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Prevalence of XPO1 and NOTCH1 Gene Mutations in CLL and its Association with Trisomy 12

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ABSTRACT

Background: Recent studies reported whole genome sequencing of Chronic Lymphocytic Leukemia (CLL) samples and found repeated mutations in the XPO1 and NOTCH1 genes. XPO1 was found mutated in 2.4% of cases, while NOTCH1 was found mutated in 12.2% or 15.1% of CLL samples.

Aim: Our aim is to detect the prevalence of XPO1 and NOTCH1 gene mutation in CLL patients and its association with different subtypes of CLL and trisomy12 positive cases.

Methods: The coding XPO1 (exons 15 and 16), and the NOTCH1 (exon 34; RefSeq NM-017617.2) mutations hotspot were analyzed by direct sequencing of genomic DNA extracted from blood mononuclear cells. Purified amplicons were subjected to conventional DNA Sanger sequencing using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, and USA). We reported the results of sequencing of XPO1 and NOTCH1 in 186 CLL cases presenting to Dammam University Hospital in Saudi Arabia.

Results: Our results confirmed frequency of XPO1 mutations. However, we found only 5 NOTCH1 mutations in 127 IGVH unmutated/ZAP70^{+} CLL samples (4%), and one mutation was found in IGVH mutated/ZAP70^{-} CLL for a total percentage of 1.5%. Because 4 of 6 mutated samples also showed trisomy 12, we sequenced NOTCH1 in an additional 77 cases with trisomy 12 CLLs, including 47 IGVH unmutated/ZAP70^{+} cases. We found 41.9% NOTCH1 mutation frequency in aggressive trisomy 12 CLL cases.

Conclusions: Our data suggest that activation of NOTCH1 plays a critical role in IGVH unmutated/ZAP70^{+} trisomy 12 CLL.

Key Words: *CLL – XPO1 – NOTCH1 – Gene mutation – Trisomy 12 – IGVH mutation – ZAP70.*

INTRODUCTION

Chronic Lymphocytic Leukemia (CLL) is the most common leukemia in adults and is characterized by the monoclonal expansion of CD5+ B cells. CLL shows clinical heterogeneity, from patients with very stable to patients with a rapidly progressive disease that is refractory to therapy. In progressive disease, there is transformation to diffuse large B-cell lymphoma, a condition known as Richter's Syndrome (RS) **[1]**.

Although biological factors, such as mutational status of IGHV genes, TP53 disruptions, chromosomal aberrations and CD38 and Zap70 expression, have been associated with clinical outcome, they do not entirely explain the molecular pathogenesis and the clinical heterogeneity of the disease. The development of new powerful sequencing technologies has made it possible to perform unprecedented detailed genetic analyses which have led to the discovery of novel genetic alterations in CLL and shed light on the understanding of this complex disease. In this way, two unexpected pathways have been identified to be mutated in CLL, and indicate that activated NOTCH1 signaling and defects in the splicing machinery play a prominent role in the development of specific subsets of CLL **[1,2]**.

The observation of a high expression of IgM in the group harboring NOTCH1 mutations also suggests that those alterations occur preferentially in cells highly responsive to external stimuli and sustaining NOTCH1 signaling **[3]**. It remains to be determined whether NOTCH1 mutations represent a primary event occurring in the first stage of transformation or a secondary event driving disease progression.

