Possible roles for activating RAS mutations in the MGUS to MM transition and in the intramedullary to extramedullary transition in some plasma cell tumors.

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Abstract
To assess a possible role in tumor progression, the occurrence and type of K- and N-RAS mutations were determined in purified tumor cells, including samples from patients with pre-malignant monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma (MM), and extramedullary plasma cell (PC) tumors (ExPCT). Immunophenotypic aberrant PCs were flow-sorted from 20 MGUS, 58 MM and 13 ExPCT patients. One RAS mutation was identified in 20 MGUS tumors (5%), in contrast to a much higher prevalence of RAS mutations in all stages of MM (about 31%). Further, oncogene analyses showed that RAS mutations are not evenly distributed among different molecular subclasses of MM, with the prevalence being increased in MM expressing cyclin D1 ($p = 0.015$), and decreased in MM with t(4;14) ($p = 0.055$). We conclude that RAS mutations often provide a genetic marker if not a causal event in the evolution of MGUS to MM. Surprisingly, RAS mutations were absent in bone marrow tumor cells from all patients with ExPCT, a result significantly different from intramedullary MM ($p = 0.001$). From 3/6 patients with paired intra- and extramedullary PCs and identical IgH sequences, RAS mutations were identified only in extramedullary PCs, suggesting a role for RAS mutations in the transition from intramedullary to extramedullary tumor.
Introduction

Multiple myeloma (MM) is an incurable B cell malignancy characterized by an uncontrolled accumulation of plasma cells (PCs), usually in the bone marrow (BM). MM often is preceded by a pre-malignant tumor called monoclonal gammopathy of undetermined significance (MGUS), which also is usually localized in the BM. Intramedullary MM may disseminate to the peripheral blood as secondary plasma cell leukemia (PCL), and in some cases can also establish tumors at extramedullary sites. Alternatively, extramedullary plasmacytoma that do not represent progression of intramedullary MM usually are indolent tumors that can be associated with intramedullary MGUS. Thus, extramedullary PC tumors (ExPCT) can be either de novo extramedullary plasmacytoma, or extramedullary MM that represents progression of intramedullary MM.

In earlier studies that used a variety of methods to analyze unpurified tumor samples, activating point mutations of the N- or K-RAS oncogene had been reported to be present in up to 50% of MM tumors, but appeared to be absent or rare in MGUS patients. In the largest reported study, RAS mutations were identified in 39% of MM tumors at the time of diagnosis regardless of stage, with a limited analysis indicating accumulation of additional RAS mutations consistent with a prevalence of nearly 50% at the time of relapse. They also found that 60% of the mutations were in N-RAS, most often at codon 61. Apart from a variable prevalence of RAS mutations reported in other early studies, there was agreement that a majority of RAS mutations involved N-RAS, most often at codon 61, and that the prevalence of RAS mutations increased with disease progression and development of PCL. However, using partially purified tumor cells for their analyses, two recent studies have investigated the prevalence and type of RAS mutations in MM at different stages. One study reported RAS mutation with a prevalence of about 50% in newly diagnosed MM, PCL and human myeloma cell lines (HMCL), more often involving K- and not N-RAS. A second study reported that N-
RAS mutations are present in all MM patients at diagnosis, suggesting that N-RAS mutations are an early, universal event in MM pathogenesis.\(^7\)

To investigate the possible role of RAS mutations in MM development we performed an analysis of the RAS mutational status in purified tumor cells from patients with MGUS, MM and extramedullary PC tumors (ExPCT). For ExPCT, BM and extramedullary tumor cells were collected simultaneously, and both analyzed.

**Study design**

**Patient selection and characterization.**

A total of 20 MGUS tumors, 58 untreated MM tumors, and 13 patients with ExPCT, all with IgG or IgA isotype, were included in the study. Criteria for inclusion in the study was the presence of PCs with an aberrant MM immunophenotype in BM aspirates, determined by flow cytometry as described below. In addition, all MGUS patients were stable over a 2-year follow-up after BM sampling, with a median time from diagnosis of 6 years (range 3 – 14). BM aspirates from 13 MM patients with extramedullary disease were included and in 10 of these patients biopsies/aspirates from extramedullary locations were simultaneously collected (Table 1). Further BM samples were obtained from 9 normal donors. All samples were obtained after informed consent and the study was approved by the Ethical Committee of Copenhagen County Denmark and was in accordance with the Helsinki Declaration. The isolation of BM mononuclear cells (BMMNC) and RNA was performed as previously described.\(^8\)

