Proliferation is a central independent prognostic factor and target for personalized and risk-adapted treatment in multiple myeloma

Dirk Hose,[1,2] Thierry Rème,[3,4] Thomas Hielscher,[5] Jérôme Moreaux,[3,4] Tobias Messner,[1] Anja Seckinger,[1] Axel Benner,[5] John D. Shaughnessy Jr,[6] Bart Barlogie,[6] Yiming Zhou,[6] Jens Hillengass,[1] Uta Bertsch,[1] Kai Neben,[1] Thomas Möhler,[1] Jean François Rossi,[3,4] Anna Jauch,[7] Bernard Klein,[3,4] and Hartmut Goldschmidt[1,2]

[1]Medizinische Klinik V, Universitätsklinikum Heidelberg, Heidelberg, Germany; [2]Nationales Centrum für Tumorerkrankungen, Heidelberg, Germany; [3]CHU Montpellier, Institute for Research in Biotherapy, Hôpital Saint-Eloi, Montpellier, France; [4]INSERM U847, Montpellier, France; [5]Abteilung für Biostatistik, Deutsches Krebsforschungszentrum Heidelberg, Heidelberg, Germany; [6]Myeloma Institute for Research and Therapy, University of Arkansas for Medical Sciences, Little Rock, AR, USA; and [7]Institut für Humangenetik, Universitätsklinikum Heidelberg, Heidelberg, Germany

Acknowledgments: the authors would like to thank Véronique Pantesco, Katrin Heimlich, Maria Dörner and Gabriele Hoock for technical assistance.

Funding: this work was supported in part by grants from the Hopp-Foundation, Germany, the University of Heidelberg, Germany, the National Center for Tumor Diseases, Heidelberg, Germany, the Tumorzentrum Heidelberg/Mannheim, Germany, the Deutsche Forschungsgemeinschaft, Bonn, Germany, the European Myeloma Stem Cell Network (MSCNET) funded within the 6th framework program of the European Community and the Ligue Nationale Contre Le Cancer, Paris, France.

Manuscript received on July 7, 2010 Revised version arrived on August 25, 2010. Manuscript accepted on September 20, 2010.

Correspondence:
Dirk Hose, Medizinische Klinik V, Universitätsklinikum Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg, Germany.
Phone: +49.6221.5639047;
Fax: +49.6221.566238.
E-mail: dirk.hose@med.uni-heidelberg.de

ABSTRACT

Background
Proliferation of malignant plasma cells is a strong adverse prognostic factor in multiple myeloma and simultaneously targetable by available (e.g. tubulin polymerase inhibitors) and upcoming (e.g. aurora kinase inhibitors) compounds.

Design and Methods
We assessed proliferation using gene expression-based indices in 757 samples including independent cohorts of 298 and 345 samples of CD138-purified myeloma cells from previously untreated patients undergoing high-dose chemotherapy, together with clinical prognostic factors, chromosomal aberrations, and gene expression-based high-risk scores.

Results
In the two cohorts, 43.3% and 39.4% of the myeloma cell samples showed a proliferation index above the median plus three standard deviations of normal bone marrow plasma cells. Malignant plasma cells of patients in advanced stages or those harboring disease progression-associated gain of 1q21 or deletion of 13q14.3 showed significantly higher proliferation indices; patients with gain of chromosome 9, 15 or 19 (hyperdiploid samples) had significantly lower proliferation indices. Proliferation correlated with the presence of chromosomal aberrations in metaphase cytogenetics. It was significantly predictive for event-free and overall survival in both cohorts, allowed highly predictive risk stratification (e.g. event-free survival 12.7 *versus* 26.2 *versus* 40.6 months, $P<0.001$) of patients, and was largely independent of clinical prognostic factors, e.g. serum β_2-microglobulin, International Staging System stage, associated high-risk chromosomal aberrations, e.g. translocation t(4;14), and gene expression-based high-risk scores.

Conclusions
Proliferation assessed by gene expression profiling, being independent of serum-β_2-microglobulin, International Staging System stage, t(4;14), and gene expression-based risk scores, is a central prognostic factor in multiple myeloma. Surrogating a biological targetable variable, gene expression-based assessment of proliferation allows selection of patients for risk-adapted anti-proliferative treatment on the background of conventional and gene expression-based risk factors.

Key words: gene expression profiling, multiple myeloma, proliferation, survival, risk-adapted treatment, risk score.

Citation: Hose D, Rème T, Hielscher T, Moreaux J, Messner T, Seckinger A, Benner A, Shaughnessy Jr. JD, Barlogie B, Zhou Y, Hillengass J, Bertsch U, Neben K, Möhler T, Rossi JF, Jauch A, Klein B, and Goldschmidt H. Proliferation is a central independent prognostic factor and target for personalized and risk-adapted treatment in multiple myeloma. Haematologica 2011;96(1):87-95. doi:10.3324/haematol.2010.030296

Introduction

Multiple myeloma is an incurable malignant disease of clonal plasma cells which accumulate in the bone marrow causing clinical signs and symptoms related to the displacement of normal hematopoiesis, formation of osteolytic bone lesions, and production of monoclonal protein.[1] Malignant plasma cells of most newly diagnosed patients are characterized by a low proliferation rate. This rate increases from early to advanced stage plasma cell dyscrasia to relapsed disease[2,3] and is one of the strongest adverse prognostic factors.[3-7] "Traditional" methods for assessing proliferation in myeloma, i.e. ^{3}H-thymidine uptake,[3,8] bromodeoxyuridine uptake,[9-11] flow-cytometric cell-cycle analysis using propidium iodide[6] or assessment of Ki-67 expression,[12] did not find widespread use. This is mainly due to the not negligible effort involved in these methods, and the assumption that similar groups of patients could be identified either by conventional prognostic factors, e.g. the International Staging System (ISS) in combination with lactate dehydrogenase (LDH), or by gene expression-based risk scores.[13,14] Whereas gene expression-based assessment of proliferation[15-18] is available for large cohorts of patients, it has been hampered by the less proven association with proliferation as a "biological" variable, as well as the assumed coverage by conventional and novel prognostic factors as stated above. Consequently, proliferation has never been the focus of an analysis in a large cohort of patients considering its association with clinical prognostic factors, chromosomal aberrations, and novel gene expression-based high-risk scores.[3-7] Although these other factors allow risk stratification, "proliferation" is of special interest, as it can be targeted by available treatments (e.g. tubulin polymerase inhibitors) and upcoming therapeutic treatment options (e.g. aurora kinase inhibitors)[18].

