FREQUENCY OF NRAS AND KRAS GENES IN NEWLY DIAGNOSED ACUTE MYELOID LEUKEMIA PATIENTS

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ABSTRACT

Objective: To determine the frequency of NRAS and KRAS mutations in newly diagnosed acute myeloid leukemia patients and correlation with their clinicopathological parameters along with prognostic impact.
Study Design: Cross-sectional study.
Place and Duration of Study: Armed Forces Institute of Haematology, Rawalpindi, from Mar 2017 to Aug 2017.
Methodology: A total of 70 acute myeloid leukemia patients were recruited in this study. Each patient was assessed by blood complete picture, bone marrow examination (aspiration and trephine biopsy) and cytochemical staining by sudan black. Flow cytometry was done for immunophenotyping of each patient. Mutation analysis of NRAS and KRAS of exon 1 and exon 2 were performed on Peripheral /bone marrow sample by PCR followed by purification and analysis of gene mutations by Sanger sequencing.
Results: Of the seventy patients, five percent were positive for NRAS mutation while no KRAS Mutation was detected in our study. Mean age of our patients was 31.5 with a SD of ± 18.9. Patients presented very high levels of WBC with an average of 83.7 ± 107 years. Regarding clinicopathological parameters all NRAS mutation positive patients and NRAS mutation negative patients there was prominent splenomegaly as well as bleeding from gums but the percentage of splenomegaly and bleeding gums was much greater in NRAS mutation patients as compared to NRAS negative patients.
Conclusion: There were limited studies regarding NRAS and KRAS mutations in Pakistan so larger sample size is required to determine frequency along with its prognostic impact.

Keywords: Frequency, KRAS gene, NRAS gene, Sanger sequencing.

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogenous genetic disorder that shows variability in terms of its molecular and genetic baselines[1]. The cooperation of mutations promoting propagation/survival and those impairing differentiation can be evaluated through pathogenesis of acute myeloid leukemia (AML)[2]. In adults, it is the commonest occurring acute leukemia with approximately 3.7 in every 100,000 individuals being affected in the West annually[3]. The median age of diagnosis in adults is 67 years[4]. The male to female ratio is around 5:3[5]. Signs and symptoms include fatigue, fever, weight loss, infections, abnormal bleeding and bruising[6].

The diagnosis of AML requires the presence of 20% blasts in the peripheral or the bone marrow as specified by the (2016) WHO Revised Classification. However, subgroups of AML with recurrent genetic abnormalitiesinvolves the presence of t(8;21), t(15;17), t(9:11) or inv (16) alone are sufficient to establish diagnosis irrespective of the Peripheral blood or Bone Marrow blast count[6]. These genetic mutations of AML are segregated into 3 classes. Class I accompanies mutations which affect signal transduction pathways, tagged with a substantial rise in proliferative advantages to leukemic clones while Class II accompanies mutations that affect transcription factors and impaired differention[7,8]. FMS-like tyrosine kinase 3 (FLT3) gene, NRAS neuroblastoma retroviral associated sequence gene (NRAS) are entities of Class I mutations. Mutations of nucleophosmin gene (NPM1), the CCAAT/ enhancer binding protein gene (CEBPA), the Wilm tumor (WT1) gene, and the additional sex

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combs like 1 (ASXL1) gene are offshoots of Class II mutations. Class III mutations associated with epigenetic regulation, including isocitrate dehydrogenase[1] (IDH1), isocitrate dehydrogenase[2] (IDH2), and ten-eleven-2 (TET2), which result in a hypermethylation phenotype with impairment of hematopoietic differen-tiation[8].

The RAS genes play substantial role in the regulatory process that affect proliferation, differentiation and apoptosis[9]. RAS proteins are among the family of protoongogenes that is mutated in leukemia.These RAS proteins are expressed by three genes: Neuroblastoma (NRAS), Harvesey (HRAS), Kristen (KRAS). The abnormalities in RAS signaling pathway lead to uncontrolled proliferation and differentiation of haemapoietic progenitor cells[10]. The NRAS gene is located on short arm of chromosome 1p13.2 while KRAS gene is located on short arm of chromosome 12p12.1. The prognostic effect of these mutations is still being evaluated and till date studies have given variable prognosis[4].

