Rapid mutational analysis of N-ras proto-oncogene in hematologic malignancies: a study of 77 Greek patients

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Background and Objectives. N-ras mutations are the most commonly detected molecular abnormalities in hematologic malignancies, especially in those of myeloid origin. Different techniques have been used to detect N-ras mutations; however, most of them are either labor intensive or provide sequence data for only a limited number of codons. Consequently, study of the N-ras oncogene has not been convenient in every day clinical practice being restricted, as a rule, to retrospective analysis of patients.

Design and Methods. In this study we used a recently developed method that enables rapid and reliable detection of mutations at the cDNA level, namely, the non-isotopic RNase cleavage assay (NIRCA). Using this method we were able to screen the N-ras oncogene rapidly and determine the incidence and prognostic significance of N-ras mutations in 77 Greek patients with acute leukemia, myelodysplastic syndromes and chronic myeloproliferative disorders, both at the presentation and during relapse or progression of the disease.

Results. Activating N-ras mutations were detected in 7 patients and our results were confirmed by direct sequencing. Interestingly, two novel alterations were identified, a mutation at codon 8 (characterized by a substitution of valine by leucine) in a patient with chronic myeloid leukemia during hematologic relapse of the disease and a polymorphism at codon 92 (1002T→C, without amino acid substitution) in a patient with chronic myelomonocytic leukemia.

Interpretation and Conclusions. A rapid and easy protocol that allows the analyses of N-ras sequences has been developed. This reverse transcription-polymerase chain reaction (RT-PCR)/NIRCA protocol can allow the study of this proto-oncogene in every day clinical practice, rapidly facilitating the validation of the diagnostic and prognostic value of N-ras mutational analyses in patients with hematologic malignancies.

Key words: N-ras, acute leukemia, myelodysplastic syndromes, chronic myeloproliferative disorders, NIRCA.

Several studies have demonstrated that genetic alterations i.e. activation of proto-oncogenes by point mutations, rearrangements or amplification, or inactivation of tumor suppressor genes, are involved in the carcinogenesis of various human tumors.[1] Among the genetic alterations identified, activation of the ras proto-oncogene family (H-ras, K-ras and N-ras) is one of the most prevalent aberrations.[2] The ras genes code for highly homologous 21kDa guanine nucleotide-binding proteins with intrinsic GTPase activity, termed $p21^{ras}$, whose cellular location is at the inner surface of the plasma membrane. They are components of intracellular signal transduction pathways converting extracellular signals to transcriptional regulation in the nucleus, and they are involved in regulating both cell growth and apoptosis.[2,3] Single nucleotide alterations at critical codons of H-, K- and N-ras genes (almost always codons 12, 13 and 61) lead to altered proteins, resulting in a constitutively activated downstream signal cascade and oncogenic transformation.[2]

Mutations of the N-ras gene are the most commonly detected molecular abnormalities in hematologic malignancies, especially in those of myeloid

origin. N-ras mutations occur in approximately 5-40% of patients with myelodysplastic syndromes (MDS),[4-10] 9-45% of patients with acute myeloid leukemia (AML),[4,11-18] 5-15% of patients with acute lymphoblastic leukemia (ALL),[4,19-21] 9-39% of patients with multiple myeloma and plasma cell leukemia,[22,23] 6% of patients with idiopathic myelofibrosis,[24] and rarely (0-3%) in patients with chronic myeloid leukemia (CML).[25-28] This wide range of N-ras mutations may reflect the small number of cases in some studies, the diversity of methods employed, as well as the heterogeneous nature of these diseases. However, although these results suggest that N-ras mutations play a fundamental role in human leukemogenesis, the relevance of these mutations for the clinical and biological behavior of the subgroups of patients exhibiting them is still controversial. Most of the recent studies examining cancer cells for the presence of ras mutations have employed amplification of the critical sequences of ras genes from DNA template using polymerase chain reaction (PCR) in conjunction with differential oligonucleotide hybridization,[7,8,10,12,15,16] or direct sequencing.[4,19] Because these techniques are labor intensive and time consuming, there were efforts by several laboratories to apply molecular techniques that could permit faster detection of mutations. Most of these recent techniques are based on altered behavior of the sequences due to single base changes, such as single-strand conformation polymorphism (SSCP),[22,29,30] and denaturing gradient gel electrophoresis (DGGE),[31] or constitute variants of restricted-fragment-length-polymorphism (RFLP).[14,18] Recently, Nakao *et al.* described a new method for the detection of N-ras mutations in patients with ALL, using special laboratory and software equipment (LightCycler technology).[19] Unfortunately, even these refined protocols had serious limitations. For example, Miyauchi *et al.* reported false-negative results in SSCP analysis in 33% of positive cases.[30] As a result, the study of the N-ras oncogene has not been convenient in every day clinical practice, being restricted, as a rule, to retrospective analysis of the patients' material.

