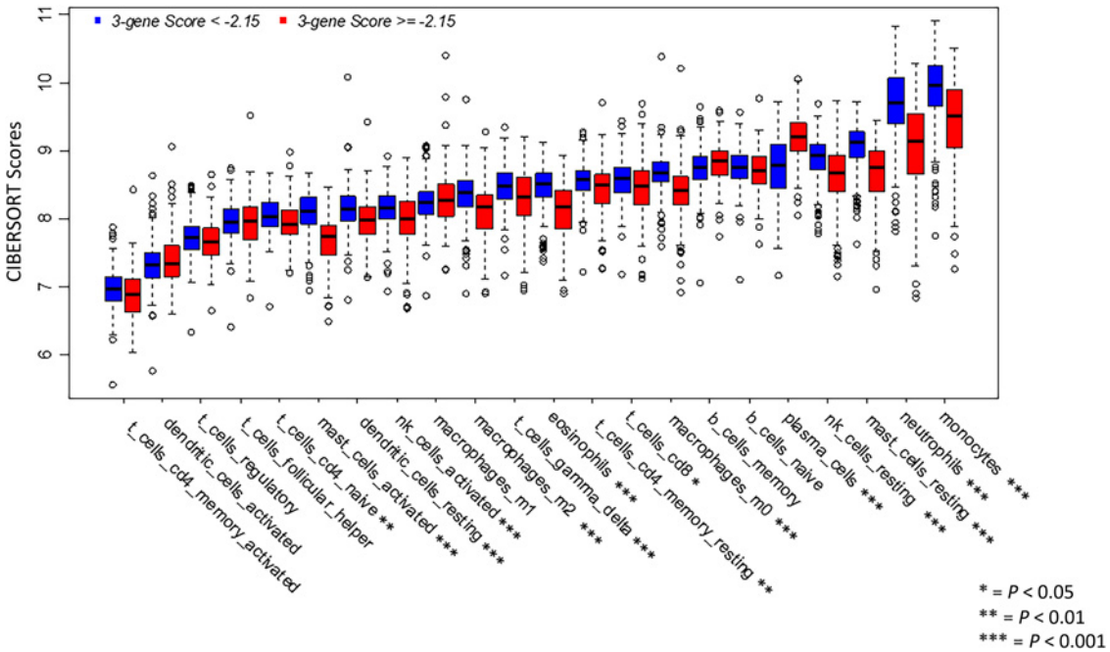

Figure 5.

High 3-gene MSC gene score is associated with reduced populations of immune cells in bone marrow of patients with multiple myeloma. The CIBERSORT analysis showed that most leukocyte populations are reduced within the microenvironment in patients with a high 3-gene MSC score compared with a low score, suggesting that a high score (>−2.15) is associated with immune cell deregulation. Expression levels of leukocyte populations that are significantly different between the 3-gene MSC high and low score are shown in Supplementary Table S5.

progression of multiple myeloma towards a more aggressive phenotype and of SMM to multiple myeloma does not solely rely on intrinsic PC factors, but are independently impacted by the biology of the surrounding microenvironment. The top gene in the MSC 3-gene score, *COL4A1*, is a type IV collagen alpha protein that is an integral component of the basement membrane where it interacts with other extracellular matrix components (22, 23). It has been shown to control the formation of new capillaries and also to regulate HIF-1α and VEGF expression, presumably by inhibiting the MAPK signaling cascade, thereby exerting an anti-angiogenic effect (24). Recent reports have suggested that *COL4A1* modulates progression of MGUS to multiple myeloma and that other members of the type IV collagen family play crucial roles in the multiple myeloma microenvironment (25, 26). The other constituents of the MSC three-gene score natriuretic peptide receptor 3 (*NPR3*) and integrin beta like 1 (*ITGBL1*) have been shown to play important roles within the extracellular matrix and a few reports suggest they contribute to tumor growth and carcinogenesis (27, 28).

In contrast to GEP70-defined low-risk patients, the tree-gene MSC score was not able to split PFS curves in GEP70-defined high-risk patients, suggesting that the biology of multiple myeloma cells in HR disease remains the main driver of outcome. When investigating further alterations in the microenvironment, we show that the prognostically adverse high three-gene MSC score is significantly associated with a reduced abundance of most of the immune cells in the bone marrow niche. The resulting immune-dysfunction could further play a crucial role in not only disease progression, but also diminished response to immunotherapies. Further research will be necessary to show how aberrant pathways of the microenvironment can be used to influence treatment decisions and could potentially be used as therapeutic targets.

Disclosure of Potential Conflicts of Interest

G.J. Morgan reports receiving speakers bureau honoraria from Janssen and is a consultant/advisory board member for Celgene, Janssen, and Takeda. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: C. Schinke, S.J. Mehdi, B. Barlogie, S. Yaccoby, G.J. Morgan

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Schinke, S.J. Mehdi, J. Epstein, S.K. Johnson, F. van Rhee, M. Zangari, S. Thanendrarajan, B. Barlogie, F.E. Davies, S. Yaccoby, G.J. Morgan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Schinke, P. Qu, A. Hoering, B. Barlogie, F.E. Davies, S. Yaccoby, G.J. Morgan

Writing, review, and/or revision of the manuscript: C. Schinke, P. Qu, A. Hoering, J. Epstein, S.K. Johnson, M. Zangari, S. Thanendrarajan, B. Barlogie, F.E. Davies, S. Yaccoby, G.J. Morgan

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Schinke, B. Barlogie, S. Yaccoby, G.J. Morgan

Study supervision: S. Yaccoby

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