Original Article
Diagnostic Hematology

Ann Lab Med 2013;33:159-166
http://dx.doi.org/10.3343/alm.2013.33.3.159
ISSN 2234-3806 • eISSN 2234-3814

ANNALS OF LABORATORY MEDICINE

N-ras Mutation Detection by Pyrosequencing in Adult Patients with Acute Myeloid Leukemia at a Single Institution

Ji Hun Jeong, M.D.[1], Soon Ho Park, M.D.[1], Mi Jung Park, M.D.[1], Moon Jin Kim, M.D.[1], Kyung Hee Kim, M.D.[1], Pil Whan Park, M.D.[1], Yiel Hea Seo, M.D.[1], Jae Hoon Lee, M.D.[2], Jinny Park, M.D.[2], Junshik Hong, M.D.[2], and Jeong Yeal Ahn, M.D.[1]

Departments of Laboratory Medicine[1] and Medical Oncology and Hematology[2], Gachon University Gil Medical Center, Incheon, Korea

Background: *N-ras* mutations are one of the most commonly detected abnormalities of myeloid origin. *N-ras* mutations result in a constitutively active N-ras protein that induces uncontrolled cell proliferation and inhibits apoptosis. We analyzed *N-ras* mutations in adult patients with AML at a particular institution and compared pyrosequencing analysis with a direct sequencing method for the detection of *N-ras* mutations.

Methods: We analyzed 90 bone marrow samples from 83 AML patients. We detected *N-ras* mutations in codons 12, 13, and 61 using the pyrosequencing method and subsequently confirmed all data by direct sequencing. Using these methods, we screened the *N-ras* mutation quantitatively and determined the incidence and characteristic of *N-ras* mutation.

Results: The incidence of *N-ras* mutation was 7.2% in adult AML patients. The patients with *N-ras* mutations showed significant higher hemoglobin levels ($P=0.022$) and an increased incidence of *FLT3* mutations ($P=0.003$). We observed 3 cases with *N-ras* mutations in codon 12 (3.6%), 2 cases in codon 13 (2.4%), and 1 case in codon 61 (1.2%). All the mutations disappeared during chemotherapy.

Conclusions: There is a low incidence (7.2%) of *N-ras* mutations in AML patients compared with other populations. Similar data is obtained by both pyrosequencing and direct sequencing. This study showed the correlation between the *N-ras* mutation and the therapeutic response. However, pyrosequencing provides quantitative data and is useful for monitoring therapeutic responses.

Key Words: *N-ras*, AML, Pyrosequencing, Bone marrow

Received: September 7, 2012
Revision received: November 13, 2012
Accepted: January 29, 2013

Corresponding author: Jeong Yeal Ahn
Department of Laboratory Medicine,
Gachon University Gil Medical Center,
21 Namdong-daero 774beon-gil,
Namdong-gu, Incheon 405-760, Korea
Tel: +82-32-460-3863
Fax: +82-32-460-3415
E-mail: jyahn@gilhospital.com

INTRODUCTION

The *N-ras* oncogene is a member of a family of membrane-associated proteins that instigate signal transduction upon ligand binding to a variety of membrane receptors. N-ras proteins regulate cell proliferation, differentiation, and apoptosis by cycling between active GTP-bound and inactive GDP-bound conformations [1, 2]. The most common mutations involve a single base substitution in codon 12, 13, or 61 and lead to constitutively active Ras. In its constitutively active, GTP-bound state, Ras induces uncontrolled cell proliferation and inhibits apoptosis [1, 2]. *N-ras* mutations have been identified in various solid tumors as well as hematologic malignancies [2]. The prognostic significance of *N-ras* mutations is still unclear. Some groups have pro-

posed that *Ras* mutations are related to a better prognosis [3], whereas others have proposed that mutated *Ras* genes are associated with short survival [4], and many others have been unable to verify the clinical significance of *Ras* mutations [5-13].

Most recent studies examining the presence of *Ras* mutations in cancer cells have been performed using PCR followed by dideoxy DNA sequencing or conformation-based separation such as single strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis, restriction fragment length polymorphism (RFLP), or HPLC [6, 8-11]. However, these techniques are labor intensive and time consuming and are therefore not suitable for routine clinical practice. Furthermore, it has been reported that SSCP analysis gives false-negative results in approximately 33% of cases identified as positive [14]. Sanger sequencing is the most commonly used sequencing method; however, it does not detect some mutant sequences that are present in the background of abundant wild-type DNA sequences. Compared with dideoxy sequencing, pyrosequencing has greater analytical sensitivity for the detection of mutant DNA mixed with wild-type DNA. Pyrosequencing is a simple, real-time, non-electrophoretic, high-throughput mutation detection assay that can be applied to large clinical studies [15-17]. However, the practical aspects and efficiency of pyrosequencing for the analysis of *N-ras* mutations have not been evaluated in clinical settings.

