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RESEARCH ARTICLE

Optimization of allele specific PCR (AS-PCR) for the early detection of FLT3 (D835Y) mutation in Acute Myeloid Leukemia patients at Tabuk, Saudi Arabia.

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Abstract

Activating mutations in FLT3 occur in ~30% of adult AML, primarily consisting of internal tandem duplication (ITD) mutations (~25%) and point mutations in the tyrosine kinase domain (~7%), at the activation loop (residue D835). The early detection of FLT3- D835Y in AML patients is clinically important for AML patients in this small region of Saudi Arabia especially for determining drug responses, prognosis, and selection of therapy. The present study was conducted on 47 specimens among which 37 were from leukemia patients (5 were newly diagnosed AML, 7 ALL and 25 MPD) and 10 healthy controls. Genomic DNA from blood was isolated using DNA QIAamp DNA Blood Mini Kit from Qiagen and stored at -20°C until PCR. The Allele Specific-PCR based assay for FLT3 (D835Y) mutation was optimized in the Lab .The mutation was detected in 2/5 cases of AML, 0/25 of MPD and 0/5 of ALL. It was observed that patients with AML possessed significantly higher frequency of FLT3 D835 mutation (2/5) than other leukemia cases and healthy controls. The D835Y Flt3-TKD mutation was not detected in any of the healthy controls (0/10). It was concluded that allele specific PCR technique permits the direct detection of FLT3 c.2503G>T (D835Y) point mutation in AML patients and acts as useful genetic technique that is very valuable for molecular diagnosis, prognosis, drug response, and predisposition to AML patients. In addition, the test was simple, fast, and inexpensive procedure that does not require any special equipment other than a thermo cycler.

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Introduction:-

Acute myeloid leukemia is a clinically and biologically heterogeneous disease and the most common cause of leukemia-related mortality in the United States, with an estimated 19,950 new cases and 10,430 deaths anticipated in 2016 (ACS 2016). Acute myeloid leukemia (AML) is also common among the Saudi Arabian population with a median age at diagnosis of 25 years (range, 0-99 years) among males and 28 years (range, 0-88 years) among females (1).

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However, patients with a high risk of relapse, as determined on the basis of cytogenetics, Cell counts on presentation and the presence of FLT3 mutation, are considered for allogeneic bone marrow transplantation. Extramedullary relapse (EMR) occurs in approximately 2% to 8% of all AML cases (2). The occurrence of FLT3 mutations of AML was associated with poor prognosis and significantly change treatment towards FLT3inhibitor (3-4). The FLT3 gene is a member of class III receptor tyrosine kinase family, including c-kit, c-fms, and the platelet-derived growth factor receptors (5). FLT3 length mutations (FLT3-LM or FLT3-ITD for “internal tandem duplication”) represent one of the most frequent genetic alterations in AML. They show a frequency of 20% to 27% in AML in adults (6) and of 10% to 16% in childhood cases (7).

FLT3 (FMS-like tyrosine kinase 3, CD135) is a type 3 receptor tyrosine kinase that plays important roles in cell survival, proliferation, and differentiation during normal hematopoiesis. Activating mutations of FLT3 are now recognized as the most common molecular abnormality in acute myeloid leukemia and may play a role in other hematologic malignancies as well (8). FLT3-LM mostly are represented by internal tandem duplications and/or insertion-deletion mutations in exons 11 and 12 of the human FLT3 gene on chromosome 13q12, which codes for the juxtamembrane domain of the FLT3 protein. The mutations are heterogeneous and consist of internal tandem duplications of 6 to 30 amino acids, resulting in an elongated FLT3 protein with constitutive PTK activity. In addition to the juxtamembrane domain mutations, mutations in the tyrosine kinase domain (FLT3-TKD) have also been described in AML (9).