In this study, we first explored gene expression-based proliferation assessment to allow determination of proliferation of primary myeloma cell samples in a clinical setting. Subsequently, we examined the prognostic relevance of proliferation in two independent cohorts of myeloma patients treated with high-dose chemotherapy and autologous stem cell transplantation and its potential relation with current risk factors, i.e. serum β$_2$-microglobulin (B2M), ISS, LDH, presence of the translocation t(4;14)[19] and gene expression-based high-risk signatures.[13,14]

Design and Methods

Patients and healthy donors

Patients presenting with previously untreated multiple myeloma (n=298) or monoclonal gammopathy of unknown significance (n=23) at the University Hospitals of Heidelberg, Germany, and Montpellier, France, and 14 healthy donors were included after written informed consent in this study approved by the institutional ethics committees (#229/2003). Patients were diagnosed and staged and their response to treatment was assessed according to standard criteria.[20-23] Two hundred and nine non-selected patients underwent frontline high-dose chemotherapy with 200 mg/m^2 melphalan and autologous stem cell transplantation. Survival data were validated by an independent cohort of 345 patients treated within the total therapy 2 protocol.[24] The patients' clinical parameters and treatment schedules are presented in *Online Supplementary Table S1*.

Samples

An overview of the samples studied is given in *Online Supplementary Table S2*. Bone marrow plasma cells,[18] peripheral CD27$^+$ memory B cells,[25] and polyclonal plasmablastic cells[26] were generated as described previously. The XG lines were generated at INSERM U847 as previously reported,[27-29] the human myeloma cell lines U266, RPMI-8226, LP-1, OPM-2, SK-MM-2, AMO-1, JJN-3, NCI-H929, KMS-12-BM, KMS-11, KMS-12-PE, KMS-18, MM1.S, JIM3, KARPAS-620, L363 and ANBL6 were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and the American Type Cell Culture (Wesel, Germany), respectively, and cultured as recommended.

Interphase fluorescence in situ hybridization

Online Supplementary Table S2 gives an overview of the probes used. Analyses were performed on CD138-purified plasma cells[18] and the presence of clonal/subclonal aberrations as well as the absolute number of chromosomal aberrations present were defined as described.[30] The score of Wuilleme *et al.*[31] was used to assess ploidy. The percentage of malignant plasma cells was surrogated by the highest percentage of all tested chromosomal aberrations within this sample.

Gene expression profiling

Gene expression profiling (GEP) was performed as reported.[18,32,33] Labeled cRNA was hybridized to U133 A+B GeneChip microarrays (Affymetrix, Santa Clara, CA, USA) for the Heidelberg/Montpellier group 1 (HM1), and U133 2.0 plus arrays for the Heidelberg/Montpellier group 2 (HM2) according to the manufacturer's instructions. Expression data for myeloma cell samples are deposited in ArrayExpress under accession numbers E-MTAB-316 (HM1, Affymetrix U133 A+B chips), E-MTAB-317 (HM2, U133 2.0 chips), E-GEOD-2658 and Gene Expression Omnibus GSE4581 [the latter two for total therapy 2 data (Little Rock group, LR) and molecular class association, respectively].

Measurement of proliferation of primary myeloma cells by propidium iodine

The plasma cell labeling index, i.e. the percentage of myeloma cells in S-phase, was determined as described previously.[18]

Statistical analysis

Details of the statistical analysis, including the calculation of the gene expression-based proliferation indices by Bergsagel *et al.*[15] (GEP-B), Shaughnessy *et al.*[17] (GEP-SH) and Hose *et al.*[18] (GPI), are provided in the *Online Supplementary Design and Methods*.

Results

Gene expression-based assessment of proliferation

All three gene expression-based proliferation indices showed comparable correlations with the labeling index of primary myeloma cells assessed by propidium iodide staining [n=66, r_s=0.42 (GPI), *Online Supplementary Figure S1*, r_s=0.48 (GEP-SH), r_s=0.52 (GEP-B), all P=0.001] and strong correlations between each other: r_s=0.98 (GPI:GEP-SH), r_s=0.96 (GPI:GEP-B), r_s=0.94 (GEP-SH:-B), all P=0.001. The unsupervised clustering based on the genes in the respective indices split into two branches, one containing normal bone marrow plasma cells, memory B cells and about half of the myeloma patients, the other containing all proliferating plasmablasts and myeloma cell lines, clustering together, as well as the other half of the myeloma patients. Myeloma cell samples in this branch showed a prolifera-

tion signature that resembled, to a variable degree, that of plasmablasts or myeloma cell lines (Figure 1). A similar distribution was found if only myeloma cell samples were clustered (*Online Supplementary Figure S2*). In the following, results are shown for the GPI, as this index gives the best prognostic information (Figure 2, *Online Supplementary Figure S3*). The corresponding results for the other indices are depicted in *Online Supplementary Figures S4 and S5*.

The GPI is significantly higher for myeloma cells than for normal plasma cells or non-proliferating memory B cells, but significantly lower than for proliferating plasmablasts or myeloma cell lines ($P<0.001$ in HM1, $P=0.001$ in HM2, Figure 3). A significant stage-dependent difference in GPI was found between myeloma cells from patients with early disease (monoclonal gammopathy of undetermined significance and Durie-Salmon stage I multiple myeloma) and those with advanced disease (Durie-Salmon stage II and III multiple myeloma) ($P<0.001$ in HM1, $P=0.02$ in HM2, Figure 3). The same observations held true for the other two indices (*Online Supplementary Figure S5*). In our series and the LR-data set, 43.3% and 39.4%, respectively, of all myeloma cell samples showed a GPI above the median plus three standard deviations of normal plasma cells, and thus a similar fraction of previously untreated patients (Figure 4). A comparable percentage of patients was identified using the other two indices (GEP-B 43.6% and 31.3%, GEP-SH 39.3% and 37.4% above the median of normal donor plasma cells in our and the LR-data, respectively).

Association of proliferation with chromosomal aberrations and molecular subgroups

Of the aberrations investigated by interphase FISH, samples with a gain of 1q21 showed a significantly higher GPI than samples without this gain (HM2 $P<0.001$, LR $P=0.001$) and, likewise, samples with a deletion of 13q14.3 showed a significantly higher GPI than samples without the deletion. A gain of 9q34, 15q22, 19q13 or 11q23 was associated with a significantly lower GPI (*Online Supplementary Table S3*). Neither the absolute number of chromosomal aberrations (median 3.0 each, n=175 patients, 8 interphase FISH probes tested) nor the presence of subclonal aberrations tested by interphase FISH was significantly different between myeloma cells showing a GPI above or below the median. However, patients within the LR-group with the presence of any chromosomal aberration, as detected by metaphase cytogenetics, showed a significantly higher GPI than patients without any chromosal aberration ($P<0.001$).

The only GEP-based group with a significantly higher median GPI was the "proliferation" group within the molecular classification of the UAMS (University of Arkansas for Medical Sciences). Patients with GPI^{high} were found throughout the entities in all GEP-based classifications (*Online Supplementary Figure S6*). The likely explanation for this is that progression to a proliferative state can develop within all the molecular entities. This in turn makes it necessary to assess proliferation alongside the GEP-based classifications.