The purpose of this study was to detect *N-ras* mutations in 83 Korean adult patients with AML and to compare the performance of a pyrosequencing kit to a direct sequencing method for the detection of *N-ras* mutations.

METHODS

1. Patients

Eighty-three adult patients that attended the Hematology and Oncology Department, Gachon University Gil Medical Center, Incheon, Korea from May 2004 to February 2011 were enrolled to analyze the biological and prognostic impact of *N-ras* mutations. The median age of the study population was 55.0 yr (range: 20.0-88.0 yr). A large proportion (43.4%; 36 patients) of patients were older than 65 yr. The male-to-female ratio was 1.3:1 (47 men: 36 women). All patients were newly diagnosed with AML, except some patient with t(15;17) who were diagnosed with acute promyelocytic leukemia (APL). Informed consent for tissue collection and the research studies was approved by the Institutional Review Board of Gachon University Gil Medical Center. The patients were observed from May 2004 to January 2012. Bone marrow (BM) aspirates and biopsy specimen slides were classified into AML subtypes according to the WHO (2008) classifications [18]. The following clinical characteristics were analyzed at diagnosis: age, sex, hemoglobin, peripheral white blood cell (WBC) count, platelet count, serum lactate dehydrogenase (LDH) concentration, cytogenetic findings, and percentage of blasts in BM aspirates. After the induction and consolidation of chemotherapy, the patients were reevaluated for clinical and cytological findings of leukemia or treatment response. Complete remission (CR) was defined as a normocellular BM containing fewer than 5% blasts.

Of the 83 patients, 58 were treated with a standard protocol, 45 patients received only standard chemotherapy, and 13 patients were treated with standard chemotherapy as well as BM transplantation (BMT). Due to the physical condition of the older patients, 10 patients were given palliative low-dose 1-β-D-arabinofuranosylcytosine (Ara-C) treatment only. During the course of this study, 7 patients were transferred to other hospitals or were lost to follow-up therapies. Another 8 patients were denied therapy.

2. Cytogenetic studies

Chromosomal analysis was performed on the BM aspirates by G-banding. Karyotypes were designated according to the International System for Human Cytogenetic Nomenclature (2009) [19].

3. Detection of somatic mutations by PCR

RNA from the BM aspirates was extracted using the Qiagen RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. After reverse transcription PCR using the Pure Extreme kit (MBI Fermentas, St Leon-Rot, Germany), gene rearrangements were analyzed using Seeplex Leukemia PML/RARa, AML/ETO and BCR/ABL kits (Seegene, Seoul, Korea). DNA from the BM aspirates was isolated using the QIAamp DNA blood mini kit (Qiagen). *FLT3* mutations were confirmed using a Seeplex *FLT3* Genotyping kit (Seegene). These *FLT3* mutations were classified as internal tandem duplication (ITD) mutations or mutation in the tyrosine kinase domain (TKD).

4. Detection of *N-ras* mutations by pyrosequencing and direct sequencing

The sample was collected from each patient at diagnosis, and follow-up samples were collected in 6 patients with mutations. We analyzed 90 BM samples from 83 AML patients to detect *N-ras* mutations in codons 12, 13, and 61 using the pyrosequencing; all data were subsequently confirmed by direct sequencing.

http://dx.doi.org/10.3343/alm.2013.33.3.159

DNA from the BM aspirates was prepared using the QIAamp DNA blood mini kit (Qiagen). Mutations in the *N-ras* were detected by pyrosequencing using a TheraScreen NRAS Pyro kit (Qiagen). PCR templates for pyrosequencing were amplified from genomic DNA (10 ng) using the Pyro Mark PCR Master mix (Qiagen) and 5 pmol of each primer in a total reaction volume of 25 μL. Following PCR amplification, the biotinylated PCR products were placed in 24-well plates and bound to streptavidin-coated sepharose beads (GE Healthcare, Piscataway, NJ, USA). The PCR products were denatured, and the non-biotinylated fragments were washed from the beads using the Pyromark Q24 Vacuum Workstation (Qiagen). The beads were then resuspended in annealing buffer (24.2 μL) containing 0.4 pmol of the sequencing primer. Pyrosequencing was performed using the Pyro Gold Q24 reagents (Qiagen), using dispensations based on the target sequence with the Pyromark Q24 system. Raw data files were imported into Pyromark Q24 software (version 2.0; Qiagen) for further analysis following pyrosequencing.