FLT3-TKD mutations are small mutations in the activation loop of FLT3, mostly representing point mutations in codon D835 or deletions of codon I836. These mutations alter the configuration of the constitutive activation, resulting in increased access of substrates and ATP that leads to autophosphorylation and subsequently triggers various intracellular signaling pathways (10). They induce constitutive tyrosine phosphorylation leading to activation of the receptor tyrosine kinase and are supposed to represent gain-of-function mutations (11). Corresponding activation loop mutations have been reported at position D816 of KIT (CD117 or proto-oncogene c-Kit) and also in other receptor tyrosine kinases. Most often, these result in replacement of aspartate at the 835th position by another amino acid (e.g. tyrosine, histidine, valine, or glutamate) (12). PCR-RFLP (restriction fragment length polymorphism) using EcoRV is one of the methods of detection for FLT3-TKD. It is important to note that digestion by EcoRV could not detect all FLT3-TKD mutations (13), however, sequence analysis showed that 50 – 68% of FLT3-TKD mutations were substitutions of the first nucleotide of codon D835, most commonly from G to T (14). Traditionally, FLT3-ITD, FLT3-TKD, and NPM1 mutations are detected by PCR followed by electrophoresis or Taqman RQ-PCR (15).

The traditional PCR technique is convenient and less expensive. High-resolution melting (HRM) curve analysis is a homogenous, closed-tube, post-PCR technique for rapidly and efficiently discovering genetic variations in DNA fragments (16), based on the sequence dependent dissociation behavior of DNA when exposed to increasing temperature. A Simplified method for the of the early detection of *FLT3*-D835Y in AML patients is clinically important in this region of Saudi Arabia especially for determining drug responses, prognosis, and selection of molecular-targeted therapy. *FLT3*- D835Y mutation may provide good predictive criteria of an unfavorable course in AML and could be used to identify patients at a high risk of relapse. This simplified strategy for detection of *FLT3*- D835Y mutations was feasible, reproducible, cheaper and simpler when compared with other methods. Our study aims to establish a conventional cost-effective detection method for *FLT3*- D835Y mutations in patients with acute myeloid leukemia (AML) in Tabuk population.

Material and methods:-

Ethical approval:-

The study was ethically approved by the Ethics committee, University of Tabuk.

Selection of Cases:-

The subjects were recruited from the hematology OPD of King Khaled hospital, Tabuk

Inclusion Criteria:-

Cytopathologically confirmed AML patients (M0-M2 and M4-M7FAB classification, or with refractory anemia with excess of blasts (RAEB) or refractory anemia with excess of blasts in transformation (RAEB-t) with an IPSS score of ≥ 1.5 Patients with therapy-related AML/RAEB/RAEB .

Sample collection:-

4ml-5ml of venous blood was collected in EDTA vials from AML patients and healthy controls.

Experimental Design:-

Genomic DNA extraction:

DNA extraction was done by using DNeasy Blood Kit (250) cat 69506 from Qiagen as per the manufactures instructions. The QIAamp DNA Blood Mini Kit provides silica-membrane-based DNA purification. The QIAamp DNA Blood Mini Kit was designed for processing up to 200µl fresh or frozen human whole blood. QIAamp Mini spin columns can be easily processed in a centrifuge or on vacuum manifolds. The DNA extract was dissolved in nuclease-free water, and stored at 4°C until use. The DNA quality and yield was assessed using Nanodrop spectrophotometer and agarose gel electrophoresis.

Allele-specific PCR for FLT3 (D835Y) point mutation:-

Allele-specific PCR was performed in a final volume of 25µL containing 5 µL of PCR-master mix purchased from Epigentek (USA), 0.25 µL of 25 pmol/µL of each primer as shown in table 1, 17.50µL of Nuclease free water. Finally 2µL of 50ng genomic DNA. The primers were designing using Primer3 software.

Table: 1 Primers designed for FLT3-TKD c.2503C>A (D835Y)

Primer		Sequence	Annealing Temp	PCR product
Forward wt	FLT3835CF1	5'-CATAGTTGGAATCACTCATGATATC-3	52.9	193bp
Reverse	FLT3835R	5'-TACTGAAGTTGAGTCTAGAAGAAAGA-3		
Forward mut	FLT3835AF2	5'-CATAGTTGGAATCACTCATGATATA-3	53.3	193bp
Reverse	FLT3835R	5'-TACTGAAGTTGAGTCTAGAAGAAAGA-3		

Preparation of PCR mix

The Methylamp Taq PCR Mix was premixed ready-to-use solution containing all reagents required for PCR (except template, primers and water). Methylamp Taq DNA polymerase ,5x Reaction Buffer: 1 0.4 M Tris-HCl, 0.1 M (NH₄)₂SO₄, 0.1% w/v Tween-20 ,12.5 mM MgCl₂: 1x PCR solution – 2.5mM MgCl₂ ,1mM dNTPs of each and remaining nuclease free ddH₂O as depicted in Table 2. Finally the 2µL of DNA was added from each patient and control separately.

