

Identification of translocation products but not K-RAS mutations in memory B cells from patients with multiple myeloma

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ABSTRACT

Background

Several laboratories have shown that cells with a memory B-cell phenotype can have the same clonotype as multiple myeloma tumor cells.

Design and Methods

The aim of this study was to determine whether some memory B cells have the same genetic alterations as their corresponding multiple myeloma malignant plasma cells. The methodology included sorting multiple myeloma or memory B cells into RNA stabilizing medium for generation of subset-specific polymerase chain reaction complementary DNA libraries from one or 100 cells.

Results

Cells with the phenotype of tumor plasma cells (CD38⁺⁺CD19⁻CD45^{+/+}CD56^{+/+++}) or memory B cells (CD38⁻/CD19⁺/CD27⁺) were isolated by flow activated cell sorting. In samples from all four patients with multiple myeloma and from two of the three with monoclonal gammopathy of undetermined significance, we identified memory B cells expressing multiple myeloma-specific oncogenes (*FGFR3*; *IGH-MMSET*; *CCND1* high) dysregulated by an *IGH* translocation in the respective tumor plasma cells. By contrast, in seven patients with multiple myeloma, each of whom had tumor plasma cells with a *K-RAS61* mutation, a total of 32,400 memory B cells were analyzed using a sensitive allele-specific, competitive blocker polymerase chain reaction assay, but no *K-RAS* mutations were identified.

Conclusions

The increased expression of a specific “early” oncogene of multiple myeloma (monoclonal gammopathy of undetermined significance) in some memory B cells suggests that dysregulation of the oncogene occurs in a precursor B-cell that can generate memory B cells and transformed plasma cells. However, if memory B cells lack “late” oncogene (*K-RAS*) mutations but express the “early” oncogene, they cannot be involved in maintaining the multiple myeloma tumor, but presumably represent a clonotypic remnant that is only partially transformed.

Key words: multiple myeloma, monoclonal gammopathy of undetermined significance, memory B cells, *K-RAS*, clonotype.

Citation: Rasmussen T, Haaber J, Dahl IM, Knudsen LM, Kerndrup GB, Lodahl M, Johnsen HE, and Kuehl M. Identification of translocation products but not *K-RAS* mutations in memory B cells from patients with multiple myeloma. *Haematologica* 2010;95(10):1730-1737.
doi:10.3324/haematol.2010.024778

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Funding: this project was supported in part by the Danish Cancer Society, grant number DP 00006 to TR and DP 22-00-0314 and DP 2002-04 to HEJ. HEJ was supported by a senior grant from the Multiple Myeloma Research Foundation 2003-4 and EU 6th FP to MSC-NET (LSHC-CT-2006-037602) and MK was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

Acknowledgment: the authors thank Kirsten Nikolajsen for technical assistance with FACS sorting.

Manuscript received on March 8, 2010. Revised version arrived on April 23, 2010. Manuscript accepted on May 17, 2010

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The online version of this article has a Supplementary Appendix

Introduction

Multiple myeloma (MM) is a mostly incurable B-cell malignancy characterized by uncontrolled growth and accumulation of MM plasma cells in the bone marrow.¹ A number of laboratories have identified clonotypic cells (i.e., cells having the same somatically mutated immunoglobulin genes as the MM cells) with a pre-plasma cell phenotype in peripheral blood, lymph nodes and bone marrow.^{2,8} Although some studies support the idea of a MM “stem-cell” or tumor-propagating cell present in a preplasmacytic compartment,⁹⁻¹³ other studies failed to confirm this possibility.¹⁴⁻¹⁶

MM oncogenesis is a multistep process, which usually is preceded by a pre-malignant tumor called monoclonal gammopathy of undetermined significance (MGUS).^{17,18} Two early pathways of MM oncogenesis, both of which are shared by MGUS and MM, have been proposed: a hyperdiploid pathway that is associated with multiple trisomies involving eight chromosomes, and a non-hyperdiploid pathway that is typically characterized by the presence of primary immunoglobulin heavy chain (*IGH*) translocations involving mostly the genes for cyclin D1 (*CCND1*) [t(11;14)] or *FGFR3/MMSET* [t(4;14)].^{19,20} Most hyperdiploid tumors have a *CCND1* low phenotype, with the levels of ectopic and bi-allelic *CCND1* expression being consistently lower than those in tumors with a t(11;14) translocation that have a *CCND1* high phenotype.²¹⁻²³

The prevalence of activating mutations of the K- and N-RAS oncogenes is about 30-40% in MM, with some studies showing a somewhat higher prevalence of N-RAS mutations and other studies a higher prevalence of K-RAS mutations.²⁴⁻²⁸ The prevalence of RAS mutations is much higher in malignant MM - independently of stage - than in pre-malignant MGUS. Significantly, however, although N-RAS mutations are somewhat less prevalent in MGUS than in MM, no K-RAS mutations have been identified in MGUS. Therefore, unlike hyperdiploidy and the *CCND1* low phenotype, or primary *IGH* translocations, K-RAS mutations must be a relatively late event in pathogenesis.^{19,20}

MM tumor plasma cells harboring a primary *IGH* translocation, multiple trisomies, or a RAS mutation should also have these genetic alterations in a potential MM “stem cell”, which is ultimately responsible for proliferation of the MM tumor. Here we demonstrate that we can identify “early” oncogenic events in some memory B cells but not “late” K-RAS61 mutations, suggesting that these cells are not tumor stem cells but rather premalignant, incompletely transformed remnants that coexist with the malignant cells but are not directly involved in tumor maintenance and proliferation.

