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Analysis of N-RAS Gene Mutations and P21N-RAS Protein Expression in Iraqi Patients with in AML

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Abstract

Background N-RAS mutations are the most commonly detected molecular abnormalities in hematologic malignancies, especially in those of myeloid origin.

Objective Current study aimed to determine the frequency of N-RAS mutation and its correlation with P21N-RAS protein expression in patients with acute myelogenous leukemia (AML) in Iraq.

Methods Peripheral blood, bone marrow aspirate and biopsy samples were taken from 58 newly diagnosed AML patients (57 de novo and 1 therapy related AML) and 30 individuals with reactive bone marrow conditions were selected as a control group. Samples screened for N-RAS gene mutations using nested PCR followed by mutation sensitive digestion analysis (MSDA), and immunohistochemical analysis of P21N-RAS protein expression by using anti N-RAS monoclonal antibody.

Results N-RAS mutations at the time of diagnosis were found in 10/58 (17.24%) and P21N-RAS expression found in 5/58 (8.62%) patients with AML. There was a significant difference (P = 0.001) in P21N-RAS expression between mutant and wild type N-RAS patients with AML. No N-RAS mutations or P21N-RAS expression detected in the control group individuals.

Conclusion It can be suggested that there is activation of RAS-signaling cascade in AML patients, this is may support their role in molecular pathogenesis of acute leukemia. Also, there was a significant difference between N-RAS gene status and P21N-RAS protein expression in patients with AML.

Keyword AML, N-RAS Mutation, MSDA, P21N-RAS expression, Digital analysis.

Introduction

RAS proteins are small GTPases that act as molecular switches, transducing extra-cellular signals from activated receptors at the cell surface to the nucleus, thus, regulating cell proliferation, survival, and differentiation. Three RAS genes encode four widely expressed isoforms: H-RAS, N-RAS, and the splice variants K-RAS4A and K-RAS4B [(1)]. The N-terminal region of the RAS proteins has a common structure which comprises a highly conserved G domain but RAS proteins differ substantially at the C-terminal end (C-terminal 40 amino acids), which is known as the hyper-variable region [(2)].

The RAS proteins possess intrinsic GTPase activity (induced hydrolysis of GTP to GDP), which normally leads to their inactivation and the control signal transduction. In tumors, a point mutation resulting in loss of the intrinsic GTPase activity and RAS proteins lock in an active state, thus, does not stop anymore to send signal stimulating cell proliferation and appears to be associated with the transforming

activity of the protein. All RAS mutations were missense point mutations occur at codons 12, 13 (exon 1) and 61 (exon 2) [3,4]. Activating mutations of N-RAS are most common among myeloid malignancies, found in approximately 20% to 40% of myelogenous leukemia (AML), myelodysplastic syndrome (MDS), chronic myelomonocytic leukemia (CML) and juvenile myelomonocytic leukemia (JMML) [1].

AML is characterized by a maturation block and accumulation of myeloid progenitor cells. Clinically, cytogenetically, and molecularly it has been recognized as a heterogeneous disorder [5]. Current study aimed to determine the frequency of N-RAS mutations and its correlation with P21N-RAS protein expression in patients with acute myelogenous leukemia (AML) in Iraq.

Methods

Fifty eight newly diagnosed untreated AML patients (57 de novo and 1 therapy related AML) and thirty individuals with reactive bone marrow (including 19 individuals presented with pyrexia of unknown origin (PUO) and 11 presented with idiopathic thrombocytopenic purpura (ITP)) served as control group were enrolled in this study at Department of Hematology, Baghdad Hospital at Baghdad Medical City for the period April 2011 to July 2012.

Current study was approved by the Local Ethics Committee of College of Medicine, Al-Nahrain University and informed consent in accordance with the Declaration of Helsinki was obtained from patients, control individuals or their legal guardians prior to the collection of samples and data.

Out of the total number of patients, 58 patients were diagnosed as having AML according to modified FAB classification system including; 6 M0, 11 M1, 21 M2, 10 M3, 4 M4, 5 M5 and 1 M6. Criteria's of selection for AML patients enrolled in current study was newly diagnosed, didn't receive treatment and were randomly collected in relation to age and gender.

Genomic DNA was extracted from peripheral blood specimens of patients and control individuals, N-RAS gene amplification was performed; briefly 1 µL of the extracted DNA was added to a 20-µL PCR reaction mixture containing 5 µL of AccuPower TLA PCR Premix, 10 pmol of each forward and reverse primer (Table 1) and 13µL of nuclease free water. The first round of PCR consisted of 30 cycles (denaturation at 94^{o}C for 30 seconds, annealing at 55^{o}C for 30 seconds, and extension at 72^{o}C for 30 seconds). 1 µL of the amplified product of the first round was then added to a second 20-µL PCR reaction mixture using 2nd set of primers for a further 30 cycles under identical conditions to the first round. Each round was preceded by heating at 95^{o}C for 10 minutes. Negative control (no DNA template) tube was included with each batch of samples analyzed. Beta globulin gene also amplified as control for amplification[6].

