A high-risk signature for patients with multiple myeloma established from the molecular classification of human myeloma cell lines

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ABSTRACT

Background
Multiple myeloma is a plasma-cell tumor with heterogeneity in molecular abnormalities and treatment response.

Design and Methods
We have assessed whether human myeloma cell lines have kept patients' heterogeneity using Affymetrix gene expression profiling of 40 human myeloma cell lines obtained with or without IL6 addition and could provide a signature for stratification of patient risk.

Results
Human myeloma cell lines, especially those derived in the presence of IL6, displayed a heterogeneity that overlaps that of the patients with multiple myeloma. Human myeloma cell lines segregated into 6 groups marked by overexpression of *MAF*, *MMSET*, *CCND1*, *FRZB* with or without overexpression of cancer testis antigens (CTA). Cell lines of CTA/MAF and MAF groups have a translocation involving C-MAF or MAFB, cell lines of groups CCND1-1 and CCND1-2like have a t(11;14) and cell lines of group MMSET have a t(4;14). The CTA/FRZB group comprises cell lines that had no or no recurrent 14q32 translocation. Expression of 248 genes accounted for human myeloma cell line molecular heterogeneity. Human myeloma cell line heterogeneity genes comprise genes with prognostic value for survival of patients making it possible to build a powerful prognostic score involving a total of 13 genes.

Conclusions
Human myeloma cell lines derived in the presence of IL6 recapitulate the molecular diversity of multiple myeloma that made it possible to design, using human myeloma cell line heterogeneity genes, a high-risk signature for patients at diagnosis. We propose this classification to be used when addressing the physiopathology of multiple myeloma with human myeloma cell lines.

Key words: multiple myeloma, molecular classification, gene expression profile.

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The online version of this article has a Supplementary Appendix.

Introduction

Multiple myeloma (MM) is a plasma-cell malignancy with a high degree of biological and genetic heterogeneity at presentation and a great variability with regards to the clinical outcome of the patients in response to chemotherapy.[1] During the last 15 years, numerous studies have pointed out the heterogeneity of both the phenotype (CD20, CD28, CD56, CD117) and chromosomal abnormalities of multiple myeloma cells (MMC) in association with patient outcome.[2-4] Chromosomal abnormalities include full or partial deletion of chromosomes 13 or 17, 1q21 amplifications, recurrent 14q32 translocations (in 40% of the patients involving either *CCND1*, *MMSET* and *FGFR3*, *CCND3*, *c-MAF* or *MAFB*) and hyperdiploidy (in 45% of patients with MM). The combined analysis of phenotype, chromosomal abnormalities and morphology has brought up the concept of "many and multiple myelomas".[5]

Bergsagel *et al.* have proposed a GEP-based molecular classification of MM taking into account the ubiquitous expression of D-type cyclins.[6] They identified an expression of one of the three cyclin D genes to be a general feature of MM and proposed a classification of patients within 8 TC (translocation/cyclin D) groups.[6,7] Using GEP of 414 newly diagnosed patients, Zhan *et al.* have proposed a molecular classification of MM into 7 groups.[8] These groups are characterized by an overexpression of genes involved in cell cycle and proliferation (PR group for proliferation), a lower expression of genes involved in bone disease (LB group for "low bone disease"), an aberrant expression of *FGFR3* and *MMSET* genes (MS group for MMSET), a hyperdiploid signature (HY group); an overexpression of *cyclin D1* or *cyclin D3* genes (CD-1 and CD-2 groups), or an overexpression of *MAF* and *MAFB* genes (MF group).[8] HY, CD-1, CD-2, and LB groups had a longer event-free survival and overall survival than the PR, MS, and MF groups. This 7 group GEP classification is a significant predictor for survival in MM. Using the same series of Affymetrix GEP data, Shaughnessy's group identified 70 genes whose upregulation or downregulation was linked with bad prognosis. A high-risk score was built delineating a subset of 13% of newly diagnosed patients with adverse prognosis.[9] This high-risk score has a strong prognostic value. The Intergroupe Français du Myélome (IFM) group reported another gene signature (IFM score) for high-risk patients.[10] Finally, hyperdiploid MM were also subclassified into 4 groups with different clinical outcomes or drug sensitivity.[11]

Obtaining human myeloma cell lines (HMCLs) is an important tool for promoting our understanding of myeloma pathogenesis and finding novel therapies. In particular, the biological studies of MM disease are often carried out with a limited set of HMCLs. General conclusions are then drawn for explaining MM disease, without really knowing the relevance of a limited set of HMCLs to MM disease *in vivo*. The immortalization of primary MMC into HMCL remains a rare event occurring only after primary MMC have undergone extensive proliferation *in vivo* and have likely escaped their bone marrow environment dependence with extramedullary proliferation. One mechanism could be the high frequency of *Myc* gene deregulation in 93% of these HMCLs.[12] Since the identification of IL-6 as a main growth factor for MMC 20 years ago,[13-15] we have obtained a large cohort of HMCLs, culturing primary MMC from patients with extramedullary proliferation with IL-6.[16] These HMCLs are heterogeneous based on phenotype, chromosomal abnormalities and growth factor responses.[17-20] Previous evaluation on a limited number of HMCLs lacking IL6-type HMCLs showed that HMCLs did not reflect MM diversity.[21,22]

Analyzing the gene expression profile (GEP) of 40 HMCLs including a majority of IL6-type HMCLs with Affymetrix U133 2.0 plus microarrays, we show here that HMCLs have kept the molecular heterogeneity of primary MMC of newly diagnosed patients. A list of 248 genes makes it possible to document HMCL biological and genetic heterogeneity. Of major interest, this HMCL heterogeneity gene list comprises genes that allow the design of powerful gene-based risk scores according to patients' treatment. This classification should be used when addressing the physiopathology of MM with HMCLs.

