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A Study of *FLT/ITD* Mutations in Cytogenetically Normal Iraqi Acute Myeloid Leukemia Patients

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Abstract: The present study was designed to shed light on the molecular effects caused by acute myeloid leukemia (AML) pathogenesis in three cases before, during and after treatment with chemotherapy (*in vitro*) in lymphocytes. It was also aimed to investigate *FLT3/ITD* point mutations in cytogenetically normal-AML region 100-300 bp compared to healthy control. The study comprised of 30 AML Iraqi patients and their ages ranged between 2.5-81 years. It included 12 females and 18 males compared with 26 healthy controls. Results revealed that the extracted DNA from 30 AML patients and amplified by PCR to obtain *FLT3/ITD* gene from exon 11 to 12 showed larger bands (470 and 460) bp in 2 patients when compared to wild type (330) bp. Among six patients, three of them displayed point mutations of deletion and substitution, while the others were normal since no mutations were detected. The percentages of mutation types were substitution 77.8% and deletion 22.2%. The types of mutations were missense mutations as (55.54%), deletion (22.22%) and nonsense (22.22%).

Keywords: AML, point mutation, *FLT/ITD*

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دراسة الطفرات الوراثية في جين *FLT/ITD* لبعض مرضى ابيضاض الدم الحاد العراقيين ذوي الخلايا الطبيعية

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الخلاصة: صممت الدراسة الحالية لتسليط الضوء على التأثير الجزيئي في خلايا الدم البيضاء الناتج عن امراضية ابيضاض الدم الحاد قبل واثناء وبعد العلاج الكيميائي. هدفت الدراسة للبحث عن الطفرات الوراثية النقطية للمورث *FLT3/ITD* في عينات طبيعية خلوياً لمرضى ابيضاض الدم الحاد وعينات من اشخاص اصحاء شملت الدراسة 30 مريضاً تتراوح اعمارهم بين 2.5–81 سنة وشملت 12 انثى و 18 ذكر اضافة الى 26 طبيعياً.

بينت النتائج بأن تضخيم الـ DNA المعزول من المرضى بطريقة تفاعل البلمرة التسلسلي للمناطق المشفرة أو الاكسونين 11 و 12 من المورث *FLT3/ITD* اعطى حزمتين هما 470 و 460 زوج قاعدي لمريضين مقارنة مع حزمة مفردة 330 زوج قاعدي للأصحاء.

بينت النتائج ايضاً وجود طفرات وحذوف في ستة من المرضى فقط بينما كانت عينات المرضى الباقين طبيعية مثلت الطفرات الوراثية الاستبدالية نسبة 77.8% واما الحذوف فمثلت 22.2%.

Introduction

Leukemia is a disease characterized by a clonal expansion of malignant blood cells. It evolves from the myeloid/granulocyte linage called Acute Myelogenous Leukemia (AML) or Lymphocytic precursors give rise to acute lymphocytic leukemia (ALL). Leukemia accounts for some 300,000 new cases each year (2.8% of all new cancer cases) and 222,000 deaths. This high ratio of deaths cases (74%) reflects poor prognosis of leukemia in many parts of the world, where the somewhat complex treatment regime required, are not available (1). There were 698 cases of leukemia in children aged 1 day –14 years registered at the Ibn Ghazwan pediatric oncology ward from 1993–2007. The number of cases ranged from 15 cases in the first year to 56 cases in the final year and reached a peak of 97 cases in 2006 (2). In 2010, approximately 12,330 people were diagnosed with AML and 8950 died (3).

AML is the most common type of acute leukemia in adults, accounting for 80% of new cases. AML is uncommon in children. The incidence increases steadily with age, with a sharp increase after the age of 45 years. ALL is the most common malignant disease affecting children, accounting for approximately 30% of all childhood cancers (4). Etiology of AML is largely unknown, but associated risk factors include ionizing radiation, earlier cytotoxic chemotherapy, exposure to benzene, and smoking (5).

