Detection of FBXW7 Mutations in Acute Lymphoblastic Leukemia for Some Iraqi Patients Using HRM-PCR

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***Abstract*——** FBXW 7 ligase is a potential tumor suppressor gene that is responsible for the ubiquitylation of proteins destined for proteasomal degradation. Not surprisingly, it was discovered that FBXW7 is deregulated in several human malignant diseases by chromosomal aberrations or point mutations .These mutations are expected to increase the activity of the NOTCH1 pathway and plays an important role in cellular division. Interestingly, inactivating mutations of FBXW7 have recently been found to be common in T-ALL. Here, we carry out mutation detection in the FBXW7 genes, in 50 Iraqi patients with acute lymphoblastic leukemia diagnosed by consultant medical staff. We determined the occurrence of mutations in FBXW7 using HRM-PCR and sequencing of PCR products as a sensitive assay for the detection of genetic mutations. As High resolution melting (HRM) curve analysis represents a fast, post-PCR high-throughput method for scanning somatic sequence alterations in target genes, in this study HRM-PCR was used to screen for FBXW7 mutations in blood samples obtained by from ALL patients examined previously for NOTCH1 mutations using HRM-PCR. DNA extracted from these samples was subjected to HRM-PCR to amplify exons 9 and 10 of FBXW7 followed by sequencing. FBXW7 genes mutations were identified in 10 % (5/50).

***Keywords*—** FBXW7 gene, Mutations, Acute Lymphoblastic Leukemia, HRM-PCR

I. Introduction

FBXW 7 (also known as Fbw7, hCdc4 and hSel10) with official full name F-box/WD40 repeat-containing protein 7 , E3 ubiquitin protein ligase is a potential tumor suppressor gene , maps to chromosome 4 (4q31.23) . FBXW7 (F-box and WD repeat domain-containing 7), an E3 ubiquitin ligase, is a component of the SCF complex (SKP1, CUL1 and Fbox protein) that is responsible for the ubiquitylation of proteins destined for proteasomal degradation . As the tumor suppressor FBXW7 targets proteins for proteosomal degradation, it has been shown to suppress NOTCH1 signaling by ubiquitination of the ICN1fragment [1 , 2]. Mutations in FBXW7 were found to abrogate NOTCH1 binding, thereby enhancing NOTCH1 signaling. FBXW7 may be of particular interest in this regard because FBXW7 substrates for ubiquitination include several prominent proto-oncogenes including NOTCH , c-Myc , JunB, , cyclin E and mTOR [3; 4 ; 5 ; 6] . As such, FBXW7 has been identified as an important tumor suppressor with loss-of-function mutations leading to chromosomal instability [7].The termination of NOTCH signaling is achieved by degradation ICN1. CDK8 phosphorylates NOTCH1 rendering it susceptible for ubiquitylation by FBXW7 (E3 ubiquitin ligase). FBXW7 specifically binds and ubiquitinates a CPD within the PEST domain [8]. It was reported that a specific loss of FBXW7 in the human stem cells (HSCs) resulted in their premature depletion due to active proliferation and p53-induced apoptosis. When p53 was suppressed, most animals developed T-ALL [9]. Interestingly, inactivating mutations of FBXW7 have recently been found to be common in T-ALL [10 , 11], but have not been observed in multiple myelomas and B-cell lymphomas [12]. These mutations are expected to increase the activity of the NOTCH1 pathway in a similar manner as the NOTCH1-PEST mutations do. Recently, the frequency of NOTCH1 and FBXW7 mutations has become interesting for their application in risk prediction [8]. O'Neil et al. (2007) founding does implicate FBXW7 mutations in both the pathogenesis of T-ALL and leukemic cell resistance to gamma secretes inhibitors (GSIs) . Therefore, the objective of this preliminary analysis was to detect and analyze presence of hotspot mutations in and FBXW7 tumor suppressor gene at exon 9 and 10 and its correlation with NOTCH1 mutations detected in previous study [13] in the same patients which might be occurring in the cases of ALL from our local Iraqi population using a HRM assay for somatic variation detection in clinical samples.

II. Patients, Materials, And Methods

Fifty Iraqi patients 34 were male and 16 female aged between 2 to 70 years (20 pre-treated, 15 relapsed and 15 under treated) had been diagnosed (by a consultant medical staff at Central Pediatric Teaching Hospital; Baghdad Teaching Hospital and The National Center of Hematology / The University of Mustansiriyah) with Acute Lymphoblastic Leukemia (ALL) were conveniences for the study after obtaining official approval with the help of medical staff supervisor of the patients in these medical centers. The chemotherapeutic agents used for treatment protocol for ALL patients subjected in this study included: Vincristine,

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Methotrexate, Cytosar, L-asparaginase, Dexamethasone (Decadron), Etoposide, Indoxan, Steroids.