Design and Methods

Human myeloma cell line (HMCLs)

XGs, NANs, BCN, MDN and SBN HMCLs were derived in our laboratories from primary myeloma cells cultured in RPMI1640 medium in the presence of 10% fetal calf serum (FCS) and 3 ng/ml recombinant IL-6, as previously described.[16-20,23-25] ANBL-6 was kindly provided by Dr Jelinek (Rochester, USA), KMS-11, KMS12-BM, KMS12-PE and KMM1 by Dr Otsuki (Okayama, Japan), JJN3 by Dr Van Riet (Bruxelles, Belgium), JIM3 by Dr MacLennan (Birmingham, UK), Karpas620 by Dr Karpas (Cambridge, UK) and MM1S by Dr S. Rosen (Chicago, USA). AMO-1, LP1, L363, NCI-H929, U266, OPM2, and SKMM2 were from DSMZ (Germany) and RPMI8226 from ATTC (USA). All HMCLs derived in our laboratories and ANBL-6 were cultured in the presence of r-IL-6. Identification of each HMCL was assessed by HLA Class I typing. Interphase FISH was performed according to our previously reported standard protocol.[26] Metaphase spreads and interphase cells were evaluated using a DM RXA2 fluorescence microscope (Leica, Bensheim, Germany). Ras and TP53 mutations were identified by direct sequencing of RT-PCR products (*Online Supplementary Table S1*). HMCL microarray data have been deposited in the ArrayExpress public database under accession numbers E-TABM-937 and E-TABM-1088.

Primary myeloma cells

Multiple myeloma cells (MMC) were purified from 206 patients with newly-diagnosed MM after written informed consent was given at the university hospitals of Heidelberg (Germany) or Montpellier (France). The study was approved by the ethics boards of Heidelberg University and Montpellier University.

These 206 patients were treated with vincristine, adriamicyne and dexamethasone (VAD), high-dose melphalan (HDM) and autologous stem cell transplantation (ASCT)[27] and were included in the following Heidelberg-Montpellier (HM) series. The .CEL files and MAS5 files have been deposited in the ArrayExpress public database under accession number E-MTAB-362. We also used Affymetrix data of a cohort of 345 purified MMC from previously untreated patients from the Arkansas Cancer Research Center (ACRC, Little Rock, AR, USA). The patients were treated with total therapy 2 including HDM and ASCT[28] and included in the following ACRC-TT2 series. These data are publicly available *via* the online Gene Expression Omnibus (Gene Expression Profile of Multiple Myeloma, accession number GSE2658. http://www.ncbi.nlm.nih.gov/geo/). After Ficoll-density gradient centrifugation, plasma cells were purified using anti-CD138 MACS microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany).

The t(4;14) translocation results in aberrant *FGFR3* expression in

70% of patients and *MMSET* spiked expression in 100% of patients.[29] Spiked *MMSET* expression has been taken as surrogate for the presence of t(4;14), as previously described.[19]

Gene expression profiling and statistical analyses

Preparation of complementary RNA (cRNA) and microarray hybridization, real-time RT-PCR and gene expression data analyses procedures are detailed in the *Online Supplementary Appendix A*.

The event free or overall survival of subgroups of patients was compared with the log rank test and survival curves computed with the Kaplan-Meier method. The prognostic values of parameters were compared with univariate or multivariate Cox's analysis. Statistical comparisons were made with Mann-Whitney, χ^2, or Student's t-tests. Statistical tests were performed with the software package SPSS 12.0 (SPSS, Chicago, IL, USA).

Results

Characteristics of HMCL cohort

Our cohort of 40 HMCLs comprises 24 reproducible HMCLs obtained adding exogenous IL-6 and FCS (23 from Montpellier/Nantes teams) and 16 HMCLs (collected from other laboratories and commonly used worldwide) that were obtained with FCS without adding exogenous IL-6. These HMCLs will be referred to as HMCLs$^{serum+IL-6}$ and HMCLs^{serum} (Table 1). A major difference between HMCLs$^{serum+IL-6}$ and HMCLs^{serum} is their dependence on the addition of exogenous IL-6 to grow *in vitro*. The growth of 16 of the 24 HMCLs$^{serum+IL\ 6}$ and that of none out of the 16 HMCLs^{serum} was strictly dependent on the addition of exogenous IL-6 *in vitro* (IL-6 dependence ++, P = 6E-5). A simple explanation is that adding exogenous IL-6 made it possible to expand both exogenous IL-6 dependent and independent primary MMC. CD45 expression was associated with IL-6 dependence as 62.5% (15 of 24) of HMCLs$^{serum+IL-6}$ did express CD45 as compared to 25% (4 of 16) of HMCLs^{serum} (P=0.027). All HMCLs but one expressed CD138, 36 of 40 expressed highly CD38, 38 of 40 did not express CD20 and 39 of 40 did not express CD19 (*Online Supplementary Table S2*). Cytogenetics was carried out in 39 HMCLs. Ninety percent of the HMCLs had a 14q32 translocation involving *MAF* (31%), *CCND1* (28%) or *MMSET* (23%) genes (Tables 1A and B). Of note, HMCLs with t(4;14) translocation rarely expressed CD45 (2 of 9) and if so only at a low level (Table 1A), as reported for primary MMC.[30] *TP53* abnormalities (point mutation, deletion, insertion, lack of expression, see details in *Online Supplementary Table S1*) were found in 65% of HMCLs (Table 1A and B). HMCLs$^{serum+IL-6}$ had a trend to have less TP53 abnormalities than HMCLs^{serum}, 58% *versus* 81% (P=0.1). *Ras* mutations (*N*-ras and *K*-ras) were found in 45% of HMCLs (Table 1B). Six HMCLs, five from the HMCLs$^{serum+IL-6}$ cohort, had neither *N*- or *K*-Ras mutation nor TP53 abnormality (Table 1 and *Online Supplementary Table S1*). There was a 4-fold increase in the frequency of translocations involving *c-MAF* or *MAFB* genes compared with those published for primary MMC of newly diagnosed patients (P<0.0001).[1] Thus, HMCLs are heterogeneous in terms of how they were obtained, IL-6 dependence, phenotype and gene abnormalities. The greatest heterogeneity is found for HMCLs$^{serum+IL-6}$ with 25% of HMCLs$^{serum+IL-6}$ with no myeloma specific recurrent translocation or no translocation *versus* 6% for HMCLs^{serum} (P=0.0002). In order to look further for HMCL heterogeneity, whole genome transcriptome analysis was performed using Affymetrix U133 2.0 plus microarrays.

