Prognostic Implication of N-RAS Gene Mutations in Egyptian Adult Acute Myeloid Leukemia

1Doaa M. Elghannam, 1Nashwa Khayrat Abousamra, 1Doaa A. Shahin, 1Enas F. Goda, 1Hanan Azzam, 2Emad Azmy, 3Manal Salah El-Din, 4Mohamed F. El-Refaei

1Department of Clinical Pathology, Hematology Unit, Faculty of Medicine, Mansoura University, 2Department of Clinical Hematology, Faculty of Medicine, Mansoura University, 3Department of Medical Oncology, Oncology Center, Mansoura University, 4Department of Molecular Biology, Institute of Genetics, Menoufiya University, Egypt.

The pathogenesis of acute myeloid leukemia (AML) involves the cooperation of mutations promoting proliferation/survival and those impairing differentiation. Point mutations of the N-RAS gene are the most frequent somatic mutations causing aberrant signal-transduction in acute myeloid leukemia (AML). The aim of the present work is to study the frequency and prognostic significance of N-RAS gene mutations (N-RASmut) in de novo Egyptian adult AML. Bone marrow specimens from 150 patients with de novo acute myeloid leukemia and controls were analyzed by genomic PCR-SSCP at codons 12, 13 (exon 1), and 61 (exon 2) for N-RAS mutations. In 12.7% (19/150) AML cases, N-RAS gene mutations were found and were observed more frequently in the FAB subtype M4eo (P = 0.028) and with codon 12, 13 (14 of 19; 73.7%). Patients with N-RAS mutation had a significant lower peripheral and marrow blasts (P = 0.004, P = 0.03) and clinical outcome did not improve more than in patients without mutation. In patients with N-RAS gene mutation vs those without, complete remission rate was (63.2% vs 56.5%; P = 0.46), resistant disease (15.8% vs 23.6%; P = 0.51), three years overall survival (44% vs 42%; P = 0.85) and disease free survival (42.1% vs 39.9%, P = 0.74). Multivariate analysis showed that age was the strongest unfavorable factor for overall survival (relative risk [RR], 1.9; P = 0.002), followed by cytogenetics (P = 0.004). FAB types, N-RAS mutation and leukocytosis were the least important. In conclusion, the frequency and spectrum of N-RAS gene mutation differ between biologically distinct subtypes of AML but do not significantly influence prognosis and clinical outcome in patients with AML.

During the last two decades, the pathophysiology picture of acute myeloid leukemia (AML) has rapidly advanced (Mrózek et al., 1997). Molecular alterations targeting the activation of proto-oncogenes and the inactivation of tumor suppressor genes (TSGs) play a key role in the development of multistage carcinogenesis (Spandios, 1986). These genetic alterations have been molecularly detectable and used for diagnosis, detection of minimal residual disease, and prediction of prognosis.