In this study we combined reverse transcription-polymerase chain reaction (RT-PCR) and the recently developed method NIRCA (non-isotopic Rnase cleavage assay) and successfully screened 77 patients with hematologic malignancies for N-ras mutations. This protocol turned out to be rapid, very simple, sensitive and reliable, thus greatly facilitating the study of N-ras mutational activation in every day clinical practice.

Design and Methods

Patients

Eighty consecutive patients with acute leukemia, MDS and chronic myeloproliferative disorders (MPD) were studied for the presence of N-ras mutations. Samples from 3 of these patients (1 with AML and 2 with MDS) failed to be amplified; thus the status of the N-ras gene was evaluated in 77 patients, 30 with acute leukemia (21 with AML, 7 with ALL, and 2 with acute biphenotypic leukemia), 27 with MDS, and 20 with MPD.

The patients with acute leukemia (18 males and 12 females) were diagnosed and classified according to the French-American-British (FAB) classification (2 patients with AML-M0, 6 with AML-M1, 1 with AML-M2, 2 with AML-M3, 3 with AML-M4, 6 with AML-M5, 1 with AML-M6, 3 with ALL-L1, 3 with ALL-L2 and 1 with ALL-L3).[32] Furthermore, one patient fulfilled the recent laboratory criteria of acute biphenotypic leukemia,[33] while another one displayed morphologic and immunologic findings of acute myeloid/NK leukemia and was also classified as suffering from acute biphenotypic leukemia. All the patients received conventional chemotherapy as defined by standard therapeutics protocols. PB and/or BM samples from the four patients who relapsed, were also analyzed during the relapse. The patients with MDS (14 males and 13 females) were diagnosed and classified on the basis of morphologic study of bone marrow and peripheral blood specimens according to the FAB classification (12 patients with refractory anemia-RA, 2 patients with refractory anemia with ringed sideroblasts-RARS, 5 with refractory anemia with excess of blasts-RAEB, 3 with refractory anemia with excess of blasts in transformation-RAEBt, and 5 patients with chronic myelomonocytic leukemia-CMML).[34] All the patients were treated by standard therapeutics protocols. Two patients displayed progression to atypical chronic myelogenous leukemia (aCML) according to the new FAB classification of CMLs,[35] and one of them displayed subsequent progression to acute leukemia, eventually. One patient displayed progression to RAEB, and eight patients to acute leukemia. PB and/or BM samples from these patients were analyzed during the progression, too. Of the 20 patients (14 males and 6 females) with MPD, fourteen patients harbored the bcr-abl rearrangement and were classified as having CML. Two patients were diagnosed as having idiopathic myelofibrosis. Two out of the four remaining bcr-abl negative patients were diagnosed as having aCML and two as unclassified

myeloproliferative disorder. All the patients were treated by standard therapeutic protocols. Seven patients displayed progression of the disease and PB and/or BM samples were analyzed during the progression, too. The promyelocytic leukemia cell line HL-60 (with known N-ras mutation at the second nucleotide of codon 61) served as the positive control and the erythroleukemia cell line K562 served as the wild-type (negative) control in our experiments.