Table 1. Sequences of DNA primers[6]

First Round N-RAS Gene Primers:
• RS 12 (Forward) 5' GCTCGCAATTAACCCTGATTAC
• RS7 (Reverse) 5' ATTCCTTTAATACAGAATATGG
Second Round N-RAS Gene Primers:
• RS6 (Forward) 5'ACTGAGTACAAACTGGTGGTGGTTGGACCA
• RS5 (Reverse) 5' GGTCAGCGGGCTACCCCTGGACCA

Mutation sensitive digestion analysis (MSDA) was used for the detection of mutations at codon 12 and codon 13. The second round PCR primers (RS6 and RS5) are both mismatched at a single base from their target sequence. This creates a 5' BstNI restriction site at codon 12 and 3' restriction site within sequence at the downstream end of the amplified DNA. If the

amplified DNA has normal sequence at the first two bases of codon 12, it is cleaved at both the 5' and 3' sites by BstNI to produce an 87 bp fragment, whereas mutant DNA with a substitution affecting either of the first two bases at codon 12 results in loss of this restriction site and thus cleaves only at the 3' site to produce a 116 bp fragment. A codon 13 mutation creates an HphI recognition site. Digestion of the 135 bp amplified fragment with this enzyme thus leads to cleavage of mutant DNA at a 5' and a 3' site to produce a 75 bp fragment, while normal sequence is digested at only the 3' position to produce a fragment of 117 bp. For both enzymes, the 3' site is always cleaved and serves as a control for the digestion [6] (Fig. 1).

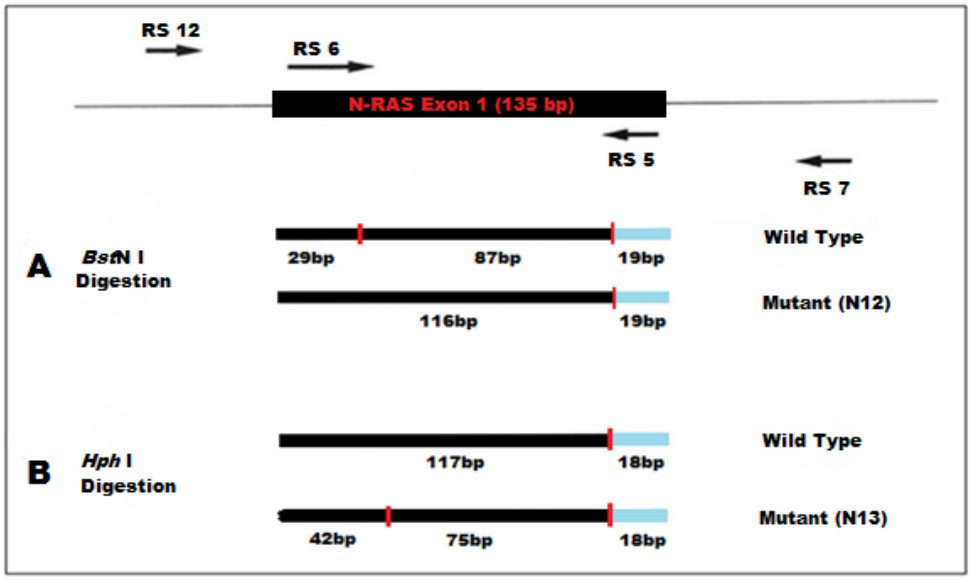

Fig. 1. Schematic illustration of the PCR-based MSDA used for the detection of codon 12 and 13 mutations. The positions of the first round primers for exon 1 (RS12 and RS7) and the second round nested primers (RS6 and RS5) are shown. (A) *Bst*NI digestion of amplified sequence for codon 12 mutations. (B) *Hph*I digestion of amplified DNA for codon 13 asp mutations [6].

PCR products were digested directly after 2nd round amplification, for codon N12 detection, 10 µl of PCR reaction mixture (about 0.1-0.5 µg of DNA), 7 µl of nuclease free water, 2 µl of NEBuffer 2 (10X) and 1µl of BstNI were mixed gently for a few seconds, then incubated at 60°C for 2 hours. The mixture was subjected to electrophoresis in 2% agarose gels containing 0.01% ethidium bromide. For codon N13 detection, 10 µl of PCR reaction mixture, 7 µl of nuclease free water, 2 µl of NEBuffer 4 (10X) and 1µl of Hph I were mixed gently for a few seconds, then incubated at 37°C for 2 hours. The mixture was subjected to electrophoresis in 2% agarose gels containing ethidium bromide [6,7] (Fig. 2-4).