The pyrosequencing data were confirmed by direct sequencing (Macrogen, Seoul, Korea). For the direct sequencing, 100 ng of genomic DNA from the patients was used for PCR with AccuPower PCR PreMix (Bioneer, Seoul, Korea). An initial preheating at 94℃ for 10 min was followed by denaturation at 94℃ for 40 sec, 58℃ for 40 sec, and 72℃ for 40 sec for 35 cycles and a final extension at 72℃ for 7 min. The primer sets used were forward primer 5′-GTGAGGCCGATATTAATCCG-3′ and reverse primer 5′-ACAGTCACGCTACTATGGCC-3′ for exon 1 to amplify codons 12 and 13 and forward primer 5′-GTTATAGATGGTGAAACCTG-3′ and reverse primer 5′-GCTCTATCTTCCCTAGTGTG-3′ for exon 2 to amplify codon 61. The PCR products were analyzed by direct sequencing using the exon 1 forward primer and the exon 2 reverse primer (Fig. 1).

5. Statistical analysis

The frequency of clinical characteristics was analyzed by Pearson's χ^2 test. Differences in mean variables were analyzed by Mann-Whitney statistics. Cross tabulation was used in comparison between direct sequencing and pyrosequencing. All statistical analyses were performed using the SPSS software package version 17.0 (SPSS Inc., Chicago, IL, USA). For all the analyses, P values <0.05 were considered statistically significant.

RESULTS

The incidence of mutation in the *N-ras* gene was 7.2% in the AML patients. Mann-Whitney statistics revealed that the group of patients with *N-ras* mutations (6 patients) showed higher hemoglobin levels ($P=0.022$), and a higher prevalence rate of *FLT3* mutations in patients with *N-ras* mutations was demonstrated by the Pearson's χ^2 test ($P=0.003$). Among the 83 patients, 15 patients had *FLT3* mutations (18.1%) and 13 of them showed ITD mutations. Two patients had TKD mutations. The incidence of *FLT3* mutations in patients with wild-type *N-ras* was 16.9% (13 of 77 patients) and 33.3% (2 of 6 patients) in patients with the *N-ras* mutation (Table 1). Other factors were not statistically correlated with the presence of *N-ras* mutations (Table 1). All patients with mutations were diagnosed with *de novo*

Table 1. Characteristics of AML patients with or without *N-ras* mutations

	With *N-ras* mutation median (range)	Without *N-ras* mutation median (range)	*P* value
Age (yr)	56.5 (47.0-81.0)	55.0 (20.0-88.0)	0.440
Hb (g/dL)	10.1 (8.4-12.5)	8.2 (3.0-14.2)	0.022
WBC ($\times 10^9$/L)	28.9 (2.3-102.1)	17.4 (210.0-541.0)	0.669
PLT ($\times 10^9$/L)	70.5 (44.0-124.0)	53.0 (5.0-848.0)	0.750
Blast in BM (%)	61.0 (18.4-92.6)	68.0 (14.8-96.0)	0.823
LDH (IU/L)	1,193.5 (397.0-2,712.0)	895.0 (234.0-15,000.0)	0.812
FLT3 mutation (incidence, %)	2 (2/6, 33.3%)	13 (13/77, 16.9%)	0.003

Abbreviations: WBC, white blood cell; PLT, platelet; BM, bone marrow; LDH, lactose dehydrogenase; *FLT3*, FMS-like tyrosine kinase 3.

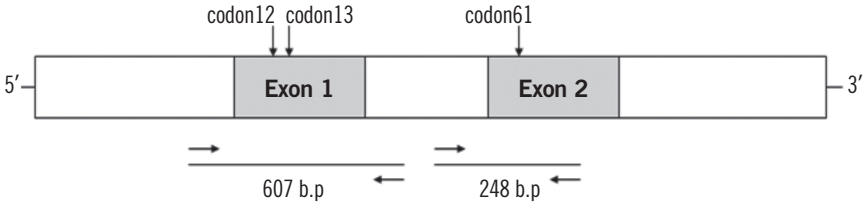

Fig. 1. Schematic representation of the location of the direct sequencing primers on the *N-ras* codons 12, 13, and 61. Codons 12 and 13 are located in exon 1, and codon 61 is in exon 2. For the detection of mutations in codon 12 and 13, PCR products of 607 nucleotides were analyzed. Mutations in codon 61 were detected by sequencing the PCR product of exon 2 (248 nucleotides).

AML. Five of 6 patients were diagnosed with AML, not otherwise specified (AML, NOS) and 4 of these 6 patients had normal karyotypes (Tables 2, 3).

We observed 3 cases with mutated *N-ras* codon 12 (3.6%), 2 cases with mutated codon 13 (2.4%), and 1 case with mutated codon 61 (1.2%). None of the patients with mutations showed homozygous mutations. The base substitution in codon 12 subsequently led to an amino acid change from wild-type glycine to serine, cysteine, or aspartic acid (G12S, G12C, and G12D). Mutations at codon 13 also induced an amino acid substitution of wild-type glycine to aspartic acid or arginine (G13D and G13R). The mutation in codon 61 caused the glutamine to arginine substitution (Q61R).