B-cell Chronic Lymphocytic Leukemia (CLL) is the most common adult leukemia in Western societies **[4]**. Genetic aberrations can be identified in the CLL samples of more than 80% of patients **[5]**. CLL cases can be subgrouped into 2 major types, aggressive or indolent, which are defined as cases that express high levels of ZAP70 and un-mutated IgH V region genes (IGHV), or low-to-negligible ZAP-70 and mutated IGHV. The most frequent recurrent genetic alterations include deletion/inactivation of 13q14 (>50%), deletion of 11q22-23 (18%), trisomy 12 (15%-18%), and deletion 17p (7%-10%) **[5]**. Two studies reported whole-genome sequencing of CLL samples and found 40 somatic mutations in 5 samples and 46 somatic mutations in 4 samples, respectively **[6,7]**. Subsequent sequencing of larger numbers of CLL samples revealed NOTCH1 mutations in 18%-20% of IGVH unmutated/ZAP70^{+} CLL samples, but only in 4%-7% of IGVH mutated/ ZAP70^{-} CLL samples **[6,7]**. One of these 2 reports also showed recurrent mutations in the XPO1 gene **[7]**. These mutations were found in 4 of 165 CLL samples or in 2.4% of cases. All these mutations were found in IGVH unmutated/ ZAP70^{+} CLL samples, and the percentage in this cohort was 4.6% **[7]**. The XPO1 gene encodes a member of the importin-β/karyopherin-β family of nuclear transport factors, namely Xpo1, which mediates nuclear export of proteins and ribonucleoprotein **[8]**. Xpo1 also is involved in the control of several cellular processes by controlling the localization of cyclin B and members of the MAPK pathway **[9]**. NOTCH1 encodes a class I trans-membrane protein functioning as a ligand-activated transcription factor **[10,11]**. On ligand binding, Notch1 undergoes several proteolytic cleavages resulting in translocation of the Notch1 intracellular domain (ICN) to the nucleus where it plays an important role in cell differentiation, proliferation, and apoptosis leading to transcriptional activation of multiple target genes, including c-Myc **[12]**. ICN contains PEST domain targeting ICN for ubiquitinylation and degradation **[10,11]**. Almost all NOTCH1 mutations in CLL are represented by the 2 base deletion frameshift resulting in a truncated constantly active protein, lacking the C-teminal PEST degradation domain Fig. (1) **[6,7]**. In addition, one frameshift insertion and 2 nonsense mutations were observed, each resulting in truncated Notch1. It has been reported that treatment with Y-secretase inhibitors induces cell growth arrest and apoptosis in different cell lines by decreasing NOTCH1 signal transduction **[13]**. Finally, antagonists that act by directly targeting the NOTCH trans-activation complex are under investigation **[14]**. These findings bring hope that these new molecular insights can be translated into new therapeutic approaches for the treatment of CLL.

In this work, we studied the prevalence of XPO1 and NOTCH1 gene mutation in CLL patients and its association with different subtypes of CLL and trisomy12.

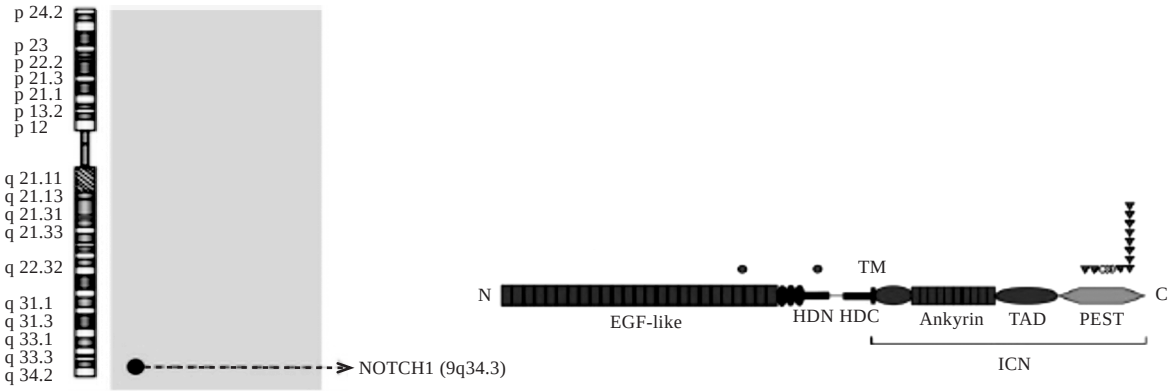


Fig. (1): Schematic representation of the Notch1 receptor on chromosome 9 and showing the PEST domain **[15,16]**.

PATIENTS AND METHODS

Patients:

The study was carried out in accordance with the institutional review board protocol approved by the Dammam University. Samples were obtained from 186 CLL patients enrolled in the CLL Research Consortium and presented to Dammam University Hospital; a written informed consent was obtained from all patients.

