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RESEARCH ARTICLE

SCREENING FOR NOTCH1 MUTATIONS IN ACUTE LYMPHOBLASTIC LEUKEMIA FOR SOME IRAQI PATIENTS USING HRM-PCR

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ABSTRACT

NOTCH families are transmembrane proteins that have dual functions as both cell surface receptors and nuclear transcriptional regulators. If NOTCH1 pathway goes awry, it contributes to cellular transformation and tumorigenesis. Here we have investigated mutations in the NOTCH1 gene, in 50 Iraqi patients with acute lymphoblastic leukemia. We determined the occurrence of mutations in NOTCH1 using HRM-PCR and sequencing of polymerase chain reaction products as a sensitive assay for the detection of genetic mutations. This study assessed the feasibility of using HRM-PCR to screen for NOTCH1 mutations in blood samples obtained by from ALL patients in routine clinical practice. Blood samples obtained from ALL patients were evaluated according to our standard clinical protocols. DNA extracted from these samples was subjected to HRM-PCR to amplify exons 26, 27 and 34 of NOTCH1 followed by sequencing. Mutations were identified in 28% (14/50) of NOTCH1 including two novel mutations. This suggests that NOTCH1 is good prognostic for ALL Iraqi patients. Further studies with a wide range of sampling to confirm these findings are required.

INTRODUCTION

The accumulation of chromosomal aberrations is one of the hallmarks of cancer cells (Peddibhotlaa *et al.*, 2009). On the other hand, induction of DNA damage in cancer cells is a well recognized therapeutic strategy for killing cancer cells (Liao *et al*., 2009). Genes and/or their products that are highly and specifically increased in leukemia could represent useful diagnostic markers for leukemia. They could also be good targets for development of novel therapeutic drugs (Mobasheri *et al.*, 2006). Therefore, many research works in recent years screen for cancer genes involved in leukemia (Karapetis *et al*., 2008; Case *et al*., 2008; Baldus *et al*., 2009 and Fielding, 2010; Lin *et al.*, 2012). NOTCH1 genes are among these genes. NOTCH1 gene (Also known as hN1; TAN1) encodes a member of the NOTCH family receptors, located on chromosome 9. The NOTCH signalling pathway is important for cell-cell communication (Fiu´za and Arias, 2007; Kopan and Ilagan, 2009). NOTCH signalling plays an important role (promote or suppress) in cell-fate determination, as well as in cell survival, cell proliferation and activation of differentiation programs (Artavanis-Tsakonas *et al*., 1999). A NOTCH1 heterodimeric proteins receptor comprised of noncovalently associated domains which are NOTCH extracellular (NEC), NOTCH transmembrane (NTM) and intracellular NOTCH (ICN) subunits (Logeat *et al*., 1998; Rand *et al*., 2000). The extracellular subunit contains (epithelial growth factor-like (EGF-like) repeats, Lin/NOTCH repeats, an N-terminal heterodimerization domain (HD-N) and a C-terminal heterodimerization domain (HD-C), while the intracellular NOTCH (ICN) contains a RAM domain, ankylin repeats, a transcription activation domain(TAD) and a proline, glutamic acid, serine, threonine- rich (PEST) domain (Leong and Karsan, 2006). PEST (Proline- Glutamate-Serine-Threonine rich) domain involved in the degradation of the intracellular part. This domain contains a WSSSSP motif and a Cdc4 phospho-degron (CPD) which is recognised by the E3 ubiquitin ligase FBXW7. In 1991, it was discovered that the chromosomal translocation t(7;9) leads to constitutive activation of NOTCH1 in human T-ALL (Ellisen *et al*., 1991). Afterwards, the gain-of-function mutation in NOTCH receptor located at heterodimerization (HI) domain-encoding locus (exon 26 and 27), transcriptional activation domain, and PEST domain (exon 34) were identified as a novel mechanism for the constitutive activation of NOTCH1 in human T-ALL (Weng *et al*., 2004). T-cell acute lymphoblastic leukemia (T-ALL) which occurs mainly via the proliferation of malignant T cell clones, accounts for 15% of ALL case in children and 20-25% of ALL cases in adults (Pui *et al.*, 2004). Overall, these are aggressive malignancies that do not respond well to

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chemotherapy and have a poorer prognosis than their B-cell counterparts (Morris *et al.*, 2008). Complex acquired genetic aberrations include chromosomal translocations (frequently involving TCR), as well as gene rearrangements and mutations resulting in abnormal expression of oncogenes like NOTCH1 may be associated with the advance and resistance to treatment of this disease (Vanura *et al*., 2009). However, NOTCH is important for directing lymphoid lineage cell fate determination and has also been implicated in HSC self-renewal. Thus, NOTCH activity may increase both self-renewal capacity and T-cell lineage commitment, which may significantly contribute to T-ALL development. Acquired NOTCH1 mutations are present in about 50% of T-ALL (Asnafi *et al*., 2009; Mansour, *et al*., 2007). More than hundred different mutations frequently involved in HD, TAD and PEST domains of NOTCH1 were reported in patients with T-ALL from many researcher groups in different countries (Zhu *et al*., 2006; Bhanushali *et al.*, 2010; Erbilgin *et al.,* 2010; Mansur *et al.*, 2011).