Immunohistochemical staining was performed on 4 mm, formalin fixed, paraffin embedded bone marrow biopsy sections (patients and control individuals) mounted on electrostatic charged, poly-L-lysine-coated slides (Fisher Scientific, USA). Sections were deparaffinized at 60ºC overnight, rehydrated. Heat induced antigen retrieval was performed with citrate buffer pH 9. Exogenous peroxidase activity was quenched in 5 minute incubation with 2% H_2O_2, sections stained with N-RAS specific antibody (dilution: 1/1500, clone: F155, Santa Cruz, USA) and incubated overnight at 4°C. Sections stained with Biotin and Streptavidin-HRP reagent. Streptavidin-biotin complex was incubated with DAB Substrate buffer. Counterstaining was performed with hematoxylin, dehydration and

mounting processes were performed and completed [8]. immunohistochemistry staining procedure was

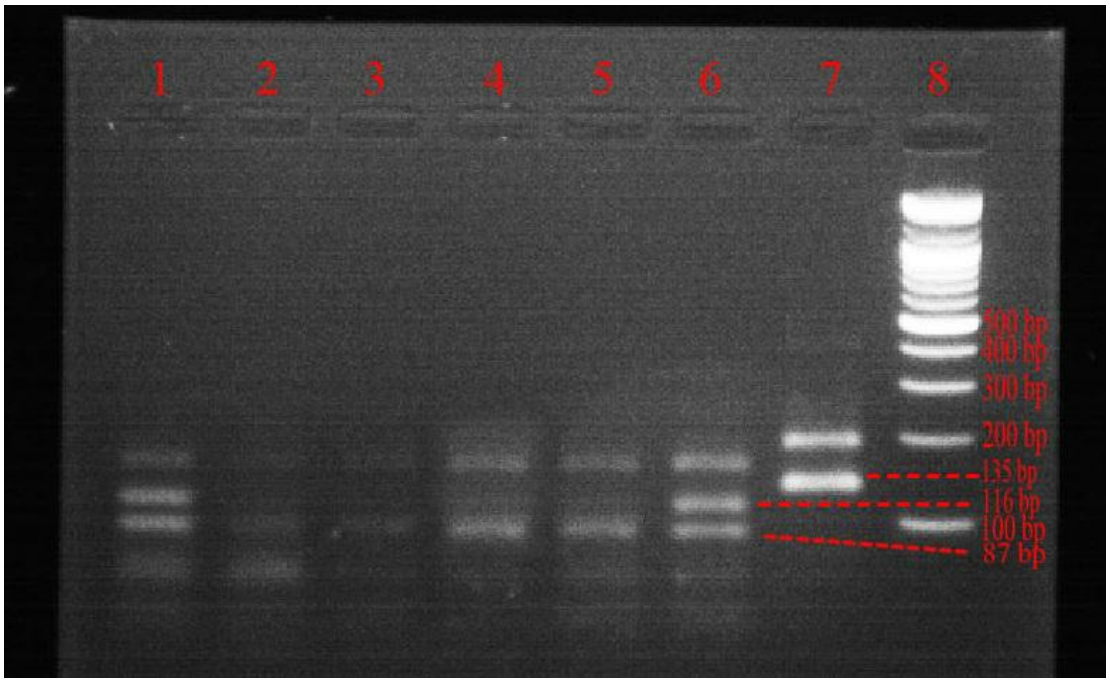

Fig. 2. Mutation sensitive digestion analysis (MSDA) from AML patients. PCR amplified DNA digested with BstNI for N-RAS codon 12 mutation. Lane 7, undigested control; lane 2, 3, 4 and 5 were wild N-RAS AML patients; Lanes 1 and 6 show AML cases with mutant N-RAS AML patents (116-bp band in lane 1, 6 was a result of N-RAS N12 mutation); lane 8, DNA size markers. Electrophoresis was done in 2% agarose gel containing ethidium bromide (final concentration 0.5 µg/ml) at (4V/cm) for 60 min.