**Flow cytometry and cell sorting.**

Cell samples were stained with 3- or 4-colours with the monoclonal antibodies CD19 FITC, CD38 FITC/APC, CD45 PERCP, CD56 PE (Becton Dickinson Immunocytometry Systems
(BDIS), San Jose, California) and CD19 Cy5 (Dako, Glostrup, Denmark), IgG1 FITC, IgG1 PE, IgG1 PerCP and IgG1 APC (BDIS) were used as negative controls. A minimum of 100,000 cells were collected and analyzed using a FACSCalibur (BDIS). Fluorescence activated cell sorting (FACS) of PCs directly to PCR tubes was performed using a FACS Vantage (BDIS) in counter mode to >96% purity.\(^8\)

**Characterizing V\(_{\text{H}}\)DJ\(_{\text{H}}\) gene rearrangements.**

The V\(_{\text{H}}\)DJ\(_{\text{H}}\) gene rearrangements representing MM clones from different locations in patients with ExPCT were identified as described previously.\(^9\)

**RAS mutation analysis by direct sequencing of FACS-purified PC lysate.**

For each patient 100 PCs with an aberrant immunophenotype were sorted directly into PCR tubes for each RAS mutational analysis. The cells were lysed and the mRNA reverse transcribed as previously described.\(^8\) Exon 1 and 2 for the N- and K-RAS genes were amplified using the primers as described\(^10\) followed by direct sequencing as previously described.\(^8\) All samples were analyzed in two independent PCR and sequencing setups, except for extramedullary samples which were analyzed only once due to a limited amount of material available.

**Allele-specific competitive blocker (ACB)-PCR for detection of K-RAS codon 61 mutations in FACS-purified PC lysate.**

We designed a ACB-PCR generating preferential amplification of the mutant allele using a mutant-specific primer (MSP) in combination with a non-extendable blocker primer (BP) having preferential binding for the wild-type allele.\(^11\) Primers for detection of mutation CAA to CAC, MSP: 5´-CTGACACAGCAGGTCTC-3´, BP: 5´-CTGACACAGCAGGTCTC-3´.
Primers for detection of mutation CAA to CTA, MSP: 5’-TCTCGACACACAGGTAT-3’, BP: 5’-TCTCGACACACAGGTA-3’. MSP primers were fluorescently-labeled with FAM at the 5’position and BP primers were modified at the 3’position with a C7 amino linker blocking polymerase elongation. MSP and BP primers were all used as forward primers in combination with a reverse primer K61B: 5’-GCTTACTGGTTCTAGAAGGCAA-3’. All primers were from MWG-Biotech AG (Aarhus, Denmark), HPLC purified and approved by MALDI-TOF MS. ACB-PCR were performed on a RT-lysate generated from FACS-purified PCs with a aberrant immunophenotype after pre-amplification with RAS specific primers: Forward: 5’-TCCATAACTTCTTGCTAAGTC-3’, reverse: 5’-GCTTACTGGTTCTAGAAGGCAA-3’, under the same conditions as described above for direct sequencing of RAS. The ACB-PCR consisted of a lower layer: MgCl₂, 25 mM, 3 μl, dNTP 10 mM each, 0.2 μl, gelatine 0.1%, 1 μl, Triton X-100, 1 μl, H₂O, 1.2 μl. Primers MSP, BP and K61B were added to give a final concentration of 0.4 μM each. Upper layer: AmpliTaq Stroffel fragment 10U/μl, 0.25 μl, 10 x Stroffel buffer 5 μl, Perfect Match PCR Enhancer (Stratagene, Aarhus, Denmark), 4 μl, final concentration 0.8U/μl, sample, (pre-amplification product) 1 μl, 5 μl, H₂O, 21.35 μl. Lower and upper layer were initial separated using AmpliWax. All above consumables were supplied by PE Applied Biosystems, (Foster city, CA.), except when otherwise mentioned. The PCR consisted of 5 min at 95°C followed by 35 cycles of 94°C, 30 sec, 55°C, 30 sec and 72°C, 1 min, followed by heating at 72°C for 10 min in a 9700 PCR machine (PE Applied Biosystems, Foster city, CA). The PCR products produced for the CTA and CAC mutations were 214 and 213 bp, respectively. After agarose gel electrophoresis products were visualized using a FUJI FLA 3000 (Fuji, Stockholm, Sweden).

Oncogene expression levels in FACS-purified PCs.
Recently, we have described an approach to examine the gene expression profile in an immunophenotypic defined population of PCs.\textsuperscript{12} Briefly, 100 PCs were FACS-sorted directly to PCR tubes, followed by global reverse transcription (RT)-PCR, generating a cDNA archive. Cyclin D1, FGFR3 and MMSET expression levels normalized to β-actin, were determined in each cDNA archived by real-time RT-PCR as described.\textsuperscript{12}

**IGH-MMSET RT-PCR.**

The *IGH*-MMSET RT-PCR was performed as described previously\textsuperscript{13} on cDNA products generated from BMMNC obtained at the time of diagnosis.

