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Detection of NOTCH1 Mutation among Chronic Lymphocytic Leukemia in Sudanese Population

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Abstract

Introduction: The proto-oncogene NOTCH1 is frequently mutated in around 10% of patients with chronic lymphocytic leukemia (CLL). The NOTCH signaling pathway in CLL cells serves a role in survival and resistance to apoptosis. The most common mutation of NOTCH1 is C.7544-7545delCT, which accounts for ~80% of all NOTCH1 mutations.

Objectives: The aim of this study was to detect the prevalence the NOTCH1 c.7541_7542delCT mutation in Sudanese patients with B- cell lymphocytic leukemia (B-CLL).

Materials and Methods: A Case-control study was conducted in Khartoum state, Sudan, during the period from April 2017 to April 2018, involved 110 CLL patients. Physical examination, complete blood count, and Immunophenotyping were performed in all patients to confirm the diagnosis. Clinical staging such as Rai and Binet were studied. Blood samples were collected from all participants; DNA was extracted by using ANALYTIKJENA Blood DNA Extraction Kit. Detection of NOTCH1 c.7544_7545delCT mutation was performed using conventional PCR-based amplification refractory mutation system (ARMS) method.

Results: The NOTCH1 c.7544-7545CT mutation was detected by AS-PCR in **46** out of **110** CLL Sudanese patients (**41.8%**).The distribution of T allele among the cases was 93.6% while the negative cases were 6.4% in cases and controls, No significant association of NOTCH1 mutation with the age and gender. Also the distribution of G allele among cases was 91% while the negative was 9%, in compare to control in which 80.9% was positive and 19.1% were negative with no significant association.

Conclusion: NOTCH1 mutations were frequently detected in B cell CLL Sudanese patients.

Keywords: B- CLL- NOTCH1- mutations- prognosis - Sudanese
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Introduction: Chronic lymphocytic leukemia (CLL) is a heterogeneous disease with highly variable clinical courses and survivals ranging from months to decades. In particular, a subset of CLL patients is known to experience a progressive symptomatic disease poorly responsive to the common immunochemotherapeutic regimens (1,2). A fraction of these high-risk CLL, overall accounting for 5%–10% of cases, can be identified by screening for TP53 mutation/ deletion,1,2 whereas an additional fraction of cases has been recently shown to bear mutations involving the NOTCH1, SF3B1 and BIRC3 genes. Overall, alterations of these genes occur in~ 20% of CLL patients at diagnosis and have significant correlations with survival in consecutive series from independent institutions (3–7). The NOTCH signaling pathway in CLL cells serves a role in

survival and resistance to apoptosis (8). The most common mutation of NOTCH1 is C.7544-7545delCT, which accounts for ~80% of all NOTCH1 mutations(8). This mutation is identified in ~10% of patients with CLL during diagnosis(9). This mutation frequently occurs in patients without immunoglobulin heavy-chain variable region (IGHV) mutations and with trisomy(10). The C.7544-7545delCT NOTCH1 mutation is a 2-bp frame shift deletion within exon 34 that produces a premature stop codon in the PEST domain, a peptide sequence rich in proline, glutamic acid, serine and threonine, which acts as a signal for protein degradation and typically limits the intensity and duration of NOTCH1 signaling(10).

The presence of this mutation has been associated with an intermediate risk of CLL and transformation to high grade lymphoma.

Mutations in the NOTCH1 gene have recently been identified as new genetic alterations associated with shorter time to- first-treatment and progression-free survival (PFS) (5,11–13). Furthermore, clinical resistance to the anti-CD20 monoclonal antibodies in CLL patients with mutated NOTCH1 was found in some clinical trials, which manifested as a lack of benefit from the addition of rituximab to fludarabine cyclophosphamide,or ofatumumab to chlorambucil (14–17). The data about the clinical impact of NOTCH1 mutations among Sudanese B – cell chronic lymphocytic patients is not previously identified, so the aim of this study was to detect the prevalence the NOTCH1 c.7541_7542delCT mutation in Sudanese patients with B- cell lymphocytic leukemia (B-CLL)., as well as, it's relation to the disease clinical impact and biological features and patient outcome.