Fms-related tyrosine kinase 3 (FLT3) is the most commonly mutated gene in human AML, and has been implicated in pathogenesis (6). FLT3 gene belongs to class III receptor tyrosine kinase and is predominnantly expressed on hematopoietic progenitor cells in the bone marrow, thymus, and lymph nodes (7). Internal tandem duplication of FLT3 gene (FLT3/ITD) is found in approximately 25-45% of adult AML and related with adverse prognosis (8; 9;10). Accordingly, and because of the increasing number of cases being affected by AML and the urgent need for better understanding for the mechanisms behind this serious disease, the present paper is evaluating the incidence of FLT3/ITD mutations in AML patients.

Materials and Methods

A total number of 30 patients, attended the Baghdad Teaching Hospital and Children Protection Hospital, were diagnosed with AML Patient's ages ranged from 2.5-81 years. Healthy control group consisted of 26 healthy human individuals of different ages (ranged from 23-57 years). No abnormalities were found in selected blood samples taken from blood donors and therefore being used as controls for comparison with blood samples taken from AML patients. Venous blood samples (5ml each) were collected from diseased individuals. Genomic DNA was extracted using the ReliaPrep™ Blood gDNA Miniprep System (11).

The DNA concentration was estimated using the Nanodrop.

The FLT3 mutation was detected using primers were provided by Integrated DNA Technologies (IDT) Company table 1 (13). PCR was carried using Master Mix: GoTaq® Green Master Mix 2X supplied by Promega Company The amplification of FLT3/ITD gene was done according to Kiyoi et al. (13) and listed in table 2. The PCR samples were sent for sequencing then the samples were compared with normal sequencing according to NCBI.

Table 1: Primes sequences used for screening and detection of FLT3/ITDs mutation

Name	Sequences	GC
12R	´5-CTT TCA GCA TTT TGA CGG CAA CC-´3	47.8٪
11F	´5-GCA ATTTTAG GTA TGA AAG CCA GC-´3	43.4٪

Table 2: The PCR reaction program

Program steps	Temperature ^{0}C	Time	No. of cycles
Preheated plate	95	4min	1
Initial denaturing	94	3 min	35
Denaturing	94	30s	
Annealing	52.8	1min	
Extension	72	2min	
Final extension	72	10min	1

Results and Discussion

The genomic DNA extracted from patients with AML and control by using Mini Prep Genomic DNA extraction kit was supplied by Promega, showed a good concentration and purity when measured using nanodrop (1-3ng /µl) and (1.1-0.9).The genomic DNA yield was different among the studied samples according to the concentration and purity. This may depend on blood freshness, storage and on the amount of WBCs in the blood samples. The PCR results revealed that identical bands related to the region were present. PCR amplified regions showed a molecular weight of 330bp as shown in figure 1. This result agreed with those obtained by Mukda et al. (16) who found that FLT3/ITD is 330bp which is identical with the current study. A genomic fragment corresponding to exon 11 to 12 of the FLT3 gene was amplified by PCR. Ten subjects of patients exhibited PCR products, two of them showed larger bands than usual ones (figure 2).

