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RESEARCH ARTICLE

High Incidence of N and K-*Ras* Activating Mutations in Multiple Myeloma and Primary Plasma Cell Leukemia at Diagnosis

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Using allele-specific amplification method (ARMS), a highly sensitive one-stage allele-specific PCR, we have evaluated the incidence of NRAS and KRAS2 activating mutations (codons 12, 13, and 61) in 62 patients with either monoclonal gammopathy of undetermined significance (MGUS) or multiple myeloma (MM), primary plasma-cell leukemia (P-PCL), and also in human myeloma cell lines (HMCL). NRAS and/or KRAS2 mutations were found in 54.5% of MM at diagnosis (but in 81% at the time of relapse), in 50% of P-PCL, and in 50% of 16 HMCL. In contrast, the occurrence of such mutations was very low in MGUS and indolent MM (12.50%). Of note, KRAS2 mutations were always more frequent than NRAS. The validity of the technique was assessed by direct sequencing of cell lines and of some patients. Multiple mutations found in two patients were confirmed by subcloning exon PCR amplification products, testing clones with our method, and sequencing them. Thus, these early mutations could play a major role in the oncogenesis of MM and P-PCL. Hum Mutat 18:212–224, 2001. © 2001 Wiley-Liss, Inc.

KEY WORDS: NRAS; KRAS2; ARMS; multiple myeloma; plasma-cell leukemia; MGUS; incidence; mutation detection

DATABASES:
NRAS – OMIM: 164790; GDB: 119457; Genbank: X02751; HGMD: NRAS
KRAS2 – OMIM: 190070; GDB: 120120; Genbank: M54968; HGMD: KRAS2

INTRODUCTION

Multiple myeloma (MM) is a very aggressive disease characterized by an excess of malignant plasma cells in the bone marrow, secreting a monoclonal immunoglobulin, i.e. the M-component [Bataille and Harousseau, 1997]. Studies devoted to the biology of myeloma cells (in comparison to that of normal plasma cells) have shown that these cells 1) are immature plasma cells (i.e., with a nucleocytoplasmic asynchrony) [Graham and Bernier, 1975]; 2) have an abnormal phenotype [Harada et al., 1993]; and 3) are unable to produce large amounts of immunoglobulins as can their normal counterpart [reviewed in Klein et al., 1995]. Thus, these cells, like many cancer cells, are blocked in their program of differentiation (and apoptosis) [Bataille and Klein, 1993]. Elegant kinetic studies performed in vivo on patients with MM have shown a low myeloma cell growth fraction at diagnosis, thus confirming that myeloma cells accumulate within the bone marrow rather than proliferate [Drewinko et al., 1981]. However, with time myeloma cells become more immature and phenotypically more abnormal, produce fewer immunoglobulins (or even none), and proliferate,

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especially outside the bone marrow [Drewinko et al., 1981; Graham and Bernier, 1975].

To clarify the pathogenesis of MM, it is critical to discover the oncogenic events involved in this initial process of differentiation blockade. Because they are involved in many cancers, activating mutations of either NRAS (MIM# 164790) or KRAS2 (MIM# 190070) were good candidates to represent such events. Initially, two studies had shown an overexpression of p21 *ras* in human myeloma cells, especially in patients with the most aggressive disease [Danova et al., 1990; Tsuchiya et al., 1988], and it has been suggested that activating mutations of *ras*-oncogenes contribute to interleukin 6-independent growth of myeloma cells and suppression of apoptosis [Billadeau et al., 1995]. For all these reasons, extensive research on *ras* mutations has been performed in patients with MM. Although activating mutations of both NRAS and KRAS2 have been detected in these patients, with some correlation with disease severity (especially for K-*ras* mutations), the percentages of mutated patients never exceeded 16.5% and 26% for KRAS2 and NRAS mutations, respectively, at diagnosis in a large series of patients [Corradini et al., 1994; Liu et al., 1996; Matozaki et al., 1991; Millar et al., 1995; Neri et al., 1989; Paquette et al., 1990; Portier et al., 1992; Tanaka et al., 1992]. Thus, activating mutations of both NRAS and KRAS2 are now considered as oncogenic events involved in later disease stages but not as early events, i.e. as causal events, in the pathogenesis of MM [Hallek et al., 1998].

The discrepancies which have been observed in *ras* mutation frequencies within hematologic malignancies came frequently from the detection limits of the method used. Several techniques frequently used, such as direct DNA sequencing or single strand configuration polymorphism, turned out to be not sensitive enough [Portier et al., 1992; Yasuga et al., 1995), or did not investigate all the mutations located at codons 12, 13, and 61 from the KRAS2 and NRAS genes. For example, DNA sequencing is a useful method for the detection of germline mutations but is useful only when the fraction of mutated alleles is greater than 20% [Bar-Eli et al., 1989]. Furthermore, the number of patients included in some studies was very low [Tanaka et al., 1992]. Of course, a great number of techniques with high sensitivity have been developed, such as mutant allele-specific amplification [Bjorheim et al., 1998; Hasegawa et al., 1995; Kohata, 1996], oligonucleotide hybridization [Paranavitana, 1998], and different enriched PCR [Khanna et al., 1999; Ronai and Minamoto, 1997; Ward et al., 1998]. These methods have described *ras* mutations in colorectal tumors but rarely in MM or other hematologic malignancies. The reference study from the Eastern Oncology Group [Liu et al., 1996] using oligonucleotide specific hybridization found more than one mutation in some patients with MM (5/160), even if they did not study the full spectrum of mutations for each patient.

