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

Thomas Rasmussen, Michael Kuehl, Marianne Lodahl, Hans E. Johnsen, and Inger Marie S. Dahl

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 premalignant monoclonal gammopathy of undetermined significance (MGUS), multiple myeloma (MM), and extramedullary plasma cell (PC) tumors (ExPCTs). 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 ($P = .015$) and decreased in MM with t(4;14) ($P = .055$). 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 ($P = .001$). From 3 of 6 patients with paired intramedullary and extramedullary PCs and identical immunoglobulin heavy chain gene (Igh) sequences, RAS mutations were identified only in extramedullary PCs, suggesting a role for RAS mutations in the transition from intramedullary to extramedullary tumor. (Blood. 2005;105:317-323)

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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 premalignant tumor called monoclonal gammopathy of undetermined significance (MGUS), which is also 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 plasmacytomas that do not represent progression of intramedullary MM are usually indolent tumors that can be associated with intramedullary MGUS. Thus, extramedullary PC tumors (ExPCTs) can be either de novo extramedullary plasmacytomas or extramedullary MM that represent 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, 2 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 (HMCLs), 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. 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 ExPCTs. For ExPCTs, BM and extramedullary tumor cells were collected simultaneously and both were analyzed.

Patients, materials, and methods

Patient selection and characterization

A total of 20 MGUS tumors, 58 untreated MM tumors, and 13 patients with ExPCTs, all with immunoglobulin G (IgG) or IgA isotype, were included in the study. Criterion for inclusion in the study was the presence of PCs with an aberrant MM immunophenotype in BM aspirates, determined by flow cytometry as described in “Flow cytometry and cell sorting.” 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 years). BM

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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 healthy 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 (BMMNCs) and RNA was performed as previously described.8

**Flow cytometry and cell sorting**

Cell samples were stained with 3 or 4 colors with the monoclonal antibodies CD19 fluorescein isothiocyanate (FITC), CD38 FITC/allophycocyanin (APC), CD45 peridinin chlorophyll A protein (PerCP), CD56 phycoerythrin (PE; Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA), and CD19 cyanin 5 (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 more than 96% purity.8

**Characterizing V\textsubscript{H}D\textsubscript{J\textsubscript{H}} gene rearrangements**

The immunoglobulin V\textsubscript{H}D\textsubscript{J\textsubscript{H}} gene rearrangements representing MM clones from different locations in patients with ExPCTs 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 mutation 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 described10 followed by direct sequencing as previously described.8 All samples were analyzed in 2 independent PCR and sequencing setups except for extramedullary samples, which were analyzed only once due to a limited amount of material available.

**Table 1. Patients with extramedullary plasma cell tumors (ExPCTs)**

<table>
<thead>
<tr>
<th>Patient name</th>
<th>ExPCT location</th>
<th>Simultaneously diagnosed</th>
<th>ExMM diagnosed</th>
<th>Medullary phase followed by extramedullary phase</th>
<th>Extramedullary phase with less than 5% extramedullary PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>ExPCT-1C</td>
<td>Orbit</td>
<td>44</td>
<td>44</td>
<td>ExPCT-2C</td>
<td>ExPCT-3C</td>
</tr>
<tr>
<td>ExPCT-4</td>
<td>Pleura</td>
<td>5</td>
<td>5</td>
<td>ExPCT-5C</td>
<td>ExPCT-6</td>
</tr>
<tr>
<td>ExPCT-7C</td>
<td>Lymph node</td>
<td>7</td>
<td>7</td>
<td>ExPCT-8</td>
<td>ExPCT-10</td>
</tr>
<tr>
<td>ExPCT-8</td>
<td>Tumor pelvis</td>
<td>42</td>
<td>42</td>
<td>ExPCT-10</td>
<td>ExPCT-10</td>
</tr>
<tr>
<td>ExPCT-10</td>
<td>Thorax, upper limb</td>
<td>8</td>
<td>8</td>
<td></td>
<td>ExPCT-10</td>
</tr>
<tr>
<td>ExPCT-1C</td>
<td>Orbit</td>
<td>44</td>
<td>44</td>
<td></td>
<td>ExPCT-3C</td>
</tr>
<tr>
<td>ExPCT-2C</td>
<td>Hypophysis</td>
<td>10</td>
<td>19</td>
<td></td>
<td>ExPCT-3C</td>
</tr>
<tr>
<td>ExPCT-5C</td>
<td>Soft tissue</td>
<td>25</td>
<td>35</td>
<td></td>
<td>ExPCT-6</td>
</tr>
<tr>
<td>ExPCT-12</td>
<td>Intraspinal-lumbosacral column</td>
<td>40</td>
<td>36</td>
<td></td>
<td>ExPCT-10</td>
</tr>
<tr>
<td>ExPCT-13C</td>
<td>Intraspinal, cervico-thoracal</td>
<td>32</td>
<td>2</td>
<td></td>
<td>ExPCT-12</td>
</tr>
<tr>
<td>ExPCT-3C</td>
<td>Ascites/peritoneum (lower limb, gastrointestinal tract, skin)</td>
<td>&lt; 5</td>
<td>1.3]</td>
<td></td>
<td>ExPCT-3C</td>
</tr>
<tr>
<td>ExPCT-6</td>
<td>Pleura</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
<td>ExPCT-6</td>
</tr>
<tr>
<td>ExPCT-10</td>
<td>(Mammæ and skin)</td>
<td>1.2</td>
<td>&lt; 5</td>
<td></td>
<td>ExPCT-10</td>
</tr>
<tr>
<td>ExPCT-12</td>
<td>Pleura (thorax wall)</td>
<td>1.1</td>
<td>0.5</td>
<td></td>
<td>ExPCT-12</td>
</tr>
</tbody>
</table>

