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The Influence of N-RAS Gene Mutations on the Response to Induction Therapy in AML Iraqi Patients

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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 influence on response to induction therapy in patients with acute myelogenous leukemia (AML) in Iraq.

Methods Peripheral blood and bone marrow samples were taken from 58 newly diagnosed AML patients and 30 individuals with reactive bone marrow were selected as a control group. Samples screened for N-RAS gene mutations using nested PCR were followed by mutation sensitive digestion analysis (MSDA).

Results N-RAS mutations at the time of diagnosis were found in 10/58 (17.24%) patients with AML and no mutation in control individuals. Patients with mutant N-RAS showed lower complete remission (CR) than wild type, the difference was not significant (60% vs. 72.92%, $P = 0.414$).

Conclusion The current results provide clues for activation of RAS-signaling cascade in AML patients, supporting their role in molecular pathogenesis of leukemia. N-RAS mutations show no influence on CR rate in AML patients. Further studies on larger scale to define the prognostic significance of N-RAS mutations are recommended.

Keyword AML, N-RAS mutation, MSDA, complete remission.

Introduction

RAS proteins are small GTPases that act as molecular switches, transducing extracellular signals from activated receptors at the cell surface to the nucleus, thus, regulating cell proliferation, survival, and differentiation. Three RAS genes encode 4 widely expressed isoforms: H-RAS, N-RAS, and the splice variants K-RAS4A and K-RAS4B [(1)].

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, does not stop anymore to send signal stimulating cell proliferation and appears to be associated with transforming activity of the protein. All RAS mutations were missense point mutations occur at codons 12, 13 (exon 1) and 61 (exon 2) [(2,3)]. 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].

AMLis characterized by a maturation block and accumulation of myeloid progenitor cells. Clinically, cytogenetically, and molecularly it has been recognized as a heterogeneous disorder [4]. Although in AML N-RAS mutations were first reported 25 year ago, the prognostic impact of N-RAS mutations is still under discussion and seems to vary from disease to disease. Several studies indicate an association with poor outcome; others found a negative prognostic impact of N-RAS mutations only in AML with favorable karyotypes; other found N-RAS mutations associated with a favorable prognosis and at last some studies could not defined a prognostic impact of N-RAS mutations [5]. The current study aimed to determine the frequency of N-RAS mutation, its influence on response to induction therapy in AML patients in Iraq.

Method

Fifty eight newly diagnosed untreated AML patients and thirty individuals with reactive bone marrow (including 19 individuals presented with pyrexia of unknown origin and 11 presented with idiopathic thrombocytopenic purpura) served as control group were enrolled in thisstudy at Department of Hematology / Baghdad Hospital at Baghdad Medical City for the period April 2011 to July 2012.

The study was approved by the 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.

DNA extraction from peripheral blood, amplification and enzyme restriction was done at the Department of Pathology/Baghdad Collage of Medicine. Genomic DNA was extracted from peripheral blood specimens of patients at time of presentation, N-RAS gene amplificationperformed; briefly 1 µL of the extracted DNA was added to a 20µL PCR reaction mixturecontaining 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

First Round N-RAS Gene Primers: [6]
• RS 12 (Forward) 5’ GCTCGCAATTAACCCTGATTAC • RS7 (Reverse) 5’ ATTCCTTTAATACAGAATATGG
Second Round N-RAS Gene Primers: [6]
• RS6 (Forward) 5’ACTGAGTACAAACTGGTGGTGGTTGGACCA • RS5 (Reverse) 5’ GGTCAGCGGGCTACCCCTGGACCA

Mutation sensitive digestion analysis (MSDA) was used for 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’ *Bst*NI restriction site at codon 12 and 3’ restriction site within sequence at the downstream end of 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 *Bst*NI 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 *Hph*I 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 (Figure 1) [6].