For this reason, we have developed a new sensitive allele-specific PCR method, derived from mutant-allele-specific amplification (MASA) for checking both NRAS and KRAS2 genes [Hasegawa et al., 1995]. We have improved the reliability of the technique by using double mismatching at the 3′ end of the primer as described in allele-specific amplification method (ARMS) [Ferrie et al., 1992]. In addition, we have used a high fidelity polymerase without nested PCR to reduce or suppress any contamination. For each patient, a positive mutation detection was obtained with first, a multiplex PCR to locate the base and the mutated codon, and second, an independent not nested simplex PCR to identify the change in base mutation.

This method gave us the opportunity to evaluate with a sensitive method the frequency of *ras* activating mutations in a cohort of patients with either MM, primary and secondary plasma cell leukemia (PCL), extramedullary plasmacytoma (EMP), or monoclonal gammopathy of undetermined significance (MGUS), as well as in human myeloma cell lines (HMCL). We report here a very high incidence of KRAS2 rather than NRAS mutations in patients with both MM and PCL at diagnosis, showing that such mutations are important events in the occurrence of these malignancies.

MATERIALS AND METHODS

Patients

Thirty three newly diagnosed patients with MM were included in the current study. All these patients had symptomatic disease, fulfilled the diagnostic criteria of the Southwest Oncology Group [Durie and Salmon, 1977], and were stratified according to the myeloma staging system of Durie and Salmon [1975]. In order to thoroughly investigate the potential importance

of N-and K-*ras* mutations in the biology of MM, we also studied available samples obtained from 1) six MGUS and two smoldering MM (SMM); 2) six MM in medullary relapse; 3) five MM in extramedullary relapse, including two with extramedullary plasmocytoma (EMP) and three with secondary PCL; and 4) 10 patients with primary PCL. These last patients had more than 20% (i.e., 2,000/μl) malignant plasma cells in their peripheral blood.

Human Myeloma Cell Lines (HMCLs)

The XGs, SBN1, and MDN HMCLs were established by ourselves [Zhang et al., 1994]; U266 and RPMI 8226 were purchased from ATCC (Rockville, MD); and OPM2, LP1, L363, and NCI H929 from DSM (Scheiwg, Germany). ANBL6 was a generous gift from D. Jelinek (Mayo Clinic, Rochester, MN), JJN3 from Dr. Van Camp (VUB, Brussels, Belgium), AMO1 from Dr. Minowada (Fujisaki Cell Center, Okayama, Japan), and Karpas 620 from Dr. Karpas (Cambridge, UK).

Serial Dilution of Mutant Cell

Mutant KRAS2 XG2 cells, and wild type KRAS2 Jurkatt cells were grown separately in an RPMI culture media with 10% fetal bovine serum. Cells were harvested (10^7) and XG2 cells were mixed with Jurkatt cells in different ratios (10%, 5%, 1%, 0.1%, 0.01%). We obtained three independent series of five dilutions in this way.

Cell Purification and DNA Extraction

Plasma cells were labeled with the B-B4 anti-CD138 monoclonal antibody as described previously [Pellat-Deceunynck et al., 1994] and isolated on columns, according to the manufacturer's instructions.

Bone marrow mononuclear cells and purified myeloma cells were collected as previously described and kept frozen at –80°C. Cells were suspended in 20 mM Tris HCl pH 7.4, 5 mM EDTA, 0.4 % SDS, 1 mg/ml proteinase K for 16 hr at 37°C. Genomic DNA was precipitated by ammonium acetate 3M final, and ethanol. Finally, DNA was rinsed in 70% ethanol, and resuspended in TE buffer (20 mM Tris HCl pH 7.4, 1mM EDTA).

Allele Specific PCR Amplification

5′ wild type PCR primers (Genosys Biotechnologies, Cambridgeshire, UK) and mutated PCR primers are described in Table 1. The reverse primers 1206L described elsewhere

TABLE 1. **Primers for KRAS2 and NRAS Genes Used in Study**

P	KRAS2 sequence	*P*	NRAS sequence
Exon 1, codons 12, 13	(Gly, Gly) ACTTGTGGTAGTTGGAGCTGGTGGC	Exon 1, codons 12, 13	(Gly, Gly) ACTGGTGGTGGTTGGAGCAGGTGGT
K12F	ACTTGTGGTAGTTGGAGCTG (Gly)	**N12F**	ACTGGTGGTGGTTGGAGCAG (Gly)
K1211	ACTTGTGGTAGTTGGAGCCC (Arg)	N12F	ACTGGTGGTGGRRGGAGCGA (Ser)
K1212	ACTTGTGGTAGTTGGAGCCT (Cys)	N1212	ACTGGTGGTGGTTGGAGCGC (Arg)
K1213	ACTTGTGGTAGTTGGAGCCA (Ser)	N1213	ACTGGTGGTGGTTGGAGCGT (Cys)
K1221	CTTGTGGTAGTTGGAGCTAC (Ala)	N1221	CTGGTGGTGGTTGGAGCAAA (Asp)
K1222	CTTGTGGTAGTTGGAGCTAT (Val)	N1222	CTGGTGGTGGTTGGAGCAAC (Ala)
K1223	CTTGTGGTAGTTGGAGCTAA (Asp)	N1223	CTGGTGGTGGTTGGAGCAAT (Val)
K1311	TTGTGGTAGTTGGAGCTGGCC (Arg)	N1311	GGTGGTGGTTGGAGCAGGCA (Ser)
K1312	TTGTGGTAGTTGGAGCTGGCT (Cys)	N1312	GGTGGTGGTTGGAGCAGGCC (Arg)
K1313	TTGTGGTAGTTGGAGCTGGCA (Ser)	N1313	GGTGGTGGTTGGAGCAGGCT (Cys)
K1321	TGTGGTAGTTGGAGCTGGTAC (Ala)	N1321	GTGGTGGTTGGAGCAGGTAA (Asp)
K1322	TGTGGTAGTTGGAGCTGGTAT (Val)	N1322	GTGGTGGTTGGAGCAGGTAC (Ala)
K1323	TGTGGTAGTTGGAGCTGGTAA (Asp)	N1323	GTGGTGGTTGGAGCAGGTAT (Val)
Exon 2, codon 61	(Gln) TATTCTCGACACACAGCAGGTCAA	Exon 2, codon 61	(Gln) CATACTGGATACAGCTGGACAA
K61F	TATTCTCGACACAGCAGGTC (Gln)	**N61F**	CATACTGGATACAGCTGGAC (Gln)
K6111	TATTCTCGACACAGCAGGCA (Lys)	N6111	CATACTGGATACAGCTGGGG (Glu)
K6112	TATTCTCGACACAGCAGGCG (Glu)	N6112	CATACTGGATACAGCTGGGA (Lys)
K6121	ATTCTCGACACAGCAGGTTC (Pro)	N6121	ATACTGGATACAGCTGGATC (Pro)
K6122	ATTCTCGACACAGCAGGTTG (Arg)	N6122	ATACTGGATACAGCTGGAAG (Arg)
K6123	ATTCTCGACACAGCAGGTTT (Leu)	N6123	ATACTGGATACAGCTGGATT (Leu)
K6131	TTCTCGACACAGCAGGTCGC (His #2)	N6131	TACTGGATACAGCTGGACTC (His #2)
K6132	TTCTCGACACAGCAGGTCGT (His #1)	N6132	TACTGGATACAGCTGGACTT (His #1)