Design and Methods

Patients and samples

Bone marrow aspirates and peripheral blood samples from 120 patients with MM, and three MGUS patients with a *CCND1* high phenotype, were collected from our biobank of diagnostic material from clinical trials. In addition, peripheral blood samples from three healthy individuals were used as controls. All samples were obtained after informed consent and the study was approved by the Ethical Committee of Copenhagen County and was performed in accordance with the Helsinki Declaration. Bone mar-

row mononuclear cells, peripheral blood mononuclear cells and RNA were isolated as reported elsewhere.^{7,28,29}

Flow cytometry and single-cell sorting

Bone marrow and peripheral blood mononuclear cells were stained with three or four colors with the monoclonal antibodies CD19-FITC, CD27-FITC/PE, CD38-FITC/APC, CD45-PERCP, CD56-PE [Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA, USA], CD19-Cy5 (Dako, Glostrup, Denmark). IgG1-FITC, IgG1-PE, IgG1-PerCP and IgG1-APC (BDIS) were used as negative controls. Fluorescence activated cell sorting (FACS) of memory B cells or plasma cells, using a FACS Vantage (BDIS) in counter mode, was performed to separate cells directly to polymerase chain reaction (PCR) tubes.^{7,29,30}

Oncogene expression levels in purified plasma cells

We have previously described an approach to examine the gene expression profile in an immunophenotypically defined population of plasma cells.^{28,31-33} Briefly, single or 100 plasma cells/memory B cells were FACS-sorted directly to PCR tubes: global reverse transcriptase (RT)-PCR was performed to generate a PCR cDNA archive. The levels of expression of *CCND1*, *FGFR3* and *MMSET*, normalized to the level of expression of β -actin, were determined by real-time RT-PCR for each archived cDNA. For quantitative reactions (qRT-PCR), the amount of cDNA was adjusted to give a Ct of 16-18 for β -actin.

IGH-MMSET reverse transcriptase polymerase chain reaction

Nested *IGH-MMSET* RT-PCR was performed as described by Malgeri *et al.*³⁴ The identity of all PCR products was confirmed by direct sequencing. The frequency of cells containing *IGH-MMSET* hybrid transcripts was estimated by limiting dilution, using the equation $f = \sum k_i / [N - \sum (k_i \times c_i / 2)]$ (where f = frequency of the target cell, k_i = the number of positive wells at the i 'th dilution, c_i = number of cells per well at dilution i and N = the total number of cells in all the wells at all dilutions).³¹

Blimp-1 and XBP-1 polymerase chain reaction

Blimp-1 and XBP-1 PCR was performed on global RT-PCR amplified products from one or 100 sorted cells using the following primers: Blimp-1, forward: 5-AGATTTAA-GCCCCTTGCCACA-3, reverse: 5-TTCAACGTCTTCTTAGGGTCTCTTG-3. XBP-1, forward: 5-TCCAGTTTTAGGTCCTTTAGTTTGC-3, reverse: 5-ATACATGAGGA-AAGAGCCCC-3. The identity of the PCR products was determined by sequencing as previously described.³²

RAS mutation analysis by direct sequencing of purified plasma cell lysate

In a previous study we identified MM patients having K-RAS61 mutations by direct sequencing of a plasma cell-lysate generated from 100 FACS-sorted immunophenotypic aberrant plasma cells (CD38⁺/CD45^{hi}/CD19⁺/CD56⁺).²⁸

Allele-specific competitive blocker polymerase chain reaction for detection of K-RAS codon 61 mutations in purified plasma cell lysate

We previously designed an allele-specific competitive blocker (ACB)-PCR generating preferential amplification of the allele carrying K-RAS61 (CAA to CAC and CAA to CTA) mutations.²⁸ The method used a mutant-specific primer in combination with a non-extendable blocker primer with preferential binding for the wild-type allele. ACB-PCR was performed on a lysate generated from 100 FACS-purified memory B cells or plasma cells with an aberrant immunophenotype.