C (in ExPCT-1C and similar constructions) indicates clonal IgH; M, RAS mutation in extramedullary but not intramedullary tumor cells; PR, partial remission; NA, not available; and CR, complete remission. *PC% determined morphologically. †For determination of PC% in extramedullary locations and when < 5% PCs were observed morphologically, aberrant PC percentages were determined by flow cytometry as described in “Patients, materials, and methods.” ‡Observation time in months after diagnosis. §Status at the observation time after diagnosis. ¶These patients were under treatment, which may result in low medullary tumor burden. ††Multiple locations, the locations in parentheses were not available for analysis.

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

We designed an ACB-PCR generating preferential amplification of the mutant allele using a mutant-specific primer (MSP) in combination with a nonextendable blocker primer (BP) having preferential binding for the wild-type allele.11 Primers for detection of mutation CAA to CAC were as follows: MSP, 5’-CTCGACACAGCGTCTTC-3’; BP, 5’-CTCGACA-CACCGGTCCTA-3’. Primers for detection of mutation CAA to CTA were as follows: MSP, 5’-TCTCGACACAGGGTAT-3’; BP, 5’-TCTCGAC-ACACGGTAA-3’. MSP primers were fluorescently labeled with 6-carboxylfluorescein (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’-GGTACTGTTCTTACAGAAGCCAA-3’. All primers were from MWG-Biotech AG (Aarhus, Denmark), high-performance liquid chromatography (HPLC) purified, and approved by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). ACB-PCRs were performed on a reverse transcription (RT) lysate generated from FACS-purified PCs with an aberrant immunophenotype after preamplification with RAS-specific primers (forward, 5’-TCCATACTTTCTGCTAAGTCTC-3’; reverse, 5’-GGTACTGTTCTTGCAAGG-3’) under the same conditions as described in the previous section for direct sequencing of RAS. The ACB-PCR consisted of a lower layer: MgCl2, 25 mM; 3 μL deoxynucleoside triphosphate (dNTP) 10 mM each; 0.2 μL, gelatin 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. The upper layer consisted of AmpliTaq Stoffel fragment 10 U/μL, 0.25 μL, 10 × Stoffel buffer 5 μL; Perfect Match PCR Enhancer (Stratagene, Aarhus, Denmark), 4 μL, final concentration 0.8 U/μL sample (preamplification product) 1 μL H2O, 21.35 μL. Lower and upper layers were initially separated using AmpliWax. All above consumables were supplied by PE Applied Biosystems (Foster City, CA), except when otherwise mentioned. The PCR consisted of 5 minutes at 95°C followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, followed by heating at 72°C for 10 minutes in a 9700 PCR machine (PE Applied Biosystems). 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. Briefly, 100 PCs were FACS sorted directly to PCR tubes, followed by global RT-PCR, generating a cDNA archive. Cyclin D1, fibroblast growth factor receptor 3 (FGFR3), and multiple myeloma SET domain (MMSET) expression levels normalized to β-actin were determined in each cDNA archived by real-time RT-PCR as described.15

IGH-MMSET RT-PCR

The IGH-MMSET RT-PCR was performed as described previously13 on cDNA products generated from BMMNCs obtained at the time of diagnosis.