For each tested mutation, primers for allele specific amplification share mismatching bases at their 3′ end. In bold, we show primer names for wild type sequence used as controls. Sequences corresponding to codons 12, 13, or 61 from the wild type K- and N-ras genes are underlined. The amino-acid modifications induced by mutations are in brackets.

[Hasegawa et al., 1995]; K6102 (5′- GCATGGCATTAGCAAAGACTC-3′); N12R (5′-GCTACCAC TGGGCCTCACCT-3′); and N61R2 (5′-TGACTTGCTATTATTGATGG-3′) were used, respectively, for amplification of the KRAS2, exon 1; KRAS2, exon 2; NRAS, exon 1; and NRAS, exon 2. The amplification multiplex and simplex were performed in the same PCR reaction mixture containing 10 mM Tris HCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 50 mM KCl, 200 mM of each deoxynucleoside triphosphate (Pharmacia, Uppsala, Sweden), 50 pmol of each primer, 1.25U of AmpliTaq Gold (Perkin-Elmer Corp., Norwalk, CT), and 100 ng of DNA. PCR was performed for 38 cycles of 0.5 min at 94°C, 1.5 min at Tm, and 1.5 min at 70°C. A step of 10 min at 94°C was performed before the cycles according to manufacturer indication for Taq Gold use. For codons 12,13, and 61 from the KRAS2 gene mutations, Tm used was 59°C; for the codons 12 and 13 from the NRAS gene it was 67°C, except for oligonucleotides 1321, 1322, 1323 63°C, and for codon 61, 54°C. Following amplification, 20 microliters of each PCR product was loaded on a 3.5% agarose gel, electrophoresed, and stained with ethidium bromide. The product length was 115 base pair (bp) for N12F-N12R amplification, 140 bp for K12F-K1206L, 106 bp for N61F-N61R2, and 210 bp for K61F-K6102.

Because of the relative large number of cycles used, we were very careful when performing the PCR. Every step (mix preparation, DNA extraction, migration electrophoresis) was performed in a separate room. As controls, we introduced blanks, i.e. a reaction tube without DNA to check reaction mix and a known DNA for validating each analysis process, from DNA extraction to electrophoresis of PCR products. We incorporated dUTP instead of dTTP in the PCR mix and used uracyl-DNA glycosylase at the beginning of each PCR to be sure that we erased PCR products from the previous PCR.

Use of the AmpliTaq Gold polymerase was also an important feature of our protocol since we could perform a hot start PCR without the inconvenient of opening PCR tubes. This thermostable polymerase is also very specific, especially in multiplex PCR conditions [Birch, 1996].

DNA Sequencing

The primers 1203 and 1204 described previously [Hasegawa et al., 1995] were used for amplification of the exon 1 of KRAS2 gene. Primers K6101 (5′-TTGAAGTAAAAGGTGCACTGA-3′), and K6102 (5′-GCATGGCATTAGCAAAGACTC-3′) were used for amplification of the exon 2 of KRAS2 gene. Primers N1F (5′-GCTGGTGTGAAATGACTGAG-3′) and N12R were used for amplification of exon 1 of NRAS gene, and N2F (5′-TTCTTACAGAAAACAAGTGG-3′) and N61R (5′-AAAATAATGACTCCTAGTACCT) for amplification of exon 2 of NRAS gene. The PCR was performed in the same reaction mixture but using a single Tm of 54°C, only 25 pmol of each oligonucleotide, and 35 cycles. Five microliters of the PCR products were analyzed on a 2% agarose gel to verify the expected size, purified on Bio-gel P100 fine (Bio-Rad, CA), sequenced on ABI377 DNA sequencer, and analyzed with Genescan, Sequence Navigator software (Applied Biosystems Inc, Foster City, CA).