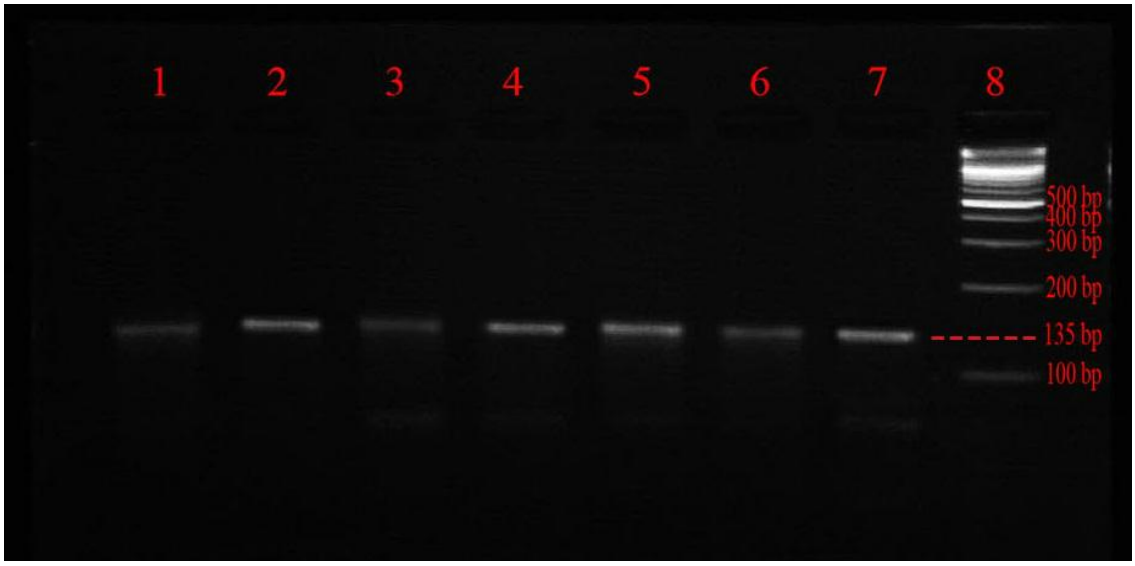

Fig. 3. Mutation sensitive digestion analysis (MSDA) from AML patients. PCR amplified DNA digested with *Hph*I for N-RAS codon 13 mutation. Lane 7, undigested control; lane 1, 2, 3, 4, 5 and 6 were wild N-RAS AML patients; Lane 8, DNA size markers.Electrophoresis was done in 2% agarose gel containing ethidium bromide (final concentration 0.5 µg/ml) at (4V/cm)for 60 min.

The cellular staining pattern for P21N-RAS was dark to light brown nuclear stain of blast cells in AML (Fig. 5). Scoring of the immune-histochemical expression was performed using specialized automated cellular image analysis system (Digimizer software v3.7.0.0 - 2010). The digital analysis software describe the expression with three parameters; which are: Intensity (intensity of staining which is negatively proportional to strength of staining), Fractional Area percentage FAP (area stained per high power field); and Digital Labeling Index DLI (calculated by multiplying the fractional area percentage by the reverse intensity, reflecting the integration of percentage of positive cells and intensity of expression) (Fig. 6).

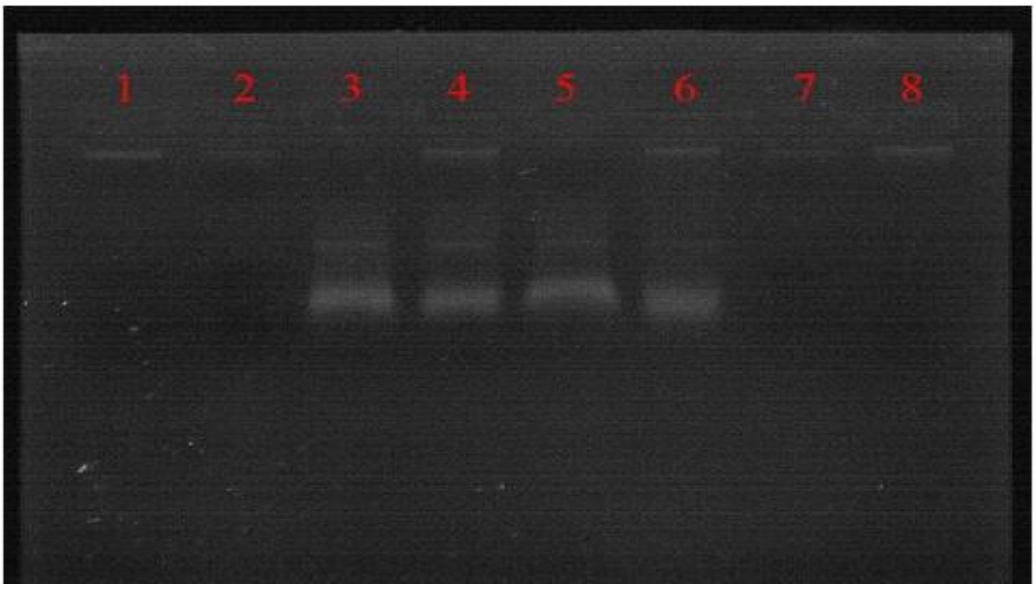

Fig. 4.BstNI and HphI enzymes digestion control (lanes 1, 3, 5 and 7 were unamplified DNA from AML patients while lanes 2, 4, 6 and 8 were unamplified DNA from control individuals). Lanes 3 and 4 contain DNA digested with BstNI. Lanes 5 and 6 contain DNA digested with HphI. Digested lanes show smear in comparison with undigested lanes, which show single bands. Electrophoresis was done in 2% agarose gel containing ethidium bromide (final concentration 0.5 µg/ml) at (4V/cm) for 60 min.