Results

Selection of bone marrow plasma cells and peripheral blood memory B cells

Normal plasma cells were identified as CD38⁺⁺/CD19⁺/CD45⁺/CD56⁻ and MM plasma cells as CD38⁺⁺ and CD19⁻ and/or CD45⁻ and/or CD56⁺. The MGUS patients analyzed often had mixtures of normal and aberrant plasma cells (MM immunophenotype) in the bone marrow, but only immunophenotypically aberrant plasma cells were FACS-sorted for further analyses, as described previously.²⁸ Memory B cells with the CD38⁻/CD19⁺/CD27⁺ immunophenotype were selected for FACS-sorting in all cases (healthy individuals, MGUS and MM), as described previously.⁷

Selected memory B cells are Blimp-1 and XBP-1 negative

MM plasma cells and memory B cells were separated based on their pattern of expression of CD antigens. However, the immunophenotype of the malignant plasma cells could be a reflection of the environment (bone marrow *versus* peripheral blood). Thus, the observed cells in the CD38⁻/CD19⁺/CD27⁺ FACS-sorted fraction with a dysregulated oncogene could be MM plasma cells with a yet unidentified aberrant immunophenotype, or simply contaminating circulating myeloma plasma cells as a result

of the sorting procedure. To exclude the above possibilities we analyzed all FACS-sorted cells for expression of Blimp-1, a transcriptional repressor important for the final differentiation of B cells into plasma cells, which is expressed in all plasma cells, but absent in memory B cells,^{35,36} and XBP-1, a second transcription factor that is unique to plasma cell differentiation.³⁷ The Blimp-1 and XBP-1 PCR assays could reproducibly detect a single FACS-sorted MM plasma cell among 100 memory B cells FACS-sorted from a healthy individual (*data not shown*). In all cases the FACS-sorted CD38⁻/CD19⁺/CD27⁺ cells (≤ 100 per tube) were negative for Blimp-1 and XBP-1, whereas all FACS-sorted CD38⁺⁺/CD19⁺/CD45⁺/CD56⁻ and CD38⁺⁺/CD19⁻/CD45⁺/CD56^{+/++} plasma cells were positive (Figure 1), indicating that an aberrant surface phenotype or contamination had no impact on the results.

Healthy memory B cells do not express CCND1 or FGFR3

Hematopoietic cells do not express *CCND1* or *FGFR3* and a complete absence of both *CCND1* and *FGFR3* expression was verified analyzing a total of 3600 healthy memory B cells isolated from the peripheral blood of three individuals, and 700 healthy plasma cells from seven individuals (Table 1 and Figures 2 and 3).^{33,38} Thus, the expression of *CCND1* or *FGFR3* in memory B cells, as found in their autologous MM plasma cells, would be a myeloma-specific event.

Table 1. Expression of dysregulated oncogenes identified in tumor plasma cells sometimes detectable in the memory B-cell compartment.

Patient ¹	Oncogene ²	Plasma cell analysis		Memory B-cell analysis		Number of cells analyzed
		Oncogene expression/ β -actin ³	Cells sorted to PCR tube ⁴	Oncogene-positive PCR tube ⁵	Oncogene expression/ β -actin ³	
MM-1	<i>CCND1</i> High	1.31	1x96	5/96	0.0062	96
MM-2	<i>CCND1</i> High	0.16	100x9	5/9	0.0017	900
MM-3	<i>FGFR3/IGH-MMSET</i>	31.05	100x7	2/7	0.0869	700
MM-4	<i>FGFR3/IGH-MMSET</i>	12.35	100x15	3/15	0.0081	1500
MM-5	<i>CCND1</i> Low	0.03	100x12	0/12	-	1200
MM-6	<i>CCND1</i> Low and <i>RAS</i>	0.01	100x9	0/9	-	900
MM-7	<i>CCND1</i> Low	0.03	100x8	0/8	-	800
MM-8	<i>CCND1</i> Low and <i>RAS</i>	0.02	100x12	0/12	-	1200
MM-9	<i>CCND1</i> Low	0.01	100x12	2/12	0.0002	1200
MM-10	<i>CCND1</i> Low	0.009	100x8	0/8	-	800
MM-11	<i>CCND1</i> Low	0.008	100x7	0/7	-	700
MM-12	<i>CCND1</i> Low	0.005	100x8	0/8	-	800
MM-13	<i>CCND1</i> Low and <i>RAS</i>	0.003	100x96	5/96	0.0003	9600
MGUS-1	<i>CCND1</i> High	1.99	100x15	4/15	0.0028	1500
MGUS-2	<i>CCND1</i> High	0.65	100x9	3/9	0.0016	900
MGUS-3	<i>CCND1</i> High	0.66	100x8	0/8	-	800
Healthy	-	-	100x12	0/12	-	1200
Healthy	-	-	100x12	0/12	-	1200
Healthy	-	-	100x12	0/12	-	1200

¹Healthy = healthy individuals. ²*CCND1* and *FGFR3* expression was determined by real-time RT-PCR analysis of global RT-PCR amplified cDNA from FACS-purified immunophenotypically aberrant plasma cells. *IGH-MMSET* transcripts were identified by RT-PCR analysis of mRNA from diagnostic bone marrow samples. *RAS* mutations were identified by direct sequencing. ³Oncogene/ β -actin identified by real-time RT-PCR in the corresponding plasma cells or memory B cells. ⁴Memory B cells were FACS-sorted directly to PCR tubes followed by global RT-PCR and a β -actin PCR test for the presence of a cDNA product. For patient MM-1 a single memory B cell was FACS-sorted to 120 PCR tubes and the β -actin cDNA product was generated in 96 PCR tubes. ⁵The number of PCR tubes with 1 or 100 memory B cells in which expression of a MM specific oncogene was identified.

