Circulating clonotypic B cells in multiple myeloma and monoclonal gammopathy of undetermined significance

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SUPPLEMENTARY METHODS

Patients and samples

Patients with MM were diagnosed according to previously described criteria by the The International Myeloma Working Group (*British Journal Hematology*. 2003;121:749-57).

Multiparameter flow cytometry immunophenotypic studies and cell purification: Erythrocyte-lysed whole PB and BM samples were stained using an 8-color stain-lyse-and-wash direct immunofluorescence technique with the following monoclonal antibody combinations - pacific blue (PacB), pacific orange (PacO), fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein/cyanin 5 (PerCPCy5), PECy7, allophycocyanin (APC), APC-hilite 7 (H7) – for PB samples: i) CD20, CD45, surface membrane (Sm)IgM, $_{Sm}$IgG, CD19, CD10, CD27, CD38; ii) CD20, CD45, $_{Sm}$IgM, $_{Sm}$IgA, CD19, CD10, CD27, CD38, and; iii) CD20, CD45, $_{Sm}$Igλ, $_{Sm}$Igκ, CD19, CD10, CD27, CD38 (for PB samples). For BM specimens, the CD45, CD138, CD38, CD56, β2-microglobulin, CD19, cytoplasmic Ig (CyIg)κ, $_{Cy}$Igλ antibody combination was used. Fluorescence measurements were performed in a FACSCanto II ™ flow cytometer (Becton/Dickinson Biosciences [BD], San Jose, CA, USA) for a total of $\geq 2 \times 10^5$ cells showing CD19 and/or CD38 expression and low-to-intermediate sideward light scatter (SSC) values using a double-step data acquisition procedure described elsewhere and the FACSDiva software (version 6.1, BD). For data analysis the Infinicyt software (Cytognos SL, Salamanca, Spain) was used.

Fluorescence-activated cell sorting (FACS) based on the above antibody combinations was used (FACSAria II ™, BD) to purify BM M-PC as well as PB $_{Sm}$IgM$^+$ naïve B-lymphocytes, non-switched $_{Sm}$IgM$^+$, switched $_{Sm}$IgA$^+$ and $_{Sm}$IgG$^+$ memory B-lymphocytes and N-PC; whenever present, circulating M-PC were also purified. The purity of the sorted M-PC and the multiple PB normal B-cell subsets was systematically

≥ 98%, and only those FACS-purified PB B-cell fractions (except M-PC), which showed no contamination (<0.01%) by circulating PB M-PC were included in the study; all other fractions were not tested (Table 1).

Analysis of *IGH* gene rearrangements of BM M-PC and CDR3 sequencing. Highly-purified BM M-PC were lysed and genomic DNA extracted and prepared for PCR using the REDExtract-N-Amp Blood PCR Kit™ (Sigma, St. Louis, MO, USA) following the recommendations of the manufacturer. Identification of the malignant *IGH* gene rearrangement was performed according to the BIOMED-2 protocol[30] slightly modified, using 4.8µL of each individual M-PC lysate (final volume of 20 µl containing 10µL of PCR ReadyMix and 7.8 pmol of each primer) per PCR reaction. Complete VDJH and incomplete DJH rearrangements were amplified by different sets of family-specific primers (VH-FR2 and DH-JH) together with the JH consensus primer, in two different multiplex PCR reactions. Reverse primers were labelled with 6-carboxyfluorescein (6-FAM). PCR reactions were performed in a Verity™ 96-Well Thermal Cycler (Applied Biosystems [AB], Foster City, CA, USA) using the following conditions: incubation at 95°C for 3 min (pre-activation) followed by 40 cycles of sequential incubation at 95°C for 45s (denaturation), 60°C for 45s (annealing), and 72°C for 90s (extension), with a final extension step for 10 min at 72°C. The PCR amplified products were denatured, the FAM-labeled single-strand DNA fragments were size-separated by high-resolution capillary electrophoresis, and identified by GeneScanning in an automated ABI PRISM 3130 Avant sequencer, using the GENEMAPPER 3.1 software (AB). A clonal population was defined according to the recently criteria defined by the EuroClonality group (Langerak et al, Leukemia 2012). In all cases, at least two tubes were run in parallel for each PCR in order to avoid pseudoclonality.