Methods

Cells. Mononuclear cells from PB and BM aspiration were separated by density gradient centrifugation and frozen at –70°C. Total RNA from 2-4×10^7 mononuclear cells was extracted using a standard guanidium thiocyanate protocol.[36] The final RNA pellet was redissolved in 30-50 μL TE buffer.

Reverse transcription and PCR. The central strategy of the utilized procedure was to generate by PCR appropriate templates for *in vitro* transcription using the T7 and SP6 RNA polymerases. We carried out the amplification of N-ras cDNA in two PCR reactions, in which the sequences of T7 and SP6 RNA promoters constituted the 5' end of the sense and antisense nested primers, respectively. Thus, the nested PCR products contained the T7 and SP6 phage promoters and under suitable conditions using the corresponding polymerases, synthesis of microgram amounts of RNA was achieved with the nested PCR product as template. The position and sequences of the utilized primers, designed with the aid of the Oligo-5 software (NBI, Plymouth, MN, USA), are shown in Table 1.

The cDNA synthesis was carried out as described previously.[37] The whole amount of synthesized cDNA was used in 100 μL PCR reaction using 200 mM of each deoxynucleoside triphosphate, 50 pmoles of each primer and 2.5 units of Taq Polymerase (Promega, WI, USA) in a PCR buffer supplied by the manufacturer. Thereafter, 1-5μL of PCR product were used for the nested PCR. Nested PCR was carried out in 50μL reaction using the same concentrations as above. The conditions of primary PCR were: 2 min at 94°C followed by 32 cycles (94°C for 90 sec, 56°C for 90 sec, 72°C for 120 sec) followed by 5 min at 72°C. The conditions of nested PCR were: 2 min at 94°C followed by 33 cycles (94°C for 60 sec, 50°C for 60 sec, 72°C for 120 sec) followed by 5 min at 72°C after the last cycle. All PCR amplifications were carried in the PCR-engine apparatus PTC-200, MJ Research, (Watertown-Mass., USA). The nested PCR products were analyzed in 1,5% TBE agarose gels.

Table 1. Sequence of primers used to amplify the N-ras cDNA.

	*Position**	*Sequence*
RAU	404-421	5' TGG CGA AAG AAT GGA AGC 3'
RAL	1594-1577	5' GGG TGG CAG AGG TGT GTT 3'
T7RA	643-655	5' TTA TAC GAC TCA CTA TAG GAG GGT CTC CAA CAT 3'
SP6RA	1222-1208	5' ATT TAG GTG ACA CTA TAG GAA CTG GCG TAT TTC TC 3'

The underlined sequences constitute the T7 and SP6 promoters.
**The numbering of N-ras cDNA is according to Hall & Brown, 1985.*[39]

Non isotopic Rnase cleavage assay (NIRCA). This method is based on a previously described protocol,[38] and was performed using the MisMatch Detect II system (Ambion, Austin, TX, USA) with some modifications. As wild type controls we used PCR product generated from the cell line K562, since we used PCR product from the cell line HL-60 as the positive control. In principle, a sense RNA product generated *in vitro* from a wild type control template, using the appropriate RNA polymerase was hybridized with the antisense RNA strand generated from either the same template (as a non-mutated control), and template generated from patients or from the HL-60 cell line (as the mutated positive control). To control the procedure, the opposite combinations, i.e. hybridization using the antisense RNA strand of the wild type sample with the sense strand generated from patient was performed. Sense and anti-sense RNA strands generated from each patient were also hybridized to each other, and thus, a group that consists of four RNA duplexes was generated for every patient. Any mismatches in the RNA-RNA hybrids were revealed by RNAse treating and separating the products in 1.5% TBE agarose gels. For every 10μL transcription reaction, 2-5 μL of nested PCR were used as template and transcription was carried out as suggested[37,38] at 37°C for 70 min. Hybridization reactions were also carried out according to manufacturer's instructions. Concerning the RNAse digestion, we determined the optimal cleavage conditions for the N-ras gene, treating the generated RNA duplexes with a combination of the three RNases in the following dilutions: 1/300 of RNase#1, 1/100 of RNase#2 and 1/150 of RNase#3. The digestion reactions were performed according to the manufacturer's instructions. Cleavage products were analyzed by electrophoresis in 1.5% TBE agarose gels.