Table 2 : Preparation of PCR mix

PCR for normal allele of FLT3-TKD c.2503C>A (D835Y)		
Reagent	1x	5 x
PCR master mix	5ul	25ul
FLT3835CF1	0.25 ul	1.25 ul
LT3835R	0.25 ul	1.25 ul
Nuclease free water	17.50 ul	87.50 ul
Total volume	23ul	125ul
PCR for mutant allele of FLT3-TKD c.2503C>A (D835Y)		
Reagent	1x	5 x
PCR master mix	5ul	25ul
FLT3835AF2	0.25 ul	1.25 ul
FLT3835R	0.25 ul	1.25 ul
Nuclease free water	17.50 ul	87.50 ul
Total volume	23ul	125ul

Thermo cycling conditions

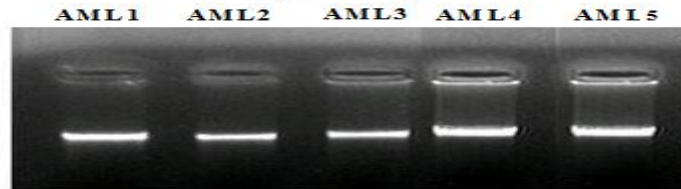
The thermo cycling conditions for the PCR amplification of Allele-specific primers for FLT3 c.2503G>T (D835Y) point mutation were initial denaturation at 95°C for 10 minutes followed by 40 cycles 95 °C for 45 sec, 57.9°C for 45 sec, and 72°C for 45 sec followed by the final extension at 72°C for 10 minutes. The amplification products were separated by electrophoresis through 2% agarose gel stained with ethidium bromide. The PCR amplification for normal as well as mutant allele of FLT3-TKD c.2503C>A (D835Y) yielded 193bp bands sizes.

Results:-

The purity of DNA extracted was checked by using Nanodrop1000 Spectrophotometer from Thermo scientific, USA. All DNA samples were screened for purity by measuring optical density (OD) at 260nm (OD₂₆₀) and 280 nm (OD₂₈₀). DNA concentration (ug/ml) was calculated based on the OD₂₆₀ reading. A ratio of ~1.8 was generally accepted as “pure” for DNA .The optimal rang of ratio obtained were 1.7-2 .The DNA 260/230: 1.7-2; Pure DNA

must have a 260/280 ratio between 1.8 and 2. The quality of DNA was checked by running in 1% gel electrophoresis as shown in Figure 1.

Figure 1. Quality check of DNA extracted from cases on 1% gel electrophoresis



Legend: Quality check of DNA
Lane 1 to lane 5 :AML 1 to AML 5

Allele-specific PCR for FLT3 (D835Y) point mutation:-

The variations in the human DNA sequence between individuals can be an indication of predisposition to disease, affect the response to drug treatment, or more directly be the fingerprint of an inheritable trait or defect. Significant efforts at improving the speed, accuracy and sensitivity of detecting such polymorphisms have led to the development of a number of powerful approaches. Sequence-specific base pairing between the strands of DNA, according to the Watson-Crick model, forms the basis of many detection systems.

Allele-specific PCR presented here allows efficient discrimination of point mutation by allele-specific PCR in a single reaction with standard PCR conditions. A common reverse primer and two forward allele-specific primers with different tails amplify two allele-specific PCR products, which are further separated by agarose gel electrophoresis. PCR specificity is improved by the introduction of a destabilizing mismatch within the 3' end of the allele-specific primers. This is a simple and inexpensive method for SNP detection that does not require intensive PCR optimization. In the present study, two primers were designed one was meant for the amplification of wild allele (C) whereas the other primer was designed to amplify the mutant allele (A) of FLT-TKD gene. But both primers generated a common band of 193bp for normal and mutant genotype as depicted in figure 2.