Proliferation and other conventional prognostic factors

As a single continuous variable the GPI was significantly predictive for event-free and overall survival in both groups (HM2 and LR). The same held true for a GPI above *versus* below the median in myeloma cells ($GPI^{high/low}$, HM1, HM2, LR; Figure 1, *Online Supplementary Figure S4*). By delineating a "high-proliferation group" above the median and two standard deviations, three significantly different proliferation groups could be distinguished (Figure 2B, *Online Supplementary Figure S3B, Online Supplementary Table S4*). The same held true if the thresholds of the HM2 group were applied to the LR data normalized with the docval-package (see *Online Supplementary Design and Methods*, *Online Supplementary Figure S7*).

It is interesting to note here that we initially started with the hypothesis that high-risk as defined by proliferation would be captured either by gene expression-based high-risk scores or conventional factors, especially B2M and LDH. As expected, B2M and LDH each allowed the delineation of a group of patients with an adverse prognosis within the LR cohort investigated here. When using the cut-offs used in the ISS, i.e. B2M 3.5 mg/dL and 5.5 mg/dL, in each case three groups of patients with significantly different overall survival rates (after 60 months: 73% *versus* 58.4% *versus* 45.8%, $P<0.001$) and event-free survival (70.7 *versus* 42.7 *versus* 32.6 months, $P<0.001$) were delineated. B2M was significantly higher only in the GPI^{high}-group ($P=0.03$), (B2M 2.7, 2.95 and 4 mg/dL in the GPI^{high}, GPI^{median} and GPI^{low} groups, respectively). The median LDH concentration was significantly higher in the GPI^{median} (172 U/l, P<0.001) and GPI^{high} (234 U/l, P<0.001) groups than in the GPI^{low} group (146 U/L). The same held true for LDH in terms of event-free survival (62.6 *versus* 18.5 months, $P<0.001$) and overall survival (after 60 months, 68.9 % *versus* 30.5 %, $P<0.001$, using an optimal threshold of LDH >249 U/L calculated by maximum log-rank statistics). When combining LDH and B2M [group 1, $B2M^{high}$ (>3.5 mg/dL), LDH^{high} (>249 U/L); group 2, only one of the two factors elevated; group 3, both factors high], three groups of patients with significantly different event-free survival (18.1 *versus* 41.8 *versus* 72.8 months, $P<0.001$) and overall survival rates (after 60 months: 24.4% *versus* 56.1% *versus* 75.4%, $P<0.001$) could be delineated. Despite LDH showing a correlation with GPI (r=0.38, $P<0.01$), of the GPI^{high}-(27 patients) and LDH^{high} / $B2M^{high}$-risk groups (27 patients), only seven patients (i.e. 26%) overlapped. One possible reason is that the GPI is a surrogate for the integral median of proliferation of the respective myeloma cell population, independently of the total number of myeloma cells, whereas the serum LDH is dependent on the LDH production of a single myeloma cell times the number of myeloma cells.

In a multivariate model, the GPI remained a prognostic factor independent of the main clinical factors used for risk stratification, i.e. B2M or ISS stage, which also showed prognostic value as single variables (Figure 2C,D, *Online Supplementary Figure S3C,D*, Table 1). In a Cox model with GPI and translocation t(4;14), both factors remained independently prognostic. The same held true if B2M was added (*Online Supplementary Table S4*).

Gene expression-based proliferation indices and expression-based high-risk scores

The gene expression-based high-risk scores of Shaughnessy *et al.*[13] (Shaughnessy-HR) and Decaux *et al.*[14] (Decaux-HR) both significantly delineated a group of patients with an adverse risk (HM1, HM2 and LR; Figure 2F,G, *Online Supplementary Figure S3F,G, Online Supplementary Table S4A*). The Shaughnessy-HR score was also significant as a continuous variable (event-free sur-

vival: HM2, $P<0.001$, LR, $P<0.001$; overall survival: HM2, $P<0.001$, LR $P<0.001$). An interesting question here is the overlap between patients identified as being at high risk by GPI and those at high risk according to the expression-based high-risk indices: in a Cox-model tested with either the Shaughnessy-HR or the Decaux-HR (both as dichotomized variables), the GPI as a continuous or dichotomized variable was mostly independently predictive for event-free and overall survival in our and the LR-data (*Online Supplementary Table S4B*) despite being significantly higher in high-risk patients than in low-risk ones (Shaughnessy-HR, P= n.a., $P<0.001$, $P<0.001$ and Decaux-HR $P=0.005$, $P<0.001$, $P<0.001$, for HM1, HM2 and LR, respectively). Likewise, there was only a partial overlap between patients identified as high risk by the HR-scores and the GPI^{high} (Table 2). This is also reflected by the limited number of genes within the HR-indices showing a correlation with the GPI, i.e. 10/70 and 5/70 genes of the Shaughnessy-HR score and 1/15 and 1/15 genes of the Decaux-HR score correlated with an r_P greater than 0.8 with the GPI in the HM2 and LR, respectively (*Online Supplementary Table S5*). Taken together, some of the genes in the risk scores are associated with proliferation, and there was a partial overlap of patients identified as high risk by the HR-scores and the GPI. The likely explanation is that the HR-scores are derived from an association of genes in "high risk" patients, i.e. patients with a short overall survival. As patients with highly proliferative myeloma cells (or high GPI) have a very adverse prognosis, it can be expected that features of the gene expression of these patients (namely proliferation genes) are also present in the high-risk score.

Overlap of high-risk scores

The percentages of patients identified as "high-risk" by conventional prognostic factors, genetic markers, gene expression-based risk scores and the GPI and the overlap of the respective groups of patients are shown in Table 2.

Discussion

Gene expression-based assessment of proliferation

Current gene expression-based proliferation indices have been constructed in different ways: genes used in the indices by Shaughnessy's group[17] and Bergsagel *et al.*[15] were selected simply for the fact of being "associated with proliferation" with only one gene overlapping between these two indices, whereas our index was constructed by selecting proliferation genes (in terms of gene ontology) differentially expressed between proliferating malignant