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

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.

Screening of *NOTCH1* mutations by HRM-PCR and sequencing

HRM was performed for mutations screening (Molecular Oncology Diagnostics Unit, Guy's & St Thomas' National Health Service Foundation Trust, London, UK) 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 to cover the mutation hotspots in NOTCH1 (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 in the N-terminal region of the heterodimerization domain(HD-N) (exon 26), the C-terminal region of the heterodimerization domain (HD-C) (exon 27), the transcriptional activation domain (TAD) (exon 34), and the PEST domain (exon 34) of the *NOTCH1* gene 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 1. A list of the *NOTCH1* gene primers sequences

Sequence of primers 5'- 3'	Name of Primer
TGCAGTTCTAAGGCTCTGCTC	NOTCH1- X26F
CGGAACCTCCGTCTCTTTTA	NOTCH1-X26R
GTCCTGACTGTGGCGTCAT	NOTCH1- X27-F
TAGCAACTGGCACAAACAGC	NOTCH1-X27-R
GCTGGCCTTTGAGACTGGC	NOTCH1- X34A-F
GCTGAGCTCACGCCAAGGT	NOTCH1- X34A-R
CAGATGCAGCAGCAGAACCTG	NOTCH1- X34B-F
AAAGGAAGCCGGGGTCTCGT	NOTCH1- X34B-R
GTGACAGCCAGTGCAACTCA	NOTCHC26- F1
GGGCTCCCGCAGGAAGTG	NOTCHC26M-R1
CTTCCTGCGGGAGCCCAG	NOTCHC26M-F2
CTGTCCAGGGGAAGGCAGT	NOTCHC26- R2
GTGACAGCCAGTGCAACTCA	NOTCHC26-F1
CCGCAGGAAAGGACCTAAGT--GGAAGGAGCTGTTGCGCAG	NOTCHC26INS-R1
TTAGGTCCTTTCCTGCGGGA--GCTCAGC	NOTCHC26INS-F2
CTGTCCAGGGGAAGGCAGT	NOTCHC26-R2
ATCAGCACTGGCCGGAGT	NOTCHC27-F1
TCTCCGGGTAGACGATGGAGCTG	NOTCHC27M-R1
CGTCTACCCGGAGATTGACAAC	NOTCHC27M-F2
CGGAGTGCCATTCAGAAAAT	NOTCHC27-R2
GTTTGAATGGTCAATGCGAGT	NOTCHC34AM-F1
TATAAGTTTTGTGGCTGCAC	NOTCHC34AM-R1
GCCACAAAACTTATAGATGCAG	NOTCHC34AM-F2
AAATTAAAATCCTCGTTCTTATTTTG	NOTCHC34AMR2
AACCACCTGCCTGGGATG	NOTCHC34AINSF1
TCAGGCAGATGGCGAAGGGTTGTA--TTGGTTCGGCACCATGC	NOTCHC34AINSR1
TTCGCCATCTGCCTGACTGCGGG--GGAGTGTGGCACCA	NOTCHC34AINSF2
TCTCCTGGGGCAGAATAGTG	NOTCHC34AINSR2
TCCCAGATGATGAGCTACCAG	NOTCHC34BINSF1
TCAGGTGTG-TCTCCTGGGGCAGA--ATAGTG	NOTCHC34BINR1
AGGAGACACACCTGAGCCCCGCC--CTGCCCACGTCGCT	NOTCHC34BINSF2
AAATTAAAATCCTCGTTCTTATTTTG	NOTCHC34BINSR2

Table 2. Frequency of mutations in NOTCH1 gene according to the groups of acute lymphoblastic leukemia patients

ALL Patients Groups	Mutated NOTCH1
Pre-treated (Total 20)	5/20 (25 %)
Under-treated (Total	2 /15 (13.33 %)
Relapsed (Total 15)	7 /15 (46.66 %)