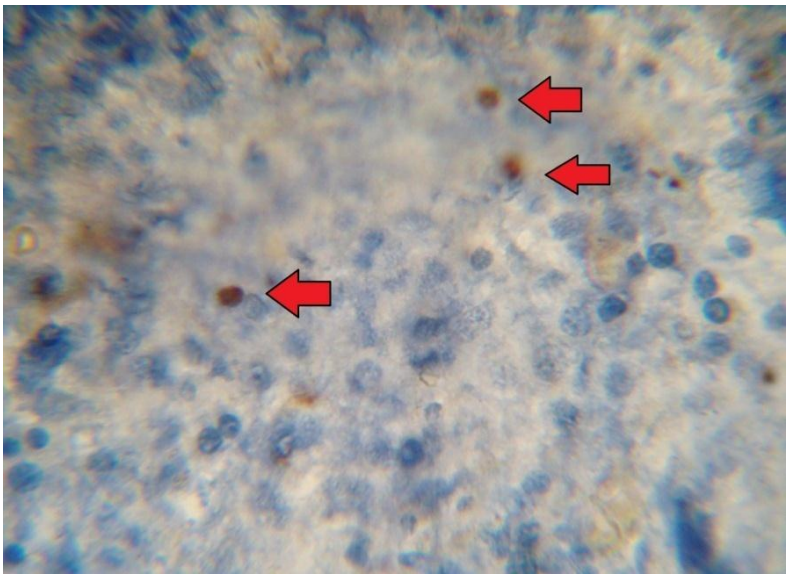

Fig. 5. Bone marrow biopsy of AML patient show positive immunohistochemical expression of P21 N-RAS (arrows refer to brownish coloration of the nucleus) (40X)