Direct sequencing. The results obtained by the

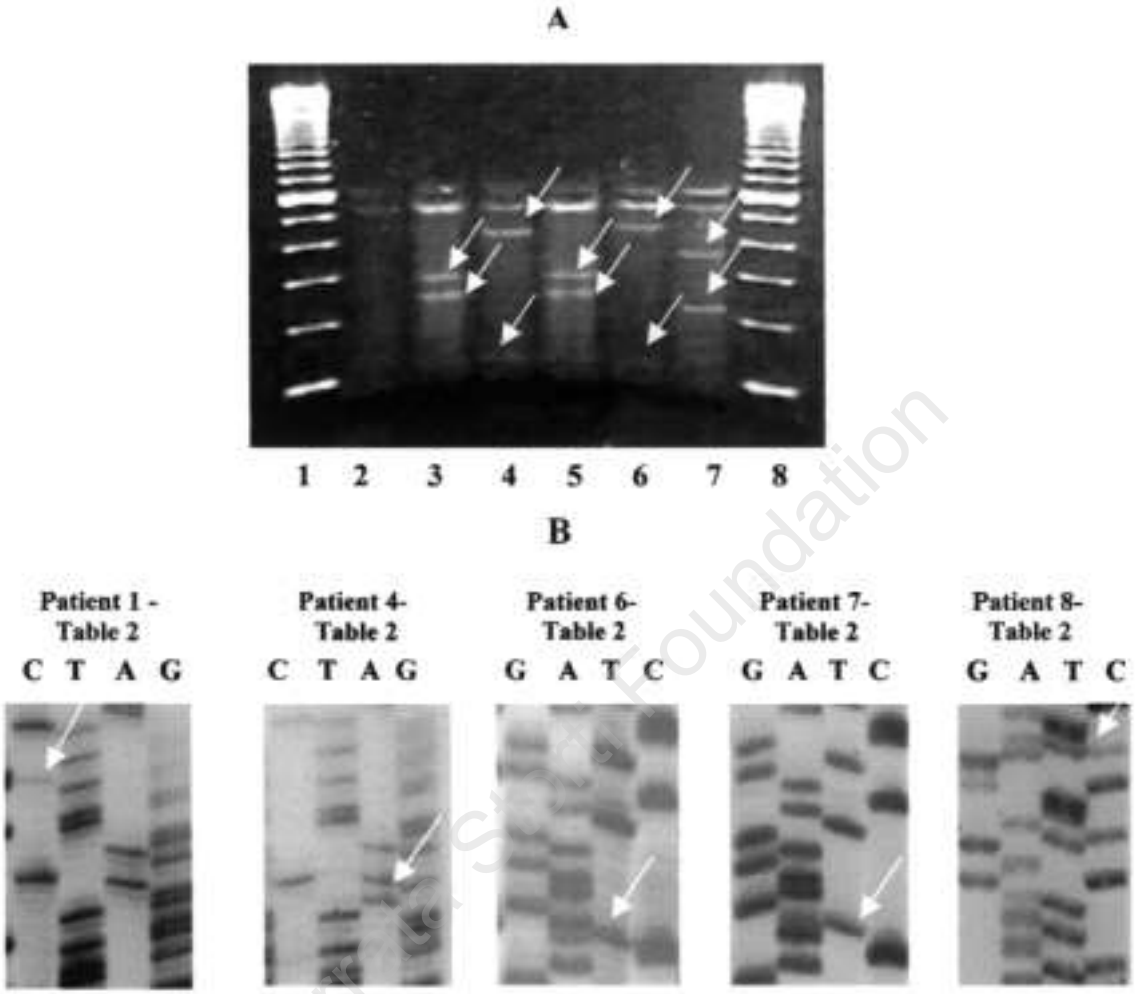

Figure 1. N-ras mutations established by RT-PCR/NIRCA and confirmed by direct sequencing. A. NIRCA digestion products of normal individual and patients with N-ras mutations, using the combination of RNases in the following dilutions: 1/300 of RNase#1, 1/100 of RNase#2 and 1/150 of RNase#3. Lanes 1 and 8: 100bp ladder molecular weight marker (GibcoBRL). Lane 2: cleavage products of hybrids generated with sense RNA from a patient without mutation and antisense from the wild type control (cell line K562). Lane 3: cleavage products of hybrids generated using sense RNA from the cell line HL-60 (with known N-ras mutation at the second nucleotide of codon 61 – positive control) and antisense from the wild type control. Lane 4: cleavage products of hybrids generated using sense RNA from patient 4 of Table 2 with a mutation at codon 12 and antisense from the wild type control. Lane 5: cleavage products of hybrids generated using sense RNA from patient 6 of Table 2 with a mutation at codon 61 and antisense from the wild type control. Lane 6: cleavage products of hybrids generated using sense RNA from patient 1 of Table 2 with the novel mutation at codon 8 and antisense from the wild type control. Lane 7: cleavage products of hybrids generated using sense RNA from patient 8 of Table 2 with the polymorphism at codon 92 and antisense from the wild type control. The arrows reveal alterations related to cleavage products at 420bp and 120bp for the mutations at exon 1, at 300bp and 260bp for the mutation at exon 2 and codon 61, and 330bp and 230bp for the polymorphism at codon 92. B. Sequencing analysis of nested PCR samples of patient 1 of Table 2 (G→C at the first nucleotide of codon 8), patient 4 of Table 2 (G→A at the second nucleotide of codon 12), patient 6 of Table 2 (A→T at the second nucleotide of codon 61), patient 7 of Table 2 (A→T at the third nucleotide of codon 61) and patient 8 of Table 2 (T→C at the third nucleotide of codon 92). The location of mutations is noted by arrows.