Two patterns of *CCND1* expression in multiple myeloma or monoclonal gammopathy of undetermined significance

Previously, we identified a high level expression of oncogenes involved in *IGH* translocations (*FGFR3/MMSET*, *CCND1*), or expression of hybrid transcripts associated with translocation t(4;14) (*IGH-MMSET*) in FACS-purified MM plasma cells.^{33,38} There are two distinct groups of *CCND1*-positive MM: the t(11;14) group, all showing high expression of *CCND1*, and a group with low to intermediate *CCND1* expression associated with hyperdiploidy, including 11q trisomy.²² To identify which MM patients belonged to the two different *CCND1* groups for further studies we determined the level of *CCND1* expression in FACS-purified plasma cells from 120 MM patients (*Online Supplementary Figure S1*). As in previous studies, which included only about half of these patients, we identified 21 (17.5%) MM patients with plasma cells showing high *CCND1* expression (*CCND1*/β-actin >0.1) indicating a t(11;14), and 30 (25%) patients with low *CCND1* expression (*CCND1*/β-actin >0.001 and <0.1).^{21,22,28,33,39} By contrast, the *CCND1*/β-actin was less than 0.0001 (but mostly <0.000001) in the other 69 cases of MM, and also in plasma cells and memory B

cells from healthy individuals (Table 1, Figure 2, and *data not shown*).

CCND1 expression in memory B cells from patients with multiple myeloma or monoclonal gammopathy of undetermined significance with t(11;14)

Two patients with MM and three with MGUS with a *CCND1* high phenotype were selected for further analyses (Table 1, MM 1-2 and MGUS 1-3).³³ Memory B cells from the two MM and three MGUS patients were FACS-purified, followed by global RT-PCR amplifications of the RNA pools from one or 100 cells (Table 1). The expression of *CCND1* was analyzed using quantitative real-time PCR assays (reproducibility shown in *Online Supplementary Figure S2*). From patient MM-1, for whom 4% of memory B cells had been previously identified as clonotypic,^{40,41} a single memory B cell was FACS-sorted into each of 120 PCR tubes, with an amplified cDNA product (a cDNA archive) generated in 96 tubes. Analysis of *CCND1* expression (Figure 2A, Table 1) revealed 5/98 positive wells, indicating that memory B cells had a similar frequency of *CCND1*⁺ cells (5%) as clonotypic cells (4%). Memory B cells from patient MM-2 were sorted into nine tubes, each containing 100 sorted cells, and *CCND1* expression was detected in five of these nine tubes (Table 1). Therefore, less than 1% of the memory B cells from patient MM-2 expressed *CCND1*, consistent with the results of a previous study in which we showed that less than 1% of the memory B cells were clonotypic in this patient.⁴¹ We also identified *CCND1*⁺ memory B cells in two out of the three *CCND1*⁺ MGUS cases, by analyzing 1500, 900, and 800 (negative case) memory B cells available for analyses (Table 1 and Figure 2B).

We observed a lower level of oncogene expression – in the range of 94 to 710 times lower – in memory B cells than in autologous MM plasma cells (Table 1). This could be due to several factors. Firstly, the expected ratio of

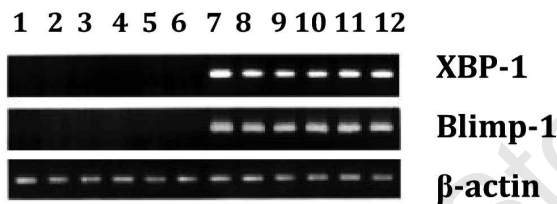


Figure 1. Blimp-1 and XBP-1 expression. Global RT-PCR products from plasma cells and memory B cells were analyzed by RT-PCR for expression of the transcription factors XBP-1 and Blimp-1. Results are shown for three myeloma patients and three healthy individuals, with 100 sorted cells per tube in each case. Lanes 1-3, oncogene-negative (healthy) memory B cells; lanes 4-6, oncogene-positive (myeloma) memory B cells; lanes 7-9, MM plasma cells; lanes 10-12, healthy plasma cells.