We performed pyrosequencing and direct sequencing for all 83 patients. The BM study was performed at initial diagnosis, and the patients who received chemotherapy were re-evaluated for evidence of leukemia or CR after chemotherapy. Seven *N-ras* mutations were detected in 6 cases (7.2%) by both sequencing methods, and there was no difference in the detection rate (Fig. 2). The percentage of *N-ras* mutant DNA assayed for codon 12, 13, and 61 mutations by pyrosequencing was 23%-49% (Table 3). In 5 patients, the *N-ras* mutation disappeared

Table 2. History of AML and cytogenetics

	N of patients (%)	With *N-ras* mutation (%)	Without *N-ras* mutation (%)
Type of AML			
De novo	78 (94.0)	6 (7.7)	72 (92.3)
s-AML	1 (1.2)	0 (0.0)	1 (100.0)
t-AML	4 (4.8)	0 (0.0)	4 (100.0)
Total	83 (100.0)	6 (7.2)	77 (92.8)
Cytogenetic subgroup			
Recurrent	12 (14.4)	0 (0.0)	12 (100.0)
Normal	42 (50.6)	4 (9.5)	38 (90.5)
Complex	11 (13.3)	0 (0.0)	11 (100.0)
Other	18 (21.7)	2 (11.1)	16 (88.9)
Total	83 (100.0)	6 (7.2)	77 (92.8)
WHO (2008) subtype			
AML, NOS	47 (56.6)	5 (10.6)	42 (89.4)
AML with recurrent genetic abnormalities	12 (14.5)	0 (0.0)	12 (100.0)
AML with MRC	20 (24.1)	1 (5.0)	19 (95.0)
t-AML	4 (4.8)	0 (0.0)	4 (100.0)
Total	83 (100.0)	6 (7.2)	77 (92.8)

Abbreviations: s-AML, secondary AML; t-AML, therapy related AML; AML with MRC, AML with myelodysplasia-related changes; AML, NOS, AML, not otherwise specified.

Table 3. Characteristics of AML patients with *N-ras* mutation and sequencing results

Sample No.	Age	Sex	*N-ras* Mutation	Direct sequencing	Pyro-sequencing	WHO classification	Karyotype	*N-ras* Mutation after induction CTx	*FLT3* mutation	Clinical course	Survival period after diagnosis
N-ras codon12 (incidence: 3.6%)											
34	59	M	G12S	G/A GT	G: 50%, A: 49%	AML with MRC	46,XY[20]	Not detected	Not detected	Follow up loss	
49	52	M	G12C	G/T GT	G: 48%, T: 49%	AML, NOS (Acute myelomonocytic leukemia)	46,XY[20]	Not done	Not detected	Deceased	3 days
71	81	F	G12D	G G/A T	G: 55%, A: 42%	AML, NOS (Acute monocytic leukemia)	46,XX[20]	Not done	D835Y	Deceased	1 month
N-ras codon13 (incidence: 2.4%)											
33	54	F	G13D	G G/A T	G: 64%, A: 36%	AML, NOS (Acute myeloid leukemia without maturation)	46,XX,der(16) t(1;16) (q21;q12.1) [14]/46,XX[6]	Not detected	Not detected	Deceased	3 months
46	67	M	G13R	G/C GT	G: 61%, C: 39%	AML, NOS (Acute myeloid leukemia with maturation)	46,XY,del(9) (q21q22) [25]/46,XY[5]	G13R* (G: 67%, C: 33%)	Not detected	Alive, complete remission	23 months
N-ras codon 61 (incidence: 1.2%)											
58	47	F	Q61R	C A/G A	A: 77%, G: 23%	AML, NOS (Acute myeloid leukemia with maturation)	46,XX[20]	Not detected	D835Y	Alive, induction failure, allo-BMT	14 months

*After induction chemotherapy, the *N-ras* mutation in codon 13 persisted and the mutated allele burden was similar to that at initial diagnosis.
AML, NOS, AML, not otherwise specified.
Abbreviations: CTx, chemotherapy; BMT, bone marrow transplantation; AML with MRC, AML with myelodysplasia-related changes; D835Y, *FLT3* kinase domain point mutation.