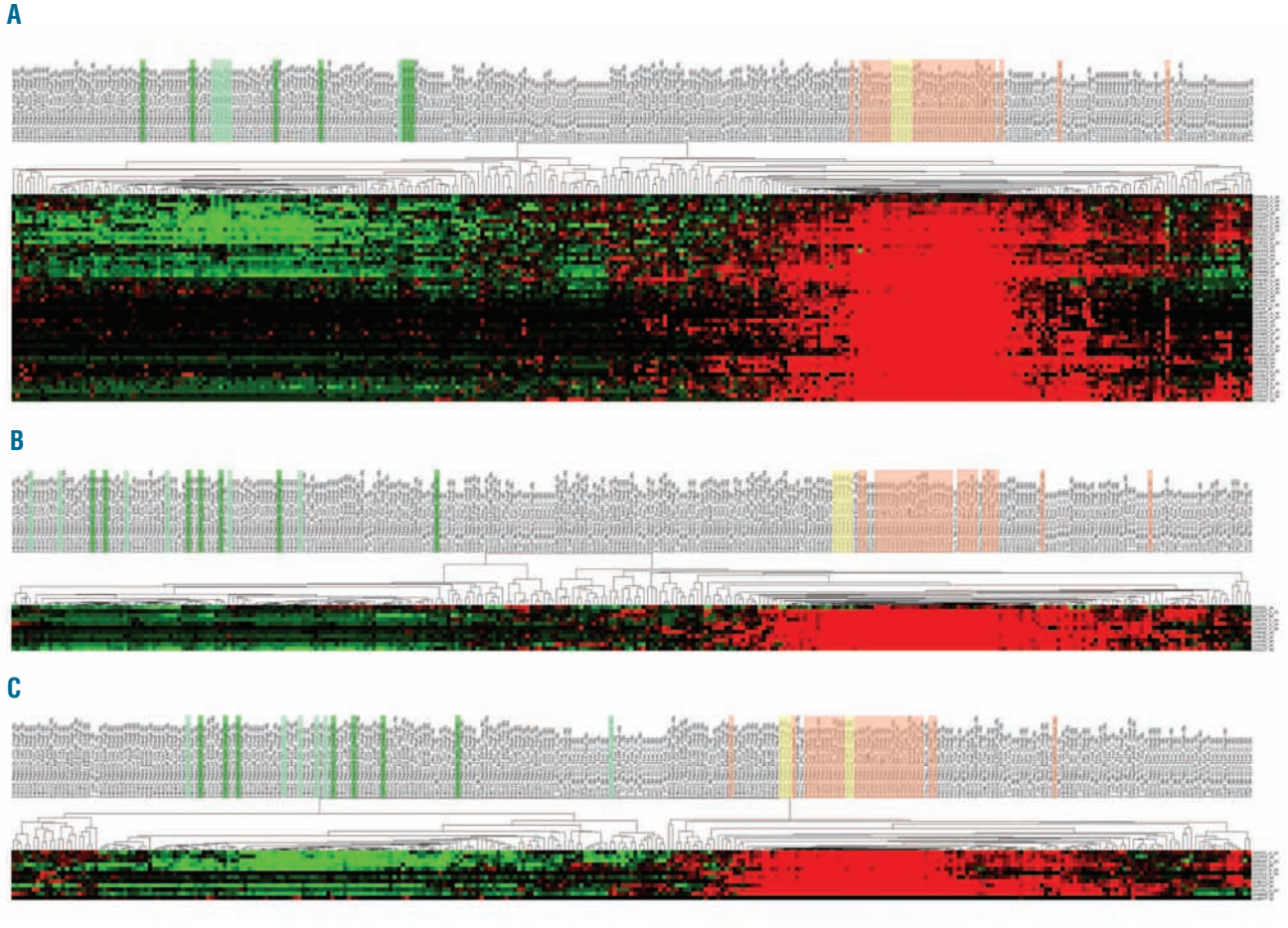

Figure 1. Unsupervised hierarchical clustering of normal bone marrow plasma cells (dark green), polyclonal plasmablastic cells (yellow), memory B cells (light green), myeloma cell lines (red) and myeloma cells (white). Clustering based on (A) the GPI of Hose *et al.*, (B) the index of Shaughnessy's group and (C) the index of Bergsagel *et al.* The data for the HM2-group are shown (see *Online Supplementary Design and Methods* for details).

(myeloma cell lines; "malignant proliferation") as well as non-malignant cells (proliferating plasmablastic cells; "benign proliferation") and non-proliferating, non-malignant cells (normal plasma cells and memory B cells),[18] therefore having a less arbitrary biological definition (Table 1 shows the genes in the three indices and *Online Supplementary Table S6* provides a comparison). Despite these methodological differences, all three indices correlated reasonably well with proliferation as determined by propidium iodide-staining (*Online Supplementary Figure* S1) and showed a very strong correlation with each other. Thus in the following, results are discussed for the GPI only. All results obtained with the GPI are in agreement with published findings: normal plasma cells and plasma