Clonal PCR products were purified with ExoSap (USB Corp, Cleveland, OH, USA) and directly sequenced in both directions in an ABI 3130 DNA sequence

analyzer using the BigDye ™ Terminator v3.1 Cycle Sequencing Kit (AB). Germline VH, DH and JH gene segments from complete *IGH* V(D)JH gene rearrangements were identified through direct comparison with the IMGT database (Lefranc et al. *Nucleic Acids Res.* 2001; Giudicelli et al. *Nucleic Acids Res.* 2004) (http://imgt.cines.fr) using the DNAPLOT software (MRC Center for Protein Engineering, Cambridge, UK). *IGH* DH and *IGH* JH germline gene segments from incomplete DH-JH gene rearrangements were identified using BLAST search in the *IGH* DH-JH germline locus sequence (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Once the segments had been identified, the N-region was highlighted for ASO-primer design due to its high specificity for each individual rearrangement, as previously described (Sarasquete et al. Haematologica 2005). If a single (clonal) peak was detected in a sample, it was sequenced as described above in order to confirm that the sequence was or not identical to the CDR3 signature of the reference sequence, indicating that the sets of primers were really specific. In order to assess the sensitivity of each patient-specific set of primers used in the ASO-PCR assay (e.g.: the lowest number of cells detected), ASO-PCR analyses were carried out in multiple tubes containing serial dilutions of ≥1 previously purified BM M-PC from individual patients. A signal was considered to be positive when a peak with the expected fragment size was detected clearly above the GeneScanning background signal in ≥ 2 replicates.

Design of ASO-primers and sensitivity of the ASO-PCR technique. ASO-primers complementary to the *IGH* VH-DH or *IGH* DH-JH junctional regions were designed using the OLIGO 6.1 software (Molecular Biology Insights, Cascade, CO, USA). Design conditions avoided primer dimer formation with ΔG >-3.5 Kcal/M, GC-rich 3' ends and T_m differences with *IGHJ* primers >2°C. Amplicon sizes were always <170 bp.

A qualitative fluorescent ASO-PCR with CDR3 specific primer and its respective 6-FAM-labeled *IGH* JH primer (intronic, allele-specific or consensus), was carried out

on purified BM M-PC from each patient; purified PB B-cells from healthy donors were studied in parallel as negative controls. ASO-PCR conditions were the same as described above. Each set of patient-specific *IGH* CDR3 primers were tested at different annealing temperatures in order to determine the maximum amplification efficiency for each pair of primers (Supplementary Table 2). In all cases, a single peak was present in the patient`s purified BM M-PC samples, while absent in purified normal PB B-cells. The single (clonal) *IGH* gene peak detected for M-PC from individual patients was sequenced as described above, and the amplified sequences systematically showed an identical CDR3 signature to those of the reference sequence, indicating that the sets of primers were highly specific.

In order to determine the sensitivity of each patient-specific set of primers used in the ASO-PCR assay (e.g.: the lowest number of cells detected), ASO-PCR analyses were performed in multiple tubes containing serial dilutions of ≥1 previously purified BM M-PC from individual patients. For this purpose, DNA from M-PC obtained from each patient was diluted into polyclonal DNA obtained from purified PB B-cells from healthy donors. Multiple replicates (range: 3 to 7) of each dilution were produced and analyzed in parallel with the ASO-PCR set of primers and technique described above. The sensitivity of the ASO-PCR assay for each pair of primers/patient was established as the highest dilution in which the clonal peak could be repeatedly detected (Supplementary Table 2). A signal was considered to be positive when a peak with the expected fragment size was detected clearly above the GeneScanning background signal in ≥ 2 replicates.

ASO-PCR analysis of purified PB subpopulations of B-cells and PC. DNA was extracted from pre-defined numbers of highly-purified PB B-cell and PC subsets, as described above, except for the volumes of the lysing solution that were lower; purified B-cells and PC were incubated with 5 µl of extraction solution (5 min, RT) to which 45 µL of neutralization solution was added. ASO-PCR conditions were as described above

and all purified cells from each cell compartment from each patient were analyzed by ASO-PCR (7 to 10 ASO-PCR replicates/patient). Presence of clonotypic B-cells within a specific B-cell or PC compartment was defined when ≥2 replicates were found to be positive. For every sample, DNA quality was evaluated by a control PCR for the thromboxane synthase gene (TBXAS1 primers: forward 5` GCCCGACATTCTGCAAGTCC 3`, Reverse 5` GGTGTTGCCGGGAAGGGTT 3`). The sensitivity of the assay was defined as the number of cells/μL of PB evaluated, based on the number of cells analysed per FACS-purified cell fraction and its absolute count in PB. Presence of clonotypic B-cells within a specific B-cell or PC compartment was defined when ≥ 2 replicates were found to be positive. For every sample, DNA quality was evaluated by a control according to the BIOMED-2 protocols.

Statistical analyses The Mann–Whitney U test was used to estimate the statistical significance of differences observed between distinct experimental groups (SPSS version 20, SPSS Inc., Chicago, IL, USA). *P* values ≤ 0.05 were considered to be associated with statistical significance.