http://dx.doi.org/10.3343/alm.2013.33.3.159

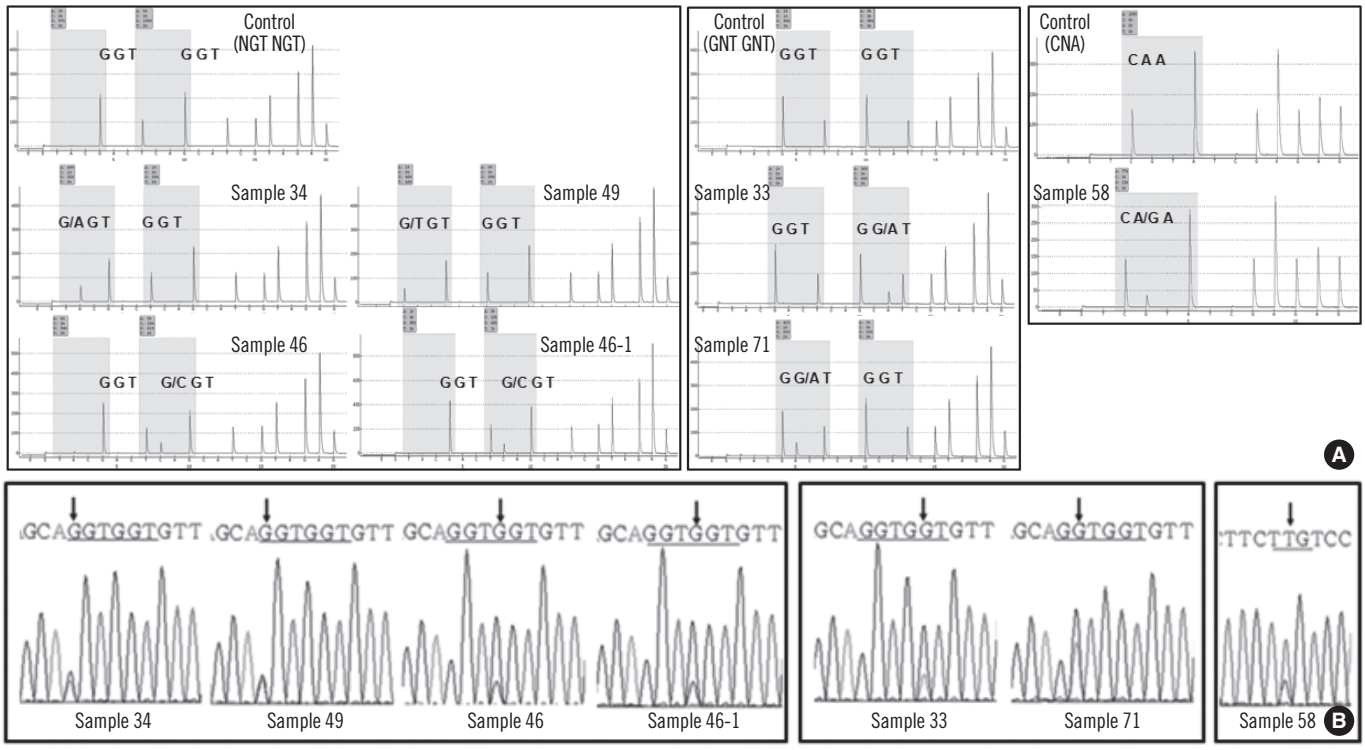

Fig. 2. *N-ras* mutations established by pyrosequencing and confirmed by direct sequencing. (A) Pyrogram showing the presence of mutation and quantitative data of mutated clones. The horizontal axis refers to the base, and the vertical axis represents the intensity of the fluorescent signal. The shaded regions represent the analyzed codon. The first box shows the analytic results of substitutions in the first base of the *N-ras* codons 12 and 13. The second box presents mutations in the second base of the *N-ras* codons 12 and 13. The third box shows a positive result for a mutation in the second base of codon 61. (B) All patient samples were confirmed by direct sequencing, and the plots showed the same results as pyrosequencing.

from the BM after the induction chemotherapy, and morphological remission in the BM was also evident. Although there was no evidence of leukemic blasts in the BM, the *N-ras* mutation in codon 13 persisted after the induction therapy in 1 patient (sample no. 46). In this patient, the mutated allele burden was similar to that at initial diagnosis (initial, 39%; after induction chemotherapy, 33%) (Table 3), and the mutation disappeared after the first consolidation therapy. Of these 6 patients, 4 died, 1 was associated with treatment failure, and only 1 patient reached CR after BMT (Table 3).

DISCUSSION

Here, we studied the incidence of *N-ras* mutations in adult patients with AML. The incidence of *N-ras* mutation was 7.2% in the adult patients with AML examined at a single Korean institution. Recent studies have shown that *N-ras* mutation is present in AML patients in the range of 7.03-20.26%, and this value is reduced in Asian countries [6-13] (Table 4).

Compared to the total patient pool in this study, the patients with *N-ras* mutations (6 patients) showed higher hemoglobin levels ($P=0.022$) and a higher prevalence of *FLT3* mutations ($P=0.003$). Other factors did not correlate with the presence of *N-ras* mutations.