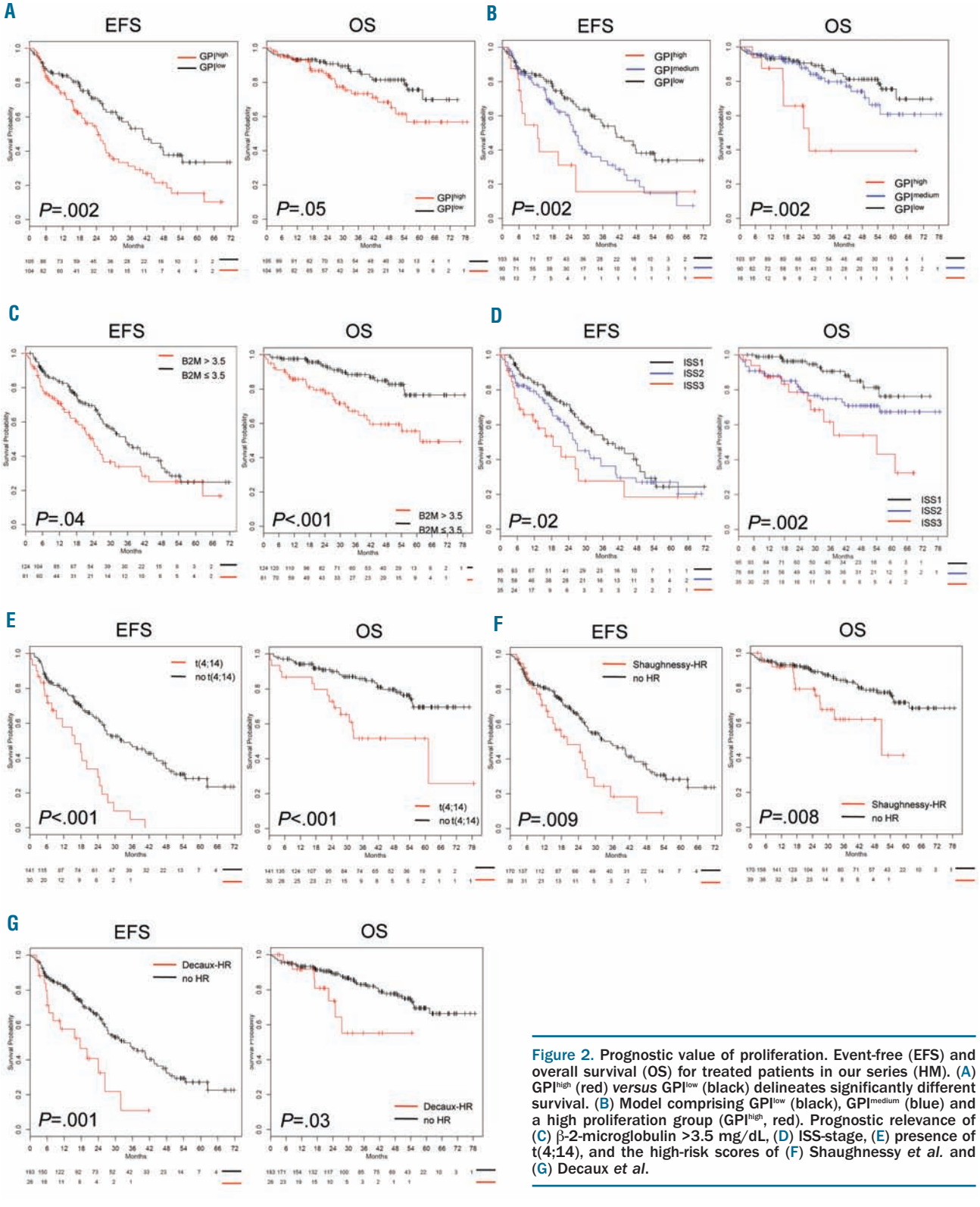

Figure 2. Prognostic value of proliferation. Event-free (EFS) and overall survival (OS) for treated patients in our series (HM). (A) GPI^{high} (red) *versus* GPI^{low} (black) delineates significantly different survival. (B) Model comprising GPI^{low} (black), GPI^{medium} (blue) and a high proliferation group (GPI^{high}, red). Prognostic relevance of (C) β-2-microglobulin >3.5 mg/dL, (D) ISS-stage, (E) presence of t(4;14), and the high-risk scores of (F) Shaughnessy *et al.* and (G) Decaux *et al.*

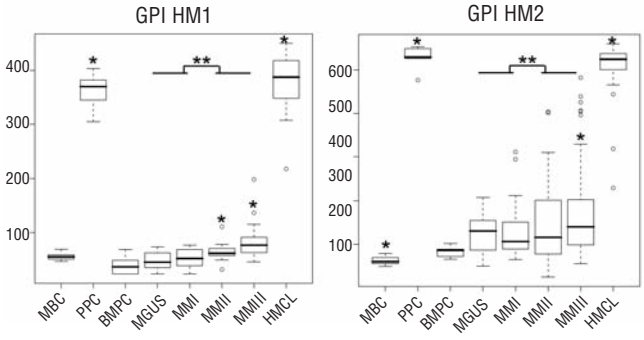

Figure 3. Gene expression-based proliferation index of memory B cells (MBC), polyclonal plasmablastic cells (PPC), normal bone marrow plasma cells (BMPC), myeloma cells (MMC) and myeloma cell lines (HMCL). MMC samples are subdivided into monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM) I/II/III according to Durie-Salmon stage. Significant differences between BMPC and other samples are indicated by one asterisk (*), and between early (MGUS/MMI) and late (MMII/III) stages by two asterisks (**).

cells of almost all patients with monoclonal gammopathy of undetermined significance do not show measurable proliferation *in vitro*.[2,34] A significantly higher but still low proliferation rate is found in most patients with newly diagnosed myeloma.[2,34] In our series (HM) and the LR one, about 40% of all myeloma cell samples had a GPI above the range of that of normal bone marrow plasma cells plus three standard deviations (Figure 4). Considering the density plot, boxplot and unsupervised clustering (Figures 1, 3 and 4, *Online Supplementary Figures S2 and S5*), proliferation values were continuously distributed with a small fraction of patients' myeloma cells showing a cell-line-like proliferation rate.

Association of proliferation with chromosomal aberrations, gene expression profiling-delineated groups of multiple myeloma, and D-type cyclin expression

The total number of aberrations as detected by interphase FISH was not significantly different in myeloma cells with a high or low GPI, so there is no indication that proliferation drives chromosomal aberrations, or *vice versa*. Nevertheless, proliferation in our series was associated with chromosomal aberrations related to disease progression, i.e. gain of 1q21 and deletion of 13q14.3.[35-37] At the same time, of all chromosomal aberrations supposedly connected with etiological groups in myeloma, only chromosomal gains associated with hyperdiploidy (i.e. chromosomes 5, 15 and 19) were associated with a significantly different (lower) proliferation rate, whereas neither t(4;14) (despite its association with deletion of 13q14) nor t(11;14) was associated with a different rate of proliferation.

Within gene expression-based classifications of myeloma (e.g. molecular classification of myeloma, TC-classification),[17,38-40] the only group showing a significantly higher proliferation rate (GPI) was the "proliferation group" within the molecular classification (*Online Supplementary Figure S6*).[17] Nevertheless, in all groups, patients' samples with high GPI could be found (*Online Supplementary Figure S6*).

D-type cyclin expression, a hallmark of myeloma,[38,39,41] was not associated with proliferation, in perfect agreement with its function as a "threshold sensor" triggering G1-entry.[42]

Collectively, these data indicate that myeloma cells in all etiological groups can proliferate, i.e. proliferation is a biological factor independent of etiology.

Proliferation is a continuous prognostic variable

Of note, the GPI was developed as a surrogate of a biological variable, without any input of prognostic information. Proliferation as assessed by plasma cell labeling indices using ^{3}H-thymidine-incorporation,[3] DNA/CD38 double-staining[6] or a BrdU-based technique[7,10,11] has frequently been shown to be of strong prognostic relevance. Here, proliferation as a continuous variable was similarly prognostic, in terms of event-free and overall survival, in all cohorts of patients (*Online Supplementary Figure S4*). This was the case if two groups were distinguished by values above or below the median, or if three groups were formed by additionally delineating a "high proliferation" group as suggested by the distribution of proliferation (Figure 4). The model remained prognostic when the thresholds from the HM2 group were used for individual *cel-files of the LR-group, normalized with the respective parameters of HM2 (*Online Supplementary Figure S7*; *Online Supplementary Design and Methods*). Using a model based on log-rank testing to define optimal thresholds of the GPI, different cut-offs for event-free survival and overall survival appeared in each cohort, which could not be explained by the existence of defined "proliferation groups". Likewise, considering the biological reasoning above, we interpret proliferation as a continuous prognostic variable that can be dichotomized or divided into three ranges for clinical risk assessment.