Gene expression profiling of the HMCLs

Comparing the gene expression profile of HMCLs$^{serum+IL-6}$ and HMCLs^{serum}, 23 genes were up-regulated in HMCLs$^{serum+IL-6}$, but none in HMCLs^{serum} (1,000 permutations, ratio ≥ 2 and FDR = 0%, *Online Supplementary Table S3A*). Among these 23 genes, 3, CD45 (PTPRC), SOCS3, and BCL6, have been reported to be induced by IL-6 in MMC or other cell lineages[31-33] (*Online Supplementary Table S3A*). The NF-κB gene index reported by Anunziata *et al.*[34] and adapted to HMCLs[35] ranged from 4.2 to 8.6 without significant difference between HMCLs$^{serum+IL-6}$ and HMCLs^{serum} (*Online Supplementary Table S4*). Only 2 genes (*MDM2* and *CDKN1A*), 2 well known TP53 targets,[36] were differentially expressed between HMCLs without and with TP53 mutations (*data not shown*). No significant differential gene expression was found between HMCLs with or without N-ras or K-ras mutations (*results not shown*).

Identification of 6 groups in human myeloma cell lines based on gene expression profiles and identification of 248 genes documenting the heterogeneity of HMCLs

The 40 HMCLs could be clustered into 6 groups using unsupervised hierarchical clustering and 4,163 probe sets with the highest variance. Within each group, the GEP of the HMCLs were significantly correlated each other (P≤0.05) but none of the 6 groups was significantly correlated to another one. Each of the 6 groups could be identified by genes known to be important markers of MM disease such as *c-MAF*, *CCND1*, *FRZB*, *MMSET*, *FGFR3* and cancer testis antigens (CTA) (*Online Supplementary Figure S1*). To further delineate the gene signature of these 6 groups, a SAM multiclass analysis was run identifying 248 HMCL heterogeneity genes differentially expressed between the 6 groups (1,000 permutations, FDR ≤ 5%) (*Online Supplementary Tables S5 to S10*). Using these 248 genes, an unsupervised hierarchical clustering grouped HMCLs into 2 major clusters and for each cluster, into 3 groups. In the first cluster, the 2 groups are characterized by translocations involving *MMSET/FGFR3*, or *MAF* loci and were termed MS and MF groups by analogy with ACRC molecular classification[8,37] (Figures 1 A and B). In the second cluster, 2 groups over-express CTA genes; one is also characterized by *MAF* translocations (termed CTA/MF) and a second one by a lack of recurrent translocations and an overexpression of FRZB (termed CTA/FRZB). This later comprises 5 HMCLs without 14q32 translocation or MM specific recurrent translocations, with an increase in odd chromosomes, and shows overexpression of *CTA* and *FRZB* genes. As 5 out of 7 HMCLs from CTA/FRZB group are of male origin, we have checked that the CTA/FRZB group was not built due to Y chromosome gene expression since deleting Y chromosome genes did not change the CTA/FRZB clustering (*data not shown*). The CTA/MF and CTA/FRZB groups comprise exogenous IL-6 dependent HMCLs except only the U266 HMCL that can grow without adding IL-6. But U266 cells produce autocrine IL-6 that drives their growth.[38] Five out of 6 HMCLs of the CTA/MF group had translocations involving *c-MAF* or *MAFB* as HMCLs of the MF group and SAM analysis between the CTA/MF and MF groups indicated that mostly CTA genes were differentially expressed (*Online Supplementary Table S3B and C*).

The 2 last groups gather HMCLs with *cyclin D1* or *D3*

translocations. One group had a typical CCND1 signature and was termed CD-1. The other one had also a CCND1 signature with expression of genes that were found in the CD-2 group (*RRAS2, ZDHHC14, MDK, DMD*). However, because this group did not over-express anchoring genes of the CD-2 group like *MS41* or *PAX5*, it was termed CD-2like (CD-2L). SAM analysis between the CD-1 and CD-2L groups indicated that among genes differentially expressed between the 2 groups, 6 (*WARS, DNAJC12, TM6SF1, ATF5, STOM, ERN1*) belong to the genes differentially expressed between CD-1 and CD-2 groups of patients.

Of note, a homologous classification of HMCLs was obtained using the 700 genes of the Arkansas group[8] allowing molecular classification of patients (*Online*

Table 1A. Characteristics of the HMCL cohort. HMCLs were obtained culturing primary myeloma cells with culture medium supplemented with FCS alone or recombinant IL-6 and FCS as indicated by the laboratory of origin.