RAS genes and their sequences are among the most-studied oncogenes, there are three functional RAS genes: N- (from a neuroblastoma cell line), K- (Kirsten), and H- (Harvey) RAS, each gene contain 4 exons, encoding homologous proteins (p21) that have the biochemical property of binding guanine nucleotides and exhibit intrinsic GTPase activity and whose cellular localization is at the inner surface of the plasma membrane (Bowen et al., 2005). They have been implicated with the transduction of signals that regulate cell proliferation, survival, and differentiation and in neoplastic transformation (Stirewalt et al., 2001). Cloning and sequence analysis of oncogenic RAS genes have revealed that the mechanism of activation involves a single base substitution that alters an amino acid of the corresponding p21 protein and thus decreases associated GTPase activity. Point mutations have been demonstrated in either codon 12, 13, or 61 of RAS genes. Moreover, in all cases investigated thus far, the mutations turned out...
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to be specific for the tumor cells and were not found in normal cells of the respective patient (Kiyoi et al., 1999; Beaupre & Kurzrock, 1999). N-RAS gene mutation seem to be the most prominent RAS mutations in patients with AML and have been reported in 11% to 30% of patients (Neubauer et al., 1994). They lead to increased activity of the RAS pathway, resulting in increased proliferation and decreased apoptosis rate in experimental systems N-RAS gene mutation was found in more mature progenitors, suggests that N-RAS mutation is most likely a post initiation event contributing to the progression/proliferation of subclones in AML. Lethally irradiated mice that received transplants of bone marrow cells infected with mutated N-RAS (N12) develop a myeloproliferative/AML-like disease (MacKenzie et al., 1999). Although N-RAS mutations in AML were first reported 18 years ago (Bos et al., 1987; Toksoz et al., 1987), the prognostic impact of N-RAS mutations is still under discussion and seems to vary from disease to disease (Coghlan et al., 1994; Padua & West, 2000; Paquette et al., 1993). In some studies, the prognostic impact of N-RAS mutations is still under discussion and seems to vary from disease to disease (Coghlan et al., 1994; Padua & West, 2000; Paquette et al., 1993). In some studies, the prognostic impact of N-RAS mutations could not be defined at all (Bowen et al., 2005; Illmer et al., 2005; Radich, 1990). Other studies did not find a significant association of N-RAS mutations with certain cytogenetic subgroups (Stirewalt et al., 2001; Neubauer et al., 1994). In contrast, others found a higher percentage of AML M4 subtypes in patients with N-RAS (Radich et al., 1990) or even demonstrated an association especially with AML M4eo/inv(16) (Reilly, 2005; Valk et al., 2004).

We have assayed the frequency of N-RAS gene mutation in newly diagnosed de novo adult AML patients by genomic PCR-SSCP method, and correlated the results to the clinical characteristics, cytomorphology, cytogenetics and prognosis when the information was available.

Materials and Methods

Our study included 150 diagnosed de novo adult AML patients representing various French-American-British (FAB) types selected from patients admitted to the Mansoura Oncology Center during 2004 to 2007. A written informed consent was obtained from the patients prior to their enrollment in this study after Institutional Review Board approval.

All patients were treated with standard protocol (Yates et al., 1982), except those with acute promyelocytic leukemia (M3) who received All-trans retinoic acid plus anthracycline (Kanamaru et al., 1995). AML patients included 110 males and 40 females with median age 49 years. Patients were diagnosed according to standard diagnostic methods including cytomorphological, cytochemical and immunological evaluation (Schoch et al., 2004).

Resistant disease (RD) was defined as more than 15% BM blasts and partial remission as 5% to 15% BM blasts after course 1. Overall survival (OS) was defined as the time from entry to death. For patients achieving first CR, disease-free survival (DFS) was defined as the time from first CR to an event (death or relapse).

In addition 30 healthy subjects with matched age and sex were selected to act as a control group for the evaluation of N-RAS gene mutations in normal subjects. All patients and control samples were analyzed for mutation in exon 1, 2 of the N-RAS gene using genomic PCR-SSCP (Single-strand conformation polymorphism) method (Kiyoi et al., 1999).

Genomic DNA was extracted from diagnostic marrow specimens using the QIAamp DNA blood mini kit for DNA extraction (QIAGEN Inc Chasworthy, CA). The concentration of extracted DNA was then measured by UV spectrophotometry (Pharmacia, Biotech, UK) at 260 & 280 nm and run on agarose gel electrophoresis 2% for detection of purity.

Separate assays were developed for mutation detection at "hot spots" in codons 12/13 (exon 1) and codon 61 (exon 2). Oligonucleotide primers used for amplifying short fragments (241 base pair [bp], exon 1; 201 bp, exon 2) were designed for PCR (Bowen et al., 2003) as follows: N12/13 [forward, 5'-GAC TGA GTA CAA ACT GGT GG-3'; reverse, 5'-TGC ATA ACT GAA TGT ATA CCC-3']; N61 [forward, 5'-CAA GTG GTT ATA GATGGTGAAACC-3'; N61 reverse, 5'-AAG ATC ATC CTT TCA GAG AAA ATA AT-3'].