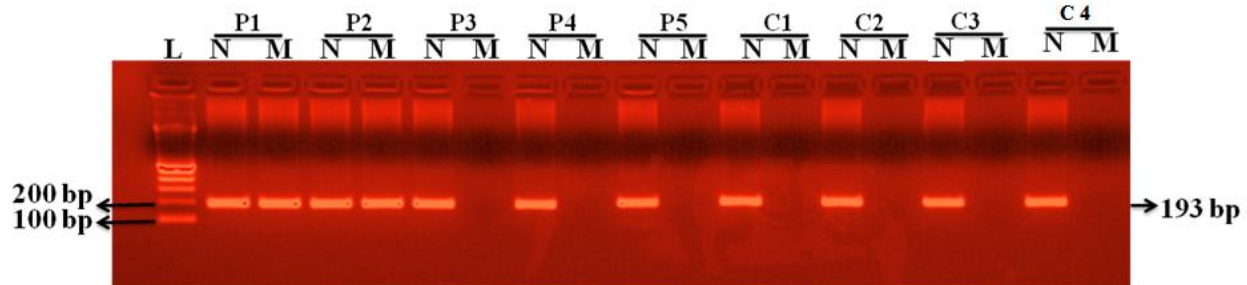


Figure 2. FLT3 835 mutation in AML patients and healthy controls:

L= 100bp Ladder, N= Normal, M=Mutant,
P1 and P2 = Positive for FLT3-TKD mutation
P3,P4,P5= Negative for FLT3-TKD mutation
C1,C2,C3 and C4 Controls samples = Negative for FLT3-TKD mutation

The Allele Specific-PCR based assay for FLT3 (D835Y) mutation was optimized in the Lab . This is the most common KD mutation at codon 835, converting aspartic acid to tyrosine (D835Y). The mutation was detected in 2/5 cases of AML, 0/25 of MPD and 0/5 of ALL cases as depicted in figure 3. It was observed that patients with AML possessed significantly higher frequency of FLT3 D835 mutation (2/5) than other leukemia cases and healthy controls. Interestingly, all the controls were found to be negative for the D835Y FLT3-TKD mutation. FMS-like tyrosine kinase 3 (FLT3) is mutated in approximately a third of acute myeloid leukemia cases, predominantly in the forms of FLT3/internal tandem duplication mutations in the juxtamembrane domain or point mutations in the kinase domain.