HMCL Name HMCL[serum+IL-6]	IL-6 dependence[1]	Origin[2]	Disease[3]	Patient sample[4]	Gender	Isotype	t(14q32 or 22q11 ;)	Target genes	Ras	TP53	CD45	HMCL classification
ANBL6	+	CO	MM	PB	F	l	t(14;16)	*c-Maf*	*wt*	*abn*	-	MF
BCN	+	MN	MM	PB	F	Gk	t(14;16)	*c-Maf*	*wt*	*wt*	-	CD-1
MDN	+	MN	PCL	PB	M	Gk	t(11;14)	*CCND1*	*mut*	*wt*	+	CD-1
NAN1	+	MN	MM	PE	M	Ak	t(14;16)	*c-Maf*	*wt*	*abn*	-	CTA/MF
NAN3	+	MN	MM	PE	F	Ak	t(4;14)	*MMSET*	*mut*	*abn*	-	MS
NAN6	+	MN	MM	PB	F	Ak	t(14;20)	*MafB*	*wt*	*abn*	+	CTA/MF
SBN	+	MN	PCT	PE	M	Al	t(14 ;?)	unknown	*wt*	*wt*	-	CTA/FRZB
XG1	++	MN	MM	PB	M	Ak	t(11;14)	*CCND1*	*mut*	*abn*	+	CTA/FRZB
XG2	++	MN	MM	PE	F	Gl	t(12;14)	unknown	*mut*	*abn*	+	CTA/FRZB
XG3	++	MN	PCL	PE	F	l	t(14 ;?)	unknown	*mut*	*wt*	+	CTA/FRZB
XG4	++	MN	MM	PB	M	Gk	t(14 ;?)	unknown	*wt*	*abn*	-	CTA/FRZB
XG5	++	MN	MM	PB	F	l	t(11;14)	*CCND1*	*wt*	*abn*	-	CD-1
XG6	++	MN	MM	PB	F	Gl	t(16;22)	*c-Maf*	*wt*	*wt*	+	CTA/MF
XG7	+	MN	MM	PB	F	Ak	t(4;14)	*MMSET*	*mut*	*wt*	+/	MS
XG10	++	MN	PCT	AF	F	Ak	t(14 ;?)	unknown	*mut*	*wt*	+	CTA/MF
XG11	++	MN	PCL	PB	F	l	t(11;14)	*CCND1*	*wt*	*abn*	+	CD-2L
XG12	++	MN	PCL	PB	F	l	t(14;16)	*c-Maf*	*mut*	*wt*	+	CTA/MF
XG13	++	MN	PCL	PB	M	Gl	t(14;16)	*c-Maf*	*wt*	*abn*	+	MF
XG14	++	MN	PCL	PB	M	k	t(11;14)	*CCND1*	*mut*	*abn*	+	MF
XG16	++	MN	PCL	PB	M	k	none	none	*mut*	*abn*	+	CTA/FRZB
XG19	++	MN	PCL	PB	F	Al	t(14;16)	*c-Maf*	*wt*	*wt*	+	CTA/MF
XG20	++	MN	PCL	PB	M	l	t(4;14)	*MMSET*	*wt*	*abn*	-	MS
XG21	++	MN	MM	PE	M	l	t(11;14)	*CCND1*	*wt*	*wt*	+	CD-1
XG24	++	MN	PCL	PB	F	Al	t(4;14)	*MMSET/FGFR3*	*mut*	*wt*	-	MS
HMCL[serum]												
AMO1	-	CO	PCT	AF	F	Ak	t(12;14)	unknown	*wt*	*wt*	+	CD-2L
JIM3	-	CO	MM	PE	F	A	t(4;14)	*MMSET/FGFR3*	*wt*	*abn*	-	MS
JJN3	-	CO	MM	PE	F	Ak	t(14;16)	*c-Maf*	*mut*	*abn*	+/-	MF
Karpas620	-	CO	PCL	PB	F	Gk	t(11;14)	*CCND1*	*mut*	*abn*	-	CD-2L
KMM1	-	CO	MM	SC	M	l	t(6;14)	*CCND3*	*mut*	*abn*	-	CD-2L
KMS11	-	CO	MM	PE	F	Gk	t(4;14)	*MMSET/FGFR3*	*wt*	*abn*	-	MS
KMS12BM	-	CO	MM	BM	F	NS	t(11;14)	*CCND1*	*wt*	*abn*	-	CD-2L
KMS12PE	-	CO	MM	PE	F	NS	t(11;14)	*CCND1*	*wt*	*abn*	-	CD-2L
L363	-	CO	PCL	PE	F	NS	t(20;22)	*MafB*	*mut*	*abn*	-	CD-2L
LP1	-	CO	MM	PB	F	Gl	t(4;14)	*MMSET/FGFR3*	*wt*	*abn*	-	MS
MM1S	-	CO	PCL	PB	F	Al	t(14;16)	*c-Maf*	*mut*	*wt*	-	MF
NCI-H929	-	CO	MM	PE	F	Ak	t(4;14)	*MMSET/FGFR3*	*mut*	*wt*	+/-	MS
OPM2	-	CO	MM	PB	F	Gl	t(4;14)	*MMSET/FGFR3*	*wt*	*abn*	-	MS
RPMI8226	-	CO	MM	PB	M	Gl	t(14;16)	*c-Maf*	*mut*	*abn*	-	MF
SKMM2	-	CO	PCL	PB	M	Gk	t(11;14)	*CCND1*	*wt*	*abn*	-	CD-1
U266	-	CO	MM	PB	M	El	t(11;14)	*CCND1*	*wt*	*abn*	+	CTA/FRZB

[1]++ if growth is strictly dependent on adding exogenous IL-6, + if dependent on adding exogenous IL-6, - if not; [2]Origin of the HMCL, MN Montpellier or Nantes, CO collected; [3]Disease at diagnosis: MM multiple myeloma, PCL plasma cell leukemia, PCT plasmacytoma: [4]Origin of the sample: AF ascitic fluid, BM bone marrow, PE pleural effusion, PB peripheral blood, SC subcutaneous. SBN, XG3 and XG4 with no recurrent translocation of the 14q32 locus, XG16 has a wild-type 14q32 locus. Target genes: target genes of the translocation. Ras: mutation (codons 12 or 13 or 61) of N or K-Ras, see details in Online Supplementary Table S1. TP53: abn abnormal (point mutations, deletion, insertion), lack of mRNA expression, see details in Online Supplementary Table S1 Phenotype was analyzed by flow cytometry (see Online Supplementary Table S2).

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Table 1B. Compared frequencies of chromosomal abnormalities, CD45 expression, TP53 abnormalities and Ras mutations in HMCLs$^{serum+IL6}$ and HMCLs^{serum}.