Supplementary table 1: Clinical and biological features of MM and MGUS patients at diagnosis

	Patient Group	
Clinical/Laboratory features	MM (n=7)	MGUS (n=3)
Age (years)	68±8	66±7
Gender		
Male/Female	6/1	1/2
Disease at presentation		3/3
MGUS	NA	3/3
Symptomatic MM	6/7	NA
Smoldering MM	1/7	NA
Isotype of M-Component		
IgG	4/7	3/3
IgA	3/7	0/3
Clinical Stage (ISS)		
I	2/7	
II	4/7	NA
III	1/7	
Hemoglobin ≤ 8.5 g/dL	1/7	0/3
β2-microglobulin> 3.5 μg/L	5/7	0/3
Albumin≥ 3.5 g/dL	4/7	0/3
Creatinine≥ 2mg/dL	0/7	0/3
% S-phase BMPC > 1.5%	0/7	0/3
% BM PC>33%	4/7	0/3
% BM M-PC>95% of all PC	7/7	0/3

BMPC: bone marrow plasma cells; NA: not applicable; M-PC: (mono)clonal PC.
ISS: international staging system for multiple myeloma

Supplementary table 2: Primers used in the ASO-PCR reaction for the *IGH* CDR3 gene sequence.

Patient ID	Sensitivity of the assay	CDR3 Sequence	Primer sense 5`3`	Primer Antisense 5`3`	Tm (°C)	Length of the PCR product
MGUS 1	≤0.1 %	**GTATTACGATCTTTTGACTGGTTATTATCTAAATTCCTAAATCGATGACA**GCCTTTGACTACTGGGGCCAGG	GGTTATTATCTAAATTCCTAAATCGATGA	CAGAGTTAAAGCAGGAGAGAGGTTGT (intronic JH4)	62	107 bp
MGUS 2	≤0.1 %	TCATCTATTACTGTGCG**CGAAAAGGGAACGC**CTTTGACAACTGGGGCCAGGGAACGT	TCATCTATTACTGTGCGCGAAAAG	CAGAGTTAAAGCAGGAGAGAGGTTGT (intronic JH4)	62	106 bp
MGUS 3	≤0.1%	TGTGCGAGAG**GTCCCCATTACAGTGACTTGACTGGATACAGTTGGTACC**TTGATTCCTGG	AGGTCCCCATTACAGTGACTTGAC	CAGAGTTAAAGCAGGAGAGAGGTTGT (allele-especific)	69	108 bp
MM 1	ND	ATTACTGTGTGAAAGA**TCGATTGTCGGATTTTTGGAGCGGTGTCCCCTTTCTTG**ACTGGGGCCAG	ATTACTGTGTGAAAGATCGATTGTCG	AGGCAGAAGGAAAGCCATCTTAC (intronic JH3)	60	120 bp
MM 2	≤0.1 %	TGTGCG**AAAGTCGTAGGGGGGTCTCATCTATTGTAGTAGTACCAGCTGCTATTGGTTTGACGGTTTT**TGCTTTTGATATCTGG	AAAGTCGTAGGGGGGTCTCATC	AGGCAGAAGGAAAGCCATCTTAC (intronic JH3)	68.5	125 bp
MM 3	≤0.1 %	AGGATATTGTA**ATAGAACCAGCTGCTAAGCCGAGCTACAACAGTACATGT**ACTACTTTGACTACT	GCTAAGCCGAGCTACAACAGTACAT	CAGAGTTAAAGCAGGAGAGAGGTTGT (intronic JH4)	68	106 bp
MM 4	≤0.06%	TGTGTATTATTGTG**GGAGAGACAAGTATGTGGTAGCGACTACTGCGG**TCGCGCA	TGTGTATTATTGTGGGAGAGACAAGTA	AGAGAGGGGGTGGTGAGGACT (intronic JH5)	62	107 bp
MM 5	≤0.2%	**GTAGAGATGACTACACCGCCGAAAAATACCCGACAATGACTACT**GGGGCCAGGGAACCCTGGTCACCGTCTCCTCA	ACCGCCGAAAAATACCCGACA	CAGAGTTAAAGCAGGAGAGAGGTTGT (intronic JH4)	60	91 bp
MM 6	≤0.001 %	**TGTTTATTACTGTGCGAAGGACAGGAACAGTGGCTGGGACAAGTGGTTCGCCCCCTGG**GGCAAGGGAACCC	TTATTACTGTGCGAAGGACAGGAA	CTTACCTGAGGAGACGGTGACC (JH consensus)	60	84 bp
MM 7	≤0.001%	TGACACGGCCTTGTATTAC**TGTGTAAAAGATCGGGGCAGCTCGTCCTTGCTCCCGACGTTAGACTTCTGG**GGCCA	GCTCGTCCTTGCTCCCGAC	CTTACCTGAGGAGACGGTGACC (JH consensus)	60	60 bp

The CDR3 sequences are shown in bold. Underline nucleotides highlight the position of sense ASO primers. ND: Not Determined due to sample shortage.