Proliferation is an independent prognostic variable

The GPI is, therefore, independent of the most prominent clinical risk factors, i.e. ISS stage and B2M concentra-

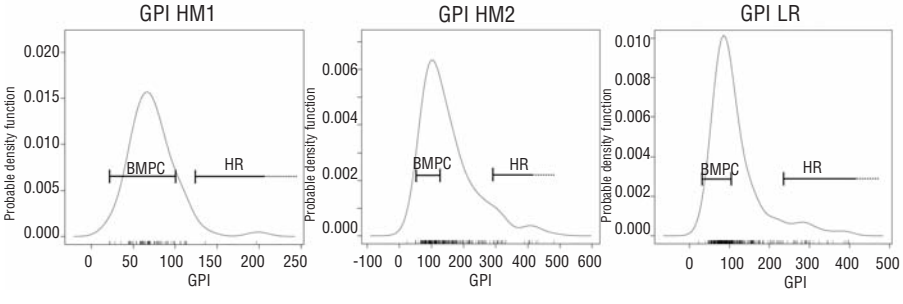

Figure 4. Distribution of proliferation. Distribution of the GPI for newly-diagnosed patients within our series (HM1 and HM2; n=298) and the Little Rock-data (LR; n=345). Horizontal bars indicate the median GPI plus three standard deviations of normal plasma cells (BMPC) and the high proliferation group (HR) defined as the median GPI of myeloma cell samples plus two standard deviations.

Probeset	Gene Symbol	Cytoband
219918_s_at	ASPM	1q31
204092_s_at	AURKA	20q13.2-q13.3
209464_at	AURKB	17p13.1
202095_s_at	BIRC5	17q25
204531_s_at	BRCA1	17q21
209642_at	BUB1	2q14
203755_at	BUB1B	15q15
203418_at	CCNA2	4q25-q31
214710_s_at	CCNB1	5q12
202705_at	CCNB2	15q22.2
203213_at	CDC2	10q21.1
202870_s_at	CDC20	1p34.1
205167_s_at	CDC25C	5q31
203967_at	CDC6	17q21.3
221520_s_at	CDCA8	1p34.3
209714_s_at	CDKN3	14q22
218542_at	CEP55 (C10orf3)	10q23.33
205394_at	CHEK1	11q24-q24
201897_s_at	CKS1B	1q21.2
204170_s_at	CKS2	9q22
203764_at	DLG7	14q22.3
38158_at	ESPL1	12q
206102_at	GINS1	20p11.21
204318_s_at	GTSE1	22q13.2-q13.3
213008_at	KIAA1794	15q26.1
204444_at	KIF11	10q24.1
219306_at	KIF15	3p21.31
218755_at	KIF20A	5q31
209408_at	KIF2C	1p34.1
204162_at	KNTC2	18p11.32
203362_s_at	MAD2L1	4q27
220651_s_at	MCM10	10p13
201930_at	MCM6	2q21
212021_s_at	MKI67	10q25-qter
212789_at	NCAPD3	11q25
218662_s_at	NCAPG	4p15.33
219588_s_at	NCAPG2	7q36.3
204641_at	NEK2	1q32.2-q41
221923_s_at	NPM1	5q35
201202_at	PCNA	20pter-p12
200886_s_at	PGAM1	10q25.3
204887_s_at	PLK4	4q28
203554_x_at	PTTG1	5q35.1
222077_s_at	RACGAP1	12q13.13
204240_s_at	SMC2	9q31.1
203145_at	SPAG5	17q11.2
205339_at	STIL	1q32; 1p32
210052_s_at	TPX2	20q11.2
202954_at	UBE2C	20q13.12
204026_s_at	ZWINT	10q21-q22

Table 1. Genes incorporated within the gene expression-based proliferation indices (GPI) and high-risk scores. Our GPI comprises genes associated with the central cell-cycle machinery, i.e. cyclins B1 (*CCNB1*), B2 (*CCNB2*) and A2 (*CCNA2*). Cyclin A2 mediates, together with *CCNE1/2* in association with *CDK2*, S-phase entry; *CCNA2*, *CCNB1* and *CCNB2*, in association with *CDK1/2*, mediate mitotic entry. The main functions of cyclin-A (together with cyclin-E) are DNA replication and centromer-duplication, of cyclin-A additionally mitosis, whereas B-type cyclins act only on mitosis. Likewise, the mitotic regulators Aurora-kinases A (*ARKA*) and B (*ARKB*) and their associated proteins TPX2 and survivin (BIRC5) can be found in the GPI. The GPI further includes members of the spindle-checkpoint (e.g. *BUB1*, *BU1B*, *CDC20*). Additional genes comprise the proliferating cell nuclear antigen (*PCNA*) and the gene coding for Ki-67 (*MKI67*). Genes overlapping with the GPI of Bergsagel *et al.* are depicted in dark gray (additionally present in this index: *TYMS*, *TK1*, *KIAA101*, *TOP2A*, *TRIP13*), those with the index by Shaughnessy's group in medium gray (additionally present in this index: *TOP2A*, *ANAPC7*, *CDCA1*). No gene is present in all three indices. Genes also present in the 70 (17) gene high-risk score of Shaughnessy *et al.* are depicted in dark orange. No gene overlaps with the 15-gene high-risk score of Decaux *et al.*

tion, each being prognostic as a single variable (*Online Supplementary Table S4B*). This independence of B2M is in agreement with published data from Greipp *et al.*[4,5] Given the lack of association, it is not surprising that the GPI is a significant prognostic factor independent of t(4;14). This independence also remained when the Cox model additionally included B2M (*Online Supplementary Table S4*). An interesting question is to what extent proliferation is independent of gene expression-based high-risk scores. Like the proliferation index, the scores of the UAMS (Shaughnessy-HR)[13] and the Francophone Myeloma Intergroup (Decaux-HR)[14] are prognostic as single variables using the published thresholds or tested as continuous variables (Figure 2F,G, *Online Supplementary Figure S3F,G*, *Online Supplementary Table S4*). Tested together with the proliferation index, a certain degree of independence was found, partly depending on which variable was tested as a continuous variable (*Online Supplementary Table S4*). However, given the partial overlap of genes and the correlations between the risk scores and the GPI, a certain interdependence is not surprising (Tables 1 and 2). In the larger LR-cohort, the Shaughnessy-HR score was independently prognostic if tested with t(4;14), B2M, and GPI in a multivariate model (*Online Supplementary Table S4B*). Thus, the GPI, presence of t(4;14), B2M concentration and the risk score seem to carry complementary prognostic information.

"High-risk" groups of patients assessed by GPI or other scores

As described above, we initially started with the hypothesis that "high-risk" status, as defined by proliferation, could be captured either by gene expression-based high-risk scores or conventional factors, especially B2M and LDH. This, however, was not the case (see results for LDH and B2M and Table 2 depicting the overlap of the respective risk assessments). This finding is a strong argument for assessing proliferation in clinical settings, alongside these clinical prognostic factors, chromosomal aberrations, and GEP-based risk stratification.

