ASXL1 MUTATION ANALYSIS IN CHRONIC MYELOID LEUKEMIA PATIENTS

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ABSTRACT

Background
In recent years, additional sex comb-like 1 (ASXL1) gene mutations have recently been linked to several myeloid cancers.

Objectives
To characterize ASXL1 mutation prevalence, determine if these abnormalities might constitute a significant event in CML prognosis, and establish the correlations if these mutations are associated with CML transformation and/or imatinib (IM) resistance. Here we designed a study to investigate ASXL1 gene that is frequently mutated in myeloid malignancies and evaluated their occurrence in a well-defined group of CML patients.

Patients and Methods
The study population consists of 80 patients diagnosed with CML under treatment with TKI (Imatinib 400, 600, 800 mg/day and Nilotinib). Ten healthy subjects were checked as controls. Depending on their molecular and/or cytogenetic response, CML patients will either be classified into imatinib-resistant or imatinib-good responders. Then the DNA was extracted depending on the Salting-Out protocol. Then genome amplification was performed on exon 12 and in the HOT spot region for the detection of somatic mutations, using conventional PCR.

Results
Nine out of 80 CML samples (11.25%) were determined to have mutations with the ASXL1 gene. We identified a novel Mutation (c.1808_1820delCCTCCTGCGGGGG S603Ffs*96) in one of the patients that has not been reported before. We also identified three other mutations (c.1933_1934del G p.G645Wfs*12, c.2047A>G p.T683A, c.1900_1922 delAGAGAGGGGGCCACCCTGCCAT E635Rfs*15).

Conclusion
Our discovery of an ASXL1 mutation, a potential tumour suppressor gene, is a significant genetic aberration in CML. Our findings suggest that ASXL1 mutations are common in patients with late stages of the disease and imatinib therapy resistance.

Keywords: Leukemia, Chronic Myeloid Leukemia, CML, ASXL1, Imatinib, a Tyrosine kinase inhibitor, TKI.

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INTRODUCTION

The haematological malignancy known as chronic myeloid leukemia (CML) is characterized by the uncontrolled expansion and accumulation of mostly myeloid cells in the bone marrow and blood \(^{(1)}\). The Philadelphia (Ph) chromosome, which is a balanced translocation between the long arms of chromosomes 9 and 22 \(t(9;22)(q34;q11)\), is a characteristic of CML \(^{(2)}\). As a molecular outcome of this translocation, the fusion protein BCR-ABL, a constitutively active tyrosine kinase, is formed \(^{(3)}\).

The Food and Drug Administration (FDA) authorized imatinib as first-line therapy for newly diagnosed CML \(^{(4)}\). Imatinib was the first inhibitor of the BCR-ABL1 tyrosine kinase, and its safety profile has been better than expected after 21 years of clinical usage \(^{(5,6)}\). Imatinib mesylate (previously STI-571) is a 2-phenylamino pyrimidine chemical that preferentially interacts with the binding site of adenosine triphosphate (ATP) of various protein tyrosine kinases, becomes the model for the intended therapy for both haematological and solid malignancies \(^{(7)}\).

BCR-ABL interacts with many cellular and genetic processes that subsequently combine to enhance the disease for its blast phase. Therefore, imatinib resistance and a poor prognosis may be caused by different mechanisms, whether they are dependent on or independent of BCR-ABL \(^{(1,8)}\).

The ASXL1 gene is localized on the region of chromosome 20q11 which is usually associated with cancer \(^{(9)}\). ASXL1 is a highly conserved gene containing an N-terminal ASX homology domain and a C-terminal plant homeodomain finger region (PHD) \(^{(10)}\). ASXL1 links to components of the polycomb repressive complex 2 (PRC2). This complex silences genes by trimethylating histone H3-lysine 27, and mutations in ASXL1 impair PRC2 function \(^{(11)}\).

The ASXL1 protein functions in the deubiquitination of different DNA and histone regulatory complexes. Several studies have identified mutations in ASXL1 in chronic and blast-phase MPNs \(^{(12)}\), and solid tumors as seen in colorectal and endometrial cancers. Exon 12 of ASXL1 is frequently mutated in hematologic malignancies \(^{(11)}\).

ASXL1 mutations in adult myeloid malignancies are clinically linked with poor prognosis and worse outcomes \(^{(9,13-15)}\). Despite that there are few studies evaluating the mutational status of ASXL1 in CML, researchers revealed that ASXL1 mutation represents a significant novel molecular aberration in CML. \(^{(9)}\) It has been demonstrated that mutations in the ASXL1 families may develop in the advanced stages of CML \(^{(16)}\).