**Statistics**

All statistics were performed using the StatView 4.5 software form Abacus Concepts, Inc. Berkeley, CA. The significance of the absence of *RAS* mutation in patients with ExPCT compared to MM was determined using the Fisher’s exact test. Mean CD56 fluorescence levels for different MM subsets were compared using an unpaired *t*-test. The significance of oncogene expression and *IGH*-MMSET hybrid transcripts in relation to the presence or absence of *RAS* mutation in PCs was determined using the Pearson Chi-square test.
Table 1. Patients with extramedullary plasma cell tumors (ExPCT).

<table>
<thead>
<tr>
<th>Patient name</th>
<th>Medullary at diagnosis of MM</th>
<th>Medullary when ExMM diagnosed</th>
<th>ExPCT tumor location</th>
<th>PC%†</th>
<th>PC%*</th>
<th>Time‡</th>
<th>Status§</th>
</tr>
</thead>
<tbody>
<tr>
<td>ExPCT-1C</td>
<td>44</td>
<td>44</td>
<td>Orbit</td>
<td>100</td>
<td>4</td>
<td>4</td>
<td>Dead</td>
</tr>
<tr>
<td>ExPCT-4</td>
<td>5</td>
<td>5</td>
<td>Pleura</td>
<td>4</td>
<td>12</td>
<td></td>
<td>Dead</td>
</tr>
<tr>
<td>ExPCT-7C</td>
<td>7</td>
<td>7</td>
<td>Lymphnode</td>
<td>100M</td>
<td>63</td>
<td></td>
<td>Dead</td>
</tr>
<tr>
<td>ExPCT-8</td>
<td>42</td>
<td>42</td>
<td>Tumor pelvis</td>
<td>6</td>
<td>48</td>
<td></td>
<td>PR</td>
</tr>
<tr>
<td>ExPCT-10</td>
<td>8</td>
<td>8</td>
<td>Thorax, upper limb</td>
<td>NA</td>
<td>42</td>
<td></td>
<td>PR</td>
</tr>
</tbody>
</table>

Simultaneously diagnosed medullary and extramedullary disease.

<table>
<thead>
<tr>
<th>Patient name</th>
<th>PC%*</th>
<th>Medullary when ExMM diagnosed</th>
<th>ExPCT tumor location</th>
<th>PC%†</th>
<th>Time‡</th>
<th>Status§</th>
</tr>
</thead>
<tbody>
<tr>
<td>ExPCT-2C</td>
<td>10</td>
<td>19</td>
<td>Hypophysis</td>
<td>12</td>
<td>17</td>
<td>Dead</td>
</tr>
<tr>
<td>ExPCT-5C</td>
<td>25</td>
<td>35</td>
<td>Soft tissue</td>
<td>13</td>
<td>71</td>
<td>Dead</td>
</tr>
<tr>
<td>ExPCT-12</td>
<td>40</td>
<td>36</td>
<td>Intraspinal-lumbosacralcoluma</td>
<td>NA</td>
<td>27</td>
<td>Dead</td>
</tr>
<tr>
<td>ExPCT-13C</td>
<td>32</td>
<td>2‡</td>
<td>Intraspinal, cervico-thoracal</td>
<td>100M</td>
<td>7</td>
<td>Dead</td>
</tr>
</tbody>
</table>

Medullary phase followed by extramedullary phase

<table>
<thead>
<tr>
<th>Patient name</th>
<th>PC%*</th>
<th>ExPCT tumor location</th>
<th>PC%†</th>
<th>Time‡</th>
<th>Status§</th>
</tr>
</thead>
<tbody>
<tr>
<td>ExPCT-3C</td>
<td>&lt;5</td>
<td>Ascites/ peritoneum</td>
<td>12M</td>
<td>80</td>
<td>Dead</td>
</tr>
<tr>
<td>ExPCT-6</td>
<td>0.5</td>
<td>Pleura</td>
<td>77</td>
<td>9</td>
<td>Dead</td>
</tr>
<tr>
<td>ExPCT-10</td>
<td>1.2</td>
<td>(Mammae and skin)‡</td>
<td>NA</td>
<td>71</td>
<td>Dead</td>
</tr>
<tr>
<td>ExPCT-12</td>
<td>1.1</td>
<td>Pleura (thorax wall)‡</td>
<td>0.6</td>
<td>12</td>
<td>CR</td>
</tr>
</tbody>
</table>

Extramedullary phase with <5%† medullary PC.

*PC% determined morphologically.
† For determination of PC% in extramedullary locations and when <5% PC were observed morphologically, aberrant PC% were determined by flow cytometry as described in study design. NA = not available.
‡ Observation time in months after diagnosis.
§ Status at the observation time after diagnosis. CR, complete remission; PR, partial remission.
¶ These patients were under treatment, which may result in low medullary tumor burden.
‡ Multiple locations, the locations in parentheses were not available for analysis.
C, clonal IgH
M, RAS mutation in extramedullary but not intramedullary tumor cells
Results

Detection of K- and N-RAS mutations.