They included 155 males and 31 females with an age range of 50-75 and a median of 65 years. For 6 of these patients, 2 time points were provided, for a total of 192 samples analyzed. The 2 time points represent different stages of the disease: The first time point was provided in a clinically indolent stage while the last time point was provided during the aggressive stage. All cases satisfied the WCLL diagnostic criteria for CLL **[17]** and were selected on the basis of: I) Untreated disease; II) Availability of biological material. Lymphocyte morphology, immunophenotype, FISH analysis and IGHV sequencing were performed as previously described **[18]**, where there were 127IGVH unmutated/ ZAP-70$^+$ CLL and 65 IGVH mutated/ZAP-70$^-$ CLL samples.

Methods:

Progression was determined by clinical parameters such as increase in spleen size, white blood count, and overall Rai stage. Aggressive status was defined as unmutated IGVH (>98% of homology to the germline), and >20% of ZAP70-positive cells. Indolent status was defined as mutated IGVH, and <20% of ZAP70-positive cells **[19]**.

Sequencing:

DNA was extracted with the DNeasy Blood and Tissue Kit (QIAGEN). The coding XPO1 (exons 15 and 16), and the NOTCH1 (exon 34; RefSeq NM-017617.2) last coding exon, which encodes the portion of the PEST domain, mutations hotspots previously identified in CLL **[20]**, were analyzed by direct sequencing of genomic DNA extracted from blood mononuclear cells. Purified amplicons were subjected to conventional DNA Sanger sequencing using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, and USA). For amplification, we used high-fidelity advantage 2 polymerase master mix (Clontech). The primer sequences were: Xpo15-16dir2: Ttaggaaatgtacttgtagtttcta, xpo15-16rev2: Gggtctctaacaagacaaaaacat; notch33dir: Acccagcctcacctggtgcaga, notch-33rev: Tcggccctggcatccacagag. Purified amplicons were subjected to conventional DNA Sanger sequencing using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, CA, and USA). If mutated peak (s) on chromatograms were as high as the Wild-Type (WT) peak, we concluded that mutations were in 100% of cells. Otherwise, mutations were found in 50% and 25% of cells accordingly.

RESULTS

Samples were obtained from 186 CLL patients, male to female ratio was 5 to 1, with an age range of 50-75 with a median of 65 years. We sequenced XPO1 coding region (exons 15 and 16) in our set of samples from 186 CLL patients. Six cases had 2 samples collected at 2 different time points, resulting in total of 192 CLL samples analyzed, 127 IGVH unmutated/ ZAP70$^+$, and 65 IGVH mutated/ZAP70. We found the E571K mutation in 4 of 192 samples (2.1%), Fig. (2). All the mutated samples were in the IGVH unmutated/ZAP70$^+$ cohort, with a frequency of 4/127 (3.1%). In addition, we found the V565I (ex16-61719490 G-A) mutation in the first and second samples collected from a patient who first had indolent disease (sample collection 1) that later became progressive (sample collection 2). The other IGVH unmutated/ZAP70$^+$ sample displayed a V520A mutation in exon 15 (ex15-61719700 [T-C], in ~ 25% of cells). In summary, we found XPO1 mutations in 6 of 127 IGVH unmutated/ZAP70$^+$ cases (4.7%), but in only 1 of 65 IGVHmutated/ZAP70$^-$ cases (1.5%).

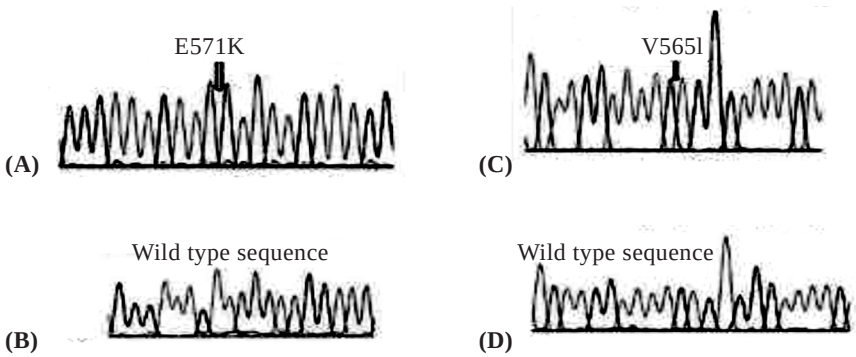


Fig. (2): XPO1 gene mutation. (A,B) Showing E571K mutation and its wild type, (C,D) Showing V565I mutation and its wild type.