As the knowledge about leukemogenesis has increased it is accepted that genetic abnormalities leading to leukemia are not only heterogenous but complex, and multiple aberrations often cooperate in a multistep process to initiate the complete leukemia phenotype. Our study will determine the frequency of NRAS and KRAS mutations in newly diagnosed AML patients and to determine the initial prognostic stratification of these patients.

METHODOLOGY

This cross-sectional study was carried out in the department of Haematology, Armed Forces Institute of Pathology, Rawalpindi from March 2017 to August 2017. Seventy patients of newly diagnosed acute myeloid leukemia were inducted by nonprobability consective sampling technique Patients of all age groups and both genders with AML were included in the study and patients having secondary leukemia, ALL and patients on chemotherapy were excluded from the study.The approval from the ethical commitee has been sought and certificate attached.

Three ml venous blood in ethylene diamine tetra acetic acid (EDTA) wascollected and blood complete counts was performed by Sysmex XE-5000 automated hematology analyzer. Blood smear was examined for differential leucocyte count and presence of blasts. Bone marrow examination was done and smears from aspirate were made and examined. Flow cytometry was carried out on peripheral blood/bone marrow aspirate of these patients .

Table-I: Thermocycle program for NRAS and KRAS Genes.

Stage	Temperature °C	Time (sec)	Cycles
NRAS Exon 1			
Initial Denaturation	94	120	25
Denaturation	94	55	25
Annealing	62	60	25
Extension	72	90	25
Final Extension	72	180	25
NRAS Exon 2			
Initial Denaturation	94	120	28
Denaturation	94	55	28
Annealing	60	60	28
Extension	72	90	28
Final Extension	72	180	28
KRAS Exon 1			
Initial Denaturation	94	120	25
Denaturation	94	55	25
Annealing	62	60	25
Extension	72	90	25
Final Extension	72	180	25
KRAS Exon 2			
Initaial Denaturation	94	120	25
Denaturation	94	55	25
Annealing	59	60	25
Extension	72	90	25
Final Extension	72	180	25

Diagnosed AML patients peripheral blood/ bone marrow samples were collected for DNA extraction. DNA extraction was carried out by GeneJet Genomic DNA Purification kit (Thermo Scientific USA). Primers were designed by Primer

Quest Tool (Integrated DNA Technologies, Iowa, USA) and synthesized by Biosearch Tech (Petaluma, CA, USA). Ready to use PCR master mix (Bioran Life Sciences, Ludwigshafen, Germany) was used for Polymerase chain reaction in a 25 ul reaction mixture containing 10 pmol of the forward and reverse primers (table-II) 12.5ul of Taq Master Mix (2X) and 100 ng of genomic DNA. Primer and amplification conditions were shown in table-I. All amplicons were visualized on 6.0% acrylamide gel followed by silver staining. Prior to sequencing, the amplified product was purified by the PureLink PCR Purification Kit, (Invitrogen, Carlsbad, CA, USA) to remove the nonspecific amplification products, primer-dimers and unused dNTPs. The sequencing was carried out by Sanger's dideoxy chain termination method using the BigDye Terminator sequencing kit v3.1 (Applied Bio-systems, Foster City, CA, USA). Polymerase chain reaction was carried out in a 20ul reaction mixture containing 10 pmol of the sequencing primer (table-I), 2ul of 5x sequencing buffer, 4ul of RR v3.1 and 100 ng of DNA. Amplification conditions are specified in table-I. Agen Court Clean SEQ (Beckman Coulter Inc., Brea, CA, USA) was used to purify the amplified product to exclude dye blobs and unused labeled ddNTPs. Genetic analyzer 3130 (Applied Biosystems) was used for data collection and sequencing analysis software v 5.2.3 (Applied Biosystems) was used to analyze the data. The observed data was compared with the NCBI reference sequence of the KRAS and NRAS gene.

RESULTS

Out of total 70 patients 38(46%) were males and 32 (54%) were females with male to female ratio of 1.5: 1. Mean age of these patients was 38 years with range between 1 to 54 years. According to FAB classification, 5% were AML M4, 15% were M1, 31% were M2, 3% were Mo, 7% M6, 9% M3 (fig-1).