Statistics

All statistics were performed using the StatView 4.5 software form Abacus Concepts (Berkeley, CA). The significance of the absence of RAS mutation in patients with ExPCTs compared with MM was determined using the Fisher 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.

Results

Detection of K- and N-RAS mutations

FACS-sorted PCs (CD38+/CD19+/CD45−/CD56−/−/+/+) were analyzed for the presence of mutations in the N-RAS and K-RAS genes (codons 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 Epstein-Barr virus (EBV)–transformed IM-9 cell line (with a N−RAS1 mutation) and the HMCL OPM-2 (without known RAS mutations) we found that spectral differences could be routinely identified when at least 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. However, to identify possible MM and MGUS patients harboring RAS mutations in subclonal populations we developed a sensitive ACB-PCR method for detection of K-RAS61 mutations (see “Patients, materials, and methods”). 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 was optimized to detect a single FACS-sorted RAS-mutated cell among nonmutated B cells (Figure 1A). K-RAS mutations were not detected in patients with N-RAS mutations (Figure 1B), and only in one case was an additional K-RAS mutation 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 with 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 with MGUS was followed for 14 years and died from an unrelated disease 3 years after the N-RAS61 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 with 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.

RAS mutations in extramedullary but not intramedullary tumor cells in 3 patients

Immunophenotypic aberrant PCs were FACS sorted from the BM aspirates obtained from 13 patients with ExPCTs 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 = .001). However, it might be biased by the possibility that some of the intramedullary tumor cells are actually premalignant MGUSs (see “Discussion”). To gain further knowledge on the difference in RAS mutations between MMs and ExPCTs we analyzed paired extramedullary and intramedullary samples. From 10 of 13 patients with ExPCTs we obtained tumor cells from extramedullary locations simultaneously with the samples from the BM (see “Patients, materials, and methods”). From 6 of these 10 patients we were able to identify a clone-specific IgH gene sequence (ExPCT-1, -7, -2, -5, -13, -3; Table 1). In all 6 cases we found identical clone-specific IgH gene sequences in the corresponding BM and extramedullary tumor cells (for an 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 an example, see Figure 2). Strikingly, despite the absence of RAS mutations in BM tumor cells, RAS mutations were found in 3 of 6 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 3 of 6 patients with different presentations of ExPCT as summarized in Table 1. In addition, where a clonal relationship could not be established, we analyzed the extramedullary PCs (patients ExPCT-4, -6, -8, -11; Table 1) without finding evidence for the presence of RAS mutations.

**MM tumor cells express more CD56 than tumor cells from patients with ExPCTs**

A strong association between the absence of CD56 expression and extramedullary spread has been described, possibly because high CD56 expression may restrict egress of tumor cells from the BM microenvironment. We therefore determined the mean CD56 fluorescence on PCs for all MM patients and patients with ExPCTs 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 median level on MM PCs without RAS mutations (median = 690; range, 3-3611; P = .0313). Three MM patients with RAS mutations were CD56+/− (mean fluorescence 5, 8, and 231), but at 1-year follow-up, 2 of the patients had stable disease and 1 patient developed PCL. The CD56 fluorescence levels were substantially higher in MM with and without RAS mutations when compared with intramedullary tumor cells from patients with ExPCTs (median = 72; range, 3-474; P = .0008 and .01, respectively).

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**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 (1)</td>
</tr>
<tr>
<td>MM Total</td>
<td>18/58 (31)</td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>4/13 (31)</td>
<td>N-61 (1), K-13 (1), K-61 (2)</td>
</tr>
<tr>
<td>Stage II</td>
<td>4/11 (36)</td>
<td>N-61 (1), K-12 (1), K-13 (1), K-61 (1)</td>
</tr>
<tr>
<td>Stage III</td>
<td>10/34 (29)</td>
<td>N-61 (4), N-13 (1), K-61 (5)</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 (1)</td>
</tr>
</tbody>
</table>

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### 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 ratios for all MGUS PCs has been reported previously, and the cyclin D1-β-actin ratios in MM PCs with and without RAS mutations are shown.