Characteristics	HMCLs		HMCLs$^{serum+IL6}$		HMCLs^{serum}	
	n	%	n	%	n	%
	40		24	57	16	43
Chromosomal abnormalities						
t(4;14)	9	22.5%	4	17%	5	31%
t(14/16) (q32/q23) and other c-MAF/MAFB translocations	12	30%	8	33%	4	25%
t(11;14)	11	27.5%	6	25%	5	31%
t(6;14)	1	2.5%	0	0%	1	6%
t(14;other)	6	15%	5	21%	1	6%
no t(14;)	1	2.5%	1	4%	0	0%
CD45+ expression (including partial expression)	19	47.5%	15	62.5%	4	25%
TP53 abnormalities	26	62%	13	54%	13	81%
N-or K-Ras mutations	18	45%	11	46%	7	44%

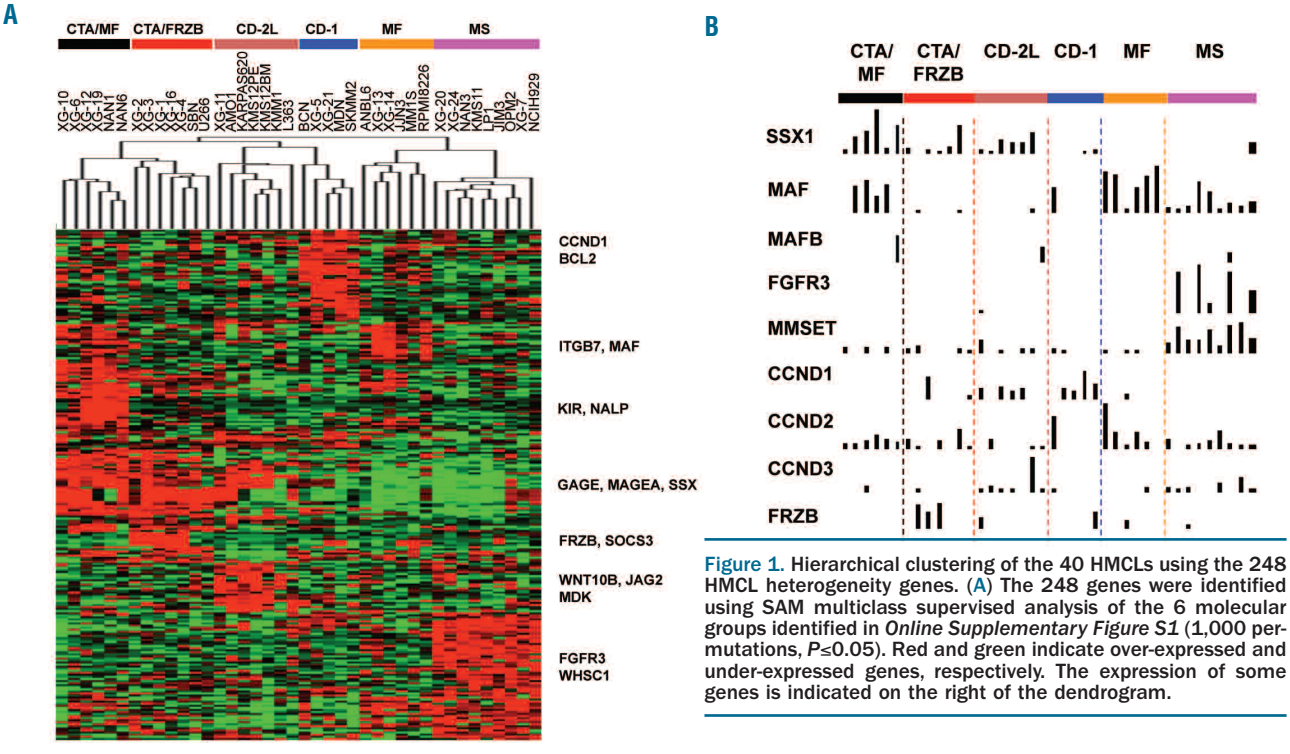

Figure 1. Hierarchical clustering of the 40 HMCLs using the 248 HMCL heterogeneity genes. (A) The 248 genes were identified using SAM multiclass supervised analysis of the 6 molecular groups identified in *Online Supplementary Figure S1* (1,000 permutations, $P \leq 0.05$). Red and green indicate over-expressed and under-expressed genes, respectively. The expression of some genes is indicated on the right of the dendrogram.

HMCLs were clustered into 2 major clusters. One cluster is split into 2 clusters and 4 groups named CTA/MF, CTA/FRZB, CD-1 and CD-2L. The other one is split into 2 groups termed as MF and MS. Name of groups was chosen by analogy with the ACRC molecular classification.[8] (B) The Affymetrix signal for each gene is proportional to the height of each bar (representing a single HMCL). Note that *spiked* expression of *CCND1*, *MAF* and *MAFB*, and *FGFR3* and *MMSET* is strongly correlated with specific subgroup designations.

Supplementary Figure S2) (P=NS).

Expression of 4 genes, *LRP12*, *TEAD1*, *MAF* and *ITGB7*, was validated by real-time RT-PCR (*Online Supplementary Figure S3*).

HMCLs have kept some of the molecular heterogeneity of newly diagnosed patients

Using the 248 genes discriminating the 6 HMCL groups, an unsupervised clustering was run with the GEP of primary MMC of 345 newly diagnosed patients of the ACRC TT2 cohort (Figure 2). Six clusters were identified with a significant correlation between samples within a given cluster ($P \leq 0.05$). This clustering of primary MMC based on HMCL heterogeneity gene signature partially overlapped the ACRC 7 group molecular classification (P=0.01).[8] Cluster 1 comprised 100% of patients of the MS (spiked MMSET expression) group, cluster 2: 71% of patients of LB (low bone disease) group, cluster 3: 100% of patients of MF (c-MAF or MAF-B overexpression) group, cluster 4: 46% of patients of PR (proliferation) group and 29% of patients of HY (hyperdiploid) group, cluster 5: 92% of patients of HY group and cluster 6: 89% of patients of CD-1 or CD-2 (cyclin D1 expression) groups (Figure 2). These data demonstrate that HMCLs have kept the molecular heterogeneity

MMC of newly diagnosed patients. Of note, 25 genes were shared between the 248 HMCL heterogeneity genes and the 350 top 50 genes making it possible to define the ACRC 7 group molecular classification.[8]

Can HMCL heterogeneity genes predict for patient survival?