To characterize ASXL1 mutation prevalence, determine if these abnormalities might constitute a significant event in CML prognosis, and establish the correlations if these mutations are associated with CML transformation and/or imatinib (IM) resistance, here we designed a study to investigate ASXL1 gene that is frequently mutated in myeloid neoplasms and evaluated their frequency in a well-defined cohort of CML patients.

METHOD AND MATERIALS

The study population consists of 80 patients diagnosed with CML under treatment with TKI (Imatinib 400,600,800 mg/day and Nilotinib). Clinical information regarding the disease phase, drug response, and smoking status of patients was collected from their clinical files. The existence of the Philadelphia chromosome (Ph+) in CML patients is a condition for inclusion within the study.

Depending on their molecular and/or cytogenetic response, CML patients will either be classified into imatinib-resistant or imatinib-good responders. Imatinib-resistant patients are those with CML who do not achieve a full cytogenetic response by 12 months and/or do not get a substantial molecular response by 18 months after starting medication. Secondary resistance is defined as the absence of the entire cytogenetic response and/or the absence of the primary molecular reaction. In addition, 10 sex and age-matched healthy individuals without a history of cancer or other chronic diseases and with normal WBC and Platelet count are included in this study.

Genome amplification was performed using conventional PCR using a specific program which was optimized after performing a gradient for the gene. Then we confirmed the PCR product by running them on agarose gel (1.5%) as shown in Figure 1. After that sequencing of the samples was done using an automated Sanger sequencing (SeqStudio) analyzer, and results were checked for any variations as shown in Figure 2.
ASXL1 Mutation Analysis in Chronic Chronic Myeloid Leukaemia Patients

Figure 1. Agarose gel (1.5%) electrophoresis showing samples’ PCR products for the ASXL1 gene. No. 1 to No. 10 were patient samples, with a 50 bp DNA ladder after them. A negative control sample at the end.

Note: The expected product size was 338 bp.


The sample sequences were viewed on a computer using Chromas software (Figure 2) and checked for any impurities and variations in the sequences. Then the sequences were BLAST-ed against the gene’s ref. seq with the accession number (NG_027868.1) using the online NCBI Blast tool to confirm the existence of any mutation. Samples with mutations were then put in some prediction tools like mutation taster, FATHMM and PredictSNP to determine if the detected mutations had deleterious effects or not. While samples with variations and improper sequences have been repeated.

Statistics

The odds ratio test was applied to examine the risk association of ASXL1 mutations with resistance to imatinib and to determine the risk association of ASXL1 mutations with disease progression. P-values < 0.05 are considered as statistically significant.

RESULTS

Mutation types, frequency, and detected polymorphisms in CML patients.

We carried out a mutational analysis of exon 12 of the ASXL1 gene in all 90 samples. 9 out of patients’ 80 samples (11.25%) were determined to have mutations with the ASXL1 gene and in 4 types. The type and frequency of mutations are shown in Table 1 and Figure 3. The most common mutation was P. G645WfsX12 (deletion-frameshift) which was detected in 6 patients (7.5%). One of the patients had a novel long mutation as frameshift -deletion. The mutations were identified only among patients with advanced phases (accelerate and blastic phase). Both CP-CML patients and healthy controls lacked any of the ASXL1 mutations. In addition, one patient had substitution G>A in c.1954 which indicates polymorphism.
Risk association ASXL1 mutations with resistance to imatinib

In this study, 100% of the patients with ASXL1 mutations were resistant to IM, and no mutations were observed in good responders to IM. There was a significant difference regarding IM resistance in patients with ASXL1 mutations compared to those without mutations, as the risk of increasing resistance in patients with mutations was 32-fold higher than in those without mutations (Table 2).

Risk association of ASXL1 mutations with disease progression

None of the patients with the mutation were in the chronic phase and mutation was observed only in advanced-phase patients. There was a very significant difference in the development of the disease progression in the comparison between the two groups with and without mutation. The risk of disease progression among the patients with ASXL1 mutation was higher (163-fold) than those without mutation group, Table 3.