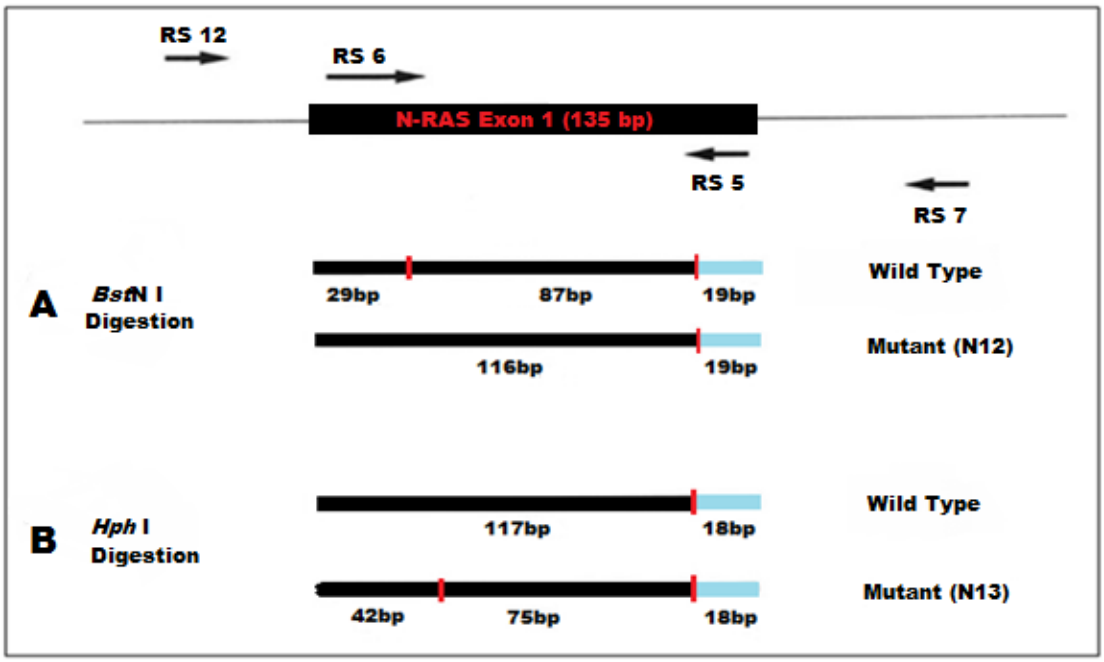

Fig. 1. Schematic illustration of the PCR-based MSDA used for the detection of codon 12 and 13 mutations. The positions of the first round primers for exon 1 (RS12 and RS7) and the second round nested primers (RS6 and RS5) are shown. (A) *Bst*NI digestion of amplified sequence for codon 12 mutations.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

PCR products were digested directly after 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 NE Buffer 2 (10X) and 1µlof *Bst*NI were mixed gently for a few seconds. Then incubated at 60°C for 2 hours and mixture were subjected to electrophoresis in 2% agarose gels containing 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 and mixture were subjected to electrophoresis in 2% agarose gels containing ethidium bromide (Figure 2, 3 and 4) [6,7].

Induction Therapy

The primary objective in treating patients with AML is to induce remission and thereafter prevent relapse. Complete remission (CR) was defined morphologically as no circulatory blasts, with absolute neutrophil count (ANC) $>1.5\times10^9$/L, and platelet count $>100\times10^9$/Land cellular marrow with blasts <5% and absence of extra medullary involvement [8]. Failure of induction was defined as less than 50% reduction in marrow blast percent from that at presentation.Patients neither in complete remission nor in failure regarded as partial response. Treatment is conventionally divided into two phases: induction and post induction [8].

Doxorubicin 30mg/m^2 per day i.v. infusion over 30 min from day 1-3 and Cytosine Arabinoside 100mg/m^2 per day i.v. infusion over 16 hours from day 1-7. For FAB group M3, doxorubicin 30mg/m^2per day i.v. infusion over 30 min was given on days 1, 3, 5, 7 (4 doses) along with All Trans Retinoic Acid (ATRA) in a dose of 45mg/m^2 per oral daily in 2 divided doses from day 1 till remission[9].