Cloning and Sequencing of PCR Products

An aliquot of amplicon prepared for direct DNA sequencing was also ligated into the vector PGEM-T (Promega, Charbonnieres, France) at 4°C overnight. A 2 μl aliquot from each ligation mixture was used to transform competent *Escherichia coli* Xl1 cells. These were plated and blue/white screened. White colonies were picked up and grown to saturation in 200 μl of TYGPN medium (50 μg/ml ampicilline) without agitation during 24 hr on 96 wells plates. Ten plates for each amplicon were prepared. Sterile glycerol was added in each well and stored at –70°C for further use. DNA plasmid was extracted using a DNA extraction method (Qiagen, GmbH, Hilden, Germany) from bacterial growth into 4 ml of LB medium.

PCR using the conditions described above but for 35 cycles on diluted plasmid DNA in water was carried out to test simultaneously for the presence of an insert and the KRAS2 and/or NRAS mutation status. Amplification products were then visualized on a 3.5 % agarose gel. After screening of plates, and for positive plates the 12 columns, and finally eight wells for a positive column, one clone by mutation from positive well was sequenced according protocol described previously. DNA sequencing was performed using the –21M13 (5′-TGTAAAACGACGGCCAGTG-3′), and for verification with M13R (5′-AGCGGATAACATTTCACAGGA-3′).

RESULTS

ARMS Test Development

We have established a very sensitive and accurate protocol for detection of NRAS and KRAS2 mutations. Our methodology is based on a multiplex codon-specific PCR followed by a nucleotide specific simplex PCR. To obtain high sensitivity, without loss of specificity, detection was performed in two steps.

First, a multiplex PCR was performed, using a set of sense-primers, on identified mutated codons, and bases concerned. Second, a simplex PCR was applied on tumor DNAs showing a positive signal, by using a single primer corresponding to each of the variant possible nucleotides. This approach differs from the previously described MASA protocol [Hasegawa et al., 1995] in the following ways:

1. The sense primers (Table 1) have been modified to create a double mismatch at the 3′ end by introducing a mutation on the penultimate base as described in the ARMS PCR [Andre et al., 1998; Ferrie et al., 1992] and recommended to have a more reliable inhibition of elongation [Nollau and Wagener, 1997]. This improvement efficiently eliminates potential false positive results by strengthening mismatching in case of mutation on the target DNA. During the development of this protocol we noticed that the choice of the penultimate must be carefully done. One patient presenting a CAA->CGA (182A>G, Q61R) mutation, at codon 61 of NRAS, also seemed to share the mutations CAA->CAC (183A>C, Q61H) and CAA->CAT (183A>T, Q61H) in the same codon. In fact, we previously used primers N6131 and N6132 ending by GC and GT respectively at their 3′ ends. These mutations were not different from mutant sequences in their penultimate base and we therefore obtained false extension of 3′-ends of primers. By choosing primers ended by TC and TT, we did not encounter signals due to misextension of the mutant-specific primer on wild type target.
2. We have only performed a one step PCR and no nested PCR. This condition reduced risk of contamination and emergence of false-positive bands as described in some enrichment polymerase chain reactions [Jacobs et al., 1999].

The presence of KRAS2 and NRAS mutations as detected by our ARMS PCR was confirmed by different ways:

1. Specificity of mutated primers was evaluated by testing cell lines using our method and by direct sequencing. Sequencing of the involved exons was performed on the eight HMCLs for which we have detected a mutation, and the last seven cell lines without mutation identified with our PCR method. Results (Table 2) showed concordance for all studied cell lines (i.e. MDN, RPMI 8226, XG2, XG6, JJN3, and XG7). For RPMI 8226 and XG2, we found the same point mutation 35G>C (G12A) in KRAS2 gene confirmed by sequencing exon 1. This mutation was different from results presented in a previous paper [Portier et al., 1992]. The mutation was tested again on purified and checked cells and confirmed our finding.

We also tested the purified myeloma cells, and MDN cell line derived from the same patient (recently isolated in our lab) and found the same mutation 38G>A (G13D, NRAS gene) in both samples.

All those cell lines were tested between four and 10 times and were used as positive controls in our method.

2. Reproducibility was assessed by testing 10 times normal DNA from peripheral blood B lymphocytes, the human T lymphoma cell lines Jurkatt, and U266, and ANBL6 HMCL without any discrepancy. We tested each mismatched primer (i.e. primers for codons 12, 13, and 61 from KRAS2 and NRAS gene). The two independent PCRs, one multiplex detecting the mutated codon and the second one detecting the affected nucleotide, were never discrepant.
3. Sensitivity was tested twice by analyzing DNA extracted from different dilutions of a mutated HMCL (XG2, sharing the 35G>C substitution in KRAS2 gene) in Jurkatt cells. In a 1/1,000 dilution we detected a mutated cell in 100% of the

TABLE 2. Comparison of Mutations Found by Direct Sequencing and Allele-Specific PCR on HMCLS and Patients