### Table 1. Mutation types, frequency, and detected polymorphisms in CML patients.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Nucleotide change</th>
<th>Protein change</th>
<th>Frequency of each mutation (n,%)</th>
<th>Total frequency in between all of the patients (N=80)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASXL1</td>
<td>c.1933-1934 del G</td>
<td>p.G645WfsX12</td>
<td>6/9 (66.67%)</td>
<td>9/80 (11.25%)</td>
</tr>
<tr>
<td>ASXL1</td>
<td>c.1900-1922 del</td>
<td>p. E635FfsX15</td>
<td>1/9 (11.11%)</td>
<td></td>
</tr>
<tr>
<td>ASXL1</td>
<td>c.2047 A&gt;G</td>
<td>p.T683A</td>
<td>1/9 (11.11%)</td>
<td></td>
</tr>
<tr>
<td>ASXL1</td>
<td>c.1808-1820 del</td>
<td>p.S603Ffsx96</td>
<td>1/9 (11.11%)</td>
<td></td>
</tr>
<tr>
<td>ASXL1</td>
<td>CCTCTGCCTGGGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASXL1</td>
<td>c.1954 G&gt;A polymorphism</td>
<td>p.G652S</td>
<td>1/80 (1.25%)</td>
<td>1.25%</td>
</tr>
</tbody>
</table>

Figure 3. Frequency of ASXL1 mutations.
**Table 2. Risk association ASXL1 mutations with resistance to imatinib.**

<table>
<thead>
<tr>
<th>ASXL1 mutation</th>
<th>IM resistance (N=35)</th>
<th>Good response (N=45)</th>
<th>P-value</th>
<th>OR, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>With mutation</td>
<td>9 (100%)</td>
<td>0 (0%)</td>
<td>0.01*</td>
<td>32.62, CI:1.82 to 583.42</td>
</tr>
<tr>
<td>ASXL1 (N=9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without mutation</td>
<td>26 (36.61%)</td>
<td>45 (63.39%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bolded: P<0.05 is significant, OR: Odds ratio; CI: confidence interval; IM, imatinib mesylate

**Table 3. Risk association of ASXL1 mutations with disease progression.**

<table>
<thead>
<tr>
<th>ASXL1 mutation</th>
<th>Chronic phase (N=64)</th>
<th>Accelerate + Blastic phase (N=16)</th>
<th>P-value</th>
<th>OR, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>With mutation</td>
<td>0 (0%)</td>
<td>9 (56.25%)</td>
<td>0.0007*</td>
<td>163, CI:8.61 to 3099</td>
</tr>
<tr>
<td>ASXL1 (N=9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without mutation</td>
<td>64 (100%)</td>
<td>7 (43.75%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bolded: P<0.05 is significant, OR: Odds ratio, CI: confidence interval

**DISCUSSION**

In recent years, it has been discovered that mutations in the ASXL1 gene are the most significant genetic alterations in a variety of myeloproliferative and/or myelodysplastic syndromes (10,23,24).

Our attention was piqued by recent reports of recurrent mutations in the putative tumour suppressor gene ASXL1, which were published in several studies. According to many studies, a wide variety of myeloid malignancies have been linked to missense, nonsense, and frameshift mutations in ASXL1, including 43% of chronic myelomonocytic leukaemia, 17% of acute myelogenous leukaemia, 15% of chronic myelogenous leukaemia, 11% of myelodysplastic syndrome, and 8% of myeloproliferative neoplasm (24, 25).

Recently, Willekens, et al. reported common mutations in addition to the BCRABL1 fusion gene impacting the ASXL1 gene in Ph-positive and Ph-negative clones of adult CML patients using a targeted deep next-generation sequencing (NGS) strategy. These mutations may contribute to the aetiology, clonal evolution, and development of CML (13).

In our study, there was a significant difference in patients with ASXL1 mutations and those without mutations regarding imatinib resistance, with the P value <0.01. Some other studies support our results and show ASXL1 mutations in those which resistant to imatinib (26).

Regarding the risk association of ASXL1 mutations with disease progression, there was a very significant difference between patients with ASXL1 mutations as compared to those without mutations, with the P value <0.007. Also, there are a lot of studies that show poor prognosis and worse outcomes in CML patients with ASXL1 mutations (9,13-16).

In conclusion, our research demonstrates a significant prevalence of ASXL1 mutations in individuals with late disease stages and treatment resistance to imatinib. Future research should examine ASXL1 mutations in larger groups of CML patients and compare results to outcomes and prognostic information. The functional role of ASXL1 mutations in the development and progression of CML should be the subject of future studies. Future treatment attempts might be enhanced with a deeper knowledge of the molecular context. Lastly, in addition to the measurement of mutation frequency of an exon or a particular part of an exon by conventional PCR, next-generation sequencing for the whole coding exons as a more reliable technique for more interpretation of the ASXL1 gene role in CML should be undertaken.

**REFERENCES**