NIRCA were verified by directly sequencing all the samples that appeared to be mutated and twenty-one of those that appeared to be normal. Nucleotide sequences were analyzed using the T7 PCR product sequencing kit (Sequenase 2.0/United States Biochemical) according to the manufacturer's instructions. Subsequently, denaturated samples were separated on 6% polyacrylamide gels containing 7M urea, followed by drying and exposure to X-ray film.

Statistical analysis. The statistical analysis was performed using the SPSS statistical program for Windows (edition 7.0). Three patients with acute leukemia were not treated in our center and thus were not counted in the statistical analysis. Furthermore, since cytogenetic data were not available from all the patients, this parameter was not statistically analyzed. Differences in the distribution of clinical and biological variables between the subgroups of the patients were analyzed by Fisher's exact test. Logistic regression analysis was used to examine the effect of the variables of interest on the response to chemotherapy in patients with acute leukemia. The same method was used in order to examine the variables of interest on the progression of the disease in patients with MDS and MPD. Patients treated by bone marrow transplantation were censored for survival at the time of such treatment. Survival was calculated from the time of diagnosis until death or through December 1999. Survival probabilities were estimated by the Kaplan-Meier method, and differences in the survival distributions between the N-ras-positive and N-ras-negative groups were tested according to the long-rank test. Cox's proportional hazards model was used in order to explore the relation of the variables of interest to the overall survival. *p* values less than 0.05 were considered statistically significant.

Results

Incidence of mutations

In the present study all the samples were analyzed on at least two separate occasions (on the same sample), incorporating positive controls, with complete concordance of the results. As shown in Tables 2 and 3 and Figure 1, mutations were detected in 7 of 77 patients. As a result, the incidence of N-ras mutations in patients with acute leukemia was 13.3% (9.5% in patients with AML and 14.3% in patients with ALL), in patients with MDS it was 7.4% and in patients with MPD it was 5% (Tables 2 and 3).