We used the same set of samples to screen for NOTCH1 mutations (exon 34) Fig. (3). Interestingly, we found only 5 mutations among 127 IGVH unmutated/ZAP70$^+$ CLL samples (4%). One mutation was found in 6/192 IGVH-mutated/ZAP70$^-$ samples for a total percentage of 3.1% (Table 1).

Table (1): NOTCH1 mutation frequency in CLL.

Sample description	Total mutation	Aggressive	Indolent
• Total samples, first set	6/192 (3.1)*	5/127 (4.0)	1/65 (1.5)
• Trisomy 12 samples in the first set	4/19 (21.1)	4/15 (26.7)	0/4 (0)
• Trisomy 12 set	23/77 (29.9)	22/47 (46.8)	1/30 (3.3)
• Total trisomy 12 samples	27/96 (28.1)	26/62 (41.9)	1/34 (2.9)

* No. (%)

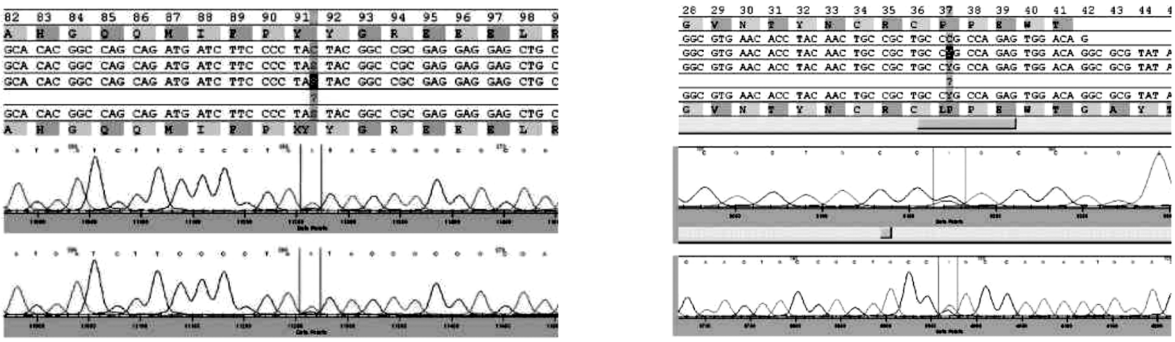
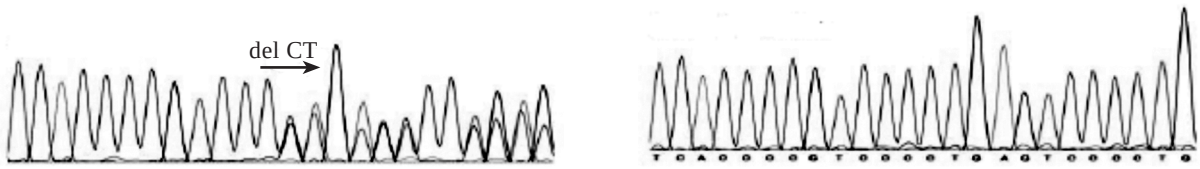


Fig. (3): DNA sequencing electropherograms demonstrating the NOTCH1 sequence mutations.

Because 4/6 samples with NOTCH1 mutations had trisomy 12 Fig. (4), we examined for NOTCH1 mutations in 77 additional cases that also had trisomy 12. This set of samples included 47 IGVH unmutated/ZAP70$^+$ aggressive cases, that were discordant for ZAP70 expression; however, they were characterized as aggressive because they were treated within 1 year of diagnosis), and 30 IGVH mutated/ZAP70$^-$ cases. Among these samples, we found NOTCH1 mutations in 22/47 (46.8%) IGVH unmutated/ZAP70$^+$ aggressive cases, but in only 1/30 (3.3%) IGVH-mutated/ZAP70$^-$ cases. Collectively, for all cases examined with trisomy 12, we found NOTCH1 mutations in 26/62 (41.9%) IGVH unmutated/ZAP70$^+$ aggressive cases, and in 1/34 (2.9%) IGVH-mutated/ZAP70$^-$ indolent cases. Twenty-five cases had mutations in NOTCH1 that were similar to those described **[6,7]** namely a heterozygous 2-bp frameshift deletion P2515fs. Two other cases had mutations resulting in Q2409stop or L2457V. All mutations were observed in 100% of cells in each sample, except in 2 cases in which the P2515fs mutation was observed in ~50% of the cells, and in one case, in ~25% of the cells.