PCR amplification was performed in 50 µl reaction, contained 50-100 ng of genomic DNA, 10 mM Tris HCl (pH 8.3), 50 mM KCl and 1.5 mM MgCl2, 200 µM of each deoxyribonucleotide triphosphate (dNTP), 2.5 units Taq polymerase, and 6% dimethylsulphoxide.
PCR conditions were as follows: (1) Exon 1, HotStar Taq (Qiagen, Valencia, CA), 0.625 U primers (12.5 pmol) of each N12/13 forward, N12/13 reverse. Denaturing at 95°C for 5 min and 94°C for 30s; annealing at 55.5°C for 1 min; extension at 72°C for 1 min for 35 cycles; final cycle at 72°C for 10 min. (2) exon 2, HotStar Taq (Qiagen), 0.625 U (12.5 pmol) of each primer N61 forward and N61 reverse. Denaturing at 95°C for 5 min and 94°C for 30s; annealing at 55.5°C for 1 min; extension at 72°C for 1 min for 35 cycles; final cycle at 72°C for 10 min.

Single-strand conformation polymorphism (SSCP) (Hayashi, 1991) was performed for PCR product to detect N-RAS gene mutations. Products were mixed with 10 volumes of loading buffer, quenched on ice immediately, and applied to 5% polyacrylamide gel electrophoresis at 50 V, overnight, stained by silver nitrates, wrapped in plastic foil. Normal gene exhibits a specific conformational pattern, while a mutant gene displays a pattern with a different electrophoretic mobility (mobility shift).

Statistical Analysis
Data analysis was performed using SPSS for window version 16.0 (SPSS, Inc, Chicago, IL, USA); we used non-parametric methods for analysis of data as some variables showed violated normality (age, CBC and Blast counts); therefore median is used to express the central tendency of quantitative variables. Differences between two groups regarding frequency was done by Chi square test. Prediction of overall survival was done using multivariate analysis applying (age, cytogenetics, FAB subtypes, WBC count, N-RAS mutation state) as a co-variants. Survival studies were done using Kaplan Meier curve and Log rank test.

Results
Mutation Frequency of N-RAS in AML Samples
Screening of N-RAS mutations around the mutational hot spots (codons 12, 13, 61) revealed extra PCR bands (mutant bands) in addition to wild bands in 19 (12.7%) of 150 AML patients, whereas 131 had only the N-RAS wild-type allele (N-RASwt). None of the patients with mutations was homozygous for the mutation, indicative negative loss of heterozygosity (-ve LOH) (figure 1).

Mutation Spectrum of N-RAS
Most mutations (14 of 19; 73.7%) were found at codon 12, 13 (exon1). Mutations at codon 61(exon 2) were detected in 5 (26.3%) of 19 patients.

Correlation of N-RAS Mutation with Cytomorphology
In the FAB subtype M4eo, N-RAS mutations were represented more frequently (9 of 29) than they were in all other subtypes (P=0.028) (Table 1). In the M3, M6 and M7 subtypes, no N-RAS mutation was detected, making N-RAS mutations highly under represented in these subtypes. In all other FAB subtypes, the distribution of N-RAS mutation did not differ significantly from each other.

N-RAS Mutation and Presenting Clinical Patient Characteristics
Primary analysis revealed that patients with N-RASmut had significantly lower percentages of peripheral (12% versus 43%, P= 0.004) and marrow blasts (61% versus 71%, P= 0.04) compared to those without N-RASmut. However, N-RAS mutation frequency did not vary significantly with age and white cell count (Table 1).

N-RAS Mutations and Cytogenetics
Based on cytogenetic findings, 128 patients were segregated into four groups: a good-risk group (n= 29) was defined by karyotype, t(8;21) or inv(16); a standard-risk group (n=
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99) by normal or other karyotype. In the cytogenetic subgroups with inv(16), N-RAS mutations occurred at higher frequencies 42% (8 of 19; \( P = 0.01 \)). In contrast, N-RAS mutations were significantly underrepresented in the groups with normal karyotype (26.3%; 5 of 19; \( P = 0.003 \)).