FACS-sorted PCs (CD38++/CD19+/CD45+/CD56−/i/CD56++) were analyzed for the presence of mutations in the N-RAS and K-RAS genes (codon 12, 13 and 61) using RT-PCR followed by direct sequencing. The MGUS patients analyzed often had mixtures of normal and aberrant PCs in the BM identified as CD38++/CD19+/CD45+/CD56− and CD38++/CD19+/CD45+/CD56++, respectively. Cells with an aberrant (MM) immunophenotype showed PC morphology and light chain restriction (data not shown). In all cases aberrant PCs were FACS-sorted to purity above 96% for RAS mutational analysis. By dilutional analysis of the EBV transformed IM-9 cell line (with a N-RAS61 mutation), in the HMCL OPM-2 (without known RAS mutations), we found that spectral differences could be routinely identified when ≥ 25% of the cells harbored a RAS mutation (data not shown), a sensitivity similar to that of direct sequencing of DNA.14 When using purified PCs for detection of RAS mutations, there is in general a good correlation between the RAS mutations detected by direct sequencing and more sensitive methods.5 However, to identify possible MM and MGUS patients harboring RAS mutations in sub-clonal populations we developed a sensitive ACB-PCR method for detection of K-RAS61 mutations (study design). Due to a limited amount of material it was not possible to analyze for all types of RAS mutations and K-RAS61 was chosen for ACB-PCR analysis as it was the most frequent mutation observed by direct sequencing. To ensure that the detected RAS mutations originated from aberrant PCs the method were optimized to detect a single FACS sorted RAS mutated cell among non-mutated B-cells (Figure 1A). K-RAS mutations were not detected in patients with N-RAS mutations (Figure 1B), and only in one case an additional K-RAS mutation were identified in PCs negative by direct sequencing (Figure 1C). No additional RAS mutations were detected among MGUS patients (Figure 1D).
A high prevalence of activating RAS mutations in MM compared to MGUS.

We determined the RAS mutational status in FACS-purified aberrant PCs from 20 stable MGUS patients and 58 BM samples obtained from untreated MM patients. Among the MGUS patients we observed a single case out of 20 with a RAS mutation in codon 61 of N-RAS (Table 2). The patient was followed with MGUS for 14 years, and died from an unrelated disease 3 years after the N-RAS 61 mutation was detected, without evidence for transformation to smoldering or active myeloma. RAS mutations were identified in 18 of 58 MM cases (31%), with comparable frequencies of RAS mutation among stage I, II and III MM patients and a slightly higher frequency of K-RAS mutations (19%) compared to N-RAS mutations (12%) (Table 2). In all cases, only a single RAS mutation was observed for each tumor, without heterogeneity between the first and second analyses. The observed low frequency of RAS mutations identified in PCs with a characteristic MM immunophenotype from stable MGUS patients (5%) is in striking contrast to a high frequency of RAS mutations at all Durie and Salmon stages of MM patients at the time of diagnosis (31%). This indicates that RAS mutations provide a molecular marker that distinguishes some MM tumors from
MGUS, and suggests that these mutations may facilitate the transition from MGUS to MM in a subset of patients.

Table 2. RAS mutations in the development of MM

<table>
<thead>
<tr>
<th>Disease stage</th>
<th>RAS mutations</th>
<th>RAS type (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGUS</td>
<td>1/20 (5%)</td>
<td>N-61</td>
</tr>
<tr>
<td>MM Total</td>
<td>18/58 (31%)</td>
<td>N-61, K-13, K-61(2)</td>
</tr>
<tr>
<td>Stage I</td>
<td>4/13 (31%)</td>
<td>N-61, K-12, K-13, K-61</td>
</tr>
<tr>
<td>Stage II</td>
<td>4/11 (36%)</td>
<td>N-61, K-61(4), N-13, K-61(5)</td>
</tr>
<tr>
<td>Stage III</td>
<td>10/34 (29%)</td>
<td></td>
</tr>
<tr>
<td>ExPCT Medullary PC</td>
<td>0/13 (0%)</td>
<td></td>
</tr>
<tr>
<td>Extramedullary PC</td>
<td>3/6 (50%)</td>
<td>K-61(2), N-61</td>
</tr>
</tbody>
</table>