We identified both *N-ras* mutation and *FLT3*-TKD mutation in 2 patients that presented with normal karyotypes. Whitman et al. [20] reported that younger adults with *de novo* CN-AML and *FLT3*-TKD mutations in D835/I836 are associated with worse outcome than those without *FLT3*-TKD mutations. A previous report by Illmer et al. [21] stated that AML patients with high Ras activity were highly sensitive to high doses of Ara-C and that Ras activity may overcome the poor prognosis in younger patients even in the presence of *FLT3* mutations. In our study, case 71 had both *N-ras* mutation and *FLT3*-TKD mutation, and she was treated with palliative low doses of Ara-C because of her old age (81 yr old); she passed away 1 month after diagnosis. The other patient with both mutations (sample no. 58) who was younger (47 yr old) showed induction failure and demonstrated CR after a high dose of Ara-C treatment and allogenic BMT. These findings are consistent with the conjecture that ag-

Table 4. Recent *N-ras* mutation studies in AML patients

Study	N of patients	Mutation incidence (%)	Prognostic effect	Median age of patients (yr [range])	Analysis method	Study population
Ritter et al. (2004) [7]	232	20.26	Not significant	55 (18-77)	PNA-based technique	Germany
Bowen et al. (2005) [6]	1,106	11.00	Not significant	<60	DHPLC, RFLP, Direct sequencing	UK
Bacher et al. (2006) [5]	2,502	10.30	Not significant	63.4 (18.3-91.8)	Melting curve analysis	Germany
Schlenk et al. (2008) [9]	872	13.00	Not significant	48 (16-60)	Direct sequencing	Germany
Stirewalt et al. (2009) [8]	140	19.00	Not significant	67 (55-88)	SSCP	USA
Ishikawa et al. (2009) [10]	144	7.60	Not significant	52 (15-80)	Direct sequencing	Japan
Shen et al. (2011) [13]	1,185	7.03	Not significant	Not shown	Chip based MALDI-TOF MS	China
Rockova et al. (2011) [12]	439	9.79	Not significant	43 (15-60)	Heteroduplex analysis	Netherland
Patel et al (2012) [11]	398	10.00	Not significant	46.5 (18-60)	Direct sequencing	USA

Abbreviations: PNA, peptide nucleic acid; DHPLC, denaturing high performance liquid chromatography; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism analysis; MALDI-TOF MS, matrix-assisted laser desorption/ionisation-time of flight mass spectrometry.

gressive treatments are required for the patients with *N-ras* and *FLT3*-TKD mutations.

In this study, all *N-ras* mutations were observed in *de novo* AML, but 94.0% of the enrolled patients were diagnosed with *de novo* AML. Therefore, the result could not reflect a relationship between the subtype of AML and the incidence of *N-ras* mutation. Two of the 6 patients were diagnosed with acute myelomonocytic/monocytic leukemia. Several studies have reported that *N-ras* mutations promote myeloid maturation defects with relative sparing of the monocyte lineage. In addition, *Ras* mutations are particularly prevalent in leukemia and preleukemia with monocytic characteristics [3, 5, 6, 22].

All point mutations in *N-ras* induced an amino acid change. In 3 patients, a G→A base substitution in codon 12 and 13 was detected; this is the most commonly reported base substitution in *Ras* mutations in AML [23, 24]. The glycine residue located in position 12 (Gly12) lies close to the "finger loop" of the GTPase activating proteins (GAP) that complements the active site of Ras. Any mutation at this position results in sterical interference with the geometry of the transition state, in which GTP is hydrolyzed in the presence of GAP fingers [1]. It has been reported that G12D shortens disease latency rather than contributing to tumorigenesis [25, 26]. We found that 2 of 3 patients with *N-ras* mutations in codon 12 died within 1 month after diagnosis; one of them had a G12D mutation. The other patient revealed re-induction failure and was lost to follow up (Table 3).

All *N-ras* mutations disappeared during chemotherapy, thereby suggesting a relationship between the presence of *N-ras* mutation and disease status. In one patient (case 46), leukemic blasts were not detected in BM, and chromosomal abnormalities present at initial diagnosis (46,XY,del(9)(q21q22)[25]/46,XY[5]) also disappeared after the induction chemotherapy. Nevertheless, the *N-ras* mutation persisted after the induction chemotherapy, and the level of the mutated gene (33%) was similar to the level at initial diagnosis (39%) (Table 3). After first consolidation chemotherapy, direct sequencing and pyrosequencing in case 46 did not detect the *N-ras* mutation. In the study by Bacher et al. [5], they concluded that *N-ras* mutations were not a good marker for follow-up control [5]. However, our study shows that monitoring *N-ras* mutations can be a more sensitive indicator of therapeutic response than morphologic or conventional cytogenetic studies.