HMCL	Gene	Nucleotide changes identified by allele-specific PCR[a]	Nucleotide changes identified by direct sequencing
JJN3	NRAS	35G>A (++)	35G>A
L363	NRAS	183A>C (+++)	183A>C
MDN	NRAS	38G>A(+++)	38G>A
RPMI 8226	KRAS2	35G>C (+++)	35G>C
XG1	NRAS	34G>C (+++)	34G>C
XG2	KRAS2	35G>C (+++)	35G>C
XG6	KRAS2	181C>G(+++)	181C>G
XG7	KRAS2	34G>T (+++)	34G>T
1 (pp[b])	KRAS2	183A>C (++)	183A>C
4 (pp)	KRAS2	34G>C (++), 34G>T (+)	34G>C
5 (pp)	NRAS	182A>G (++), 182A>T (++)	182A>G
7 (BM[c], 20%[d])	KRAS2	35G>C (+++), 183A>C (+++)	Wild-type
11 (pp)	NRAS	182A>T (++), 183A>C (+)	Wild-type
12 (pp)	KRAS2	37G>T (+++)	Wild-type
13 (pp)	KRAS2	183A>C (+++)	183A>C
14 (BM[c], 12%[d])	KRAS	181C>A (+++), 182A>C (++)	Wild-type
18 (pp)	KRAS2	35G>T (+++), 35G>C (+)	35G>T
19 (pp)	KRAS2	**37G>C (+++), 182A>T (+++)**	182A>T
	NRAS	**183A>C (+++), 183A>T (+++)**	Wild-type
28 (BM[c], 7%[d])	NRAS2	182A>G (++)	Wild-type
37 (BM[c], 43%[d])	KRAS2	35G>A (++), 38G>A (++)	Wild-type
39 (pp)	NRAS	38G>A (+++)	38G>A
44 (pp)	KRAS	**182A>G (+++), 181C>A (+++)**	182A>G
45 (pp)	KRAS2	34G>A (+++), 35G>C (+++), 35G>T (++)	34G>A, 35G>C
58 (pp)	NRAS	183A>T (+++)	183A>T
59 (pp)	NRAS	37G>C (+++)	37G>C
64 (BM, ?[d])	NRAS	181C>A (+++)	Wild-type

[a]Intensity of PCR evaluation: +++, strong; ++, average; +, weak.
[b]pp, Purified plasmocytes.
[c]BM, Whole bone marrow.
[d]% of bone marrow plasmocytosis.
Mutations in bold type identified by subcloning of exon amplification PCR products.

samples tested and in a 1/10,000 dilution we detected mutated cells in 33% of the samples (Fig. 1).

Comparison of Allele-Specific PCR and Direct Sequencing of Tumor Derived DNA

Eighteen patients showing a mutation by PCR were also evaluated by direct sequencing of PCR amplified DNA. We have particularly selected patients showing multiple mutations. DNA was extracted from bone marrow and purified myeloma cells. KRAS2 and NRAS genotyping by multiplex PCR and direct sequencing were in good agreement for DNA obtained from purified myeloma cells (Table 2). Mutations were detected in this population 11 times for 13 DNA

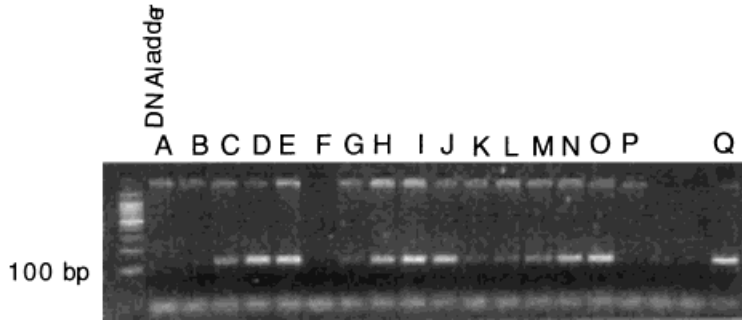

FIGURE 1. Sensitivity of allele-specific PCR evaluated for the 35G>C (G12A) mutation in K-ras gene. We have tested three series of mutant XG2 in Jurkatt cell-line at several concentrations (0.01%, 0.1%, 1%, 5%, and 10%), lanes A to E, F to J, and K to O, with mutant primer K1221. For two series we observed a sensitivity of one mutated cell detected among 1,000 normal cells. In the third series, sensitivity was improved to 1/10,000 (K). We have repeated this experiment three times and have obtained the same results. As controls, we amplified Blank (lane P), and DNA from cell line XG2 (lane Q).

samples analyzed from purified plasma cells. In each case, a single mutation was detected by sequencing, except in patient 45, where a double mutation on the same codon was suspected. For patients found mutated by our method (five cases) using DNA extracted from whole bone marrow, none was confirmed by sequencing. We tested these discrepancies again using both multiplex and simplex PCRs and found the same results.

Cloning and Sequencing of PCR Products

To enhance information on the validity of our method we subcloned exons from patients 19 and 44 showing four and two mutations, respectively. In each case, direct sequencing showed one mutation only (Table 2). For each patient 960 clones were tested in 96 wells plates in four steps. First, a mini-prep per plate was made and screened by our method with allele specific PCR. We performed the experiment on one mini-prep per plate obtained by pooling aliquot of each well in 4 ml of LB. For positive plates selected, the same protocol was used to test each column of the plate, and finally each well for a positive column. In the last step, we tested by sequencing clones positive for mutation. For the two patients, we found all the mutations given by our method as shown in Figure 2 for patient 19.

