Mutational Activation of N- and K-ras Oncogenes in Plasma Cell Dyscrasias

By Paolo Corradini, Marco Ladetto, Claudia Voena, Antonio Palumbo, Giorgio Inghirami, Daniel M. Knowles, Mario Boccadoro, and Alessandro Pileri

The frequency of N- and K-ras oncogene mutations was investigated in plasma cell dyscrasias. Genomic DNAs from 128 patients were selected for this study: 30 monoclonal gammopathies of undetermined significance, 8 solitary plasmacytomas, 77 multiple myelomas (MM), and 13 plasma cell leukemias (PCL). A two-step experimental approach was devised. All samples were screened for mutations by single-strand conformation polymorphism analysis. DNA fragments displaying an altered electrophoretic mobility were further studied by direct sequencing to confirm and characterize the nature of the mutations. Ras mutations are not randomly distributed because they are detectable only in MM (9%) and PCL (30.7%). N-ras codons 12, 13, and 61 and K-ras codon 12 were found to be mutated, but N-ras codon 61 mutation was the most frequent finding (63.8%). In conclusion, ras mutations were found in PCL, and in a subset of MM characterized by advanced-stage disease and adverse prognostic parameters. Furthermore, based on our findings, it is possible to speculate that ras mutations represent a late molecular lesion in the process of multistep carcinogenesis.

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PLASMA CELL DYSCRASIAS are a group of diseases characterized by the expansion of plasma cells producing monoclonal Ig. Monoclonal gammapathy of undetermined significance (MGUS), solitary plasmacytoma (SP), multiple myeloma (MM), and plasma cell leukemia (PCL) are the clinical entities forming this group. MGUS is a benign disorder even if, in a large series with 20 years of follow-up, 24.5% of patients developed MM.1 SP is characterized by indolent behavior, with a median survival that can exceed 10 years, whereas MM and PCL follow an aggressive clinical course.2

The pathogenesis of these diseases is still unknown, and the role of some dominantly acting proto-oncogenes, such as bcl-1, bcl-2, c-myc, Moloney leukemia virus integration-4 (MLVI-4), N-ras, K-ras, and H-ras, has been investigated only in MM.3,4 So far, the molecular lesions more frequently detected in MM patients are activating point mutations involving the N- and K-ras oncogenes.5-7

The family of ras oncogenes consists of three related genes: H-, K-, and N-ras. They encode proteins of 21 Kd (p21) with GTPase activity that are located at the inner surface of the cell membrane. These oncogenes acquire their transforming potential when point mutations occur at the codons 12, 13, or 61, resulting in single amino acid substitution.10 Activated ras genes have been identified in several human cancers, including some hematologic malignancies such as acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), myelodysplasias, and blast crisis of chronic myelogenous leukemia (CML).11-15 However, among more differentiated lymphoid tumors, such as chronic lymphocytic leukemia (CLL), hairy cell leukemia (HCL), and non-Hodgkin's lymphoma (NHL), only MM was found to harbor activated ras genes.16

Several reasons prompted us to investigate ras oncogenes in plasma cell dyscrasias. First, the incidence of ras mutations has been reported with some discrepancies.17,18 Second, we wanted to correlate its incidence with several clinical parameters in a large panel of MM cases. Third, the presence of these mutations has never been evaluated in MGUS, SP, or PCL. Finally, because these oncogenes have been already detected in some human precancerous lesions (eg, adenomas and myelodysplasias), indicating that they participate in the early stages of tumorigenesis, we wanted to determine the temporal relationship between ras activation and the onset of an aggressive plasma cell dyscrasia.

MATERIALS AND METHODS

Patients and nucleic acid extraction. We selected for this study 128 patients with various forms of plasma cell dyscrasias. These patients included 77 patients with MM, 13 with PCL, 30 with MGUS, and 8 with SP. All the patients were studied at diagnosis, except for 24 with MM that were in the relapse phase (Table 1). Bone marrow or tissue samples (plasmacytomas) were collected during standard diagnostic procedures. Genomic DNA was purified by proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation.17

Oligonucleotide primers. All the oligonucleotides used for polymerase chain reaction (PCR) amplification and direct sequencing were chemically synthesized using a 391 PCR-MATE EP, DNA Synthesizer (Applied Biosystems, Foster City, CA) on a 0.2 µmol/L scale, according to the users’ manual. The oligonucleotide primers for N- and K-ras genes are the same as reported previously.16,18