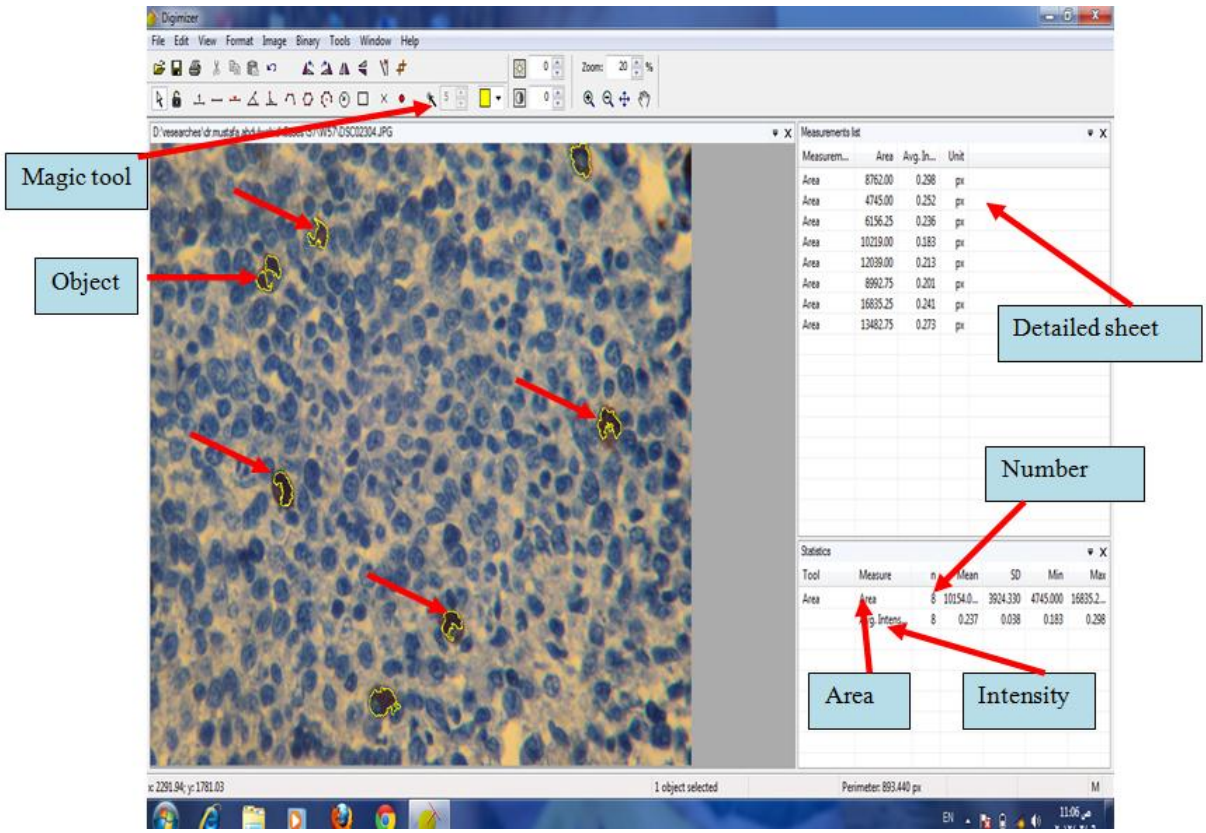

Fig. 6. Image analysis in Digimizer software v3.7.0.0

Data were analyzed using SPSS program (Statistical Package for Social Sciences) version 16 and Microsoft Office Excel 2007. Numeric data were expressed as (mean ± SE) and frequency was used to express discrete data. Student T-test was used to analyze numeric data while Chi-square and test was used to analyze discrete data. Values were considered statically significant when ($P < 0.05$).

Receiver operator characteristic (ROC) analysis was performed to determine the cut-off value for P21N-RAS protein expression at the time of diagnosis, anything above it was considered positive, and anything below it, was considered as negative (no expression).

Results

Out of 58 patients, There were 33 (56.89%) male patients and 25 (43.10%) female patients with a M:F ratio 1.3:1, mean age was 41.57 ± 2.53 year (age range was 13-75). Out of 30 individuals in control group, 18 (60%) individuals were males and 12 (40%) were females with a M:F ratio 1.5:1. The mean age was (38.77 ± 2.93) year (age range was 16-70 year).N-RAS mutations were found in 10 out of 58 (17.24%) of AML patients ($P = 0.091$). All mutations were in codon 12 and no mutation in codon 13. No mutations were detected in control group individuals. In AML patients, the mean P21N-RAS expression intensity was (0.637 ± 0.098), FAP was (4.904 ± 0.830) and DLI (10.714 ± 1.758). No P21N-RAS expression detected in control individual (0 out of 30) (Table 2).

There was no significant difference in patient's gender ($P = 0.855$) and mean age between mutant and wild type N-RAS AML patients (40.20 vs. 41.85, $P = 0.407$). The mean WBC count was significantly higher (54.33 vs. 31.25 x 10^9/L, $P = 0.033$) and the mean bone marrow blast percentage was significantly lower (56.50 vs. 69.31%, $P = 0.025$) in patients with mutated N-RAS than that of patients with wild type N-RAS (Table 3).

Table 2. Characteristics of patients and control individuals enrolled in present study

Characteristic		AML Patients (N = 58)	Control Individuals (N = 30)
Age (Year)	Mean Range	41.57 ± 2.53 13-75	38.77 ± 2.93 16-70
Gender	Male Female M:F ratio	33 25 1.3:1	18 12 1.5:1
Diagnosis		AML-M0 = 6 AML-M1 = 11 AML-M2 = 21 AML-M3 = 10 AML-M4 = 4 AML-M5 = 5 AML-M6 = 1	PUO = 19 ITP = 11
N-RAS Gene Status		Mutant = 10 Wild Type = 48	Mutant = 0 Wild Type = 30
WBC Count (10^9/L)		35.23 ± 6.60	6.34 ± 0.53
Platelets Count (10^9/L)		47.14 ± 7.22	183.73 ± 31.90
PCV percentage (%)		25.76 ± 0.82	38.40 ± 0.28
Peripheral Blood Blast percentage (%)		49.03 ± 4.26	0.00 ± 0.00
Bone Marrow Blast percentage (%)		67.10 ± 3.22	1.53 ± 0.09
Anemia		62.07	0.00
Fever (%)		44.83	63.33
Bleeding Tendency (%)		41.38	36.67
Weight Loss (%)		12.07	0.00
Splenomegaly (%)		36.21	0.00
Hepatomegaly (%)		25.86	0.00
Lymphadenopathy (%)		18.97	0.00
Intensity		0.637 ± 0.098	0.00 ± 0.00
FAP		4.904 ± 0.830	0.00 ± 0.00
DLI		10.714 ± 1.758	0.00 ± 0.00

Table 3. Clinical and hematological parameters in patients with mutant and wild type N-RAS gene