**RAS mutations in extramedullary but not intramedullary tumor cells in three patients.**

Immunophenotypic aberrant PCs were FACS-sorted from the BM aspirates obtained from 13 patients with ExPCT, and analyzed for the presence of RAS mutations. Surprisingly, RAS mutations were absent in the intramedullary tumors cells for all 13 patients. When comparing this observation to the frequency of RAS mutations in newly diagnosed MM patients from the work of Liu et al., Bezieau et al. and the present study (98/251, 39%), this difference is highly significant (p = 0.001). However, it might be biased by the possibility that some of the intramedullary tumor cells are actually pre-malignant MGUS (see Discussion). To gain further knowledge on the difference in RAS mutations between MM and ExPCT we analyzed paired extramedullary and intramedullary samples. From 10/13 patients with ExPCT, we obtained tumor cells from extramedullary locations simultaneously with the samples from the BM (see study design). From 6 of these 10 patients we were able to identify a clone-specific IgH gene sequence (ExPCT 1, 7, 2, 5, 13, 3 in Table 1). In all 6 cases we found identical clone-specific IgH gene sequences in the corresponding BM and extramedullary tumor cells (for one example, see Figure 2). Extramedullary and BM tumor cells from the 6 patients with
identical IgH gene sequences were flow-sorted and the RAS mutational status was determined (for one example see Figure 2). Strikingly, despite the absence of RAS mutations in BM tumor cells, RAS mutations were found in three of six extramedullary tumor cell samples (localized in ascites, lymph node, and intraspinal tumor; ExPCT-3, 7, 13; Table 1). Only a single RAS mutation was observed for each patient. Thus, RAS mutations were exclusively observed in extramedullary PCs from three of six patients with different presentation of ExPCT as summarized in Table 1. In addition, where a clonal relationship could not be establish we analyzed the extramedullary PCs (patients ExPCT 4, 6, 8, 11 in Table 1) without finding evidence for the presence of RAS mutations.

>>>Figure 2<<<

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A

CD38

CD45

BM

LN

B

V_{ab} V_{bc} V_{cd} V_{de} V_{ef} V_{fg} V_{gh} V_{hi} V_{ij} P

N

M

BM

LN

C

CTCTCT CTCTCT CTCTCT CTCT

BM

LN

---

12
MM tumor cells express more CD56 than tumor cells from patients with ExPCT.

A strong association between the absence of CD56 expression and extramedullary spread has been described\(^9,15,16\), possibly because high CD56 expression may restrict egress of tumor cells from the BM microenvironment.\(^{17}\) We therefore determined the mean CD56 fluorescence on PCs for all MM patients and patients with ExPCT included in the study (Figure 3). The mean CD56 fluorescence levels on MM PCs with a \(RAS\) mutation (median = 1172, range 3 - 3323) were significantly higher than the mean level on MM PCs without \(RAS\) mutations (median = 609, range 3 – 3611, \(p = 0.0313\)). Three MM patients with \(RAS\) mutations were CD56 negative/low (mean fluorescence 5, 8 and 231), but at one-year follow-up two of the patients had stable disease and one patient developed PCL. The CD56 fluorescence levels were substantially higher in MM with and without \(RAS\) mutations when compared to intramedullary tumor cells from patients with ExPCT (median = 72, range 3 - 474), \(p = 0.0008\) and 0.01 respectively.

>>>Figure 3<<<

\[\text{RAS\ mutation in relation to other recurrent genetic alterations in MM.}\]

PCs from all MGUS patients, 17 MM with a \(RAS\) mutation and 37 MM patients without a \(RAS\) mutation were FACS-purified using the same sorting gate as described for the analysis
of RAS mutations and the expression level of cyclin D1, FGFR3, MMSET and β-actin were determined. The oncogene/β-actin ratio for all MGUS PCs has been reported previously, the cyclin D1/β-actin ratio in MM PCs with and without RAS mutations are shown in Figure 4. Cyclin D1 positive MM belong to 2 distinct groups, the t(11;14) group all showing high expression of cyclin D1 and a group with low to intermediate cyclin D1 expression mainly associated with 11q polysomy. Among the 54 MM cases analyzed 9 (17%) showed a high and 13 (24%) showed a low to intermediate cyclin D1 expression level (Figure 4). Most interestingly the MGUS patient harboring a N-RAS mutation showed very high expression of cyclin D1 (cyclin D1/β-actin = 0.658) indicating a t(11;14) translocation. For the MM patients a correlation between RAS mutational status and expression of cyclin D1 and the presence of the t(4;14) translocation were investigated (Table 3). The presence of RAS mutations was significantly associated with cyclin D1 expression (p = 0.015). The cyclin D1 positive group was divided into high (RAS = 5/17 versus no RAS = 4/37) and low to intermediate (RAS = 6/17 versus no RAS = 7/37) expressing groups (Figure 4) resulting in a less evident association with RAS mutations (p = 0.088 and p = 0.191 for high and low to intermediate, respectively), probably due to the loss of statistical power. Importantly, as shown in Figure 4 a similar distribution between patients with high and low to intermediate cyclin D1 expression was observed in both the cohorts with and without a RAS mutation. Thus, RAS mutations show an association with the presence of cyclin D1 expression and not the mechanisms behind the dysregulation. We also observed a clear tendency for RAS mutations to be less frequent in the t(4;14) MM subset (p = 0.055). In contrast, the occurrence of RAS mutations was not significantly different in the negative group (Table 3).
Table 3. RAS mutations and other recurrent genetic alterations in MM.