Gene expression-based assessment of proliferation in a clinical setting

As "traditional" means of assessing proliferation have not found widespread clinical use, why should a gene expression-based approach? There are various reasons. (i) Proliferation is a central independent risk factor in myeloma, with only partial overlap with other "high-risk scores", and thus of high clinical interest (Table 2). (ii) Proliferation is unique due to the fact that it is not only an adverse prognostic factor and, therefore, enables risk-adapted treatment, but it can also be directly counteracted by anti-proliferative treatment. Within the context of clinical trials, compounds such as mitotic inhibitors, e.g. inhibitors of aurora kinase[18] or Eg5-Kinesin,[43] could be added in a personalized manner to the standard treatment regimens of patients selected due to a high GPI, with the hypothesis that this would result in higher response rates in these patients. Likewise, it is tempting to speculate that these patients might benefit from prolonged maintenance treatment after high-dose therapy and autologous stem cell transplantation. This issue will be addressed within the recently started phase III MM5 trial of the German Myeloma Multicenter Group (EudraCT No. 2010-019173-16). (iii) GEP can be performed routinely and is done in more than 80% of therapy-requiring patients in our institutions, and thus allows assessment of proliferation within large patient cohorts alongside high-risk scores. This, in turn, overcomes the need for additional laborious investigations on the background of limited numbers of malignant plasma cells available, and allows the assessment of proliferation from data already acquired.

Conclusion

Proliferation of malignant plasma cells, assessable by gene expression-based proliferation indices, is a central

Table 2. Delineation of "high risk" patients by the respective variable and scores. In each row, first the percentage of high-risk patients as identified by the respective factor is given. The next fields give the percentage of patients who are also identified by the other factors. Example: GPI identifies 7.7% of patients as high-risk, 21.4% of whom are also identified by the presence of t(4;14) as high-risk. GPI, gene-expression based proliferation index; HR, high-risk score; ISS, International Staging System; LDH, lactate dehydrogenase; t(4;14), presence of a translocation t(4;14); del 17p, deletion of 17p13.

	% (as high risk)	GPI high	Shaughnessy-HR	Decaux-HR	ISS	LDH	t(414)	del17p	NONE of others
GPI high	7.7	---	68.8	68.8	50.0	56.3	21.4	28.6	18.8
Shaughnessy-HR	18.7	28.2	---	35.9	25.6	35.9	43.8	18.8	21.9
Decaux-HR	12.4	42.3	53.8	---	38.5	42.3	26.1	18.2	31.8
ISS	17.0	22.9	28.6	28.6	---	51.4	13.0	22.7	36.4
LDH	20.2	19.0	33.3	23.8	42.9	---	12.5	17.4	26.1
t(4;14)	17.5	10.0	46.7	20.0	10.0	10.0	---	17.9	35.7
del17p	10.2	11.1	22.2	14.8	18.5	14.8	19.2	---	57.7
None	22.7	---	---	---	---	---	---	---	---

biological and prognostic factor in multiple myeloma, independent of B2M, ISS-stage, high risk-associated chromosomal aberrations, and gene expression-based risk-scores. Information on the GPI would enable the use of directly anti-proliferative treatment to be limited to patients showing an increased GPI, in turn providing the means for a personalized, risk-adapted, tailored treatment approach.

Authorship and Disclosures

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

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

References

1. Kyle RA, Rajkumar SV. Multiple myeloma. N Engl J Med. 2004;351(18):1860-73.
2. Witzig TE, Timm M, Larson D, Therneau T, Greipp PR. Measurement of apoptosis and proliferation of bone marrow plasma cells in patients with plasma cell proliferative disorders. Br J Haematol. 1999;104 (1):131-7.
3. Boccadoro M, Gavarotti P, Fossati G, Pileri A, Marmont F, Neretto G, et al. Low plasma cell 3(H) thymidine incorporation in monoclonal gammopathy of undetermined significance (MGUS), smouldering myeloma and remission phase myeloma: a reliable indicator of patients not requiring therapy. Br J Haematol. 1984;58(4):689-96.
4 Greipp PR, Lust JA, O'Fallon WM, Katzmann JA, Witzig TE, Kyle RA. Plasma cell labeling index and beta 2-microglobulin predict survival independent of thymidine kinase and C-reactive protein in multiple myeloma. Blood. 1993;81(12):3382-7.
5. Greipp PR, Katzmann JA, O'Fallon WM, Kyle RA. Value of beta 2-microglobulin level and plasma cell labeling indices as prognostic factors in patients with newly diagnosed myeloma. Blood. 1988;72(1): 219-23.
6. San Miguel JF, Garcia-Sanz R, Gonzalez M, Moro MJ, Hernandez JM, Ortega F, et al. A new staging system for multiple myeloma based on the number of S-phase plasma cells. Blood. 1995;85(2):448-55.
7. Steensma DP, Gertz MA, Greipp PR, Kyle RA, Lacy MQ, Lust JA, et al. A high bone marrow plasma cell labeling index in stable plateau-phase multiple myeloma is a marker for early disease progression and death. Blood. 2001;97(8):2522-3.
8. Latreille J, Barlogie B, Johnston D, Drewinko B, Alexanian R. Ploidy and proliferative characteristics in monoclonal gammopathies. Blood. 1982;59(1):43-51.
9. Schambeck CM, Wick M, Bartl R, Lamerz R, Fateh-Moghadam A. Plasma cell proliferation in monoclonal gammopathies: measurement using BU-1 antibody in flow cytometry and microscopy: comparison with serum thymidine kinase. J Clin Pathol. 1995;48(5):477-81.
10. Lokhorst HM, Boom SE, Bast BJ, Ballieux RE. Determination of the plasma cell labelling index with bromodeoxyuridine in a double fluorescence technique. Br J Haematol. 1986;64(2):271-5.
11. Greipp PR, Witzig TE, Gonchoroff NJ, Habermann TM, Katzmann JA, O'Fallon WM, et al. Immunofluorescence labeling indices in myeloma and related monoclonal gammopathies. Mayo Clin Proc. 1987;62 (11):969-77.
12. Alexandrakis MG, Passam FH, Kyriakou DS, Dambaki K, Niniraki M, Stathopoulos E. Ki-67 proliferation index: correlation with prognostic parameters and outcome in multiple myeloma. Am J Clin Oncol. 2004;27(1):8-13.
13. Shaughnessy JD, Jr., Zhan F, Burington BE, Huang Y, Colla S, Hanamura I, et al. A validated gene expression model of high-risk multiple myeloma is defined by deregulated expression of genes mapping to chromosome 1. Blood. 2007;109(6):2276-84.
14. Decaux O, Lode L, Magrangeas F, Charbonnel C, Gouraud W, Jezequel P, et al. Prediction of survival in multiple myeloma based on gene expression profiles reveals cell cycle and chromosomal instability signatures in high-risk patients and hyperdiploid signatures in low-risk patients: a study of the Intergroupe Francophone du Myelome. J Clin Oncol. 2008;26(29):4798-805.
15. Bergsagel PL, Kuehl WM, Zhan F, Sawyer J, Barlogie B, Shaughnessy J Jr. Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma. Blood. 2005; 106(1):296-303.
16. Perou CM, Jeffrey SS, van de Rijn M, Rees CA, Eisen MB, Ross DT, et al. Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. Proc Natl Acad Sci USA. 1999;96(16):9212-7.
17. Zhan F, Huang Y, Colla S, Stewart JP, Hanamura I, Gupta S, et al. The molecular classification of multiple myeloma. Blood. 2006;108(6):2020-8.
18. Hose D, Reme T, Meissner T, Moreaux J, Seckinger A, Lewis J, et al. Inhibition of aurora kinases for tailored risk-adapted treatment of multiple myeloma. Blood. 2009;113(18):4331-40.
19. Avet-Loiseau H, Attal M, Moreau P, Charbonnel C, Garban F, Hulin C, et al. Genetic abnormalities and survival in multiple myeloma: the experience of the Intergroupe Francophone du Myelome. Blood. 2007;109(8):3489-95.
20. Greipp PR, San Miguel J, Durie BG, Crowley JJ, Barlogie B, Blade J, et al. International staging system for multiple myeloma. J Clin Oncol. 2005;23(15):3412-20.
21. Durie BG. Staging and kinetics of multiple myeloma. Semin Oncol. 1986;13(3):300-9.
22. Durie BG, Salmon SE. A clinical staging system for multiple myeloma. Correlation of measured myeloma cell mass with presenting clinical features, response to treatment,