Single-strand conformation polymorphism (SSCP) analysis. SSCP analysis was performed according to an adapted version of a previously published method.19,20 Briefly, 100 ng of genomic DNA was amplified in a 10 µL reaction, adding 10 pmol of each primer, 0.5 U of Taq DNA polymerase (Promega, Madison, WI), 1 µCi of [α-32P]dCTP (specific activity, 3000 Ci/mmole), 2.5 µmol/L dNTPs (Pharmacia LKB Biotechnology, Uppsala, Sweden), 10 µmol/L Tris-HCl (pH 8.8), 50 µmol/L KCl, 1 mmol/L MgCl2, 0.01% gelatin, final concentration. Thirty cycles of denaturation (94°C), annealing (56°C to 60°C depending on primer melting temperature), and extension (72°C) were performed on an automated heat-block (DNA thermal cycler; MJ Research, Boston, MA), followed by a 7-minute final extension at 72°C. Two microliters of the 10 µL reaction mixture was diluted 1:18 in 0.1% NaDodSO4/10 mmol/L EDTA and further mixed 1:1 with a sequencing stop solution containing 20 mmol/L NaOH.
RAS MUTATIONS IN MULTIPLE MYELOMA

Fig 1. SSCP analysis of N- and K-ras oncogene mutations in plasma cell dyscrasias. PCR-amplified fragments corresponding to codons 12, 13, and 61 were amplified from genomic DNA, denatured by heat, and run on a 6% acrylamide gel containing 10% glycerol. Representative cases of MM and PCL are shown. Samples were scored positive for mutations when bands different from the normal control (N) were detectable. Arrowheads point to bands of the mutated samples.

Samples were heated at 95°C for 5 minutes, chilled on ice for 3 minutes, and then 3 μL was loaded onto a 6% acrylamide/TBE gel containing 10% (vol/vol) glycerol. Gels were run at 8 W for 12 to 15 hours at room temperature. Autoradiography films were exposed at −70°C with intensifying screens for varying periods of time.

Direct sequencing of PCR products. Amplifications were performed using 100 ng of genomic DNA, 20 pmol of each primer, and 2.5 U of Taq DNA polymerase (Promega) in a 50 μL final volume reaction. The number of cycles, thermocycling conditions, and reaction buffer were as described above. The PCR products were electrophoresed through a 2% low melting point (LMP) agarose (GIBCO BRL, Gaithersburg, MD), the LMP slice was melted at 68°C, and DNA was phenol extracted and ethanol precipitated. N- and K-ras, 5' and 3' primers, were labeled with [γ-32P]ATP using T4 polynucleotide kinase (Promega). Direct sequencing reactions of both strands were performed using the Promega fmol sequencing system according to the manufacturer’s instructions. The sequencing reactions were performed in a thermal cycler at 58°C annealing temperature for 9 cycles. Reaction products were run on a 6% acrylamide/urea gel, fixed in 10% acetic acid, and then exposed at −70°C with intensifying screens for varying periods of time.

RESULTS

Genomic DNAs from 128 patients with plasma cell dyscrasias were analyzed for mutations of the N- and K-ras on-
Table 1. Frequency of Ras Oncogene Mutations in Plasma Cell Dyscrasias

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Positive/Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGUS</td>
<td>0/30</td>
</tr>
<tr>
<td>SP</td>
<td>0/8</td>
</tr>
<tr>
<td>MM</td>
<td>7/77</td>
</tr>
<tr>
<td>PCL</td>
<td>4/13</td>
</tr>
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</table>

Abbreviation: SP, solitary plasmacytoma.

cogenes (Table 1). All DNAs extracted from these specimens were amplified using pairs of oligonucleotide primers flanking the genomic regions spanning codons 12, 13, or 61 of the N-ras and K-ras genes.

A two-step experimental approach was devised. All samples were screened for mutations within the N-ras and K-ras genes by SSCP analysis. DNA fragments displaying an altered electrophoretic mobility by SSCP analysis (Fig 1) were subsequently reamplified and studied by direct sequencing to confirm and characterize the nature of the mutations. The SSCP technique can detect sequence changes affecting one or more nucleotides in a defined stretch of DNA. This method, for 150- to 200-bp fragments, has a sensitivity level of at least 1%.20 A dilution experiment was performed to confirm the presence of mutant DNA.