(A) **(B)**

Fig. (4): NOTCHI1 mutation in CLL trisomy 12 patient: A) Chromatogram of a heterozygous CT coding sequence deletion at position chromosome 9:138510470-71. B) Wild type of NOTCHI1 gene.

DISCUSSION

In the current study, we found XPO1 mutations in 6 of 127 IGVH unmutated/ZAP70$^+$ cases (4.7%), but in only 1 of 65 IGVH mutated/ZAP70$^-$ cases (1.5%). These data confirmed previously reported results **[7]**.

Notch1 mutations were independent of gender, thus suggesting that Notch1 mutations might be an important marker of unfavorable prognosis in both male and female CLL patients. Interestingly, we found only 5 mutations among 127 IGVH unmutated/ZAP70$^+$ CLL samples (4%). All these changes were previously de-

scribed 2-bp frame-shift deletion P2515fs, resulting in truncated Notch1 protein **[6,7]**. One mutation was found in 6/192 IGVH-mutated/ ZAP70^{-} samples for a total percentage of 3.1% (Table 1). These results show 4- to 5-fold lower Notch1 mutation frequency in IGVH unmutated/ZAP70^{+} CLL compared with previous reports (4% vs 18%-20%) **[6,7]**, suggesting that Notch1 mutations may not be as prevalent as previously reported which may be due to ethnic variation.

In fact, the first studies reported a high frequency of Notch1 mutations in IGVH unmutated cases and in aggressive clinical phases of CLL as chemo-refractory with disease progression towards transformation into RS. A significant adverse impact on outcome has also been reported independently of other clinico-biological features, including TP53 alterations and unmutated IGHV genes, as Notch1 positive patients showed a significantly shorter overall survival, a shorter time to progression and a high risk of RS **[21]**.

Analyses on larger number of patients and on specific subgroups of patients have now documented a particularly high frequency of NOTCH1 mutation in CLL cases harboring trisomy 12 (+12), one of the cytogenetic alterations recurrently observed in CLL and classically associated with an intermediate prognosis **[22]**. Del Giudice and colleagues documented a high frequency of Notch1 mutations (30%) in CLL cases harboring trisomy 12 as the sole cytogenetic abnormality **[21]**. Importantly, this study also revealed a significant shortening of survival in the Notch1 mutation positive patients, refining the intermediate prognosis of CLL cases with trisomy 12. Moreover, the study highlighted that the presence of Notch1 mutations in +12 CLL cases is associated with a peculiar gene-expression profile characterized by an over representation of cell cycle related genes that are located on chromosome 12.

Although 2 previous studies reported high mutation frequency for NOTCH1 in IGVH unmutated/ZAP70^{+} CLL **[6,7]**, in our set of samples we only observed 4% frequency. On the other hand, our data suggest that almost half of IGVH unmutated/ZAP70^{+} trisomy 12 CLL patients (41.9%) harbor Notch1 mutations, indicating that Notch1 activation is strongly associated with trisomy 12. These differences could be explained, at least in part, by the fact that previous reports did not specifically study Notch1 mutations in trisomy 12 CLL, and did not specify how many trisomy 12 samples were present in their sample pools. All NOTCH1 mutations, except one, resulted in a truncated protein, lacking the C-terminal PEST degradation domain, rendering it constitutively active **[6,7]**. Functional significance of L2457V mutation remains to be elucidated.

In conclusion, Notch1 represents a new target of genetic lesions that could be involved in the pathogenesis of CLL and identifies a subgroup of patients with poor prognosis. Considering the high frequency of Notch1 mutations in a subgroup of patients harboring trisomy 12 and the prognostic implications of this, these mutations should be evaluated at diagnosis and progression. As Notch1 represents a new therapeutic target in CLL, future studies should evaluate the sensitivity of Notch1 mutation positive CLL cases to Notch1 inhibitors, as has been documented in T-ALL.

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