Parameter	Mutant N-RAS (N=48)	Wild Type N-RAS (N=10)	P
Gender (%)	Male 18.18 Female 16	Male 81.82 Female 84	0.828
Age (year)	40.20 ± 6.27	41.85 ± 2.80	0.407
WBC (10^9/L)	54.33 ± 9.19	31.25 ± 7.64	**0.033**
Hematocrit Percentage (%)	24.50 ± 1.36	26.02 ± 0.95	0.185
Platelets Count (10^9/L)	45.80 ± 15.24	47.42 ± 8.12	0.463
Peripheral Blood Blast Percentage (%)	38.50 ± 3.74	51.23 ± 5.10	**0.028**
Bone Marrow Blast Percentage (%)	56.50 ± 5.12	69.31 ± 3.68	**0.025**
Anemia	80%	58.33%	0.199
Bleeding Tendency	40%	41.67%	0.922
Fever	40%	45.83%	0.736
Weight Loss	30%	8.33%	0.056
Splenomegaly	50%	33.33%	0.318
Hepatomegaly	40%	22.92%	0.262
Lymphadenopathy	20%	18.75%	0.927

Regarding the distribution of N-RAS mutations within AML subtype according to FAB Classification; the mutations were detected in 1 out of 4 (25.00%) patients with M4 (*P* = 0.670), followed by M5, 1 out of 5 (20.00%) patients (*P* = 0.864), then M2, 4 out of 21 (19.05%) patients (*P* = 0.784), M1, 2 out of 11 (18.18%) patients (*P* = 0.926), M0, 1 out of 6 (16.67%) patients (*P* = 0.969), M3, 1 out of 10 (10.00%) patients (*P* = 0.837) and no mutations were detected in M6 patient(0 out of 1) (*P* = 0.605). There was no significant difference in N-RAS mutation among different AML FAB subtype (*P* = 0.105).

There was significant difference between patients with mutant and wild type N-RAS in Intensity (0.210 vs. 0.726, *P* = 0.044), FAP (6.785 vs. 4.512, P = 0.012) and DLI (32.310 vs. 6.215, *P* = < 0.001)(Table 4).

Table 4. Correlations between N-RAS mutations and Immunohistochemical expression in AML patients

Digital Parameter	Wild type N-RAS			Mutated N-RAS			*P* value
	N	Mean	SE	N	Mean	SE	
Intensity	48	0.726	0.113	10	0.210	0.051	**x 0.044**
FAP	48	4.512	0.350	10	6.785	0.952	**x 0.012**
DLI	48	6.215	0.964	10	32.310	7.687	**< 0.001**

By applying ROC curve analysis between mutant and wild type N-RAS in AML patients, DLI cut off value were 21.414 with acceptable sensitivity (71.4%) and acceptable specificity (100%)(Table 5 and Fig. 7).

Table 5. Receiver operator characteristic curve analysis between AML patients and control group

	Intensity	FAP	DLI
Cut Off Value	0.45	14.16	31.47
Sensitivity (%)	71.4	71.4	71.4
Specificity (%)	81.6	100	100

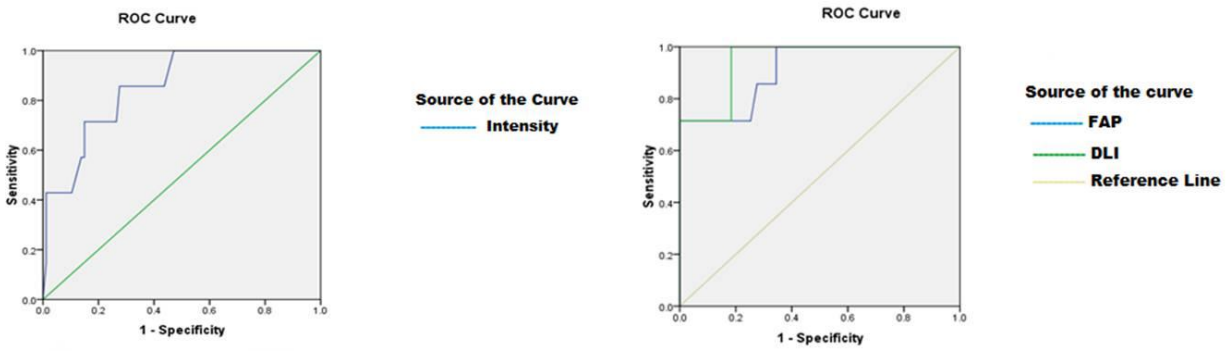

Fig. 7. Sensitivity and specificity of FAP, DLI and intensity between mutant and wild type N-RAS

By applying cut-off value, P21N-RAS expression found in 5 (8.62%) out of 58 AML patients, positive expression was found in 4 (40%) out of 10 in mutant N-RAS and in 1 (2.08%) out of 48 wild N-RAS, there was significant difference (*P* = 0.001) in P21N-RAS expression between mutant and wild type N-RAS AML patients (Table 6).