### Table 2. Ras Mutations in Plasma Cell Dyscrasias

<table>
<thead>
<tr>
<th>Sample</th>
<th>Diagnosis</th>
<th>Ras Gene</th>
<th>Codon</th>
<th>Mutation</th>
<th>Amino Acid Substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>M22</td>
<td>MM</td>
<td>N</td>
<td>12</td>
<td>GGT → GAT</td>
<td>Gly → Asp</td>
</tr>
<tr>
<td>M93</td>
<td>MM</td>
<td>N</td>
<td>12</td>
<td>GGT → GCT</td>
<td>Gly → Ala</td>
</tr>
<tr>
<td>M27</td>
<td>PCL</td>
<td>N</td>
<td>13</td>
<td>GGT → TGT</td>
<td>Gly → Cys</td>
</tr>
<tr>
<td>M28</td>
<td>MM</td>
<td>N</td>
<td>61</td>
<td>CAA → CGA</td>
<td>Gln → Arg</td>
</tr>
<tr>
<td>M91</td>
<td>MM</td>
<td>N</td>
<td>61</td>
<td>CAA → AAA</td>
<td>Gln → Lys</td>
</tr>
<tr>
<td>M19</td>
<td>MM</td>
<td>N</td>
<td>61</td>
<td>CAA → CAT</td>
<td>Gln → His</td>
</tr>
<tr>
<td>M89</td>
<td>MM</td>
<td>N</td>
<td>61</td>
<td>CAA → CTA</td>
<td>Gln → Leu</td>
</tr>
<tr>
<td>M61</td>
<td>PCL</td>
<td>N</td>
<td>61</td>
<td>CAA → AAA</td>
<td>Gln → Lys</td>
</tr>
<tr>
<td>M90</td>
<td>MM</td>
<td>N</td>
<td>61</td>
<td>CAA → CTA</td>
<td>Gln → Leu</td>
</tr>
<tr>
<td>M117</td>
<td>PCL</td>
<td>N</td>
<td>61</td>
<td>CAA → CAT</td>
<td>Gln → His</td>
</tr>
<tr>
<td>M88</td>
<td>PCL</td>
<td>K</td>
<td>12</td>
<td>GGT → CGT</td>
<td>Gly → Arg</td>
</tr>
</tbody>
</table>

Fig 2. *N-ras* codon 61 mutations detected by direct sequencing of PCR-amplified fragments. Mutated samples are matched to a control DNA. Noncoding strands are shown. Arrows point to bands corresponding to mutated base pairs.
method’s sensitivity under our experimental conditions (data not shown).

The results of SSCP and sequencing analysis are summarized in Table 2. The overall frequency of ras mutations among plasma cell dyscrasias is 8.6% (11 of 128). They are not randomly distributed because they are detectable only in PCL (30.7%) and MM (9%). Mutations were found in 5 of 53 (9.4%) MM cases at diagnosis and 2 of 24 (8.3%) cases at relapse. N-ras codons 12, 13, and 61 and K-ras codon 12 were found to be mutated, although N-ras codon 61 was the most frequent finding (7 of 11 [63.6%]). The lack of detectable mutations in the negative case was not due to insufficient representation of tumor cells, because all the samples contained more than 5% malignant cells, whereas the threshold of sensitivity of the method is 1%. Moreover, to evaluate the presence of SSCP false-negative samples, 40 negative cases were analyzed by direct sequencing (8 PCL, 4 SP, and 28 MM), showing a 100% concordance with SSCP results.

To confirm the presence and determine the nature of the mutations, the nucleotide sequence of the involved exons was determined by direct sequencing of both strands. A heterogeneous pattern of mutation was observed including C → W, G + C, G + T, and A + T transversions, and G + A transitions (Figs 2 and 3). Purine-pyrimidine transversions were the most frequent finding (9 of 11 [81.8%]). These

<p>| Table 3. Correlation Between Ras Mutations and Prognostic Parameters in MM |
|-----------------------------|-----------------|---------------|-----|-----|-----|</p>
<table>
<thead>
<tr>
<th>Patient</th>
<th>Stage</th>
<th>Albumin (g/L)</th>
<th>LI (%)</th>
<th>CRP (mg/L)</th>
<th>β2-Microglobulin (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M22</td>
<td>III A</td>
<td>29</td>
<td>0.5</td>
<td>117</td>
<td>5.5</td>
</tr>
<tr>
<td>M93</td>
<td>III B</td>
<td>47</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M28</td>
<td>III A</td>
<td>31</td>
<td>4</td>
<td>13</td>
<td>7.9</td>
</tr>
<tr>
<td>M91</td>
<td>III B</td>
<td>34</td>
<td>0.8</td>
<td>50</td>
<td>41.9</td>
</tr>
<tr>
<td>M19</td>
<td>III A</td>
<td>25</td>
<td>ND</td>
<td>121.9</td>
<td>13</td>
</tr>
<tr>
<td>M89</td>
<td>III B</td>
<td>50</td>
<td>2.7</td>
<td>6</td>
<td>11.5</td>
</tr>
<tr>
<td>M90</td>
<td>III A</td>
<td>34</td>
<td>1.2</td>
<td>10.7</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Adverse prognostic factors were albumin less than 30 g/L, LI greater than 2%, CRP greater than 6 mg/L, and β2-microglobulin greater than 6 mg/L.