N-ras mutations were mainly observed at codons 12, 13 and 61, and the most common type of mutation, that was observed in three patients, was a substitution of glycine by aspartic acid at codon 12 of the gene (Table 2, Figure 1). This type of mutation was observed in three patients with AML, two with *de novo* AML and one after transformation of MDS, with monocytic phenotype of blasts. It is noteworthy that in this last patient the mutation was identified only during the transformation into acute leukemia (17 months after the diagnosis of MDS), in parallel with absence of the expression of the normal allele of the gene (Figure 1B). N-ras mutations at codon 61 (Figure 1) were observed only in two patients with acute leukemia who displayed immunophenotypic markers of lymphoid lineage. Moreover, two novel alterations were identified, a mutation at codon 8 (characterized by a substitution of valine by leucine) (Table 2, Figure 1) in a patient with CML during the hematologic relapse of the disease and a polymorphism at codon 92 (1002T→C,[39] without amino acid substitution) in a patient with chronic myelomonocytic leukemia (CMML) (Table 2, Figure 1). Interestingly, retrospective analysis of the patient with CML using genetic material at diagnosis and 42 months later (when the patient was in hematologic remission, receiving interferon-α treatment) demonstrated absence of mutations.

All the samples previously recorded as being normal by NIRCA were confirmed as being normal on

Table 2. Summary of the N-ras mutations of the patients in this study.

#	*Diagnosis*	*Codon*	*Nucleotide substitution*	*Amino acid substitution*
1	CML	8	GTG → CTG	Val → Leu
2	AML-M4	12	GGT → GAT	Gly → Asp
3	AML-M5	12	GGT → GAT	Gly → Asp
4	CMML -AML-M5	12	GGT → GAT	Gly → Asp
5	RAEB	13	GGT → AGT	Gly → Ser
6	Acute myeloid/NK leukemia	61	CAA → CTA	Gln →Leu
7	ALL-L2	61	CAA → CAT	Gln → His
8	CMML	92	GAT → GAC	Asp → Asp

Abbreviations: CML: chronic myeloid leukemia; CMML: chronic myelomonocytic leukemia; AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; RAEB: refractory anemia with excess of blasts.

Table 3. Clinical and laboratory features of the patients of this study.

	#	Mean age (years) (Std.Dev)	Mean Hb (g/dL) (Std.Dev)	Mean WBC (/10^8/μL) (Std.Dev)	Mean PLT (/10^8/μL) (Std.Dev)	Mean of BM blast (Std.Dev)	Mean of survival (Std.Dev)	Response to CTH CR	Resistant disease	Transformation to AML
Patients with AML	21	56.9 (15.5)	9.5 (2.2)	44354.3 (51652.2)	81285.7 (83549.5)	72.3 (18.7)	10.6 (17.5)	13	8	
with N-ras mutation	2	58.5 (12.0)	10.5 (0.7)	41050 (50841)	59250 (49378.6)	68 (21.7)	23.25 (21.7)	1	1	
without N-ras mutation	19	56.7 (16.1)	9.4 (2.2)	44702.1 (53098.4)	91769.2 (81428.9)	74.5 (17.9)	5.3 (4.4)	12	7	
Patients with ALL and acute biphenotyping leukemia	9	32.1 (21.1)	10.3 (2.8)	15733.3 (13650.3)	90777.8 (68390)	74.6 (13.1)	13.5 (8.9)	5*	1*	
with N-ras mutation	2	47 (9.9)	8.0 (1.4)	14900 (15414.9)	75500 (78488.9)	85 (9.8)	16.5 (8.7)	2	0	
without N-ras mutation	7	27.8 (21.9)	11.0 (2.8)	15971.4 (14440.9)	95142.9 (71480.6)	71.7 (12.9)	7.5 (7.7)	3*	1*	
Patients with MDS	27	66.1 (112.2)	10.3 (2.0)	9678.5 (12617.8)	162074.3 (106635.2)		36 (29.8)			9 (33.3%)
with N-ras mutation	2	69 (9.8)	8.5 (4.9)	16500 (16263.5)	246000 (288500)		14.5 (7.7)			1 (50%)
without N-ras mutation	25	65.8 (11.4)	10.4 (1.7)	9132.8 (12540.7)	155360 (90646.2)		37.7 30.3)			8 (32%)
Patients with MPD	20	50.3 (18.5)	11.2 (1.9)	100363 (75987.2)	548100 (544046)		39.9 (32.2)			7 35%)
with N-ras mutation	1	45	9	205000 -	320000		69			_
without N-ras mutation	19	49.3 (18.5)	11.1 (1.9)	102487 (77456.9)	519053 (542787)		35.3 (25.6)			7 (36.8%)