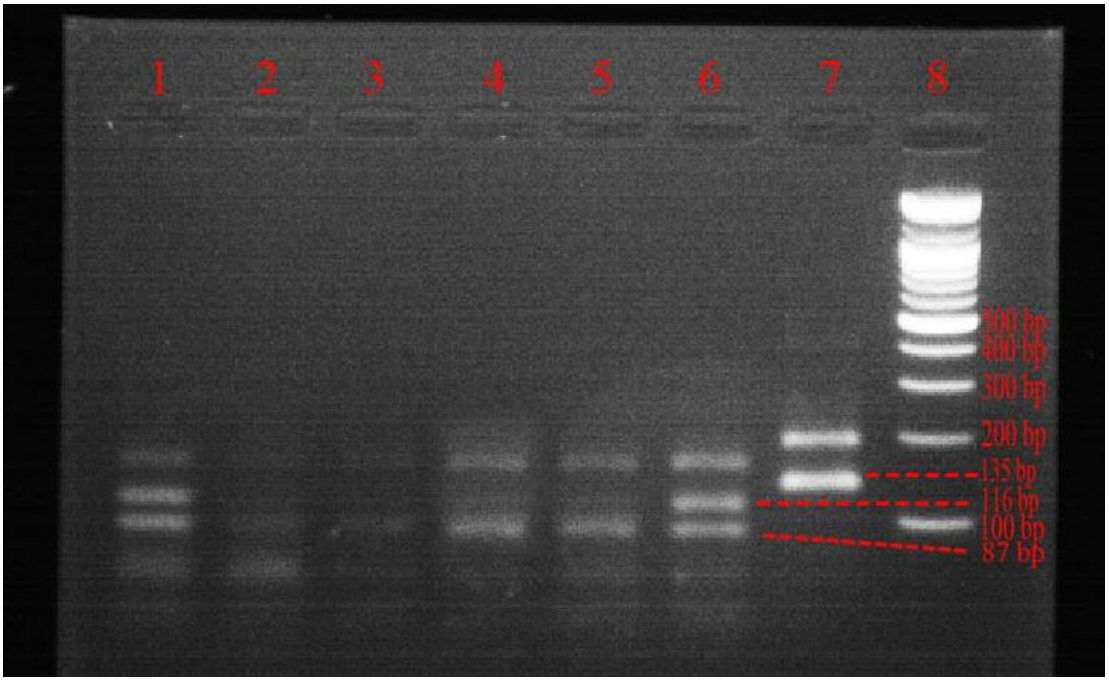

Fig. 2. Mutation sensitive digestion analysis (MSDA) from AML patients. PCR amplified DNA digested with BstNI for N-RAS codon 12 mutation. Lane 7, undigested control; lane 2, 3, 4 and 5 were wild N-RAS AML patients; Lanes 1 and 6 show AML cases with mutant N-RAS AML patents (116-bp band in lane 1, 6 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.

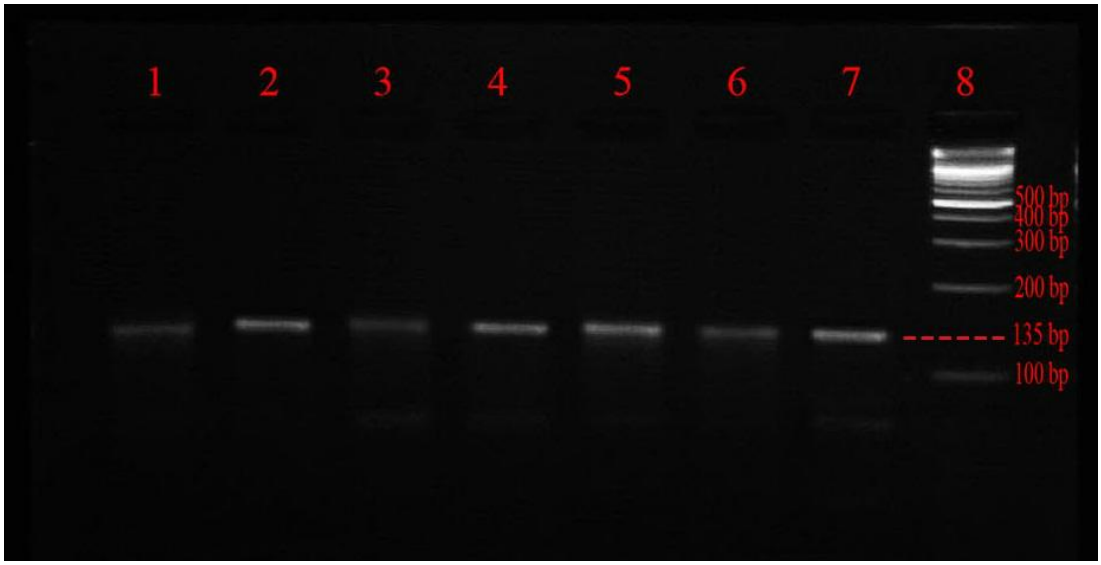

Fig. 3. Mutation sensitive digestion analysis (MSDA) from AML patients. PCR amplified DNA digested with HphI for N-RAS codon 13 mutation. Lane 7, undigested control; lane 1, 2, 3, 4, 5 and 6 were wild N-RAS AML patients; Lane 8, DNA size markers.*Electrophoresis was done in 2% agarose gel containing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