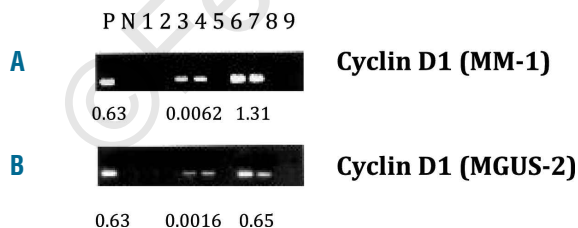


Figure 2. Oncogene analyses of plasma cells and memory B cells: (A) from a *CCND1*-positive MM patient (MM-1) and (B) from a *CCND1*-positive MGUS patient (MGUS-2). Global RT-PCR products were screened for *CCND1* expression using real-time PCR, with 100 sorted cells per tube in each case. *CCND1*-positive or negative real-time PCR products were run on a gel and bands corresponding to *CCND1* were excised from the gel and sequenced. The oncogene/β-actin ratio determined by real-time PCR is shown below each positive case. P, positive control; N, negative control (H₂O); lanes 1 and 2, healthy memory B cells; lanes 3 and 4, oncogene-positive memory B cells; lane 5, RT control; lanes 6 and 7, myeloma plasma cells; lanes 8 and 9, healthy plasma cells.

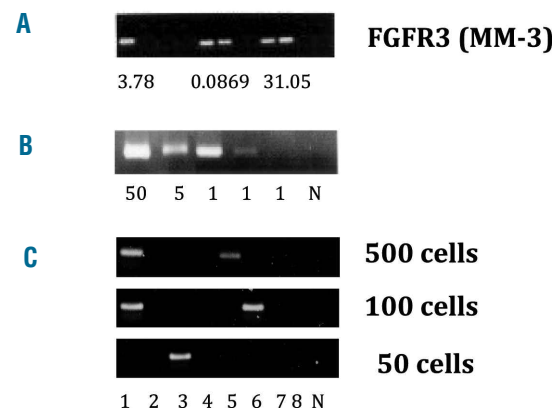


Figure 3. Identification of *IGH-MMSET* hybrid transcripts in memory B cells. (A) The *FGFR3*-positive MM patient (MM-3) was analyzed as described in Figure 2. (B) The CD38⁺⁺/CD19⁺/CD56⁺⁺ plasma cells were flow-sorted directly to PCR tubes in numbers of 50, 5 and 1 cell/PCR tube followed by nested RT-PCR for *IGH-MMSET* hybrid transcripts. (C) RT-PCR analysis of *IGH-MMSET* hybrid transcripts in sorted CD19⁺/CD38⁺/CD27⁺ memory B cells. Memory B cells were flow sorted in numbers of 500, 100 and 50 cells/PCR tube, with eight PCR tubes for each cell number followed by RT-PCR analysis of *IGH-MMSET* hybrid transcripts. N = negative control (H₂O).

oncogene-positive cells to normal cells is nearly 100% when sorting plasma cells, but usually 0 or 1 of 100 when sorting memory B cells (except for patient MM-1, Table 1). Secondly, as the dysregulation of the oncogenes is believed to be under the control of an *IGH* enhancer, oncogene expression could be further induced and increased upon plasma cell differentiation.

Expression of *FGFR3* and *MMSET* hybrid transcripts in memory B cells

Two patients (MM-3 and MM-4) had MM tumors with t(4;14) translocations, as determined by ectopic and enhanced expression of *FGFR3*, and also *MMSET* hybrid transcripts. For both patients, we demonstrated increased expression of *FGFR3* in about 0.1 to 1% of purified memory B cells (Table 1 and Figure 3A), consistent with the presence of less than 1% clonotypic memory B cells in these patients, as determined previously.⁴¹ For patient MM-3, there was enough material to enable us to perform a limiting dilution experiment to verify the presence and frequency of *IGH-MMSET* hybrid transcript-positive cells in the memory B-cell pool. *IGH-MMSET* hybrid transcripts were detected in single flow-sorted plasma cells (Figure 3B). *IGH-MMSET* hybrid transcripts were detected in 2/8, 2/8 and 1/8 PCR analyses with 500, 100, 50 cells/PCR tube, respectively (Figure 3C). Limiting dilution statistical analysis of the results shown in Figure 3C gave an estimated frequency of 0.1% *IGH-MMSET*-positive memory B cells, similar to the frequency of *FGFR3*-expressing memory B cells in this patient.

CCND1 low memory B cells were present in only two of nine patients with *CCND1* low multiple myeloma

Memory B cells were FACS-sorted from nine MM patients belonging to the *CCND1* low group (Table 1, MM 5 - 13). In two of these MM patients, *CCND1*⁺ memory B cells were identified in 2/12 and 5/96 PCR tubes with 100 cells/tube (Table 1, patients MM-9 and MM-13). However, in the other seven of nine patients with *CCND1* low MM, we did not detect *CCND1* expression in any of the purified memory B cell pools.