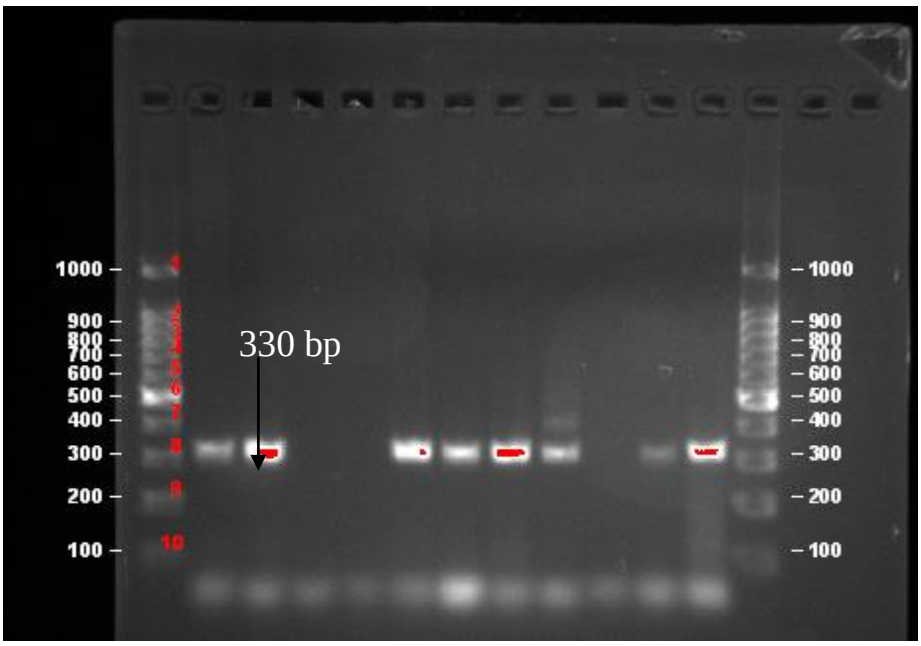
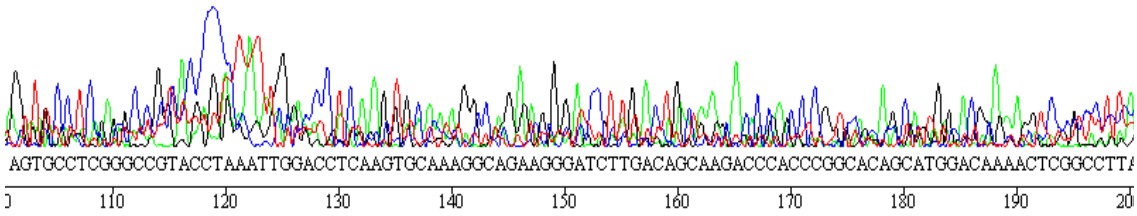
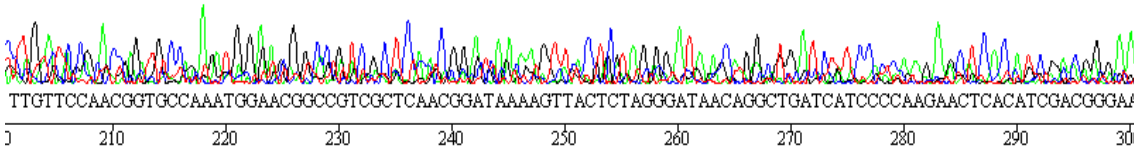
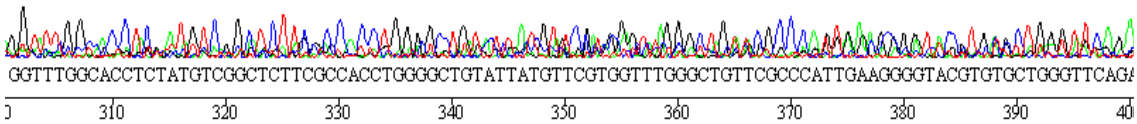


Figure 1: PCR products using Gel Documentation System of FLT3/ITD Region electrophoresis conducted on 2%agarose gel at 100 volt for 10 min and 50 volt 40 min