In this study, pyrosequencing and direct sequencing showed same efficiencies with respect to their ability to detect mutations. During the therapeutic period, the number of leukemic cells decreases, and, in some cases, it is difficult to discriminate these cells from normal early precursor cells. A low limit of detection (LOD) of pyrosequencing is important in cases with rare mutations as well as in follow-up studies for early detection of relapse in patients with AML [17, 27]. Athanasios et al. [16] determined that the LOD for Sanger sequencing, pyrosequencing, and melting curve analysis is approximately 15-20%, 5%, and 10% mutant alleles, respectively. Another advantage of pyrosequencing is that it can read a starting sequence from the first nucleotide adjacent to a pyrosequencing primer without a noise signal. In addition, pyrosequencing is a simple, one-step method that is efficient because 24 samples can be analyzed in <40 min. Pyrosequencing is a simple method compared with Sanger sequencing and does not require a separate purification step or gel electrophoresis after PCR. However, there is a limitation in pyrosequencing kits because only a few positions (≤40-50 nucleotides) can be simultaneously analyzed, and it costs more

http://dx.doi.org/10.3343/alm.2013.33.3.159

than other methods (100,000-150,000 KRW/sample) [17, 28, 29].

In our study, the numbers of patients and *N-ras* mutation cases were too small to make any statistical conclusions. In addition, because the patients received different treatments, it was difficult to compare the outcome and prognostic effect of the *N-ras* mutations. Many factors may contribute to the clinical outcome associated with the presence of *N-ras* mutations, and our analysis investigated only a few of them.

This study is important because there are no recent reports analyzing the presence of *N-ras* mutations in the Korean patients with hematologic malignancies. This study identified the incidence of *N-ras* mutations (7.2%) in patients with AML at a single Korean institution and revealed the relationship between the presence of *N-ras* mutation and the therapeutic response. We also evaluated the pyrosequencing kit for *N-ras* mutation detection as a confirmatory test to identify specific mutations. The same efficiency with respect to the detection of *N-ras* mutations was determined for pyrosequencing and direct sequencing. Because pyrosequencing provides quantitative data and information regarding the mutation type, it is a useful tool for detecting malignant cells and for following patients' progress. Finally, it may be appropriate for large-scale applications in clinical settings for the analysis of hematologic malignancies.

Authors' Disclosures of Potential Conflicts of Interest

The QIAamp DNA blood mini kits, TheraScreen NRAS Pyro kits, and the expenses of direct sequencing were supported by the QIAGEN Korea and Samil Pharmacy & Trading Company.

Acknowledgement

The authors thank Ki Hoon Sung, M.D. for his assistance in statistical analysis.