Table 6. P21N-RAS expression in AML patients

Parameter	P21N-RAS Expression		*P* value
	Positive	Negative	
Mutant N-RAS (N = 10)	4	6	**0.001**
Wild N-RAS (N = 48)	1	47	

Discussion

The clinical significance of RAS mutations has not been uniformly established, current study screened of 58 newly diagnosed AML patients with PCR-MSDA.Mutations in the N-RAS gene were found in 10 out of 58 (17.24%). This result confirms previous investigations that reported a prevalence of N-RAS mutations of (4 - 21%) in AML patients [3,9-12]. Discrepancy in RAS mutation frequency among various reports result from fact that criteria for selection of AML patients differ between various studies. N-RAS frequency in studies analyzed only de novo AML was lower than studies select AML that arose from proven MDS which is more frequently associated with N-RAS mutations [10]. Also the difference in RAS mutation frequency may explained by number of cases involved, method of screening, number of exon examined (codons 12, 13 in exon 1, codon 61 in exon 2) and type of RAS mutation (N, K and H-RAS) analyzed [12].

All N-RAS mutation detected in codon 12 (100%) and no mutation detected in codon 13, these finding were in agreement with previous studies [10,13]. Primary analyses revealed a statistically significant differencein the peripheral, bone marrow blast counts, WBC count between mutant and wild type N-RAS mutations (*P* = 0.028, *P* = 0.025, *P* = 0.033 respectively), however no significant differences had been found between the two groups with respect to age, gender, platelet count, hematocrit percentage and clinical outcomes. These findings are in agreement with those reported in literatures [3,9,10,13-15]. Mutation of the N-RAS gene affects the biology of AML. Transfection of various cell types with mutant RAS genes has been shown to stimulate secretion of interleukin-3, granulocyte, and granulocyte macrophage colony stimulating factors, leading to autonomous growth through an autocrine mechanism, increasing peripheral WBC count [16].

The highest frequency of N-RAS mutation in M4 in current study corresponded with most of the previously published studies [9,10,12]. N-RAS mutation is most likely a one event contributing to the progression/proliferation of sub-clones in AML, selection and expansion of RAS mutant clones may provide a differentiative stimulus toward the monocytic lineage [3], Van Kamp study also suggested that N-RAS mutation preferentially influences hematopoiesis to myelomonocytic differentiation or myelo-monocytic cells are more susceptible for acquiring an N-RAS mutation since N-RAS mutations are more likely to develop in cells of myelomonocytic differentiation [17]. This may be consistent with the overrepresentation of RAS mutation in M4/M5 subtypes.The low frequency of N-RAS mutation in M3 (10%) in current study corresponded with Bowen study, N-RAS mutation is relatively underrepresented in M3 where FLT3 ITD is overrepresented, both RAS mutation and FLT3 ITD are rarely present in the same tumor [3]. In the present study, P21N-RAS expression was found in 5 (8.62%) out of 58 AML patients, 4 (40%) out of 10 patients with mutant N-RAS and 1 (2.08%) out of 48 patients with wild type N-RAS show positive P21N-RAS expression. There was a significant difference (*P* = 0.001) in P21N-RAS expression between mutant and wild type N-RAS patients with AML. That is to say, there is a correlation between N-RAS gene mutations and protein expression. This finding was in agreement with previous studies [18-20].

This discrepancy between the N-RAS immuno-histochemical analysis results in different studies

might be explained by difference in the fixative used in bone marrow biopsies processing (formalin was used in current study while Bouin's solution and B5 was used in other studies). Positive P21 expression in one AML patient with wild type N-RAS may explain by the fact presence of other mutated codons that had not been screened in current study (e.g. codon 61).

Although that HphI enzyme digested the unamplified DNA, it failed to digest a 3' end of the amplified DNA (that served as a control for enzyme function) (Figure 4). Current study suggested that this negative result is not due to failure of the primer system to detect mutations in the digested PCR product but the predominance of digestion resistant band as mentioned in previous report.

Bashey and Todd studies describe an overrepresentation of the singly digested band, which is caused by the formation of restriction enzyme resistant hetero-duplexes between mutant and normal strands which are mismatched at a single base only [6,15]. In addition to that, the reverse Allele specific restriction analysis (ASRA) method described by Todd and Iland fails to demonstrate the presence or absence of wild type alleles, since a digestion resistant band merely indicates the lack of a specific mutation rather than the presence of wild type sequences [21].

In conclusion, previous results provide clues for activation of RAS-signaling cascade in AML patients, supporting their role in molecular pathogenesis of leukemia. Also, there was a significant correlation between P21N-RAS protein expression and N-RAS gene status. Thus, Immunohistochemical analysis of the P21N-RAS in blast cells of patients with AML may demonstrate the N-RAS gene expression. More studies on larger scale are required to explore P21N-RAS expression as a prognostic marker in myeloid malignancies.

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