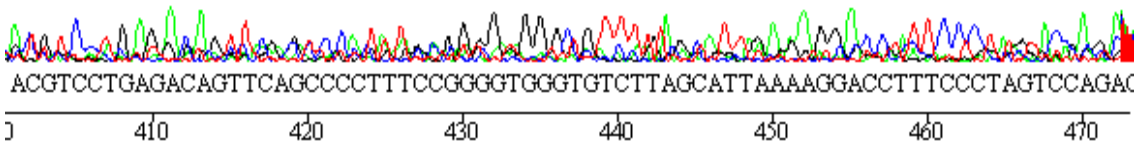
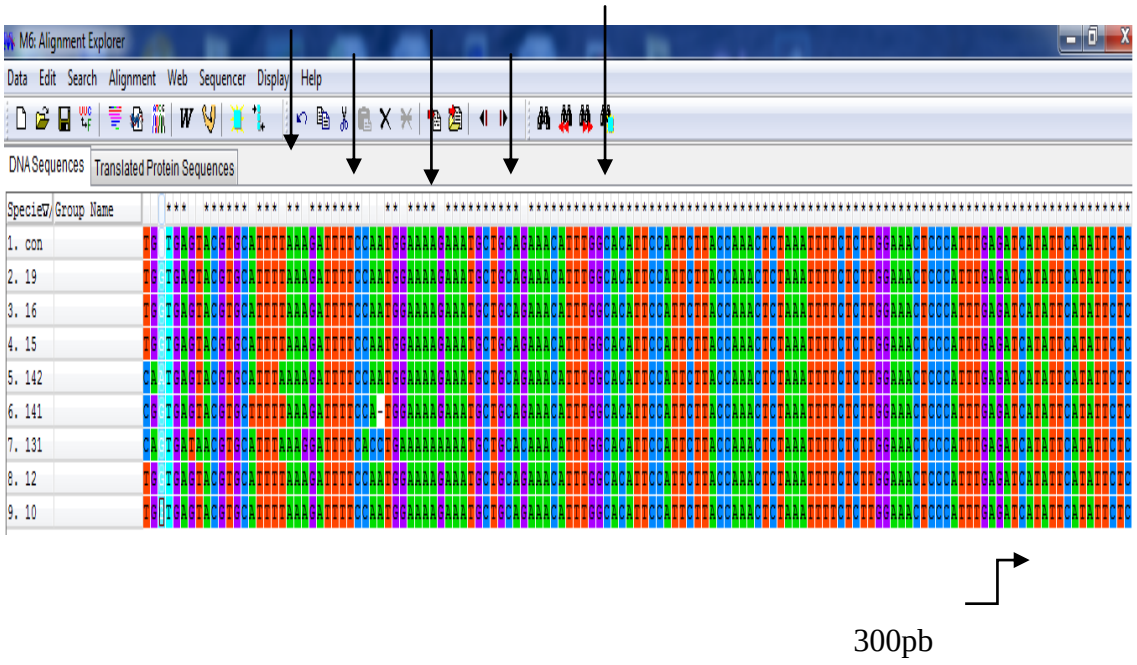


Figure 2: Chromatogram showing FLT3/ITD gene sequence which is larger than the wild type

FLT3/ITDs point mutations in AML patient were undetectable during diagnosis by routine polymerase chain reaction (PCR).The PCR products of the gene FLT3/ITD were screened by sequencing AML patients, results were directly compared with Iraqi healthy control by using the program Mega 6 (figure 3). Levis (17) reported that FLT3 internal tandem duplication (ITD) mutations predict unfavorable outcome. FLT3/ITD is an unstable aberration and may be lost or acquired at relapse.



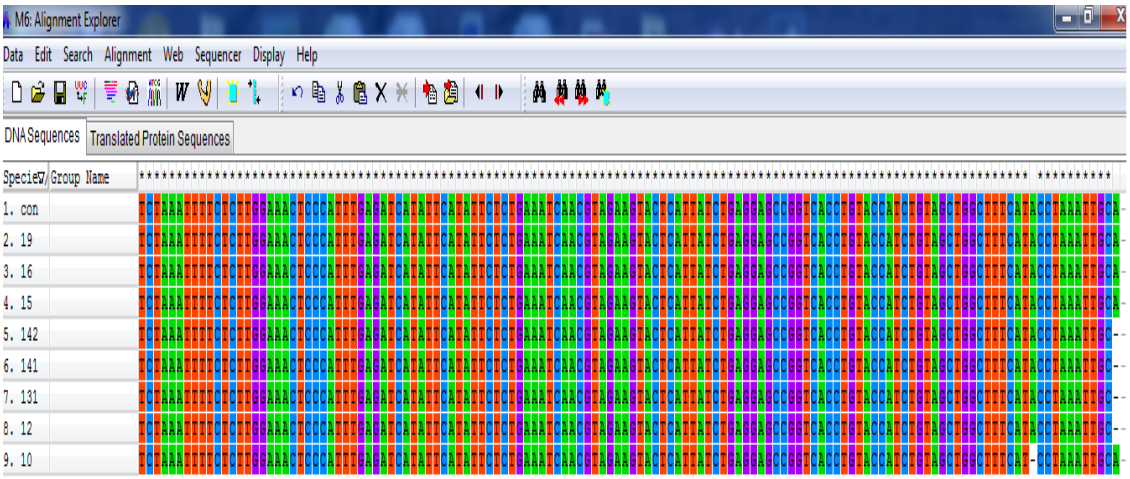


Figure 3: FLT3/ITDs point mutations as illustrated by Mega 6, compared with Iraqi healthy control. Arrows indicate the region of point mutation

The current study utilized forward primer for sequencing the FLT3 ITD in each case, six samples out of 10 were compared with healthy control at the (99 - 300) Pb regions. Among six patients, three of them displayed point mutations of deletion and substitution while others were normal since no mutations were detected (table 4). Samples that showed mutations were not received any treatment, while others were already took chemotherapy. This result agrees with those of Nakao et al. (18); Yamamoto et al. (19); Naoe and Kiyoi (20) who reported that internal tandem duplication (ITD) of base pairs within the juxta membrane coding portion or point mutations in the second kinase domain occurs in approximately 30% of patients with newly diagnosed AML leading to constitutive activation of the FLT3 gene on chromosome 13q12.