On molecular analysis NRAS mutations were detected in 4 (5.7%) of patients whereas KRAS mutations was not detected in any of these patients. Out of NRAS positive patients 3 were

Table-II: Primers for pre- and post-sequencing PCR.

Primer	Gene
FORWARD 5′- AAGGTACTGGTGGAGTATTTGATAG -3′ REVERSE 5′- CTTGAAACCCAAGGTACATTTCAG -3′	KRAS Exon 1
FORWARD 5′-CATCTTTGGAGCAGGAACAATG-3′ REVERSE 5′-TGCATGGCATTAGCAAAGACTC-3′	KRAS Exon 2
FORWARD 5′-TCCAGAAGTGTGAGGCCGAT-3′ REVERSE 5′-GATCCGACAAGTGAGAGACAGG-3′	NRAS Exon 1
FORWARD 5′-CCAGATAGGCAGAAATGGGCT-3′ REVERSE 5′-ATCTTCCCTAGTGTGGTAACCT-3′	NRAS Exon 2
Sequencing Primer	
FORWARD 5′- AAGGTACTGGTGGAGTATTTGATAG -3′	KRAS Exon 1
FORWARD 5′-CATCTTTGGAGCAGGAACAATG-3′	KRAS Exon 2
FORWARD 5′-TCCAGAAGTGTGAGGCCGAT-3′	NRAS Exon 1
FORWARD 5′-CCAGATAGGCAGAAATGGGCT-3′	NRAS Exon 2

Table-III: Clinocohaematological comparison of mutation positive and mutation negative patients.

	Patients with NRAS (n=4)	Patients without NRAS (n=66)
Male gender %	75%	46%
Bleeding gums % of patients	100%	27.2%
Splenomegaly % of patients	100%	12.1%
MeanWBC count x 10^9/l	98.6	36.3
MeanHb g/dl	7.2	9.8
MeanPlatelet count x 10^9/ l	31	64
Bone marrow Blasts %	40%	89%

males and 1 was female On FAB type classification, 3 patients had AML M4 while 1 was diagnosed as having AML FAB type M6. Clinico-haematological characteristics of patients with and without NRAS are compared in table-III. In NRAS Mutation positive cases there is missense mutation where (G>A) as shown on sequencing results (fig-2) with the following NCBI No:

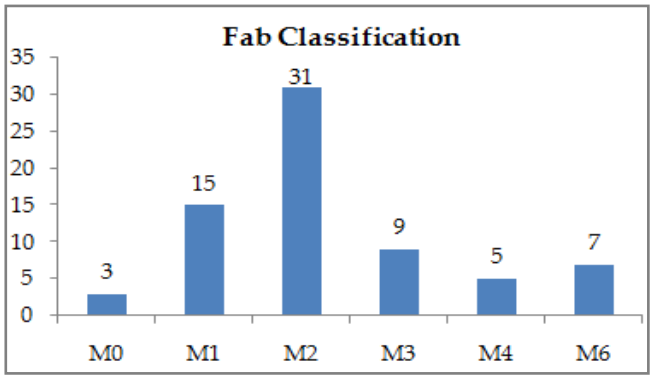

Figure-1: Percentage of patients according to French-American-British (FAB) classification.

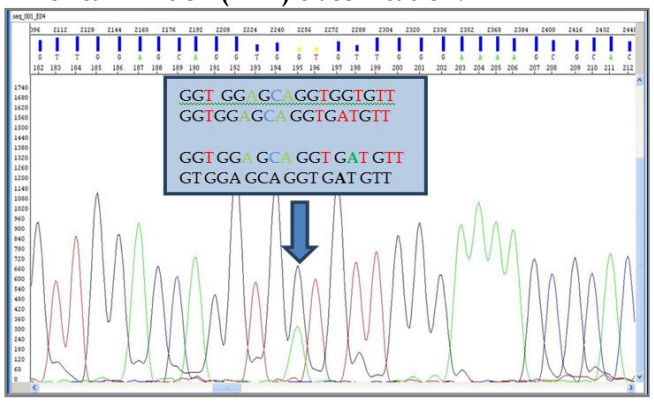

Figure-2: Chromatogram of NRAS mutation positive patient showing replacement of guanine to adenine.