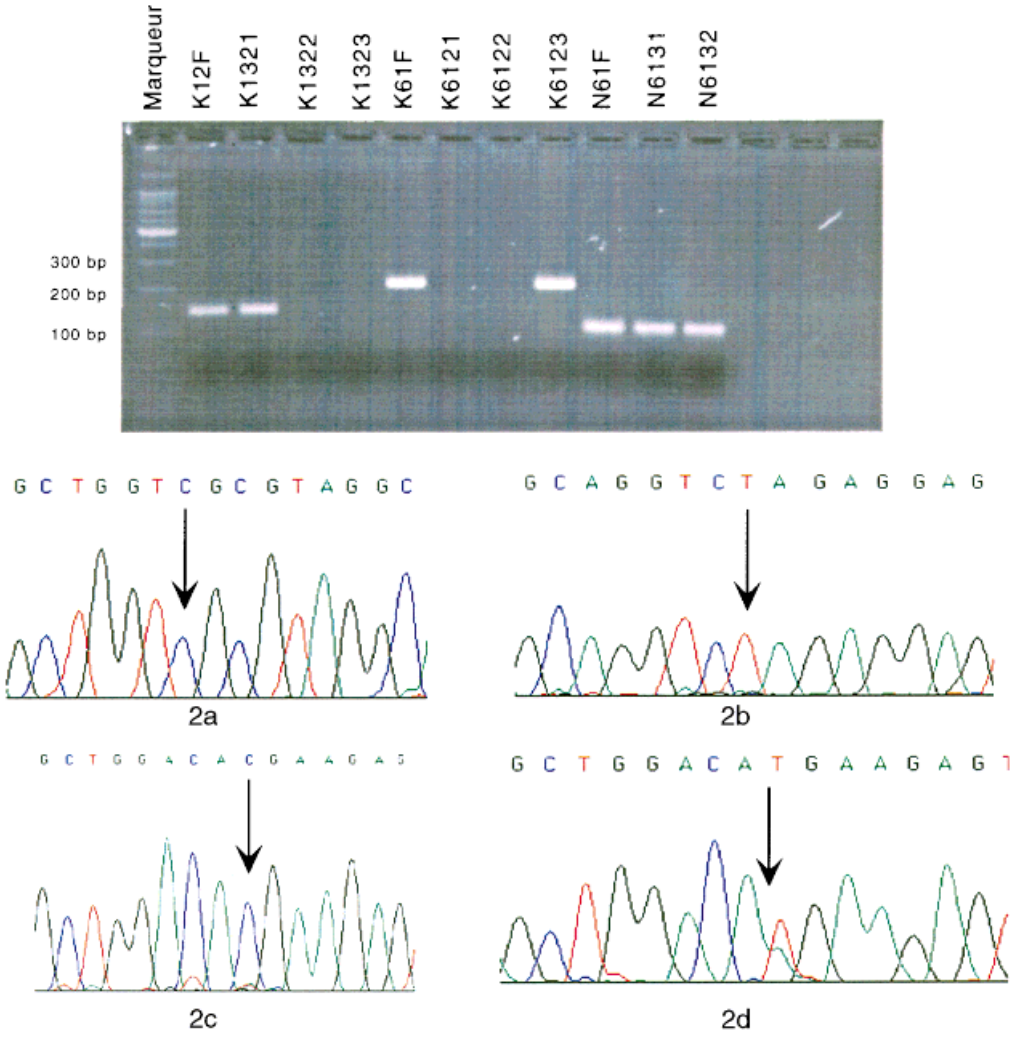

FIGURE 2. **Results from ARMS PCR and electrophoragrams from sequencing of clones for patient 19. The four mutations identified by ARMS PCR were confirmed by sequencing of clones. Mutation GGC->CGC in codon 12 (a), and CAA->CTA (b) from K-ras gene, and mutation CAA->CAC and CAA->CAT (c,d) in codon 61 from N-ras gene. Direct sequencing has only shown mutation CAA->CAT. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

These results confirm that detection limits of current sequencing methods were very low.

High Incidence of KRAS2 Rather Than NRAS Mutations in Newly Diagnosed Patients With MM

NRAS and/or KRAS2 mutations were investigated in 33 consecutive patients with MM. Detailed results are shown in Table 3. Out of 33 completely evaluable patients, NRAS and/or KRAS2 activating mutations were found in 18 cases (54.5%), with a mean of 1.6 mutations per mutated patient including two patients (5 and 19) showing four mutations. Three other patients showed evidence of two mutations. Overall, NRAS mutations (23% of evaluable cases) were less frequent than KRAS2 mutations (33%).

High Incidence of K- Rather Than N-*ras* Mutations in Patients With Primary PCL

NRAS and/or KRAS2 mutations were investigated in 10 patients. NRAS and/or KRAS2 activating mutations were found in five cases (50%), a percentage identical to that of newly diagnosed MM. A mean of 1.8 mutations per mutated patient was found. Thus combining MM and primary PCL at diagnosis, 23 out of 43 evaluable patients (54%) presented *ras* mutations, with a mean of 1.7 mutations per mutated patient, more frequently KRAS2 than NRAS mutations, that is 32.5% versus 23.25%.

Activating *ras* Mutations in Patients With Progressive Disease and in HMCL

NRAS mutations, codon 61 were found in three out of 10 patients with progressive disease (30%). KRAS2 mutations were found in five out of 11 patients (45.5%). By and large, NRAS and/or KRAS2 mutations were detected in nine out of 11 evaluable patients (81%), with a mean of 1.2 mutations per mutated patient. The study was incomplete for NRAS, codon 12 and 13 because of insufficient material.

Sixteen HMCL were investigated (see the Materials and Methods section). The results are detailed in Table 3 showing that 50% of HMCL had *ras* mutations. Of note, NRAS mutations, codon 61 were found in only one of 16 HMCL (6%). HMCL without mutation are AMO1, ANBL6, JURKATT, KARPASS 620, LP1, OPM2, U266, and XG5.

Low Frequency of *ras* Mutations in Monoclonal Gammopathy of Undetermined Significance (MGUS) and Indolent Myeloma (SMM)

Only one patient out of six MGUS and two SMM analyzed showed one mutation located in NRAS gene 181C>A (Q61K). Of course, recruitment was poor compared to the other stages of disease, but showed clearly the low level of *ras* mutations in those patients, i.e., one out of eight (12.5%).

Overall Results

Out of 54 evaluable patients in all codons with either MM or PCL, NRAS and/or KRAS2 mutations were found in 32 cases (59%). Of note, KRAS2 mutations (20 cases) were more frequent than NRAS mutations (14 cases), 37% versus 26%. Of major interest, we found 10/54 (18.5%) of patients showing multiple *ras* mutations. Multiple mutations of the *ras* gene have been described for colorectal cancer and correlated to the advanced stage of tumor [Moerkerk et al., 1994].