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**Figure 2. Genetic analyses on flow-sorted PCs.** (A) CD38 versus CD38 flow dot blot of BMMNCs and lymph nodes (LN) from a patient with extramedullary myeloma (ExMM-8; Table 3). The PCs from both locations expressed high levels of CD56 and were CD19+ (data not shown). The sort gate used for generating pure CD38+/−/CD45+/−/CD56+/−/CD19− PCs to RAS mutational analysis is shown. (B) The V(H)DJH gene rearrangements representing the MM clone were identified by RT-PCR using consensus V(H) and J(H) primers. Among BMMNCs a polyclonal V(H)D1 (originating from normal B cells) and a clonal V(H)D1 band was identified. The LN population consisted of a V(H)D1 clone. To verify clonal identity the V(H)D1 gene was sequenced, showing identical complementarity-determining region (CDR) I, II, and III sequences. The CDRIII sequence is shown in panel B. (C) Direct sequencing of RAS RT-PCR products generated from BM- and LN-localized CD38+/−/CD45+/−/CD56+/−/CD19− PCs 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. Arrows show places of mutations.

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**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 patients, the mean CD56 fluorescence for the major populations of aberrant BM-localized PCs is shown. Error bars represent the 90th and 10th percentiles. solid line indicates values above and below the 90th and 10th percentiles.
in Figure 4. Cyclin D1–positive MMs 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 ratio = 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 = .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 = .088 and P = .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 = .055). In contrast, the occurrence of RAS mutations was not significantly different in the negative group (Table 3).

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 (eg, interleukin 6 [IL-6], insulinlike growth factor 1 [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.

Our study confirms the observation reported by Liu et al that the prevalence of RAS mutations is independent of clinical stage. In addition, we observed a slightly higher prevalence of K-RAS mutations compared with N-RAS mutations, a result that is similar to the results for MM tumors and cell lines reported by Bezieau et al and also to results reported for MM cell lines by Chesi et al. By contrast, a significantly higher prevalence of N-RAS versus K-RAS mutations was reported in the large study of Liu et al, as well as in other earlier and smaller studies that used different methods to detect RAS mutations in unpurified tumor samples.

Apart from the possibility of technical differences, we have no explanation for the preponderance of N- versus K-RAS mutations in earlier studies or the different prevalence of combined K- and N-RAS mutations in this study (31%) versus the studies of Liu et al (39%) and Bezieau et al (51%). A comparison of ACB-PCR and direct sequencing used in this study revealed only one additional MM case with a K-RAS61 mutation and no additional MGUS cases. Bezieau et al found a similar result when using enriched PC samples in a comparison between allele-specific amplification method (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 an MM-specific immunophenotype, enhancing the likelihood of detecting RAS mutations in relevant cells. When using a sensitive detection for K-RAS61 mutations we find no evidence for subclonal PC populations with RAS mutations. However, we cannot exclude the existence of clonal subpopulation 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 et al 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.

Apart from our studies, few other studies have attempted to clarify the RAS mutational status in MGUS patients, although 2 studies should be cited. First, Corradini et al 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 identified 1 of 6 MGUS tumors harboring an N-RAS61 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 N-RAS61 mutation.

Table 3. RAS mutations and other recurrent genetic alterations in MM

<table>
<thead>
<tr>
<th>RAS Mutation</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>
<tr>
<td>P</td>
<td>.015</td>
<td>.055</td>
<td>.272</td>
</tr>
</tbody>
</table>

*Translocation t(4;14) identified by both IGH/MMSET and FGFR3/MMSET expression (see “Patients, materials, and methods”).†PCs without a detectable cyclin D1, FGFR3, MMSET, or IGH-MMSET expression.
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 6 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 premalignant MGUS (5%). Similar to results reported by others, we have also 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 premalignant 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. Interestingly, we observed an increased prevalence of RAS mutations in MM patients with cyclin D1 expression. The 2 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 was 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 has 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.

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 significantly lower frequency among MGUS than MM patients. In contrast, the t(11;14) involving cyclin D1 has been detected at a significantly lower frequency among MGUS than MM patients.12,28,29 In addition, virtually all HMCLs, in which mutations were observed in the t(4;14) MM subset (Table 3), are derived from the t(4;14) MM subset.30 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.15-17 This aberrant phenotype is present at the MGUS stage, suggesting that the predisposition for developing extramedullary disease is determined early in MM oncogenesis.31

For 7 of the 13 patients with ExPCtS shown in Table 1 there appears to be coexistence of intramedullary MGUS (patients with ≤10% BM PCs) and extramedullary tumor. For 2 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 intramedullary 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 3 patients with RAS mutations. However, regardless of the pathway, the end result indicates that in all 3 cases, the tumor cells in the intramedullary and extramedul- lary 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 3 MM patients and in 16 of 40 PCL cases. In addition, virtually all HMCLs, 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 coexist with these various extramedullary tumors.

In summary, although much remains to be learned, it appears that RAS mutations might have 2 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 intramedullary 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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**References**

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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