*Abbreviations: Hb: Hemoglobin, WBC: White Blood Count, PLT: Platelets, BM: Bone marrow, CTH: chemotherapy for acute leukemia, CR: complete remission, AML: acute myeloid leukemia, ALL: acute lymphoblastic leukemia, MDS: myelodysplastic syndromes, MPD: myeloproliferative disorders. #The survival is estimated in months. *Three patients with acute leukemia were not treated in our center and thus were not counted in this analysis.*

direct sequencing, thus demonstrating the absence of false negative results. However, N-ras mutations indicated by NIRCA in two patients with acute leukemia and two patients with MDS were not confirmed by direct sequencing, thus indicating that either this protocol is not immune to false positive results or that the sensitivity of NIRCA might exceed slightly that of direct PCR sequencing. However, as has been described and confirmed by us previously,[37,38] the sensitivity of NIRCA is almost similar to that of direct PCR sequencing (namely between 15-25%). Subsequently, the results of these four samples were eventually considered as false positive, and were included in the ras-negative groups in the statistical analysis. A similar practice has also been followed in similar instances in the literature.[14]

Prognostic significance of mutations

No significant differences were established between the N-ras positive and the N-ras negative groups either in patients with acute leukemia (p=0.663 for response to chemotherapy and p=0.389 for overall survival) or MDS (p=0.564 for progression of the disease and p=0.081 for overall survival). On the other hand, considering that only one patient with MPD exhibited an N-ras mutation, it was not practical to estimate differences between the N-ras positive and the N-ras negative groups.

Discussion

Ras mutations play a fundamental role in the development and progression of hematologic and other malignancies.[3,40,41] In this study, which is the

largest published study of the incidence of N-ras mutations in Greek patients with hematologic malignancies, we found N-ras mutations in 7 of 77 patients analyzed. Mutations were identified both in patients with acute leukemia and preleukemic states, namely MDS and MPD, indicating that ras mutations could be either an early or a late event in the multistep procedure of leukemogensis. Moreover, one patient with CMML (case 4 in Table 2) displayed the N-ras mutation at transformation of the disease to acute leukemia, and not during the MDS phase. Thus, we can speculate that the N-ras mutation was the causative event of transformation. Moreover, absence of expression of the normal allele of N-ras was established in the same patient, in parallel with the presence of mutation (Figure 1B). This event is infrequent, although it has been demonstrated in patients with leukemia.[42] However, we detected N-ras mutations in patients in preleukemic states (MDS and MPD) which did not progress eventually into overt leukemia. Additionally, ras mutational activation has been described in hematologically normal individuals after or without exposure to cytotoxic chemotherapy or occupational exposure to petrochemicals.[43-47] Thus, we can speculate that the activation of N-ras gene alone is not sufficient to cause malignant disease and additional molecular lesions are required for the full malignant phenotype. Furthermore, in a patient with CML a novel mutation at codon 8 was identified. Although N-ras mutations were mainly observed at codons 12, 13 and 61, several studies have demonstrated mutations at adjacent codons, such as 11,[4] 15,[48] 59,[49] and 60.[17] It is obvious that NIRCA provides the capability to detect such mutations, and this is an important advantage of the method. Moreover, the codon 8 is located in a domain (constituting amino acid residues 5-22) of the N-ras gene with guanine nucleotide (GTP/GDP) capacity.[50] Thus, although the precise role of codon 8 mutations has not been estimated until now, we can speculate that such mutations would result in conformational change of the $p21^{ras}$ to its active state, resulting in a constitutively activated downstream signal cascade and oncogenic transformation.