TABLE 3. NRAS and KRAS2 Mutation Detection in Patients With Different Diagnoses

RAS	MM		PPCL		Relapse		MGUS/SMM		HMCL	
gene/codon	Pos/n	%	Pos/n	%	Pos/n	%	Pos/n	%	Pos/n	%
K12	9/33	27.30	3/10	30	1/11	9	0/8	0	3/16	18.75
K13	2/33	6	0/10	0	1/11	9	0/8	0	0/16	0
K61	7/33	21.20	0/10	0	4/11	36	0/8	0	1/15	6.25
N12	0/27	0	0/10	0	0/7	0	0/8	0	2/15	12.50
N13	0/27	0	1/10	10	1/7	14	0/8	0	1/15	6.25
N61	7/30	23.30	2/10	20	3/10	30	1/8	12.5	1/15	6.25
Overall	54.50%		50%		81%		12.50%		50%	

Results for ras mutations detection by our method in a series of patients with multiple myeloma (MM), primary plasma-cell leukemia (P-PCL), patients in relapse, monoclonal gammopathy of undetermined significance (MGUS), indolent myeloma (SMM), and human myeloma cell lines (HMCL). This table indicates the number of mutations identified for each codon from N and K-ras genes. Overall results indicate the percentage of patients showing at least one mutation in N or K-ras genes.

DISCUSSION

Since 1989, eight studies have been devoted to the research of activating *ras* mutations in patients with malignant plasma-cell disorders (mainly MM) and HMCL [Corradini et al., 1993; Liu et al., 1996; Matozaki et al., 1991; Millar et al., 1995; Neri et al., 1989; Paquette et al., 1990; Portier et al., 1992; Tanaka et al., 1992]. As outlined, NRAS mutations were found in 19% (ranges: 0–50%) of the patients at diagnosis. In these studies, NRAS codon 6l was the most frequently involved and the C<u>A</u>A/C<u>G</u>A substitution the most frequent one. Of note, KRAS2 mutations have been investigated in six out of eight of these studies and appeared less frequently than that of NRAS [Corradini et al., 1993; Liu et al., 1996; Neri et al., 1989; Paquette et al., 1990; Portier et al., 1992; Tanaka et al., 1992]. Indeed, they were detected in 9% (ranges: 0–16.5%) of the patients at diagnosis. In two studies, the percentages of mutated patients increased in those with extramedullary disease, but this did not reach statistical significance [Corradini et al., 1993; Portier et al., 1992]. Thus, all these studies have shown that a mean of 28% of previously untreated patients had activating mutations of *ras* (rather NRAS than KRAS2). No significant correlation emerged from these studies between such mutations and disease severity, except for the most recent one showing that patients with KRAS2 (but not NRAS) mutations at diagnosis had a shorter survival span [Liu et al., 1996]. Also of note, is that generally few patients had more than one mutation involving either NRAS or KRAS2.

The aims of our study were to develop validated tests for all point mutations from NRAS and KRAS2 genes located in codon 12, 13, and 61 and to apply them for reappraising the frequency and the potential role of NRAS and KRAS2 activating mutations in 62 malignant plasma-cell disorders, including MGUS, SMM, MM, primary PCL, EMP, and secondary PCL and in 15 immortalized tumors (i.e., HMCL). We have chosen ARMS because this technique has already been successfully applied elsewhere, such as for detection of mutations in hemochromatosis [Andre et al., 1998] or in cystic fibrosis [Ferrie et al., 1992] and is now commonly used in routine diagnosis in many laboratories. The suitability of ARMS PCR is demonstrated in many applications performed in different ways such as prenatal screening for cystic fibrosis [Bradley et al., 1998], gene therapy for the discrimination between expression of endogenous CFTR and introduced transgene [Thorpe and Porteous, 1999], detection of the most common G6PD gene mutations [Du et al., 1999], diagnosis of autosomal recessive spinal muscular atrophy [Ravard-Goulvestre et al., 1999], and detection of KRAS2 mutations in colorectal cancer [Fox et al., 1998]. A recent study [Tonks et al., 1999] has compared three methods: sequence specific oligotyping (SSO), reverse dot blot, and ARMS PCR for molecular typing of HLA class I. A great preference is shown for the last technique. However, care must be taken in the choice of the penultimate mismatching nucleotide, since it may give wrong positive results in testing neighboring nucleotides, as in this study.

As part of the test validation process we used sequencing of all HMCLs and direct sequencing of 18 patients, 10 of them showing more than one mutation. This approach to directly sequence PCR amplicons verified the ARMS result in all cell lines tested, and in five of eight patients showing a single mutation. For patients showing more than one mutation, direct sequencing has demonstrated the presence of a single mutation in approximately half of the tumor DNA samples investigated. For patient 45 only, we could perceive a second minor mutation by direct sequencing. Interestingly, when the analyses were performed on exons amplified from whole bone marrow instead of purified plasma cells, we never observed mutations by direct sequencing. In these cases, the mutant sequences could account for only a small proportion of the total DNA, and DNA sequencing was inappropriate for validating the ARMS results. A second stage of validation in which cloned amplicons of KRAS2 exon 1 and 2 for patient 19, and amplicons of NRAS exon 2 for patient 19 and 44 were sequenced confirmed this observation. In these cases, the DNA sequence gave similar results to those derived by ARMS, i.e. identification of four mutations for patient 19, and two mutations for patient 44. Sensitivity is quite good since one mutated cell can be detected among 1,000 normal cells in routine with 38 cycles without nested PCR. Therefore, the methodology we propose is a protocol providing a good level of sensitivity, comparable to a recently described reliable procedure using PCR/LDR [Khanna et al., 1999]. It should also