**N-RAS Mutational Status: Clinical Outcome**

N-RAS mutations had a non significant improved clinical outcome. Complete remission (CR) rate (\( N-RAS^{mut} \), 63.2%; \( N-RAS^{wt} \), 56.5%; \( P = 0.46 \)), resistant disease (\( N-RAS^{mut} \), 15.8%; \( N-RAS^{wt} \), 23.6%; \( P = 0.51 \)), 3 years survival (\( N-RAS^{mut} \), 47.4%; \( N-RAS^{wt} \), 42%; \( P = 0.85 \)), disease free survival (\( N-RAS^{mut} \), 42.1%; \( N-RAS^{wt} \), 38.9%, \( P = 0.74 \)) (Table 2). Multivariate analysis showed that age was the strongest unfavorable factor (relative risk [RR], 1.9; \( P = 0.002 \)), followed by cytogenetics (\( P = 0.004 \)). FAB types, NRAS mutation and leukocytosis were less important (Table 3).

<table>
<thead>
<tr>
<th>Table 1. Clinical Characteristics of Patients by Mutation Status.</th>
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<tbody>
<tr>
<td><strong>Total</strong></td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>n=150</td>
</tr>
<tr>
<td>Age in years (range)*</td>
</tr>
<tr>
<td>Marrow blasts (range)*</td>
</tr>
<tr>
<td>WBC ((10^3/\mu L)) (range)*</td>
</tr>
<tr>
<td>Peripheral blasts (range)*</td>
</tr>
<tr>
<td>FAB types</td>
</tr>
<tr>
<td>M4eo</td>
</tr>
<tr>
<td>Other FAB types</td>
</tr>
<tr>
<td>Cytogenetics(n=128)</td>
</tr>
<tr>
<td>t(8;21)</td>
</tr>
<tr>
<td>Inv(16)</td>
</tr>
<tr>
<td>Others</td>
</tr>
<tr>
<td>Normal</td>
</tr>
</tbody>
</table>

*Median values are indicated in age and WBC. Number of cases is shown in FAB and cytogenetics

\*P value <0.05 is considered as significant. NS=not significant.

<table>
<thead>
<tr>
<th>Table 2. Clinical Outcome Data by N-RAS Mutation Status.</th>
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<tbody>
<tr>
<td><strong>N-RAS mutant</strong></td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>n= (19)</td>
</tr>
<tr>
<td>Complete remission</td>
</tr>
<tr>
<td>Resistant disease</td>
</tr>
<tr>
<td>3-years disease free survival</td>
</tr>
<tr>
<td>3-years survival</td>
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\*P value <0.05 is considered as significant. NS=not significant.
Table 3. Overall Survival as Dependent Parameter Studied with other Covariates (Multivariate Analysis).

<table>
<thead>
<tr>
<th>Covariate</th>
<th>P value</th>
<th>Relative Risk (95% CI)</th>
</tr>
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<tbody>
<tr>
<td>Age in years</td>
<td>0.002</td>
<td>1.9 (1.2-2.6)</td>
</tr>
<tr>
<td>Cytogenetics*</td>
<td>0.004</td>
<td>2.6 (1.5-4.6)</td>
</tr>
<tr>
<td>FAB other than M2</td>
<td>NS</td>
<td>0.6 (0.02-0.7)</td>
</tr>
<tr>
<td>WBC count &gt;100 x 10^3/L</td>
<td>NS</td>
<td>0.51 (0.07-0.6)</td>
</tr>
<tr>
<td>N-RAS gene mutation</td>
<td>NS</td>
<td>0.17 (0.08-0.32)</td>
</tr>
</tbody>
</table>

*P value <0.05 is considered as significant. NS=not significant.