Methods: Study Population: This study was a cross-sectional study, conducted in Khartoum state, Sudan, in the period from April 2017 to April 2018, a total of 110 patients with Chronic Lymphocytic Leukemia. Patients were obtained at Flow Cytometry Laboratory for Leukemia & Lymphoma Diagnosis, they were referred for Immunophenotype diagnosis.

Patient's diagnosis was done based on clinical history, physical examination and complete blood count. The peripheral blood is important to demonstrate morphological abnormalities and immunophenotypic criteria. Nevertheless, B lymphocyte ≥5000×109 /l, considered as a positive in our diagnosis according to International Workshop on Chronic Lymphocytic Leukemia (1) . The stage of the Chronic Lymphocytic Leukemia was assessed by Rai and Binet (18,19), classification . All patients were newly diagnosed without any previous B-CLL treatment; As explained in our previous work (20).

Sample collection: Four ml of peripheral Blood samples were collected from all patients and divided equally in two tubes; one tube for Complete blood count and Immunophenotype and another tube for molecular analysis. Also, 2ml of whole blood from control group in (EDTA) for molecular analysis.

Determination of Blood Count: Two ml of peripheral blood (PB) were withdrawn from each patient; these samples were collected in EDTA containing tubes for Complete blood count. All results such total WBC, Absolute lymphocyte

count, Hemoglobin level, RBC and platelets were recorded. And a blood smear stained by May Grunwald Giemsa was obtained for all patients.

DNA extraction: After confirmed immunopheno typing of patients, genomic DNA was extracted from all blood samples collected from patients and controls by using *ANALYTIKJENA* Blood DNA Extraction Kit (Germany) (REF-845-KS-1020050), according to the manufacturer's instructions. After DNA extraction DNA quality was evaluated, the β-globin gene amplification was used to assess the quality of DNA in all extracted samples, as previously described (21). All specimens for β-globin gene were Successful amplification, [Primers shown in Supplementary Table-1]. DNA quantification was done after DNA extraction by using a NanoDrop spectrophotometer. Then DNA samples were routinely stored at -20℃.

NOTCH1 c.7544_7545delCT mutation analysis:

The presence of NOTCH1 c.7544_7545delCT mutation was detected by ARMS using primers and PCR parameters developed by Fabbri et al (3) with litile modifications .

Two separated PCR reaction mixtures of 20 µl were prepared for each sample (one for detection of the wild type allele and the other for the detection of mutant-type allele). PCR was performed by using 4µl 5× HOT FIREPol Blend (2,14–16) Master Mix, (Solis BioDyne, Estonia), Cat. No. 04-25-00125), 2µl of genomic DNA, 0.5 µl of each primer, and 13 µl distilled water.

The thermocycling condition for the wild-type allele including 95 °C for 2 min followed by 30 cycles at 95 °C for 30 s, 57.4 °C for 30 s, 72 °C for 30 s with a final extension at 72 °C for 5 min in TECHNE Tc-412-UK PCR Thermal Cycler 96 well. A touchdown PCR was developed to detect the mutant -type allele. The initial denaturation step was 2 min in duration at 95°C, followed by 15 cycles of 30 s at 95°C, 30 s at 62°C (decreasing 0.5 °C per cycle), and 20 s at 72°C, followed by 20 cycles of 30 s at 95°C, 30 s at 55.6°C, and 20 s at 72°C, with a final extension step of 5 min at 72°C,.Amplified PCR products and 50 bp DNA ladder (iNtRON BIOTECHNOLOGY, KOREA), were separated on 2% agarose gel and visualized after staining with ethidium bromide. Amplicon 283 bp indicates the wild-type allele was observed, Amplicon of size 183 bp indicates the mutant-type allele was observed.