Activating *K-RAS61* mutations are absent in memory B cells

FACS-sorted MM plasma cells (CD38⁺/CD19⁻/CD45^{-/ll} CD56^{+/+++}) were analyzed previously for the presence of mutations in the *N-RAS* and *K-RAS* genes using RT-PCR followed by direct sequencing, enabling the identification of patients who had aberrant MM plasma cells with a *RAS* mutation at the time of diagnosis.²⁸ From seven MM patients with a known CAC or CTA *K-RAS61* mutation in their MM plasma cells, CD38⁺/CD19⁻/CD27⁺ memory cells from peripheral blood mononuclear cells were FACS-sorted to produce 100 cells/PCR tube, then subjected to ACB-PCR for detection of *K-RAS61* mutations. As a positive control for the ACB-PCR method, a single *RAS*-mutated MM cell was FACS-sorted into 100 non-mutated healthy B cells in each experiment (Figure 4A), confirming that it was possible to efficiently detect a single cell containing the *K-RAS61* mutation in the presence of 100 cells without the mutation. Different numbers of CD38⁺/CD19⁻/CD27⁺ cells were available from the obtained blood samples, ranging from 300 to 12,000 cells, so that a total of 32,400 CD38⁺/CD19⁻/CD27⁺ cells were analyzed (Figures 4B and 5B). However, all 32,400 memory B cells

analyzed from seven different MM patients with *RAS* mutations in their plasma cells were found to be negative for *K-RAS61* mutations in the memory B-cell populations (Figures 4B and 5B).

The presence of *CCND1* low memory B cells lacking *K-RAS61* mutations

To directly compare *CCND1* expression and *KRAS61* mutations in the same pools of memory B cells, we FACS-sorted memory B cells from patient MM-13 who had MM plasma cells with both *CCND1* low expression and a *K-RAS61* mutation (Table 1). A total of 9600 memory B cells were FACS-sorted into 96 PCR tubes with 100 cells/tube (Table 1, MM-13), and the cell lysate was split into two aliquots, one for global RT-PCR followed by real-time PCR for *CCND1* and one for ACB-PCR detection of *K-RAS61* mutations. Memory B cells expressing *CCND1* were identified in 5/96 PCR tubes (Figure 5A, Table 1). However, *RAS* mutations were absent in all memory B

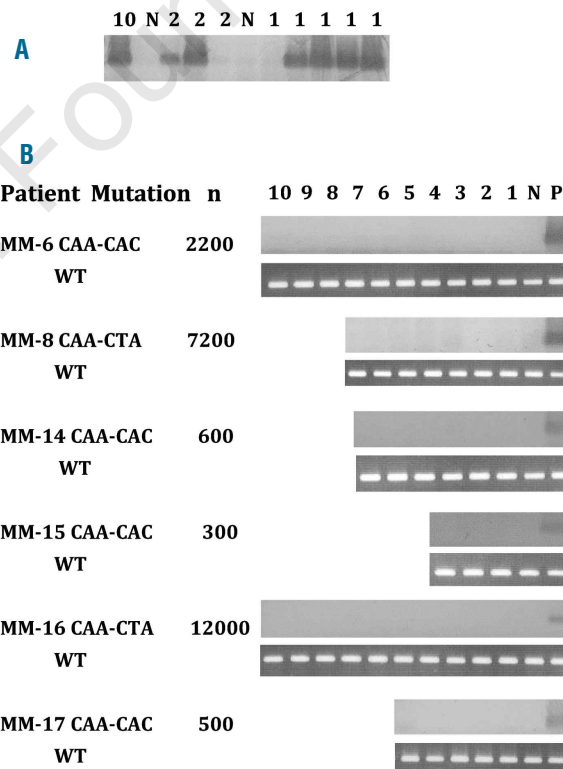


Figure 4. Screening memory B cells for *K-RAS61* mutations. (A) Sensitivity of ACB-PCR when analyzing FACS-sorted plasma cells. FACS-sorting of 1, 2 or 10 MM plasma cells from a patient with a *K-RAS61* mutation to PCR tubes containing 100 FACS-sorted CD19⁺ B cells from a healthy individual. The results are only shown for the CAA to CAC mutation, but similar results was obtained for the CAA to CTA mutation. In four out of five PCR tubes with a single FACS-sorted *K-RAS61*⁺ MM plasma cell among 100 healthy B cells, a *K-RAS61*⁺ MM plasma cell was detected. (B) From six patients with a *K-RAS61* mutation, 100 CD19⁺/CD38⁺/CD27⁺ memory B cells were FACS sorted for each ACB-PCR analysis. For each of the MM patients the *K-RAS61* mutation type is shown and the total number of CD19⁺/CD38⁺/CD27⁺ memory B cells available for analysis is given (n). P = positive control, 100 FACS-sorted plasma cells from a patient with a *K-RAS61* mutation. N = negative control, 100 FACS-sorted CD19⁺ B cells from a healthy donor. WT = wild-type, an analysis for wild-type *RAS* was performed for each FACS-sorted memory B-cell population as a positive control.

cells FACS-sorted from patient MM-13, including the tubes containing memory B cells that expressed *CCND1* (Figure 5B).