A. Genomic DNA preparation

Genomic DNA was prepared from blood samples (2-5 mL) of patients with ALL using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at _20°C until use.

B. Screening of FBXW7 mutations by HRM-PCR and sequencing

As these experiments are not available in Iraq, DNA samples send to Dr. Khalid Tobal (Molecular Oncology Diagnostics Unit, Guy's & St Thomas' National Health Service Foundation Trust, London, UK). HRM was performed for mutations screening in the presence of the cyto 9 dye and LightCycler 480 (Roche Diagnostics) and all reactions were performed in duplicate. Primer sets which were designed through primer 3 plus software Primer sequences by Dr. Khalid Tobal (Molecular Oncology Diagnostics Unit, Guy's & St Thomas' National Health Service Foundation Trust, London, UK) to cover the mutation hotspots in FBXW7 genes (Table 1). The reaction mixture included (2.0 µl TBE Buffer, 1.28 µl MgCl2 (25mM) , 0.35 dNTPs , 0.25 Enzyme (Ampitaq Gold polymerase), 10.5 µl PCR grade water , 1.0 µl Syto 9 HRM dye , 0.62 µl Primer Mix , 4.0 µl DNA) with a final volume of 20 µl . The reactions were run on a Verti 96 well thermal cycler (Applied Biosystems) according to Sequence DT-55 cycle-sequencing cycling program.

Samples with aberrant melting curves were directly sequenced from the HRM product . Mutations were identified by sequencing with API 3730 sequencer (Applied Biosystem,USA) for HRM-PCR-amplified DNA fragments using the big dye terminator v3.1 ready reaction cycle sequencing kit (Applied Biosystems, USA).

TABLE I
A LIST OF THE PRIMERS .THE PRIMERS SEQUENCES BELOW IS WRITTEN 5' TO 3'

Sequence of primers 5'- 3'	Name of Primer
AACCTTGACTAAATCTACCATGTT	BXWX10F
CTGGATCAGCAATTTGACAGTG	BXWX10R
TGGTATGTAGCCACACCCAAT	BXWC9F1
GCATACAACACACAGTGGAAGTATGC	BXWC9R1
ACTTCCACTGTGTGTTGTATGCATCT	BXWC9F2
ACAGAACAACAAAAAGGATTAGAGA	BXWC9R2
TTCCCATTCCCTTATTATGTTTAT	BXWC10F1
TGGCATCTAGAGAACCGCTAACAACTCTGC	BXWC10R1
AGCGGTTCTCTAGATGCCACTCTTAGGG	BXWC10F2
TTTCTTCATGCCAATTTTAACG	BXWC10R2

III. RESULTS AND DISCUSSION

The HRM-PCR and sequencing protocol was optimized to amplify and sequence exon 9 and 10 of FBXW7 in order to detect FBXW7 mutations. Sequencing was successful for all amplified sequences.

The frequency of mutations according to the groups of ALL patients is illustrated in table (2). As it is suspected, the results for the FBXW7 gene reveal an elevation in the mutations frequency in relapsed group. However, relapsed group of ALL patients is at high risk and failed for current maximal therapy (associated with poor outcome). Undertreated patients who received and responded to at least the 1st line treatment then the leukemic clone would be reduced significantly. Clappier *et al.* (2011) reported that genomic studies in human ALL have revealed clonal heterogeneity at diagnosis and clonal evolution at relapse. Thus, the majority of ALL cases exhibit substantial changes in genetic alterations from diagnostic to relapse; which suggest that these findings may contribute to the design of novel strategies to prevent or treat relapse.

TABLE II
FREQUENCY OF MUTATIONS IN FBXW7 GENE ACCORDING TO THE GROUPS OF ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS

ALL Patients Groups	Mutated FBXW7
Pre-treated (Total 20)	0/20
Under-treated (Total 15)	0/15
Relapsed (Total 15)	5 /15 (33.33%)

Comparing the results of NOTCH1 gene in ALL patients in the previous study with the results of FBXW7 gene in this study for the same ALL patients, the frequency of NOTCH1 gene 14/50 (28%) was followed by FBXW7 5/50 (10%), It is argued that this results of mutations screening, are close to the results of others [15; 16; 17]. This suggests that NOTCH1 are good prognostic for ALL.