**Discussion**

Characterization of molecular mutations in AML is of importance to better understand the biology of the disease, to better assess risks in individual patients, and to better detect minimal residual disease (MRD). Previous reports stated that RAS gene mutation is a relatively frequent molecular event in AML and in MDS (Schoch et al., 2005) occurring most commonly in N-RAS, followed by K-RAS, and least common in H-RAS genes. This pattern is different from solid tumors such as gastrointestinal tumors in which K-RAS is most commonly mutated. This greater transforming capacity for N-RAS mutation in hematopoietic cells (Beghini et al., 2000) and/or the predominance of N-RAS p21 protein in myeloid cells, leading to selective pressure for N-RAS (compared with K-RAS or H-RAS) gene mutation (Panagopoulos et al., 1996). All of these reports motivated us to study the frequency of N-RAS mutations in our AML patients.

N-RAS mutations were found in 13% of study-patients. Our result corroborate previous studies which reported N-RAS mutations ranging from 11.4% to 13.9% (Bowen et al., 2005; Radich et al., 1990), while other reported a higher frequency of 19% and 28% (Stirewalt et al., 2001; Paquette et al., 1993).

Primary analysis revealed that patients with N-RAS mutation had significantly lower percentages of peripheral (12%) and marrow blasts (61%) than in patients without N-RAS mutation (43%, 71% respectively). No significant differences were found between the two groups with respect to age and WBC counts as reported elsewhere (Bowen et al., 2005). Other studies reported higher WBC counts, lower percentage of blasts, and lower percentage of CD34+ blasts in patients with N-RAS mutations compared to patients without N-RAS mutations (Stirewalt et al., 2001). In this study, the correlation of N-RAS mutations and FAB subtypes demonstrated an association between frequency of N-RAS mutation and biologically distinct subtypes of AML. Patients with AML M4eo had a significantly higher frequency of N-RAS mutations, three times more than in patients with other FAB types. In contrast, we did not find a similar association in AML M4 with N-RAS mutations as reported in other studies (Illmer et al., 2005; Bacher et al., 2006). Although such association between N-RAS mutations and the FAB subtype M4 or between K-RAS and AML M4 was reported (Radich, 1990; Bowen et al., 2005), in these studies, however, M4 and M4eo were not analyzed separately, therefore, the observed association may be due to the inclusion of M4eo patients in the analysis. Data obtained from in vitro experiments suggest that mutant RAS gene promotes a myeloid maturation defect, with relative sparing of the monocyte-macrophage lineage (Darley & Burnett, 1999). This may be consistent with the
overrepresentation of N-RAS mutation in M4/M5 FAB types.

In our study, more than 40% of N-RAS mutation was detected in patients with inv(16), this result is in agreement with most of the previously published studies which reported a percentage of N-RAS mutation ranging from 26% to 37.6% (Illmer et al., 2005; Valk et al., 2004; Beghini et al., 2000; Bacher et al., 2006). Only one study by Panagopoulos et al. (1996) found N-RAS mutations in a small subset of 1 of 8 patients with inv(16), this observation may be due to the small number of cases studied.

The prognostic significance of the N-RAS gene mutation is a matter of controversy. Generally, RAS gene mutation is associated with tumor progression and with a poor prognosis such as in solid tumors and acute lymphoblastic leukemia (ALL) (Slebos et al., 1987; Lübbert et al., 1990). In this study, CR rate, DFS and OS were higher in patients with N-RAS mutations than patients without the mutations, although these differences did not attain statistical significance, such results may indicate that N-RAS mutation had a favourable impact on clinical outcome. In this study, multivariate analysis for the prediction of overall survival showed that prognosis in AML was not influenced by N-RAS mutations as demonstrated elsewhere (Bowen et al., 2005; Illmer et al., 2005; Radich et al., 1990).

In contrast, Kiyoi et al., (1999) found that N-RAS mutations represent an unfavorable prognostic parameter in patients with AML with favorable karyotype, while AML with intermediate or unfavorable karyotype, prognosis was not influenced by N-RAS mutations.

In summary, we found a clear pattern of N-RAS mutation in defined subgroups of patients with AML. However, because of the lack of a clear prognostic value of the N-RAS mutations, studies including a large number of cases with different FAB types and molecular aberrations must be considered to definitively answer the question of the prognostic impact of N-RAS mutations.

References