<table>
<thead>
<tr>
<th></th>
<th>Cyclin D1</th>
<th>FGFR3/MMSET</th>
<th>Negative†</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAS mutation n = 17</td>
<td>11/17 (65%)</td>
<td>0/17 (0%)</td>
<td>6/17 (35%)</td>
</tr>
<tr>
<td>No RAS mutation n = 37</td>
<td>11/37 (30%)</td>
<td>7/37 (19%)</td>
<td>19/37 (51%)</td>
</tr>
</tbody>
</table>

*p = 0.015 0.055 0.272

*Translocation t(4;14) identified by both IGH-MMSET and FGFR3/MMSET expression: see study design.
†PCs without a detectable cyclin D1, FGFR3, MMSET or IGH-MMSET expression.

Discussion

Activating RAS mutations mark if not mediate the MGUS to MM transition

For many kinds of tumors it is well established that activating mutations of H-, K- or N-RAS contribute to tumorigenesis. More specifically, transfection of activated RAS into EBV-immortalized B lymphoblastoid cells not only transforms these cells, but also triggers plasmacytoid differentiation. In addition, a number of studies show that activation of the RAS pathway by various cytokines (e.g., IL-6, IGF-1) is important for growth and survival of MM cells. Moreover, it appears that mutually exclusive activating mutations of K- or N-RAS, or FGFR3 in MM tumors or cell lines that have dysregulated expression of FGFR3 due
to a t(4;14) translocation, can at least partially substitute for the cytokines that affect growth and survival.\textsuperscript{25,26}

As indicated above, our study confirms the observation reported by Liu and co-workers\textsuperscript{2} that the prevalence of RAS mutations is independent of clinical stage. In addition, we observed a slightly higher prevalence of K-RAS mutations compared to N-RAS mutations, a result that is similar to the results for MM tumors and cell lines reported by Bezieau et al.\textsuperscript{5}, and also to results reported for MM cell lines by Chesi et al.\textsuperscript{25} By contrast, a significantly higher prevalence of N-RAS vs. K-RAS mutations was reported in the large study of Liu and co-workers, as well as in other earlier and smaller studies that used different methods to detect RAS mutations in unpurified tumor samples.\textsuperscript{1,27} Apart from the possibility of technical differences, we have no explanation for the preponderance of N- vs. K-RAS mutations in earlier studies, or the different prevalence of combined K- and N-RAS mutations in this study (31\%) vs. the study of Liu et al.\textsuperscript{2} (39\%) and Bezieau et al.\textsuperscript{5} (50\%). A comparison of ACB-PCR and direct sequencing used in this study revealed only one additional MM case with a K-RAS\textsubscript{61} mutation and no additional MGUS cases. Bezieau et al.\textsuperscript{5} found a similar result when using enriched PC samples in a comparison between ARMS and direct sequencing. For both MM and MGUS patients included in this study a clonal selection on the cellular level was introduced prior to the RAS mutational analysis by FACS sorting of only aberrant PCs with a MM specific immunophenotype, enhancing the likelihood of detecting RAS mutations in relevant cells. When using a sensitive detection for K-RAS\textsubscript{61} mutations we find no evidence for sub-clonal PC populations with RAS mutations. However, we cannot exclude the existence of clonal sub-population with other types of RAS mutations accounting for less than 25\% of the immunophenotypic aberrant PC population. Surprisingly, there is a recent report by Kalakonda and co-workers\textsuperscript{7} that one or more N-RAS mutations are present in all MM tumors, whereas K-RAS mutations are exceedingly rare (1/34 tumors). Apart from the
possibility of technical problems based on the use of a unique and very sensitive PCR assay that can detect mutations present in a very small fraction of cells within a tumor, we cannot explain this latter result, but only note that it is totally inconsistent with all other studies of RAS mutations in MM.\textsuperscript{27}

Apart from our studies, few other studies have attempted to clarify the RAS mutational status in MGUS patients, although two studies should be cited. First, Corradini et al.\textsuperscript{4} analyzed 30 MGUS cases without detecting RAS mutations. However, since these samples were not purified, the proportion of MGUS tumor cells versus normal PCs and other kinds of normal BM cells was not known, making it impossible to make meaningful conclusions about this negative result. Second, using a highly sensitive and mutation specific PCR method, Bezieau et al.\textsuperscript{5} identified one of six MGUS tumors harboring an N-RAS 61 mutation. Unfortunately, they did not report if this mutation was present in most or only a small fraction of the MGUS tumor cells. It is possible, for example, that this particular MGUS tumor may have contained a small subclonal population of malignant MM cells that had an N61 RAS mutation, and had not yet outgrown the MGUS tumor cells. More importantly, it is not possible to accurately compare the frequency of RAS mutations in MM versus MGUS with only six MGUS samples analyzed.