FBXW7 mutations are present in 5 cases (all at relapsed group) out of 50 ALL patients (10%), alone in 1 case, and in association with NOTCH1 mutations in 4 cases (Figure 1). Overall, 15 cases (30%) are classified as NOTCH1 and/or FBXW7 mutated and 35 cases (70%) as a wild type for both genes (NOTCH1 and FBXW7).

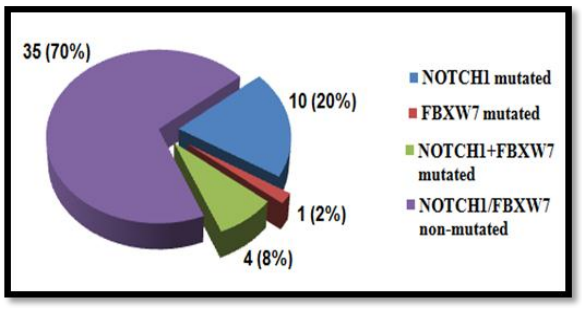

Fig 1: Frequency of NOTCH1 and FBXW7 mutations in acute lymphoblastic leukemia patients

TABLE III
ANALYSIS OF FBXW7 MUTATIONS

Patient Code	Years age Group	Group of ALL Patient	Exon	Nucleotide position	Nucleotide change	predicted amino acid	Type of Mutation
Pt. 11	11-20	Relapsed	9	c.1393 c.1394	**Substitution** (GCG> GAC)	R 465 T	**Missense**
Pt. 5	11-20	Relapsed	9	c.1393	**Substitution** (GCG> GTG)	R 465 C*	**Missense**
Pt. 9	11-20	Relapsed	9	c.1393	**Substitution** (GCG> GTG)	R 465 C*	**Missense**
Pt. 7	11-20	Relapsed	9	c.1393	**Substitution** (GCG> GTG)	R 465 C*	**Missense**
Pt. 8	11-20	Relapsed	10	c.1436	**Substitution** (TCG> TCT)	R 479 L	**Missense**

c. : Nucleotide Position ; R:Arginine ; T : Threonine ; C* : Cysteine ; L : Leucine

Table 3 shows that c.1393 in exon 9 is a hot spot for FBXW7 mutations in ALL Iraqi patients according to our results. Mutations in the conserved FBXW7-binding pocket (residues R465 and R479) have been shown to abrogate NOTCH1 binding, thereby enhancing NOTCH1 activity. About 12% of pediatric T-ALL patients had FBXW7 mutations at initial diagnosis [18]. In this study FBXW7 mutations are identified in a near percentage of cases of adult ALL (10%). FBXW7 mutations have been primarily associated with the HD domain of NOTCH1, resulting in signal amplification, whereas FBXW7 mutations in the PEST domain are less frequent [10] . It is proposed that mutations in the PEST domain relieve mutational pressure on FBXW7 (1). In contrast to studies in pediatric T-ALL , which found no coexisting mutations in the NOTCH1 PEST domain and the FBXW7 gene [18,11] , this study identified two patients with combined PEST and FBXW7 mutations . NOTCH1 mutations are an ideal target for pharmacological interventions, e.g., gamma-secretase inhibitors that prevent the generation of the ICN1 fragment and thereby suppress NOTCH1 activity [19]. Importantly, mutations in FBXW7 are associated with resistance to gamma-secretase inhibitor treatment, as disruptions of FBXW7 function maintain NOTCH1 signaling [11]. Therefore, the identification of FBXW7 mutations in one case as alone FBXW7 mutation of all patients and in four of cases with mutated NOTCH1 signaling need to be taken into account when choosing targeted therapies. As for other leukemic subtypes, tailoring the therapeutic approaches based on the molecular alteration is critical for treatment optimization in T-ALL.

Mutational analyses of NOTCH1 and FBXW7 are, at present, still exploratory. Recently, Asnafi *et al.* (2009) showed a favorable prognostic impact of NOTCH1 and FBXW7 mutations in adult T-ALL; thus, compared with previously identified oncogenic or immunophenotypic markers, NOTCH1 and/or FBXW7 mutations identify two major groups, which justifies prospective screening and therapeutic stratification. Furthermore, the future use of specific inhibitors of activated NOTCH1 may be based on the mutational profile within the NOTCH1 signaling pathway [17]. However, the associations displayed by these pathological alterations already aid disease stratification and should improve intelligent therapeutic targeting [21]. Further studies on wide range of samples to confirm these findings are required.

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