Several techniques have been used to analyze the N-ras oncogene but they have several limitations and vary in their sensitivity and reliability. The most widely used method is amplification of genomic DNA by PCR followed by oligonucleotide hybridization; however this can yield ambiguous signals, since it cannot reveal possible mutations in adjacent codons. In this study we used RT-PCR to amplify the N-ras gene and NIRCA in order to detect the possible point mutations in patients with hematologic malignancies. RT-PCR/NIRCA offers several advantages over previous techniques: it is inexpensive, reproducible and non-radioactive, it does not require special equipment, and results can be obtained at latest within 1-2 days. Additionally, NIRCA detects the entire amplified portion of the gene and therefore novel alterations can be detected, as occurred in two patients of our study (cases 1 and 8 of Table 2). In conclusion, RT-PCR/NIRCA allows inexpensive and rapid screening of N-ras mutations, and could be used as the first step selection of samples that can be further analyzed using a direct approach, such as sequencing, which will accurately identify the type of alteration. Thus, this method could make N-ras gene study possible in every day clinical practice in patients with hematologic malignancies.

It is noteworthy that in our study all the patients with AML carrying N-ras mutations displayed both the same type of mutation (a G→A transition at the second nucleotide of codon 12) and monocytic phenotype of blasts (Table 2). Although there was no evidence of an association of N-ras activation with a particular subtype of AML in some studies,[15,16,18] several studies displayed a relationship between the presence of N-ras mutations and monocytoid features in leukemic cells.[11,17] Furthermore, Maher *et al.* established that a number of hematopoietic cell lines undergo spontaneous monocytic differentiation in response to mutant ras expression,[51] while Yunis *et al.* found that the MDS patients carrying an N-ras mutation have an abnormal monocytic component at diagnosis or during disease evolution.[10] Conclusively, it is very possible that in myeloid disorders an N-ras mutation may be preferentially associated with myelomonocytic differentiation.

N-ras mutations have been previously described in ALL patients. The most commonly identified genetic alteration in these studies involved G→A transitions in codons 12 and 13 of exon 1.[19-21] Interestingly, the two mutations we identified among the patients with immunophenotypic markers of lymphoid lineage displayed mutation in codon 61 of exon 2 (cases 6 and 7 of Table 2). It would be interesting to evaluate whether this apparent difference could be due to the fact that the patients we analyzed were adults, whereas the patients analyzed previously were primarily pediatric cases. It is possible that, as in AML patients,[52,53] a difference might exist in the incidence and the type of ras mutations between pedi-

atric and adult patients with ALL. Additionally, mutations at codon 61 of the N-ras proto-oncogene have been described in melanoma, a malignant disorder presumably induced by ultraviolet exposure in adult patients;[54] thus, it is possible that different environmental exposures of particular primitive hematopoietic cells could play a role in these age differences.

In conclusion, it is possible that some of the uncertainties concerning the diagnostic and prognostic value of ras mutations in patients with hematologic malignancies are due to the relatively small number of the patients so far detected as carrying mutations. The RT-PCR/NIRCA protocol described herein could overcome this problem and could allow the study of this proto-oncogene in every day clinical practice, thus facilitating the validation of the diagnostic and prognostic value of N-ras mutational analyses in patients with hematologic malignancies.

Contributions and Acknowledgments

MS was primarily responsible for this work, from conception to submitted manuscript, he wrote the paper and he should be considered as the principal author. The remaining authors qualified for authorship according to the World Association of Medical Editors (WAME) criteria, and have taken specific responsibility for the following parts of the content: KA, EP and PT: participants in laboratory experiments; DJ and VT: participants in the collection of clinical data; CA: performed the statistical analysis; GK and PS: optimization of experimetnal procedures and revising the article critically for important intellectual content. The authors are listed according to a criterion of decreasing individual contribution to the work, with the following exception: the last author, KR had a major role as senior author in designing the study and interpreting the data.

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Potential implications for clinical practice

The assay described allows rapid mutational analysis of N-ras proto-oncogene in clinical practice. This may in turn lead to identification of any diagnostic or prognostic implication.[55-58]

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