Our study provides the first rigorous and definitive evidence that the combined prevalence of activating K- plus N-RAS mutations is much higher in malignant MM (31\%) than in pre-malignant MGUS (5\%). Similar to results reported by others, we also have shown that the prevalence of RAS mutations is independent of the stage of MM, although these studies do not preclude the possibility that there could be a small accumulation of additional RAS mutations during progression of malignant MM. Although the accumulation of RAS mutations in MM may suggest that MGUS patients harboring RAS mutations have an increased possibility for transformation to overt MM, obviously, the only definitive way to
document the timing of RAS mutations, would be to study the linear pathogenesis of individual tumors. In this study, we included the criteria that all MGUS patients should be stable over a 2-year follow-up after BM sampling for RAS mutational analysis, ensuring that none of the MGUS patients analyzed were at a stage in the transition from MGUS to MM. At a minimum, our results show that mutations in K- or N-RAS provide an intrinsic molecular marker that distinguishes some MM tumors from MGUS. Although the occurrence of RAS mutations in MGUS appears to be neither necessary nor sufficient for conversion to MM in all cases, the apparent accumulation of RAS mutations at the transition from pre-malignant MGUS to malignant MM suggests a causal role in this transition.

**Increased frequency of RAS mutations among cyclin D1 positive MM patients**

The observed frequency of MM patients with high (17%) and low to intermediate (24%) cyclin D1 expression is similar to other studies using real-time RT-PCR.\textsuperscript{12,18,19} Interestingly, we observed an increased prevalence of RAS mutations in MM patients with cyclin D1 expression. The two distinctive cyclin D1 groups observed in MM were equally represented in patients with and without a RAS mutation (Figure 4), suggesting that RAS mutations are associated with the presence of cyclin D1 expression and not the mechanisms behind the dysregulation. In contrast, a reduced prevalence of RAS mutations were observed in the t(4;14) MM subset (Table 3). One explanation is that the combination of unmutated FGFR3 and an FGF ligand in the BM microenvironment have a similar function as a RAS mutation. Significantly, RAS and FGFR3 mutations, which are mutually exclusive in MM patients, appear to play an analogous oncogenic role in MM progression, as suggested in a recent study.\textsuperscript{25} Furthermore, a possible role of the t(4;14) in the transformation of MGUS to MM has been suggested, since the t(4;14) has been detected at a significant lower frequency among MGUS than MM patients.\textsuperscript{12,28,29} In contrast, the t(11;14) involving cyclin D1 has been
detected with the same frequency in MGUS and MM patients. Thus, in the evolution of cyclin D1⁺ MM, RAS mutations may be important for transformation of MGUS to MM, whereas the t(4;14) dysregulates several oncogenes one of which (FGFR3) reduces the need for a RAS mutations in the transformation of MGUS to MM.

**Activating RAS mutations may influence the extra- versus intramedullary phenotype**

In this study as well as in work published previously by ourselves and others, we have described the absence or low expression of CD56 on both BM-localized and extramedullary PCs in patients with extramedullary disease, showing a strong association between the absence of CD56 expression and extramedullary spread. CD56 on tumor cells is thought to mediate homotypic adhesion to a number of BM cells, and seems to restrict egress from BM. A subset of MGUS/MM patients may be predisposed for developing extramedullary disease. Support for this hypothesis comes from the fact that although the mechanisms that determine CD56 expression are not well understood, approximately 20 – 25% of MM patients have a CD56 negative aberrant immunophenotype, and nearly 50% of the CD56 negative subset have extramedullary disease compared to 7% of the CD56 positive subset. The CD56 expression is absent *de novo* on all malignant PCs at diagnosis of PCL and remains constant over the disease’s course on MM PCs. This aberrant phenotype is present at the MGUS stage, suggesting that the predisposition for developing extramedullary disease is determined early in MM oncogenesis.