Discussion

All MM tumors analyzed have stable V(D)J rearrangements with an extensive accumulation of somatic mutations in the complementarity determining regions, and an absence of intra-clonal somatic mutation.^{42,43} These findings support the idea that MM cells are derived from germinal center or post-germinal center B cells. A normal germinal center B cell that has undergone repeated cycles of somatic hypermutation and antigen selection can differentiate into either a memory B cell or a terminally differentiated, long-lived plasma cell that has undergone productive IgH switch recombination, and is typically found in the bone marrow.⁴⁴ A number of laboratories have identified clonotypic CD19⁺ cells in the peripheral blood that share identical V(D)J sequences with the MM tumor cells in the same patient.²⁻⁸ Some clonotypic CD19⁺ cells are pre-switch, expressing IgM, whereas others have switched to the same IgH isotype as the CD138⁺ MM tumor cells. We previously identified a CD19⁺/CD27⁺/CD38⁻ subset of clonotypic cells in the peripheral blood which fulfills the immunophenotypic characteristics of a memory B cell.^{7,40,44}

Recently, it has been shown that most – if not all – MM tumors are generated from premalignant MGUS tumors.^{17,18} Primary *IGH* translocations, which are shared by MGUS and MM tumors, mostly have breakpoints that fall within or near *IGH* switch regions.^{45,46} It is, therefore, thought that these translocations, which are present in about 40% of MM tumors, occur as a result of errors in

IgH switch recombination, which takes place mostly in germinal center or post-germinal center B cells. Since hyperdiploidy and cyclin D dysregulation, an apparently unifying event in the pathogenesis of MM, are present in both MGUS and MM, it seems likely that these early oncogenic events may also occur in germinal center or post-germinal center B cells, although this needs to be documented.^{20,21}

It is possible that either fully or incompletely transformed cells can generate progeny with memory B cell or plasmablast/plasma cell phenotypes. Hence, there are several possible explanations for the presence of clonotypic B cells in the peripheral blood of MM patients. First, the clonotypic B cells could be incompletely transformed memory B cells that have undergone only some of the very early oncogenic events, and perhaps these cells have some proliferative or survival advantage compared to their normal counterparts. Second, the clonotypic B cells could be the MM “stem/tumor-propagating cell” that has a critical role in the maintenance of the MM tumor, and includes the full spectrum of oncogenic changes as the MM cells with a plasma cell phenotype. Third, the clonotypic B cells could be phenotypic variants of the MM tumor cells that have lost some plasma cell markers and gained some B-cell markers, possibly related to the loss of contact with the bone marrow microenvironment. In principle, any of these three types of clonotypic B cells could generate a clonotypic tumor in an immunodeficient xenogenic model. However, the first type of clonotypic B cell would differ from the other two types by sharing early but not late oncogenic events with the CD38⁺ MM tumor cells.

Our study was designed to isolate the CD38⁻CD19⁺CD27⁺ memory B cells from the peripheral blood of patients with MM and MGUS and sort them into pools containing from one up to 100 cells, from which a global PCR cDNA archive was made. Our results suggest that sorted cells were not significantly contaminated by MM tumor cells. First, we were unable to detect Blimp-1 or XBP-1 transcripts using an assay that could detect one normal plasma cell or one MM aberrant plasma cell in a pool of 100 purified memory B cells. Secondly, we could not detect *CCND1* expression in the purified memory B-cell population isolated from seven of nine MM patients in whom the tumor cells had a *CCND1* low phenotype. Third, we could not detect *K-RAS61* mutant transcripts in the purified memory B-cell populations isolated from all seven MM patients whose MM tumor cells contained the *K-RAS61* mutation. The lack of significant contamination of the memory B-cell populations most likely reflects the fact that there were very few CD38⁺ MM tumor cells in the peripheral blood.^{1,47} In addition, the FACS sorting procedure would be expected to exclude CD38⁺CD19⁻ cells from the purified CD38⁻CD19⁺CD27⁺ memory B-cell population more effectively than immunomagnetic selection of CD19⁺ cells.

As summarized in the *Results* section, we detected ectopic *FGFR3* expression in the memory B-cell population of both MM patients who had a t(4;14) translocation, and were also able to confirm this result in one of these two patients by identifying *MMSET* hybrid transcripts that represent direct products of the t(4;14) translocation, a result reported previously for a single MM patient.¹⁴ We also detected *CCND1* expression in peripheral blood memory B-cell populations derived from both MM patients and two of three MGUS patients with a *CCND1*