Abbreviations: LI, labeling index; CRP, C-reactive protein; ND, not determined.

Fig 3. N- and K-ras codon 12/13 mutations detected by direct sequencing of PCR-amplified fragments. Mutated samples are matched to a control DNA. Noncoding strands are shown for cases M22, M27, and M93; coding strand is shown for case M88. Arrows point to bands corresponding to mutated base pairs.
changes led to the replacement of glycine (N- and K-ras codons 12 and 13) and glutamine (N-ras codon 61) residues with several different amino acids (Table 2).

The relatively small percentage of ras mutations in MM did not allow a survival analysis. Ras mutations were detected only in stage III MM (7 of 37 [19%]). In addition, a prognostic evaluation was performed in all but 1 patient, in whom data were not available (M93). All the examined patients, except M90, had two or more concordant adverse prognostic parameters, as shown in Table 3. Patients M19, M22, and M28 were already dead when the analysis was performed. The median follow-up for censored patients was 20 months (range, 17 to 46 months).

Mutations were frequently detected in PCL that are characterized by an extremely high proliferative activity and a short survival. Our panel included all primary PCL except for M27 patient, who presented the leukemic transformation of an MM. The latter, together with 3 primary PCL, were found to harbor ras mutations.

**DISCUSSION**

We have investigated the mutational activation of N- and K-ras oncogenes and its correlation with clinical parameters in plasma cell dyscrasias. Recent studies have suggested that these oncogenes may play a role in myeloma pathogenesis. N- and K-ras oncogenes were found to be mutated in previous studies. In addition, the introduction of activated N-ras genes into Epstein-Barr virus (EBV)-immortalized human B lymphoblasts resulted in their malignant transformation and terminal differentiation into clonal plasma cells.

Our study indicates that ras mutations are peculiar to aggressive forms of plasma cell dyscrasias. The relatively low incidence of mutations in MM is confirmed, whereas the high incidence in PCL represents a novel finding. It should be noted that, although the SSCP technique might slightly underestimate the frequency of mutations, our findings are in agreement with the percentage found by Paquette et al in a smaller panel of cases.

The presence of activated ras genes appears to be characteristic of immature lymphoid neoplasms, and the discovery of ras mutations in MM and PCL apparently contradicts this finding. However, a possible explanation can derive from the studies indicating that human myeloma originates from the B- or even the pre-B-lymphocyte compartment. The mechanism of occurrence of such mutations remains unknown, and the considerable proportion of different amino acid substitutions may suggest that B cells are exposed to a variety of molecular events capable of inducing many different ras mutations. However, the relatively low frequency of ras mutations in MM argues against a general pathogenetic role for this oncogene in such a disease. Moreover, the low incidence at relapse rules out the possible mutagenic effect played by chemotherapy treatment.

Correlations between ras mutations and several clinical parameters were considered in MM only (Table 4), because PCL have a well-defined unfavorable clinical outcome. Our panel included 26 stage I and II cases, but mutations were detected only in stage III patients. In addition, although the small number of cases did not allow survival analysis, the presence of ras mutations was associated with adverse prognostic parameters, as shown in Table 3. These mutations identify a subset of myeloma patients characterized by an advanced stage and the simultaneous presence of adverse prognostic parameters.

Plasma cell dyscrasias represent a model of multistep carcinogenesis, and they can be divided according to different tumor progression steps corresponding to defined clinical entities, ranging from the benign gammopathy to the aggressive leukemic form. In breast and colon adenomas as well as in myelodysplasias, ras genes are frequently altered, leading to the general concept that such oncogenes act early in the multistep carcinogenesis. This role appears not to apply to plasma cell dyscrasias. The absence of mutations in MGUS indicates that they cannot be considered "precancerous lesions" similar to adenomas or myelodysplasias. Moreover, the detection of such mutations only in stage III MM and PCL suggests that ras activation most likely plays a more important role in tumor progression rather than in tumor initiation. The high frequency of ras mutations in PCL led us to speculate that these mutations probably represent a late molecular lesion conferring additional proliferative advantage to tumor cells. However, to better define the role and timing of ras gene activation, longitudinal studies on individual patients are required.

In conclusion, our work indicates that ras oncogene activation is not a general pathogenetic event for plasma cell dyscrasias, and that its presence is typical of advanced-stage MM and PCL.

**REFERENCES**

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