Statistical analysis: Patient's data was analyzed using the statistical package for social sciences (SPSS) version 16.0 software (Chicago, IL, USA). Numerical data was summarized as mean and stander deviation and N (%) of study participants, respectively. Chi Square test was used for analyzing qualitative data. Calculation of odds ratio (OR) with confidence interval (CI) for risk estimation was done by Logistic regression analysis.

Table-1: The primers sequence for NOTCH1 c.7544_7545delCT mutation

Primers	Sequence
ForMUT	5′-TCCTCACCCCGTCCCGA-3
ForC	5′-GTGACCG- CAGCCCAGTT-3′’
Rev	5′-AAGGCTTGGGAAAGGAAGC-3
β globin-GH20 (Forward)	5’-GAAGAGCCAAGGACAGGTAC-3’
β globin-PC04 (Reverse)	5’-CAACTTCATCCACGTTCACC-3’

*For MUT: Forward specific for the mutant allele, ForC: Forward common for both mutant and wild-type alleles, Rev: common reverse primer

Results: Mutational Analysis: The NOTCH1 c.7544-7545CT mutation was detected by AS-PCR in 46 out of 110 CLL Patients (41.8%) figure (1). No significant association of NOTCH1 mutation with the age and gender respectively), No significant association of NOTCH1 mutation with the age and gender (P.value=0.133, 0.203 respectively) (Table-2). On the other side regarding organomegally; there was a significant association between NOTCH1 mutation and hepatomegaly (P.value=0.005) (Table-3).

Hematological analysis: Concerning hematological parameters there is no significant differences between B cell CLL patients with NOTCH1 mutations and those without (P.value>0.05 for all) (Table-4).

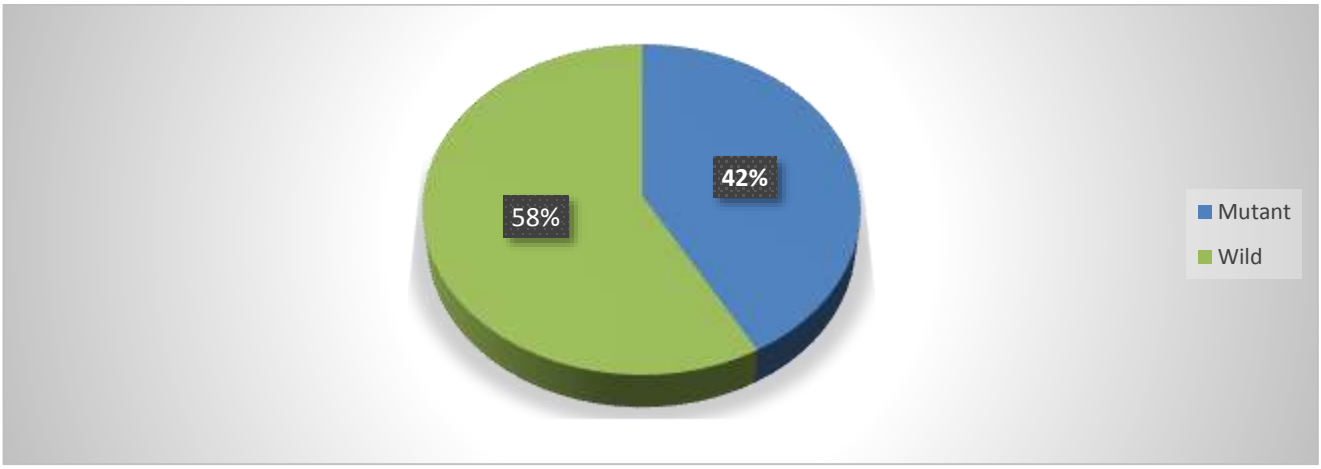

Figure 1-: Distribution of Genotype NOTCH1

Table-2: Association of Genotype NOTCH1 with gender

		Genotype NOTCH1		Total	P. value	Odd ratio (95%CI)
		Mutant	Wild			
Gender	Male	36 (32.7%)	43 (39.1%)	79 (71.8%)	0.203	1.758 (0.734 to 4.212)
	Female	10 (9.1%)	21 (19.1%)	31 (28.2%)		
Total		46 (41.8%)	64 (58.2%)	110 (100.0%)		