Table 3. FLT3/ITDs showing point mutations detected in AML patients DNA and those showed no mutation

No. of patients sample	State of treatment	Wilde type	Mutant type	Change in amino acids	Site of N.A.	Type of mutation	Effect on translation
10	Before	ACC	_CC	Deletion300	300	Deletion	Frame shift
12	After	ND	ND	ND	ND	ND	ND
13	Before	ATG AGT TTA CAA TGG AGA	_CG ATA TAA ACC TGA AAA	Deletion99&Substitution100 Ser/Ile Leu/Stop codon Gln/Thr Trp/Stop codon Arg/Lys	99,100 106,107 118 130 134 139	Deletion Substitution Substitution Substitution	Frame shift Missense Nonsense Missense Nonsense Missense

		GAA	CAA	Glu/Gln	150	Substitution Substitution Substitution	Missense
16	After	ND	ND	ND	ND	ND	ND
19	After	ND	ND	ND	ND	ND	ND
14	Before	TTT	TTA	Phe/Leu	111	Substitution	Missense

ND: Not detected mutation in this region; N.A. Nucleic acid

Analysis of FLT3/ITD gene by sequencing six patients exhibited the existence of many genetic alterations. Nine mutations in 3 patients were detected in patients before taking any treatment. Two types of point mutations

namely, Deletion and Substitution were present.

Table 4 shows the percentages of mutation types that displayed substitution (77.8%) and deletion (22.2%). Bianchini et al. (21) reported additional nucleotide changes were discovered; in total, 14 sequence variations were identified: 7 of 34 (21%) for ITDs in exon 14;2 of 34 (6%) for point mutations in exon 20; 1 of 34 (3%) for a new point mutation in exon 16; and 4 of 34 (12%) for polymorphisms in exons 13 and 14.

Table 4. Percentages of mutation types in AML patient groups

Type of mutation	Percentage
Substitution	77.8
Deletion	22.2

Previous study was conducted by of Ravandi et al. (22) found that the incidence in FLT3/ITD mutations was exhibited in 64% of patients during their first cycle of taking azacytidine and sorafenib as a chemotherapy.

Mutation in FLT3/ITD gene affects the regulation of apoptosis and proliferation. Table 5 shows that there was a missense mutation (55.54%) causing impact on phenotype that leads to replacement in amino acids. The deletion mutations lead to frame shift; there was about (22.22%) in this study. These mutations resulted in a completely different translation (defect protein) FLT3 plays an important role in stem cell proliferation, differentiation, and survival. In normal hematopoiesis, FLT3 ligand binding to the FLT3 receptor causes dimerization of the receptor, autophosphorylation, activation of tyrosine kinase, and induction of multiple intracellular signaling pathways, which are involved in cell proliferation and leukemo-genesis (23).

FMS-like tyrosine kinase 3, FLT3, is a member of the class III receptor tyrosine kinase family which also includes platelet-derived growth factor receptor (PDGFR) and stem cell growth

factor receptor, c-Kit. FLT3 is normally expressed on immature hematopoietic progenitor cells and contributes to proliferation, survival, and differentiation. Upon stimulation with FLT3 ligand (FL), FLT3 forms a homodimer and autophosphorylates itself resulting in the activation of downstream signaling cascades (24; 25).

Table 5. Percentages of FLT3/ITD mutation effect type

Effect of mutation	Percentage
Missense	55.54
Frame shift	22.23
Nonsense	22.23

It has been concluded from the current study that two AML patients developed large bands after DNA sequencing compared with controls. Point mutations were detected including deletion and substitution causing missense, nonsense and deletion.

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