For seven of the 13 patients with ExPCT shown in Table 1, there appears to be coexistence of intramedullary MGUS (patients with ≤10% BM PCs) and extramedullary tumor. For two of these patients (ExPCT-3 and ExPCT-7), a RAS mutation is present in the extramedullary but not the intramedullary tumor cells. Based on the observed differences in CD56 expression levels (Figure 3), RAS mutations accumulating in CD56⁺ PCs may allow
transformation and/or tumor expansion but not dissemination outside the BM because high CD56 adhesion restricts regress from BM. However, in the CD56−/low subset the RAS mutated PCs are not retained by CD56 adhesion allowing dissemination of the RAS mutated PC clone. We have shown that the intra- and extramedullary PCs from paired samples obtained simultaneously have a clonal relationship (identical IgH gene sequences) and that PCs without RAS mutations accumulated in the BM in contrast to extramedullary clonal relatives that sometimes have a RAS mutation. This gives definitive proof that the intramedullary located tumor cells (IgH specific sequence and germ line RAS) were precursors of the extramedullary located tumor cells (IgH specific sequence and mutated RAS). Unfortunately, we are unable to unequivocally resolve the pathways that cause a similar outcome in all three patients with RAS mutations. However, regardless of the pathway, the end result indicates that in all three cases, the tumor cells in the intramedullary and extramedullary sites do not participate in a simple ongoing equilibrium.

Others have reported the presence of RAS mutation in extramedullary tumors present in pleural effusions in three MM patients, and in 16/40 PCL cases. In addition, virtually all HMCL, in which the prevalence of RAS mutations is about 45%, are derived from extramedullary MM. Unfortunately, however, there are no published reports of the RAS mutational status of the intramedullary tumor cells that co-exist with these various extramedullary tumors.

In summary, although much remains to be learned, it appears that RAS mutations might have two kinds of roles in plasma cell tumors. First, although they appear to be neither necessary nor sufficient for progression of MGUS to MM, they seem to provide a marker and possibly a causal event of this transition in at least some instances. Second, they may mark or influence the intra- versus extramedullary fate of the tumor cell clone. In addition RAS
mutations are not evenly distributed among different molecular subclasses of MM but seem to be important for the evolution of cyclin D1 positive MM.

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FIGURE LEGENDS

Figure 1
Detection of K-\textit{RAS61} mutations using ACB-PCR. A. Sensitivity of ACB-PCR when analyzing FACS-sorted PCs. FACS-sorting of 1, 2 or 10 MM PCs from a patient with a K-\textit{RAS61} mutation to PCR tubes containing 100 FACS-sorted CD19$^+$ B-cells from a normal donor. In 4/5 PCR tubes with a single FACS-sorted K-\textit{RAS61}$^+$ MM PC among 100 normal B-cells a K-\textit{RAS61}$^+$ MM PC was detected. B. From all patients with an N-\textit{RAS61} mutation 100 PCs were analyzed. C. Patients where \textit{RAS} mutations were undetectable by direct sequencing were analyzed (10 cases shown) and a single patient was identified as positive. All MGUS patients were analyzed and all were found to be negative (10 cases shown). P = positive control, 100 FACS-sorted PCs from a patient with a K-\textit{RAS61} mutation. N = negative control, 100 FACS-sorted CD19$^+$ B-cells from a normal donor.

Figure 2
Genetic analyses on flow-sorted PCs. A CD45 versus CD38 flow dot-plot of BMMNC and lymphnode (LN) from a patient with extramedullary myeloma (ExMM-8, Table 3). The PCs from both locations expressed high levels of CD56 and was CD19$^-$ (data not shown). The sort gate used for generating pure CD38$^{++}$/CD45$^-$/CD56$^{++}$/CD19$^-$ PC to \textit{RAS} mutational analysis is shown. B. The V$_{H}$DJ$_{H}$ gene rearrangements representing the MM clone was identified by RT-PCR using consensus V$_{H}$ and J$_{H}$ primers. Among BMMNC a polyclonal V$_{H3}$ (originating from normal B cells) and a clonal V$_{H2}$ band was identified. The LN population consisted of a V$_{H2}$ clone. To verify clonal identity the V$_{H}$DJ$_{H}$ gene was sequenced, showing identical CDRI, II and III sequences. The CDRIII sequence is shown below. C. Direct sequencing of \textit{RAS} RT-PCR products generated from BM and LN localized CD38$^{++}$/CD45$$/CD56$^{++}$/CD19$^-$ PC
purified by flow sorting. The reverse sequence is shown including codon K-RAS61, with a single K-RAS61 mutation CAA to CGA in the LN.

Figure 3
CD56 expression and RAS mutations. The CD56 mean fluorescence was determined on CD38+/CD45−/CD19− gated PCs from all MM and ExMM patients included in the study. MM patients were divided based on the RAS mutational status. For ExMM patient the mean CD56 fluorescence for the major populations of BM-localized PCs is shown.

Figure 4
Cyclin D1 expression in MM PCs with and without RAS mutations. The actual cyclin D1/β-actin ratio was determined in FACS-purified MM PCs with and without RAS mutations using real-time PCR as describe in Study design. PCs with high (cyclin D1/β-actin ratio >0.1) and low to intermediate (cyclin D1/β-actin ratio <0.1) cyclin D1 expression were identified.
Possible roles for activating RAS mutations in the MGUS to MM transition and in the intramedullary to extramedullary transition in some plasma cell tumors

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