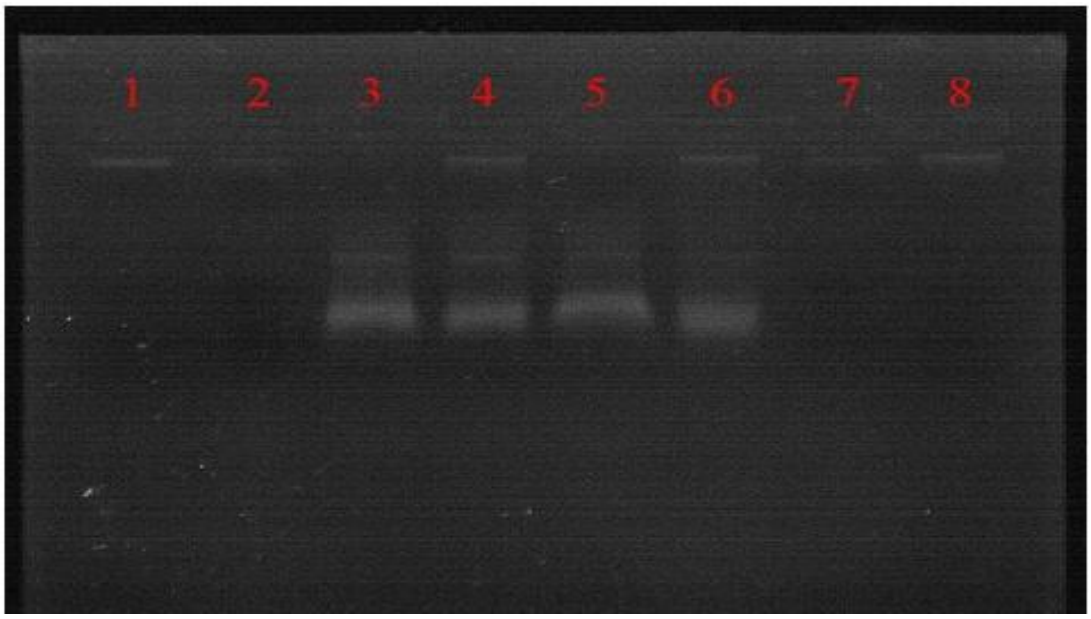

Fig. 4. BstNI and HphI enzymes digestion control (lanes 1, 3, 5 and 7 were unamplified DNA from AML patients while lanes 2, 4, 6 and 8 were unamplified DNA from control individuals). Lanes 3 and 4 contain DNA digested with BstNI. Lanes 5 and 6 contain DNA digested with HphI. Digested lanes show smear 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.

AML patients pathology reports were retrieved again from archive of Department of Hematology/Teaching Laboratories at Baghdad Medical City) near the end of the study in order to assess patients' response to induction therapy (in term of complete remission, partial remission or failure) after 2-3 weeks from induction.

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 statistically significant when ($P < 0.05$).

Results

Out of 58 patients with AML, there were 33 (56.89%) males and 25 (43.10%) females 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, there were 18 (60%) males and 12 (40%) females with a M:F ratio 1.5:1, mean age was 38.77 ± 2.93 year (age range was 16-70).

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.

There was no significant difference in patient's gender ($P = 0.855$) and mean age between mutant and wild type N-RAS AML patients (40.2 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. There was no significant difference in N-RAS mutation among different AML FAB subtype ($P = 0.105$) (rest of results summarized in table 2 and 3).

Regarding response to induction therapy, forty eight (82.76%) patients had received 3 and 7 induction regimen while ten (17.24%) patients with AML-M3 received ATRA as induction regimen.