be compared to other protocols which are either very sensitive but not very reliable, such as most of the allele specific PCR including MASA [Hasegawa et al., 1995]; quite reliable but not very sensitive, such as DNA sequencing or restriction enzymes digests [Ahuja et al., 1990; Corradini et al., 1993; Portier et al., 1992; Sawada et al., 1989]; or isotopic, such as oligonucleotide hybridization [Tanaka et al., 1992]. Due to the reliable sensitivity of our approach, we can detect several mutations in a single sample showing intratumor heterogeneity for *ras* mutations in some cases. This feature is very interesting since it allows detection of an emerging clone in a malignant or premalignant tissue. Although some authors have suggested that highly sensitive methods of detection of KRAS2 mutant in tumor DNA could obscure differences between patients with mutation present throughout the tumor and those in whom the mutation is only present in a small subpopulation [Gazdar and Virmani, 1998], these emerging cells, sharing a *ras* mutation, will be more aggressive than the original cells, and will condition the prognosis.

Finally, the methodology we propose is nonisotopic, quite simple and, compared to other techniques, does not require procedures such as enzymatic digestion or ligation, and it is amenable to automation. Furthermore, we propose the simultaneous analysis of NRAS and KRAS2 genes, the most pertinent *ras* genes in MM, when other protocols only test one of these two genes. In the current study, 53.50% of our patients with either MM or PCL had NRAS and/or KRAS2 mutations. This rate is the highest ever published by ourselves [Portier et al., 1992] or others [Corradini et al., 1993; Liu et al., 1996; Matozaki et al., 1991; Millar et al., 1995; Neri et al., 1989; Paquette et al., 1990; Tanaka et al., 1992] on similar populations of patients. These results are mainly explained by the high sensitivity of the method we used compared to the previous ones. We have evaluated our sensitivity to at least one mutated cell out of 1,000. The method is sensitive enough to detect mutations and does not need to use highly purified myeloma cells. Furthermore, combination of our protocol with plasma cell purification should also provide a very sensitive test. Our new approach not only increases the rate of detection of mutated patients but also enables the detection of more than one mutation in a significant proportion of patients, i.e. 10 out of 54 (18.50%). It is noteworthy that Losi et al. [1992] have detected 71% of mutations in colon cancer by using a similar technical approach for checking KRAS2 mutations at codons 12 and 13, i.e. MASA. All these data put together show that this allele-specific PCR method is highly sensitive and allows the detection of even a few mutants in a cell population. These results also indicate that the incidence of NRAS and KRAS2 mutations has to be re-evaluated in human cancers using more sensitive approaches than the previous ones. A similar high incidence of the same mutation has been recently described in human melanoma and explained by the mutagenic effects of UV radiation on the T-T- dinucleotide [Ball et al., 1994]. However, such a mechanism is unlikely in MM. Thus, further research is necessary to clarify this point.

Regardless of the mechanisms of *ras* mutations in MM, our data are highly relevant to the biology of this disease. During the early stages of the disease, myeloma cells rather accumulate within the bone marrow than they proliferate. Paradoxically, they produce low levels of immunoglobulins in comparison with their normal counterpart, but they overexpress some plasma-cell antigens like CD138. Thus, they are characterized by an aberrant program of differentiation, with a delayed program of cell death. Later, myeloma cells become less differentiated, producing less immunoglobulins but they proliferate more with increased cell loss in relation to increased apoptosis. The early occurrence of NRAS and KRAS2 mutations in myeloma cells could be responsible for these abnormalities. Indeed, it is worth noting that *ras*-oncogene transformation of human B lymphoblasts has been associated with 1) a downregulation of immunoglobulin gene expression, but 2) an upregulation of plasma-cell antigens like CD38 and PCA-1 in two studies [Seremetis et al., 1989; Sirinian et al., 1993]. Furthermore, *ras*-oncogene transformation of IL-6 dependent malignant plasmablasts has been associated with a survival advantage in the case of IL-6 starvation (with NRAS but not KRAS2 transfection) and with a growth advantage (in both experimental situations) [Billadeau et al., 1995; Billadeau et al., 1997]. It has eventually been shown that the expression of *ras* oncogenes

in cultured human cells could stabilize the mRNA of some critical genes like interleukin-6 (IL-6) [Billadeau et al., 1997]. A clear association between *ras* gene activation and expression of IL-6 has already been demonstrated in acute myeloid leukemia [Lubbert et al., 1993]. Taking all these data together, we can suggest that NRAS and KRAS2 mutations, which are almost frequently observed in MM, could be an early event 1) blocking the immunoglobulin gene expression in these cells while upregulating that of plasma-cell antigens (CD38, PCA-1, syndecan-1?), and 2) giving these cells a survival advantage (including an autocrine pathway through stabilization of IL-6 mRNA). K-*ras* mutations could also lead to the increase of the differentiation blockade and growth advantage, considering its prognostic value evaluated by Liu et al. [1996].

Our data open new avenues for the management of MM. The research on *ras* mutations in individuals with MGUS could delineate a subset of patients actually presenting an early MM. In a similar way, considering the specificity and sensitivity (10^{-3}) of this approach, the same research could be performed in myeloma patients to detect residual myeloma cells, either in the bone marrow or in the peripheral blood after intensive treatment. Of course it could be applied to other models, such as solid tumors. We have already used it successfully for colon tumors, and melanoma (data not shown).

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