Given that the 248 HMCL heterogeneity genes could be indicators of primary MMC heterogeneity, we examined whether some of these genes could predict for patient survival. We used the Heidelberg-Montpellier (HM) cohort of 206 patients treated within or similar to GMMG-HD3 trial[27] and the ACRC-TT2 cohort of 345 patients treated with total therapy 2 (*Online Supplementary Table S11*).[28] Seven out of the 248 HMCL heterogeneity genes, *TEAD1*, *CLEC11A*, *LRP12*, *MMSET*, *FGFR3*, *NUDT11* and *KIAA1671*, had bad prognostic value for both EFS and OAS in the HM cohort and for OAS in the ACRC-TT2 cohort (*Online Supplementary Figure S4A* and *B*). All 7 genes were over-expressed in the MS group, t(4;14), and 3 of 7, *LRP12*, *TEAD1*, *NUDT11*, were significantly over-expressed in the PR group of patients (*Online Supplementary Figure S5*). To combine the prognostic information of these 7 genes, a simple staging was built scoring patients from 0 to 7 (patients with 0/7 genes present, 1/7, 2/7, …, 7/7), running Kaplan-Meier analyses with the 8 patient groups, grouping together groups with no prognostic difference, and thus obtaining 3 major patient groups with different EFS in the HM cohort and in OAS in both the HM and the ACRC-TT2 series. Group 1 comprises patients whose MMC expressed none or 1 bad prognosis gene, group 2 patients expressing 2 to 4 bad prognosis genes and group 3 at least 5 (Figure 3A). Group 1 represented 71% of the HM patients and 73% of the ACRC-TT2 patients; group 2, 21% and 22% of HM or ACRC-TT2 patients, respectively; and group 3, 8% and 5% of HM and ACRC-TT2 patients (Figure 3A and *Online Supplementary Figure S6*). Group 3 was associated with the worst prognosis in the HM and the ACRC-TT2 cohorts (30.5 months and 41 months in the HM and the ACRC-TT2 cohorts, respectively) (Figure 3B and C).

At least 2 gene scores based on microarray GEP were predictive for patients' event free or overall survival, the high risk score (HRS) with 70 genes[9] and the Intergroupe Francais du Myelome (IFM) score with 15 genes.[10] The HMCL score gene list shares no gene with the HRS or IFM score gene lists. When compared together in multivariate Cox's analysis, only the HMCL score was significant unlike HRS and/or IFM scores in the HM cohort. In ACRC-TT2, HMCL score

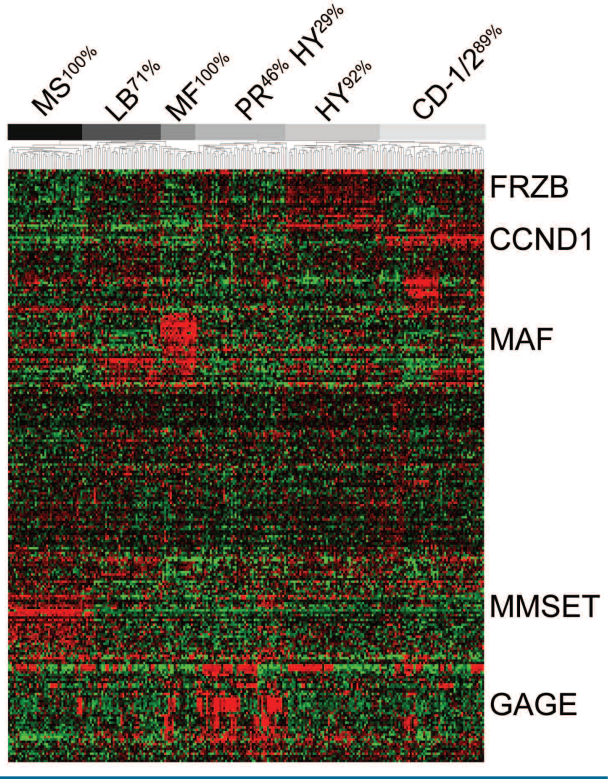

Figure 2. Unsupervised clustering of the gene expression profiling of primary myeloma cells of newly diagnosed patients using the 248 HMCL heterogeneity genes. The Affymetrix gene expression profiles of purified myeloma cells from 345 newly diagnosed patients were publicly available from the ACRC. An unsupervised clustering of the 345 samples (columns) using the 248 HMCL heterogeneity genes (lines) makes it possible to cluster samples into 6 major groups defined by the different gray color scale horizontal histograms and arrows. The percentages above the horizontal histograms indicate the overlap of this clustering with the previously published ACRC 7 molecular group classification.[8] MS:MMSET, LB: low bone disease, MF: MAF, PR:proliferation, HY:hyperdiploid, CD1/2: CD-1 + CD-2