REFERENCES

1. Malumbres M and Barbacid M. RAS oncogenes: the first 30 years. Nat Rev Cancer 2003;3:459-65.
2. Karnoub AE and Weinberg RA. Ras oncogenes: split personalities. Nat Rev Mol Cell Biol 2008;9:517-31.
3. Neubauer A, Dodge RK, George SL, Davey FR, Silver RT, Schiffer CA, et al. Prognostic importance of mutations in the ras proto-oncogenes in de novo acute myeloid leukemia. Blood 1994;83:1603-11.
4. De Melo MB, Lorand-Metze I, Lima CS, Saad ST, Costa FF. N-ras gene point mutations in Brazilian acute myelogenous leukemia patients correlate with a poor prognosis. Leuk Lymphoma 1997;24:309-17.
5. Bacher U, Haferlach T, Schoch C, Kern W, Schnittger S. Implications of NRAS mutations in AML: a study of 2502 patients. Blood 2006;107:3847-53.
6. Bowen DT, Frew ME, Hills R, Gale RE, Wheatley K, Groves MJ, et al. RAS mutation in acute myeloid leukemia is associated with distinct cytogenetic subgroups but does not influence outcome in patients younger than 60 years. Blood 2005;106:2113-9.
7. Ritter M, Kim TD, Lisske P, Thiede C, Schaich M, Neubauer A. Prognostic significance of N-RAS and K-RAS mutations in 232 patients with acute myeloid leukemia. Haematologica 2004;89:1397-9.
8. Stirewalt DL, Kopecky KJ, Meshinchi S, Appelbaum FR, Slovak ML, Willman CL, et al. FLT3, RAS, and TP53 mutations in elderly patients with acute myeloid leukemia. Blood 2001;97:3589-95.
9. Schlenk RF, Döhner K, Krauter J, Fröhling S, Corbacioglu A, Bullinger L, et al. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. N Engl J Med 2008;358:1909-18.
10. Ishikawa Y, Kiyoi H, Tsujimura A, Miyawaki S, Miyazaki Y, Kuriyama K, et al. Comprehensive analysis of cooperative gene mutations between class I and class II in de novo acute myeloid leukemia. Eur J Haematol 2009;83:90-8.
11. Patel JP, Gönen M, Figueroa ME, Fernandez H, Sun Z, Racevskis J, et al. Prognostic relevance of integrated genetic profiling in acute myeloid leukemia. N Engl J Med 2012;366:1079-89.
12. Rockova V, Abbas S, Wouters BJ, Erpelinck CA, Beverloo HB, Delwel R, et al. Risk stratification of intermediate-risk acute myeloid leukemia: integrative analysis of a multitude of gene mutation and gene expression markers. Blood 2011;118:1069-76.
13. Shen Y, Zhu YM, Fan X, Shi JY, Wang QR, Yan XJ, et al. Gene mutation patterns and their prognostic impact in a cohort of 1185 patients with acute myeloid leukemia. Blood 2011;118:5593-603.
14. Miyauchi J, Asada M, Sasaki M, Tsunematsu Y, Kojima S, Mizutani S, et al. Mutations of the N-ras gene in juvenile chronic myelogenous leukemia. Blood 1994;83:2248-54.
15. Ahmadian A, Ehn M, Hober S. Pyrosequencing: history, biochemistry and future. Clin Chim Acta 2006;363:83-94.
16. Tsiatis AC, Norris-Kirby A, Rich RG, Hafez MJ, Gocke CD, Eshleman JR, et al. Comparison of Sanger sequencing, pyrosequencing, and melting curve analysis for the detection of KRAS mutations: diagnostic and clinical implications. J Mol Diagn 2010;12:425-32.
17. Ogino S, Kawasaki T, Brahmandam M, Yan L, Cantor M, Namgyal C, et al. Sensitive sequencing method for KRAS mutation detection by Pyrosequencing. J Mol Diagn 2005;7:413-21.
18. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, et al. eds. WHO classification of tumours of haematopoietic and lymphoid tissues. 4th ed. Lyon: IARC, 2008:110-55.
19. Shaffer LG, Slovak ML, Campbell LJ, eds. ISCN 2009: An international system for cytogenetic nomenclature (2009). Basel: S Karger, 2009.
20. Whitman SP, Ruppert AS, Radmacher MD, Mrózek K, Paschka P, Langer C, et al. FLT3 D835/I836 mutations are associated with poor disease-free survival and a distinct gene-expression signature among younger adults with de novo cytogenetically normal acute myeloid leukemia lacking FLT3 internal tandem duplications. Blood 2008;111:1552-9.
21. Illmer T, Thiede C, Fredersdorf A, Stadler S, Neubauer A, Ehninger G, et al. Activation of the RAS pathway is predictive for a chemosensitive phenotype of acute myelogenous leukemia blasts. Clin Cancer Res 2005;11:3217-24.
22. Darley RL and Burnett AK. Mutant RAS inhibits neutrophil but not macrophage differentiation and allows continued growth of neutrophil precursors. Exp Hematol 1999;27:1599-608.
23. Speletas M, Arvanitidi K, Tzoanopoulos D, Tsironidou V, Pardali E, Ag-

geli C, et al. Rapid mutational analysis of N-ras proto-oncogene in hematologic malignancies: study of 77 Greek patients. Haematologica 2001;86:918-27.
24. Auewarakul CU, Lauhakirti D, Tocharoentanaphol C. Frequency of RAS gene mutation and its cooperative genetic events in Southeast Asian adult acute myeloid leukemia. Eur J Haematol 2006;77:51-6.
25. Tyner JW, Erickson H, Deininger MW, Willis SG, Eide CA, Levine RL, et al. High-throughput sequencing screen reveals novel, transforming RAS mutations in myeloid leukemia patients. Blood 2009;113:1749-55.
26. Chou FS, Wunderlich M, Griesinger A, Mulloy JC. N-Ras(G12D) induces features of stepwise transformation in preleukemic human umbilical cord blood cultures expressing the AML1-ETO fusion gene. Blood 2011; 117:2237-40.
27. Sundström M, Edlund K, Lindell M, Glimelius B, Birgisson H, Micke P, et al. KRAS analysis in colorectal carcinoma: analytical aspects of Pyrosequencing and allele-specific PCR in clinical practice. BMC Cancer 2010;10:660.
28. Voelkerding KV, Dames SA, Durtschi JD. Next-generation sequencing: from basic research to diagnostics. Clin Chem 2009;55:641-58.
29. Sivertsson A, Platz A, Hansson J, Lundeberg J. Pyrosequencing as an alternative to single-strand conformation polymorphism analysis for detection of N-ras mutations in human melanoma metastases. Clin Chem 2002;48:2164-70.

http://dx.doi.org/10.3343/alm.2013.33.3.159