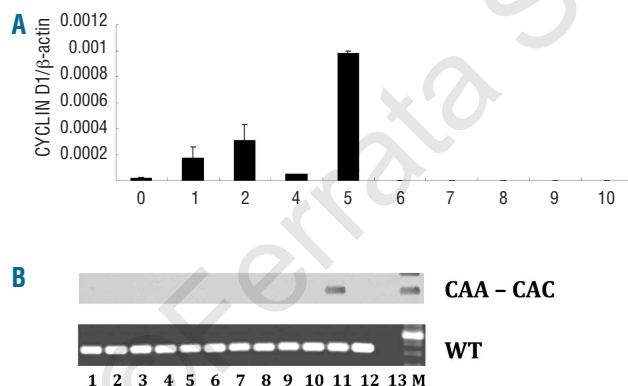


Figure 5. Simultaneous screening for *CCND1* expression and *K-RAS61* mutations. A cell lysate obtained from 100 FACS-sorted memory B cells from patient MM-13 was split into two aliquots, one for global RT-PCR followed by real-time PCR for *CCND1* and one for ACB-PCR detection of *K-RAS61* mutations. (A) In 5/96 PCR tubes *CCND1*⁺ memory B cells were identified, the measured *CCND1* level is shown (n. 1-5). N. 6-10 show five representative negative *CCND1* measurements. (B) Each memory B-cell lysate was analyzed for the *KRAS61* CAA to CAC mutation present in the patients' MM plasma cells. As a control for the presence of cDNA, each cell lysate was analyzed for the presence of wild-type (WT) *RAS*. Lanes 1-5, *CCND1*-positive memory B cells (see *Online Supplementary Figure 2A*). Lanes 6-10, *CCND1*-negative memory B cells. Lane 11, positive control, 100 FACS-sorted plasma cells from patient MM-13 with a *K-RAS61* mutation. Lane 12, negative control, 100 FACS-sorted CD19⁺ B cells from a healthy donor. Lane 13, negative control (H₂O). M = molecular size marker. *RAS* mutations were absent from all memory B cells analyzed.

D1 high phenotype which is associated with t(11;14) in their MM or MGUS tumor cells. It is possible that the third MGUS patient had memory B cells with *CCND1* expression, but that these were not present in the 800 memory B cells that we were able to analyze. Significantly, for the four cases of MM above, the fraction of memory B cells expressing a translocation-associated oncogene product was the same as the fraction of clonotypic memory B cells, as reported previously for the same patients.⁴¹ We were unable to detect *CCND1* expression in the memory B cells derived from seven of nine MM patients with a *CCND1* low phenotype, which is almost invariably associated with hyperdiploidy. Importantly, in all cases, the dysregulated oncogene identified in memory B cells was the same as that found in the corresponding MM tumor.

In contrast to the identification of oncogene products dysregulated by translocations in the memory B cells from all four MM patients and two of three MGUS patients, we were unable to identify *K-RAS61* mutations in the memory B cells of all seven patients with *K-RAS61* mutations in their CD38⁺ MM tumor cells. Moreover, in one patient who had a MM tumor with a *CCND1* low phenotype and a *K-RAS61* mutation, we could not identify the *K-RAS61* mutation in several pools of memory B cells that expressed *CCND1*.

Although there is little information about oncogenic changes in clonotypic B cells, it is important to mention a prior study that appears to be in disagreement with our results. Zojer *et al.*⁴⁷ identified a few percent of purified CD19⁺ peripheral blood B cells containing both early (*RB1* deletion, trisomy 11) and late (*P53* deletion) karyotypic abnormalities that are shared with CD138⁺ MM tumor cells. However, the authors estimated that the immunobead purification of CD19⁺ cells averaged about 95% so that the low fraction of cells with karyotypic abnormalities might represent contaminating CD138⁺ MM tumor cells. In fact, this possibility seems to be supported by their finding that the rare karyotypically abnormal cells in the CD19-selected population expressed the same high levels of cytoplasmic immunoglobulin as found in CD138⁺ MM tumor cells. In addition, their late event (*P53* deletion) is associated with advanced tumors that have a poor prognosis, and may be more likely to be

found in the peripheral blood.^{1,20}

There are two conflicting albeit potentially reconcilable views about the phenotype of a MM “stem/tumor-propagating” cell. On the one hand, Epstein *et al.* have shown that CD138⁺ cells can undergo substantial proliferation and several rounds of serial transplantation in ectopic bone in SCID-hu or SCID-rab immunodeficient models, whereas CD38⁺ or CD138⁺ cells do not proliferate significantly in this model.^{15,16} On the other hand, Matsui, Pilarski and others have shown that CD38⁺CD19⁺CD27⁺ cells - but not CD38⁺ or CD138⁺ cells - can form *in vitro* clones, or *in vivo* tumors that give rise to CD138⁺ cells in immunodeficient NOD/SCID mice.^{10,11,13} So, how do our results fit into this unresolved issue? Given that others have identified clonotypic B cells that express IgM,^{2,3} it is not surprising that we have found that some memory B cells can share early oncogenic events with MM tumor cells but not *K-RAS61* mutations. Although we found no evidence for a MM “stem/tumor-propagating” cell with a memory B-cell phenotype in peripheral blood, it is possible that this cell might be found mainly in another location, or exist at too low a level in peripheral blood to be detected by our assays.

In conclusion, our results strongly suggest the existence of clonotypic memory B cells in the peripheral blood that have “early” (ectopic translocation products) but not “late” (*K-RAS61* mutations) oncogenic events. Memory B cells with this genotype must represent pre-malignant, incompletely transformed remnants that could have some proliferative advantage compared to normal memory B cells. It remains to be determined whether clonotypic B cells that can be cloned *in vitro* or can form tumors in NOD/SCID mice are truly MM “stem/tumor-propagating” cells or phenotypically aberrant MM tumor cells that share essentially all early and late oncogenic changes with MM tumor cells.

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.

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