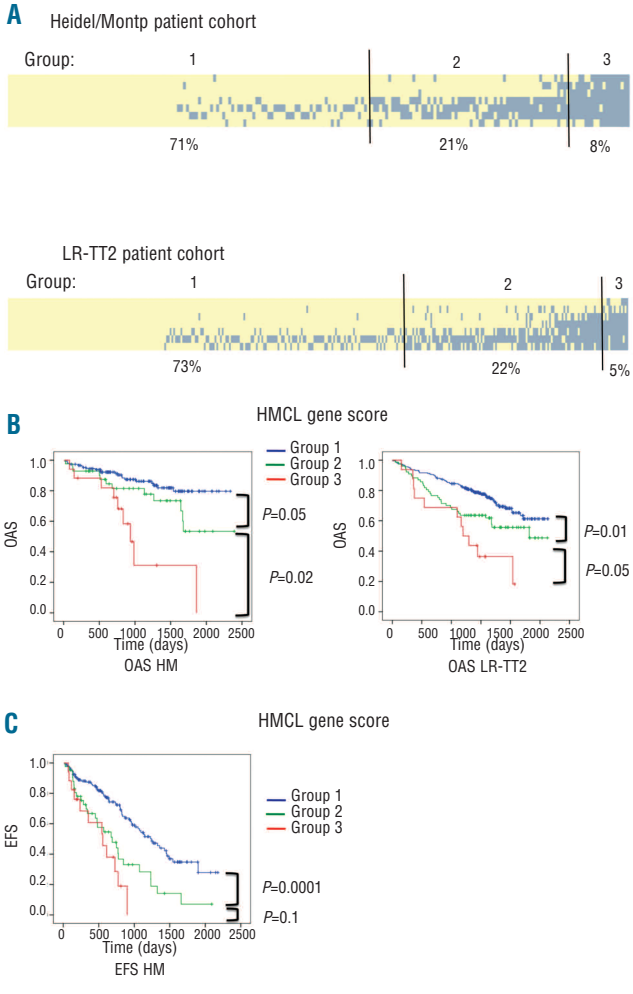

Figure 3. Seven gene prognostic score. (A) Distribution of the patients from the HM and ACRC-TT2 cohorts according to the 7-gene HMCL score. (B) Kaplan-Meier estimates of overall survival and event-free survival (C) of low risk patients (blue), intermediate risk patients (green) and high risk patients (red) according to our 7-gene HMCL score.

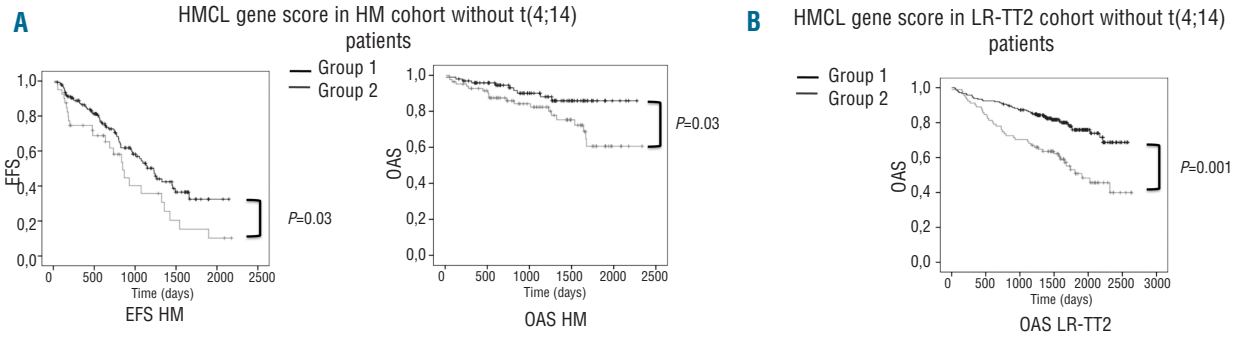

Figure 4. Kaplan-Meier estimates of overall survival and event-free survival of patients without t(4;14) according to a 6-HMCL gene score in the HM (A) or ACRC-TT2 cohort (B).

and HRS were significant in multivariate Cox's analysis, IFM score being no more significant (*Online Supplementary Table S12*). The prognostic value of the HMCL score is not hardly surprising since it identified 2 groups of patients which are already known to have poor prognosis for patients treated with VAD and then HDM and ASCT: patients with t(4;14) and patients with a proliferation gene signature.

We were interested to use the HMCL gene list to define prognostic patients' group, patients with t(4;14) who are already known to have a poor prognosis according to this treatment protocol were deleted and the same methodology was run. A list of 6 prognostic genes was obtained: *FSTL5*, *GAGE1*, *GAGE12*, *BCHE*, *HOOK3* and *LOC283352*. These were different from the previous 7 prognostic genes and it was possible to separate patients without t(4;14) in 2 groups with a different prognosis (Figure 4A and B and *Online Supplementary Figure S7*). Compared to these 2 groups, patients with t(4;14) had a worse prognosis (*Online Supplementary Figure S7*). *GAGE1* and *GAGE12* were over-expressed in the PR group, *BCHE* in the CD-1 group, *LOC283352* in the HY and the PR groups, and *FSTL5* in the HY group. *HOOK3* was not significantly over-expressed by one of the 7 groups of patients (*data not shown*). This second HMCL gene score strengthens interest in using the HMCL gene list to define prognostic patients' groups in MM.

Discussion

In this study, we have investigated the molecular heterogeneity of a large cohort of HMCLs and it reflects part of the molecular heterogeneity of primary MMC of newly-diagnosed patients. This heterogeneity of MM is evidenced by various genetic abnormalities, in particular 5 MM specific recurrent translocations, and the finding of 7 molecular groups using unsupervised classification of high-throughput gene expression data by the ACRC group.[8] This HMCL heterogeneity is in complete contrast with previous findings showing that HMCLs were not representative of MM diversity.[21,22] But both previous studies used a limited number of HMCLs mainly of serum-type only. Using only HMCL information, a 248-HMCL heterogeneity gene list was found allowing classification of MMC of newly-diagnosed patients with a significant overlap with previously-reported ACRC molecular MMC classification.[8] Given this overlapping, the ACRC nomenclature was mainly used to identify HMCL groups. In addition, this 248 HMCL heterogeneity gene list comprises genes that can identify patients who respond poorly to a given treatment. Several simple and logical conclusions can be drawn. First, the method of obtaining HMCLs greatly influences their molecular heterogeneity. The greatest heterogeneity was found within HMCLs$^{serum+IL-6}$, i.e. obtained with IL-6 and serum. In the presence of IL-6 and serum, MMC with or without myeloma-specific recurrent translocations have been immortalized, whereas, in the presence of serum, mostly MMC with recurrent translocations (involving *cyclin D1*, *MAF* or *MMSET/FGR3* genes) (14/16 HMCL^{serum}) have been immortalized. This suggests that addition of exogenous IL-6 has made it possible to expand both exogenous IL-6 dependent and independent primary MMC. An immediate conclusion is to go on obtaining HMCLs culturing primary MMC with all known myeloma growth factors (at least IGF-1, IL-6, APRIL, IL-21, HGF) to avoid selection of primary MMC responsive to one specific growth factor only.[19,20,39,40]