RESULTS AND DISCUSSION

The frequency of mutations according to the groups of ALL patients is illustrated in Table (2). As it is suspected, the results revealed an elevation in the mutations frequency in

relapsed group followed by pre-treated group. However, relapsed group of ALL patients is at high risk and failed for current maximal therapy (associated with poor outcome), while undertreated patients who received and responded to at least

insertion mutation at 7652-7653 (ins.LSAAAEPEGP); one case has insertion mutation at c.7381-7382 (ins.THL) and one case has insertion mutation at c.7697-7698 (ins.DEHAEm PDQ). Interestingly, no NOTCH1 exon 27 and 34A mutations were found. Table 3 showed that c.4796 in exon 26(HD-N

Table 3. Analysis of NOTCH1 mutations

Patient code	Age group	Group of ALL Patient	Exon (Domain)	Nucleotide Position	Nucleotide Change	predicted amino acid change
Pt.11	11-20	Relapsed	26 (HD-N)	c.4720	Substitution (GCT>GAT)	L 1573 M
Pt.13	21-30	Pre-treated	26 (HD-N)	c.4705	Substitution (GCT>GAT)	L 1568 M
Pt.14	21-30	Relapsed	26 (HD-N)	c.4912	Substitution (CGC>CAC)	A* 1639 T*
Pt. 9	11-20	Relapsed	26 (HD-N)	c.4796	Substitution (GCT>GCC)	L 1599 P
Pt.19	11-20	Pre-treated	26 (HD-N)	c.4796	Substitution (GCT>GCC)	L 1599 P
Pt.5	11-20	Relapsed	26 (HD-N)	c.4796	Substitution (GCT>GCC)	L 1599 P
			34B (PEST)	c.7615-c.7616	Ins 22 Amino acid	Frame shift
Pt.8	11-20	Relapsed	26 (HD-N)	c.4796	Substitution (GCT>GCC)	L 1599 P
			34B (PEST)	c.7618-7619	Ins 8 Amino acids	Frame shift
Pt.32	11-20	Under-treated	26 (HD-N)	c.4586- 4787	Ins 7 Amino acids	Frame shift
Pt.3	11-20	Pre-treated	34B (PEST)	c.7615-c.7616	Ins 22 Amino acids	Frame shift
Pt.17	31-40	Relapsd	34B (PEST)	c.7381-c.7382	Ins 3 Amino acids	Frame shift
Pt.24	21-30	Pre-treated	34B (PEST)	c.7652- 7653	Ins 10 Amino acids	Frame shift
Pt.27	11-20	Pre-treated	34B (PEST)	c.7652- 7653	Ins 10 Amino acids	Frame shift
Pt.28	11-20	Pre-treated	34B (PEST)	c.7652- 7653	Ins 10 Amino acids	Frame shift
Pt.29	11-20	Under-treated	34B (PEST)	c.7652- 7653	Ins 10 Amino acids	Frame shift

c. : Nucleotide Position; ins : Insertion; HD-N : Heterodimerization-N Domain; PEST : Proline,Glutamic acid, Serine,Theonine-rich domain, L : Leucine; P : Proline; M : Methionine; A* : Alanine; T*: Threonine

the 1st line treatment then the leukaemic 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. The HRM-PCR and sequencing protocol was optimised to amplify and sequence exons 26, 27 and 34 of NOTCH1 in order to detect NOTCH1 mutations. Sequencing was successful for all amplified sequences. Overall, NOTCH1 mutations were identified in 14(28%) of the 50 patients with ALL. HD N-terminal (exon 26) mutations were detected in 8 patients: 6 cases alone and two patients had more than one mutation, and had co-existing HD and PEST domain mutations. While, PEST (exon 34A) mutations were detected in 8 cases: 6 cases as a unique mutation and 2 cases in the HD domain, point mutations were predominant: two point mutations are previously mentioned c.4796 (L1599P) and c.4918 (A1639T) (Wang *et al.*, 2011 and COSMIC database : http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=bygene&ln=NOTCH1), two novel mutations (c.4705 -L1568M and c.4720-L1573M) and one novel insertion mutation at c.4586-4587(Ins. QLKQRDT) in HD N-terminal domain. While in PEST domain insertion mutations were predominant and novel in all cases : 2 cases have insertion mutation at c.7615-7616 (ins.MRSIRNLRRSGELSDEH AEPDQ); 4 cases have domain) and c.7652-7653 in the exon 34B (PEST domain) resemble a hot spots for NOTCH1 mutations in ALL Iraqi patients according to our results. 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 (Shih and Wang, 2007). As for other leukemic subtypes, tailoring the therapeutic approaches based on the molecular alteration is critical for treatment optimization in T-ALL.

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