Table 2. Correlation between N-RAS mutation and clinical-hematological parameters

Parameter		Mutant N-RAS N = 10	Wild Type N-RAS N = 48	*P*
Gender	Male	18.18%	81.82%	0.828
	Female	16%	84%	
Age (Mean ± SE)		40.20 ± 6.27 year	41.85 ± 2.80 year	0.407
WBC (Mean ± SE)		54.33 ± 9.19 x 10^9/L	31.25 ± 7.64 x 10^9/L.	**0.033**
Hematocrit Percentage (Mean ± SE)		24.50 ± 1.36 %	26.02 ± 0.95 %	0.185
Platelets Count (Mean ± SE)		45.80 ± 15.24 x 10^9/L	47.42 ± 8.12 x 10^9/L	0.463
BM Blast Percentage(Mean ± SE)		56.50 ± 5.12 %	69.31 ± 3.68 %	**0.025**
PB Blast Percentage(Mean ± SE)		38.50 ± 3.74 %	51.23 ± 5.10 %	**0.028**
Complete Remission (CR)		60%	72.92%	0.414
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

BM = bone marrow, PB = peripheral blood

Table 3. Distribution of N-RAS mutations within AML subtype according to FAB classification

FAB Classification	Mutant N-RAS		Wild N-RAS		Number of Cases	*P* value
	No.	%	No.	%		
AML-M0	1	16.67	5	83.33	6	0.969
AML-M1	2	18.18	9	81.82	11	0.926
AML-M2	4	19.05	17	80.95	21	0.784
AML-M3	1	10.00	9	90.00	10	0.837
AML-M4	1	25.00	3	75.00	4	0.67
AML-M5	1	20.00	4	80.00	5	0.864
AML-M6	0	0.00	1	100.00	1	0.605
Total	10	17.24 %	48	82.76 %	58	0.105

Thirty five (60.34%) patients achieved CR, six (10.34%) patients achieved partial remission, fourteen (24.14%) patients failed to achieve CR and there was no data available about 3 (5.17%) patients.Five (50.0%) out of 10 AML patients with mutant N-RAS and 30 (62.5%) out of 48 AML patients with wild type N-RAS achieved CR. One (10.0%) out of 10 AML patients with mutant N-RAS and 5 (10.42%) out of 48 AML patients with wild type N-RAS achieved partial remission. Four (40.0%) out of 10 patients with mutant N-RAS and 10 (20.83%) out of 48 patients with wild type N-RAS failed to achieve CR. No data was available about 3 (6.25%) out of 48 patients with wild type N-RAS. There was no significant difference (*P* = 0.501) in CR rate between patients with mutant and wild type N-RAS.

Discussion

The clinical significance of RAS mutations has not been uniformly established.In current study, N-RAS gene mutations were found in 17.24% of patients with AML. This result confirm previous reports that recognized a frequency of N-RAS

mutations to be in between (9-21%) in patients with AML [2,5,11-13]. 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 denovo AML was lower than studies select AML that arose from proven MDS which is more frequently associated with N-RAS mutations [11]. 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 [13]. All N-RAS mutation detected in codon 12 (100%) and no mutation detected in codon 13, these finding were in agreement with previous studies [5,11-14].

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). 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 [16].

Analyses revealed a statistically significant association between bone marrow blast percentage, WBC count and N-RAS mutation (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 were in agreement with those reported in previous literatures [2,5,11,14,17,18].

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 granulocytemacrophage colony stimulating factors leading to autonomous growth through an autocrine mechanism, increasing peripheral WBC count [19].

The highest frequency of N-RAS mutation in M4 in current study corresponded with most of the previously published studiesm [5,11,13]. N-RAS mutation is most likely a postinitiation 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 [2], 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 [20]. This may be consistent with the overrepresentation of RAS mutation in M4/M5 FAB types.

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 [2].

Current study showed that response to induction therapy was comparable to Alwan study and Lowenberg study were reported CR rate to be (70 - 80%) [8,9].

CR rate in mutant N-RAS patients was lower than wild type N-RAS patients, but the difference was not significant (P = 0.414). Published reports addressing the clinical significance of RAS mutations in patients with AML are inconclusive. Whereas some studies observed that the presence of N-RAS mutation did not significantly influence CR rate [2,15]. Others observed a significantly lower CR rate compared with patients without N-RAS mutation [12,21]. Third

group reported a beneficial clinical effect of RAS mutations in patients with AML in response to high dose cytarabine therapy (HiDAC) [22,23]. Last group did not show that patients with RAS mutations had significantly different outcomes [24]. This discrepancy between these studies findings may be explained by differences in the intensity of the chemotherapy protocols in use to treat group of patients and the number of cases analyzed [25].

In conclusion, N-RAS mutations show no influence on CR rate in AML patients. Further studies on larger scale to define the prognostic significance of N-RAS mutations were recommended.

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