HMCLs^{serum} were mostly CD45- (12/16 HMCLs^{serum}) and respond to IGF-1 or IL-21.[20,41] In contrast, HMCLs$^{serum+IL-6}$ were CD45^{+} or CD45^{-} (with a proportion close to that reported in newly-diagnosed patients, 2 of 3 and one of 3, respectively[42] and CD45^{+} HMCLs are not stimulated by IGF-1 or IL21 alone. *C-MAF* or *MAF-B* translocated HMCLs were highly represented within the HMCLs$^{serum+IL-6}$ cohort (8 of 24 HMCLs$^{serum+IL-6}$) suggesting that MMC over-expressing *MAF* genes are highly sensitive to paracrine IL-6. Besides the heterogeneity due to culture method of parental primary MMC, the molecular heterogeneity of HMCLs is built on Ig translocation occurring in parental primary MMC and on *CTA* gene expression. Myeloma cells frequently express CTA although the mechanisms deregulating CTA expression remain pending.[43] *CTA* gene expression is epigenetically regulated in various cancers.[44,45] More recently, Walker *et al.* have shown that the transition of normal PC and MGUS stage to MM is associated with DNA hypomethylation, but the transition of intramedullary stage to PCL or HMCL stage is associated with DNA hypermethylation.[46] The CTA signature in *CTA^{pos}* HMCLs could be an indicator of higher hypomethylation status than in *CTA^{neg}* HMCLs.[45]

All HMCLs with t(4;14) translocation under-express *CTA*. One possible explanation is that the deregulation induced by t(4;14) yields to a drug resistance and poor prognosis, making CTA deregulation unnecessary. Importantly, pri-

mary MMC with t(4;14) over-express IGF1R and IGF1[19,47] probably making their immortalization without IL-6 easier. On the other hand, 4 of 7 HMCLs of the CTA/FRZB group have no myeloma-specific recurrent translocations. This CTA/FRZB group expresses *CTA* genes and *FRZB* that could fit with one of the 4 reported hyperdiploid groups of patients over-expressing *CTA* genes.[11] Of note, all these HMCLs over-express EDNRB which is also over-expressed by the hyperdiploid group of patients.[37] However, they have numerous genetic abnormalities, eventually induced secondarily at relapse or *in vitro*, and this means it is not possible to ascertain whether they originate from hyperdiploid MMCs.

Regarding *cyclin D* or *MAF* translocations, HMCLs both belong to 2 groups. HMCLs of the CD-2L group were mostly IL-6 independent HMCLs (6 of 7) and expressed some CTA in contrast to those of the CD-1 group (one of 5 $HMCLs^{IL\text{-}6}$). Ingenuity analysis of genes differentially expressed between the CD-1 and CD-2L groups showed an upregulation of genes related to the lipid metabolism (*data not shown*). CTA/MAF HMCLs are all IL-6 dependent. An explanation was that adding IL-6 could be critical to immortalize primary MMC from these patients. In agreement, we found that primary MMC from MF patients under-expressed *IL-6* gene but over-expressed both chains of IL-6R complex suggesting that MMC from MF patients are more dependent on paracrine IL-6.[19]

Using the 248 HMCL heterogeneity genes, we have found 7 genes (*CLEC11A, FGFR3, KIAA1671, LRP12, MMSET, NUDT11, TEAD1*) that make it possible to build an HMCL score predicting survival in newly diagnosed patients with MM treated with high-dose chemotherapy. This HMCL score segregates MM patients into 3 groups with low, intermediate and high risk. These 7 HMCL genes share no gene with those of 2 previously reported gene-based risk scores, the HRS and the IFM scores.[9,10] This HMCL score was shown to be more potent than IFM score in the 2 independent patient cohorts, more potent than HRS in our patient cohort and remains independent of HRS in ACRC-TT2 series (*Online Supplementary Table S12*). This is remarkable because this list of 248 HMCL heterogeneity genes has been obtained with cells cultured for many years *in vitro*, from a minor subset of patients with extramedullary proliferation. Not surprisingly, the 2 patient groups with poor prognosis comprise mainly patients with t(4;14) and patients with a proliferation gene signature, 2 groups already identified with poor prognosis in patients treated with HDM and ASCT. To further investigate the interest in using the HMCL gene list to define prognostic patient groups, the same methodology was applied to patient cohorts without patients with t(4;14) already known to have a poor prognosis, making it possible to separate patients without t(4;14) in 2 different prognosis groups (Figure 4A and B). Thus, this 248 HMCL heterogeneity gene list, defined independently of primary MMC information, comprises genes that could help to define patients who respond poorly to a given treatment.

Another important conclusion regarding the current data is the interest in but also the danger of using HMCLs to study MM biology. Primary MMC are not easy to obtain in sufficient amounts and do not survive well in culture *in vitro*.[16,25] This is the reason why the majority of the studies of MM disease physiopathology used a limited set of cell lines and a final validation with 1-5 samples of primary MMC, often from patients with extramedullary proliferation. Given the 6 group molecular heterogeneity of HMCLs we reported here using 248 genes, it should be important to recommend that investigators classify the HMCLs they used in one of the groups and to indicate that the extension of HMCL-derived concepts to MM disease should be limited at least to patient subgroups. In this study, the role and influence of microenvironment on HMCL gene expression profile was not addressed. It was demonstrated that stromal interactions could influence expression patterns of MMC.[48-50] Further analysis of GEP modification by coculture should be of major interest to identify pathways activated by the microenvironment in relation to MM diversity.

In conclusion, this study has shown that HMCLs obtained with IL-6 and serum cover a large part of the molecular heterogeneity of primary MMC. A 248 gene list defining HMCLs heterogeneity contains new prognostic genes. This representative cohort could help identify common critical pathways and new therapeutic targets for molecular and chromosome based myeloma subgroups.

Authorship and Disclosures

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