Table-3: Association of NOTCH1 with the Organomegally

		Genotype NOTCH1		Total	P. value
		Mutant	Wild		
Lymphadenopathy	Absent	10 (9.1%)	22 (20.0%)	32 (29.1%)	**0.150**
	Present	36 (32.7%)	42 (38.2%)	78 (70.9%)	
Splenomegaly	Absent	24 (21.8%)	32 (29.1%)	56 (50.9%)	**0.822**
	Present	22 (20.0%)	32 (29.1%)	54 (49.1%)	
Hepatomegaly	Absent	45 (40.9%)	51 (46.4%)	96 (87.3%)	**0.005***
	Present	1 (0.9%)	13 (11.8%)	14 (12.7%)	

Table-4: Comparisons of patient's hematological data according to NOTCH1 mutation

Parameter	Genotype NOTCH1		P. value
	Mutant (n=46)	Wild (n=64)	
TWBCs	90.8 ± 73.1	94.4 ± 77.6	0.808
RBCs	3.7 ± 0.9	3.7 ± 0.9	0.909
PLTs	185.3 ± 93.0	192.1 ± 113.3	0.739
HB	11.3 ± 2.6	11.0 ± 2.4	0.550
Granulocytes	12.2 ± 7.1	12.4 ± 7.9	0.889
Monocytes	2.9 ± 1.9	2.4 ± 1.9	0.130
Lymphocytes	84.9 ± 8.3	85.2 ± 9.0	0.819
Absolute Lymphocyte x103/ul	79.4 ± 68.2	84.3 ± 73.2	0.724
Absolute B Lymphocyte x103/ul	71.1 ± 65.3	74.9 ± 68.4	0.765

Discussion: Chronic lymphocytic leukemia (CLL) has a high incidence in Europe and North America, intermediate in Africa and low in Asia (24). Chronic lymphocytic leukemia (CLL) is a disease that affects older people in particular, and it is often determined by the elderly, Young people rarely experience clinical symptoms that are very heterogeneous, Leukemia originates initially through changes or mutations. In the genetic material, it affects the programmed cell death of blood cells. Diagnosis: made by blood counts, blood spots, and immunoglobulin enlarged B-lymphocytes that define cloned B-cell clusters that carry CD5 antigen as well as typical B-cell markers. (23)

Certain gene mutations, including Notch homolog1, translocation-associated (Drosophila) (NOTCH1) are known biomarkers for CLL prognosis. (8)

NOTCH1 mutations were detected in 41.8% of B cell CLL cases; and this figure is higher than was previously reported 24% (Van Vlierberghe et al., 2013), 10% of another study (Sportoletti P., et al 2014). There was No statistically significant differences in age and gender in relation to NOTCH1 mutations in B cell CLL cases, this may be due to difference in race and in clinical conditions of our Sudanese patients which may have more progressive disease due to the suggested difference in the molecular and biological behaviors.In consistence with data reported by Villamor et al., (2013) there was No other significant variations in the hematologic & clinical data in the relation to NOTCH1 mutation were detected; Except for organomegally in which revealed a significant association between NOTCH1 mutation and hepatomegaly(***P.valu =0.005***) .

Conclusion: NOTCH1 mutations were frequently detected in B cell CLL Sudanese patients. NOTCH1 mutations may had harmful effect on patient outcome in B- CLL patients; so identification of NOTCH1 mutations in B-CLL patients at diagnosis is recommended for better stratification and may aid in patient management and treatment.

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