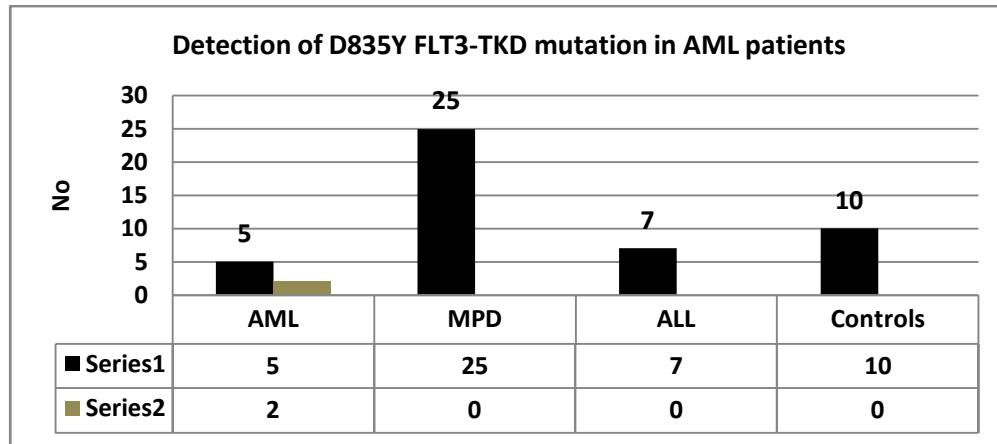


Figure 3. D835Y FLT3-TKD mutation in different subjects

For routine diagnosis, this characteristic of AS-PCR means that it is a very time-efficient method. It is concluded that allele specific PCR that permits the direct detection of FLT3 c.2503G>T (D835Y) point mutation in AML patients and acts as useful genetic markers for molecular diagnosis, prognosis, drug response, and predisposition to AML patients. The test is therefore a simple, fast, and inexpensive procedure that does not entail any special equipment other than a thermo cycler. Other mutations have also been seen like D835V, D835E, and D835H, converting aspartic acid to valine, glutamic acid, and histidine at residue 835, respectively, mutations converting glycine to glutamic acid at residue 831 (G831E) and arginine to glutamine at residue 834 (R834Q), as well as the deletion of isoleucine at residue 836. They induce constitutive tyrosine phosphorylation leading to activation of the receptor tyrosine kinase and are supposed to represent gain-of-function mutations.(19) Corresponding activation loop mutations have been reported at position D816 of KIT and also in other receptor tyrosine kinases, eg, RET. (20). On dimerization by FL stimulation, wild-type FLT3 activates a series of downstream signaling targets, including the p85 subunit of phosphoinositide 3-kinase (PI3K), growth factor receptor-bound protein 2 (GRB2), proto-oncogene tyrosine-protein kinase SRC, and SH2-containing inositol phosphatase (SHIP), suggesting its involvement in the PI3K and mitogen-activated protein kinases (RAS-MAPK) pathway.

Discussion:-

The studies on the frequencies of the FLT3-TKD mutations in AML were performed by Abu-Duhier *et al* (n = 97), (21) Yamamoto *et al* (n = 429),(22) Thiede *et al* (n = 979),(23) Moreno *et al* (n = 208),(24) and Fröhling *et al* (n = 224),(25) with AML showing a normal karyotype. According to these studies the FLT3 (D835Y) mutation has an incidence of 5.8% to 7.7% in AML patients and thus are less frequent than the FLT3-length mutation (FLT3-LM) also referred to as FLT3-ITD (internal tandem duplication)(26). Functionally, FLT3/D835Y bone marrow (BM) showed an equivalent or slightly higher engraftment ability compared with wild-type mice, and both are higher than that of the FLT3/ITD mice. These results suggest that the more quiescent HSC pool is not affected by signaling through the FLT3/D835Y mutations and that the FLT3/D835Y mutation does not mobilize the functional HSCs capable of long-term engraftment into cell cycle. According to these studies, FLT3-TKD show an incidence of 5.8% to 7.7% in AML and thus are less frequent than FLT3-length mutation (FLT3-LM) (26). Given the prevalence and propensity for poor outcome in AML patients harboring FLT3 mutations, a sustained effort has been underway to develop targeted inhibitors of the receptor-tyrosine kinase (FLT3-RTK). A variety of compounds have entered clinical trials and some have met with success.

Several FLT3 tyrosine kinase inhibitors have demonstrated *in vitro* and *in vivo* activity (28). The signaling properties of both mutation subtypes were suggested by Choudhary *et al* (29) who showed that FLT3-length mutation (FLT3-LM) gain a function over ligand-activated FLT3-wild-type, which is not gained by FLT3-TKD mutations. Gene-expression profiling with microarray analysis showed different gene-expression patterns for FLT3-LM and for FLT3-TKD positive AML. (30)

FLT3-length mutation (FLT3-LM) and FLT3-TKD mutations should be regarded as two biologically and prognostically different mutations within a single gene. The identification of new markers of leukemia and the use of increasingly sophisticated technologies for detection of important mutations should further facilitate routine monitoring of prognosis, MRD and elucidate the features of drug-resistant leukemic cells. The previous studies confirm that FLT3 activating mutations also occur in a significant percentage of AML patients. Early detection of

FLT3 mutations and an intensification of induction therapy might be useful for this group of patients to overcome the poor prognosis. Simplified methodologies for the early detection of FLT3- D835Y mutation in AML patients was clinically important for AML patients in a small region of Saudi Arabia especially for determining drug responses, prognosis, and selection of molecular-targeted therapy.

Conclusion:-

The allele specific PCR optimization can be potentially useful for the detection of FLT3-TKI (D835Y) gene mutation in AML patients. The test is a simple, fast, and inexpensive procedure that does not entail any special equipment other than a thermocycler and therefore, can be easily optimized in any laboratory for routine use.

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