and survival. Cancer. 1975;36(3):842-54.
23. Blade J, Samson D, Reece D, Apperley J, Bjorkstrand B, Gahrton G, et al. Criteria for evaluating disease response and progression in patients with multiple myeloma treated by high-dose therapy and haemopoietic stem cell transplantation. Myeloma Subcommittee of the EBMT. European Group for Blood and Marrow Transplant. Br J Haematol. 1998;102(5): 1115-23.
24. Barlogie B, Tricot G, Rasmussen E, Anaissie E, van Rhee F, Zangari M, et al. Total therapy 2 without thalidomide in comparison with total therapy 1: role of intensified induction and posttransplantation consolidation therapies. Blood. 2006;107(7):2633-8.
25. Moreaux J, Cremer FW, Reme T, Raab M, Mahtouk K, Kaukel P, et al. The level of TACI gene expression in myeloma cells is associated with a signature of microenvironment dependence versus a plasmablastic signature. Blood. 2005;106(3):1021-30.
26. Tarte K, De Vos J, Thykjaer T, Zhan F, Fiol G, Costes V, et al. Generation of polyclonal plasmablasts from peripheral blood B cells: a normal counterpart of malignant plasmablasts. Blood. 2002;100(4):1113-22.
27. Zhang XG, Gaillard JP, Robillard N, Lu ZY, Gu ZJ, Jourdan M, et al. Reproducible obtaining of human myeloma cell lines as a model for tumor stem cell study in human multiple myeloma. Blood. 1994;83(12): 3654-63.
28. Gu ZJ, De Vos J, Rebouissou C, Jourdan M, Zhang XG, Rossi JF, et al. Agonist anti-gp130 transducer monoclonal antibodies are human myeloma cell survival and growth factors. Leukemia. 2000;14(1):188-97.
29. Rebouissou C, Wijdenes J, Autissier P, Tarte K, Costes V, Liautard J, et al. A gp130 interleukin-6 transducer-dependent SCID model of human multiple myeloma. Blood. 1998;91(12):4727-37.
30. Cremer FW, Bila J, Buck I, Kartal M, Hose D, Ittrich C, et al. Delineation of distinct subgroups of multiple myeloma and a model for clonal evolution based on interphase cytogenetics. Genes Chromosomes Cancer. 2005;44(2):194-203.
31. Wuilleme S, Robillard N, Lode L, Magrangeas F, Beris H, Harousseau JL, et al. Ploidy, as detected by fluorescence in situ hybridization, defines different subgroups in multiple myeloma. Leukemia. 2005; 19(2):275-8.
32. Hose D, Moreaux J, Meissner T, Seckinger A, Goldschmidt H, Benner A, et al. Induction of angiogenesis by normal and malignant plasma cells. Blood. 2009;114(1): 128-43.
33. Seckinger A, Meissner T, Moreaux J, Goldschmidt H, Fuhler GM, Benner A, et al. Bone morphogenic protein 6: a member of a novel class of prognostic factors expressed by normal and malignant plasma cells inhibiting proliferation and angiogenesis. Oncogene. 2009;28(44):3866-79.
34. Drewinko B, Alexanian R, Boyer H, Barlogie B, Rubinow SI. The growth fraction of human myeloma cells. Blood. 1981; 57(2):333-8.
35. Fonseca R, Barlogie B, Bataille R, Bastard C, Bergsagel PL, Chesi M, et al. Genetics and cytogenetics of multiple myeloma: a workshop report. Cancer Res. 2004;64(4):1546-58.
36. Hanamura I, Stewart JP, Huang Y, Zhan F, Santra M, Sawyer JR, et al. Frequent gain of chromosome band 1q21 in plasma-cell dyscrasias detected by fluorescence in situ hybridization: incidence increases from MGUS to relapsed myeloma and is related to prognosis and disease progression following tandem stem-cell transplantation. Blood. 2006;108(5):1724-32.
37. Zojer N, Konigsberg R, Ackermann J, Fritz E, Dallinger S, Kromer E, et al. Deletion of 13q14 remains an independent adverse prognostic variable in multiple myeloma despite its frequent detection by interphase fluorescence in situ hybridization. Blood. 2000;95(6):1925-30.
38. Hose D, Rossi J-F, Ittrich C, de Vos J, Reme T, Benner A, et al. A new molecular classification of multiple myeloma (MM) using gene expression profiling (GEP) and fluorescence in situ hybridisation as predictor for event free survival (EFS). [abstract]. Blood. 2004;104(11):25a.
39. Hose D, Rossi J-F, Ittrich C, DeVos J, Rème T, Benner A, et al. Molecular classification of multiple myeloma (MM) based on gene expression profiling (GEP) and fluorescence in situ hybridisation (FISH) is an independent predictor for event free survival (EFS). [abstract]. Blood. 2005;106 (111):150a.
40. Bergsagel PL, Kuehl WM. Molecular pathogenesis and a consequent classification of multiple myeloma. J Clin Oncol. 2005; 23(26):6333-8.
41. Bergsagel PL, Kuehl WM. Critical roles for immunoglobulin translocations and cyclin D dysregulation in multiple myeloma. Immunol Rev. 2003;194:96-104.
42. Murray AW. Recycling the cell cycle: cyclins revisited. Cell. 2004;116(2):221-34.
43. Duhl DM, Renhowe PA. Inhibitors of kinesin motor proteins–research and clinical progress. Curr Opin Drug Discov Devel. 2005;8(4):431-6.