NM_002524.4 (NRAS): c.35G>A (p.Gly12Asp) G>A {Glycine >Aspartic acid}.

After diagnosis, these patients were treated with standard induction chemotherapy for AML with D3A7. Four patients received induction. However, one patient died during induction. Bone marrow examination was done of other 3 patients which showed complete haematological remission.

DISCUSSION

RAS genes are among those investigational mutations that is not included in WHO Classification 2016. There are three types of RAS genes: Harvesey (HRAS), Kristen (KRAS), and Neuroblastoma (NRAS). These proteins are GT Pases (Guanosine triphosphatase) which control cellular proliferation along with cell survival. These RAS proteins are strictly supervised by guanine nucleotide exchange factors (GEF) that aids in promoting GDP dissociation and GTP binding, and GT Pase-activating proteins (GAP) that stimulate the intrinsic GTPase activity of RAS to switch off signaling[10].

Ras genes regulate cellular proliferation by binding of a ligand to a variety of receptors and regulate cellular proliferation by cycling between inactive state ie (GDP) and active state (GTP). Once RAS is activated it causes phosphorylation of mitogen activated protein kinase (MAPK) or (RAF), which causes further phosphorylation of series of kinases mitogen activated protein kinases (MAPK) leading to cellular proliferation. So if the RAS is mutated there will be eventually enhanced cellular proliferation[10]. These pathogenetic mechanisms suggest that the presence of NRAS and KRAS may have a prognostic significance. Today, in the era of targeted therapy, knowledge of these mutations may be beneficial in tailoring treatment plans for these AML patients. However, larger sample is required to establish the exact frequency of RAS mutations. Nevertheles, longer followup is required to determine the prognosis of these mutations.

In our study, frequency of NRAS mutations was 5%. Similar results have been reported by Nageswara *et al* in a study conducted in India[11]. However Renniville *et al* has reported a higher frequency of 10% in French population[12]. Bowen *et al* conducted a study in UK which showed frequency of NRAS mutation of about 11% however this is showing a much higher frequency of NRAS mutations in the Western population[2]. KRAS mutation was not detected in our study while same study conducted by Fernandez *et al* also showed no KRAS mutation positivity[15]. Similar study conducted in French population by Renniville *et al* which showed frequency of KRAS mutation of 5%[1] that is higher than our study. So

larger studies are needed to establish the frequency of KRAS mutations in our population. Regarding NRAS mutation positive patients, all cases have raised white cell count, with the cut off limit of 50 x 10^9/l as compared to mutation negative group and it correlates with the study conducted by Nageswara *et al*[11] Platelet count in mutation positive patients is low in our study that is also comparable with same study conducted by Nageswara *et al* in India[11]. Yohe in USA which showed NRAS mutations positivity of about 8% that is slightly higher than my study and KRAS mutations was detected in 2% of cases and according to this study no clear effect on prognosis can be established however more studies is required for establishing the exact prognostic impact regarding NRAS and KRAS mutations[13]. In my study mutation positive cases are of FAB classification AML M4 that correlates with study conducted by others[2,8].

NRAS Mutation detected in our study showed base substitution from G>A and this base substitution correlates with study conducted by Bowen[2] and same study by Nageswara *et al*[11].

Regarding KRAS mutations,no mutation was detected in our study and the one done by Reniville KRAS mutations was detected in 5% of case. In a study conducted by Ahmad *et al* in Egypt showed that KRAS mutation was most commonly positive in patients with AML M4[14] Similar study was conducted by Neubauer *et al* which showed more NRAS mutation positi-vity in AML M4[17,18].

In our study additional mutations were not considered which was studied by Krauth *et al* in which patients who are RUNX1-RUNX1T1 positive were also positive for additional mutations including NRAS, KRAS, ASXL1[16].

According to study conducted by Yohe no clear prognostic impact while in our study one patient died after induction while three showed complete haematological response but exact prognostic effect cannot be determined in our study which need to be done on larger sample[13].

CONCLUSION

There are few limitations in our study first is regarding sample size which is not large and bigger sample size is required for determining the frequency along with its prognosis. In Pakistan very limited studies have been done regarding these RAS mutations and much larger sample size is required.

CONFLICT OF INTEREST

This study has no conflict of interest to be declared by any author.

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