Detection of Imatinib Resistance in Chronic Myeloid Leukaemia Using Allele-specific Oligonucleotide Polymerase Chain Reaction in Sudan

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Abstract: A descriptive cross-sectional study was conducted on chronic myeloid leukemia (CML) patients who attended to the Radio-isotope center in Khartoum (RICK), Sudan; in the period from June 2006 up to December 2008. 50 cDNA samples from Philadelphia positive CML patients; were chosen from DNAs of the laboratory of the RICK; including both males and females and of different ages, and which were treated with different therapeutic approaches including Gleevec. Two mutations; namely T315I and E255K which were thought to cause clinical resistance to Gleevec in CML patients after an initial response; were screened using Allele Specific Oligonucleotide Polymerase Chain Reaction (ASO-PCR). Five patients (10%) were found positive for T315I mutant gene, and only one patient (2%) was positive for E255K. The appearance of clinical response in patients who had a mutation was explained as the mutations having different levels of resistance. Patients who showed signs of clinical resistance, inspite of negative results for both mutations screened, were thought to had mutations other than T315I or E255K; or to resistance mechanisms other than mutations. The study found two patients who had the T315I mutant, and who received no treatment until the time of sampling, fulfilling the assumption that such mutations can exist before initiation of the drug.

Key words: Imatinib, Chronic Myeloid Leukemia, Resistance, Allele-specific oligonucleotide polymerase chain reaction, Gleevec

INTRODUCTION

Chronic myeloid leukaemia is a slow-growing cancer of the white blood cells (Goldman JM, 2007). It is one of a group of diseases called the myeloproliferative disorders, and can be defined as a clonal disease that results from an acquired genetic change in a pluripotential haemopoietic stem cell. This altered stem cell proliferates and generates a population of differentiated cells that gradually replaces normal haemopoiesis and leads to a greatly expanded total myeloid mass (Hoffbrand A.V. et al, 1999).

The exact cause of CML is not known (Hoffbrand A.V. et al, 1999). The risk of developing CML is slightly but significantly increased by exposure to high doses of irradiation (Medifocus Guidebook, 2009). The acquired genetic defect in CML is the so-called Philadelphia chromosome (Ph.chromosome) which is a translocation between two chromosomes; 9 and 22 resulting into that part of the BCR ("breakpoint cluster region") gene from chromosome 22 (region q11) is fused with part of the ABL gene on chromosome 9 (region q34) (Emmanuel C. Besa, 2009). This leads to a protein of p210 or sometimes p185 which is a tyrosine kinase thought to speed up cell division and inhibits DNA repair, causing genomic instability and potentially causing the feared blast crisis in CML (Ghanei M., Vosoghi Ali A., 2002). CML occurs from childhood to old age.

It accounts for approximately 15 percent of all cases of leukemia or approximately 4600 new cases per year. The incidence of CML appears to be constant world-wide (Hoffbrand A.V. et al, 1999, Medifocus Guidebook, 2009). It occurs in about 1.0 to 1.5 per 100 000 of the population in all countries where statistics areadequate (Ghanei M., Vosoghi Ali A., 2002). It is rare below the age of 20 years, but occurs in all decades, with a median age of onset of 40 – 50 years (Ghanei M., Vosoghi Ali A., 2002). About 2 million people worldwide are living with chronic myeloid leukemia (Hoffbrand A.V. et al, 1999). In Sudan, CML accounts for 35.5% of all adult leukemias (Tambal A.M.A. et al., 1996). Other detailed information about prevalence and incidence of CML in Sudan were not found.

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MATERIALS AND METHODS

Time and Location of Study:
The study was conducted from June 2006 to December 2008 as a descriptive cross-sectional hospital-based study in the Radio-isotope center Khartoum (RICK), in Khartoum state, Sudan.

Study Subjects:
Diagnosed Philadelphia Positive CML patients, attended or in-patient of The RICK – Sudan. The study included both sexes, irrespective of age, ethnic group, previous treatment nor the duration of the disease. An approval of the RICK was warranted. No extra blood samples were taken from patients; but the blood samples needed for routine follow ups.

Data Collection:
Data were collected from the RICK registration books, and the request forms attended to the RICK laboratory.

Specimen Collection and Preparation:
Using sterile disposable DNA free vacutainers, 3 ml of venous blood were drawn from the anticubital vein under aseptic conditions. RNA was isolated from blood samples; and a cDNA was achieved using Reverse transcription – PCR; and stored at -4ºC for the purpose of further techniques, from which 50 samples were chosen according to data extracted.

Laboratory Examination:
Allele Specific Oligonucleotide -Polymerase Chain Reaction (ASO-PCR) for two mutant genes; T315I and E255K, was performed according to Sambrook et al (Sambrook J, Russell D.W., 2001), and Willis et al (Willis S.G. et al, 2005).

PCR Amplification:
ASO-PCR for detection of T315I and E255K mutations was done. A master mix was prepared, and the total volume per tube was 25 µl. The number of tubes was multiplied by each volume of each reagent; then these figures were added together with the total volume of the DNA of all tubes. The resultant figure was subtracted from the total volume of the pre-mix to give the volume of H2O. Volumes of reagents per tube were: 10×Buffer: 2.5 µl, dNTPs: 0.5 µl, Primer 315R (reverse for T315I, see Table 1 below): 0.75 µl, Primer Bcr 267 F(forward for T315I, see Table 1 below): 0.75 µl, Mgcl2 (50mM):1.5 µl, Taq polymerase 0.25 µl. Similar calculations were done for the E255K mutant gene, but different primers were used. 255K T3R was the reverse primer, while Bcr2 687F was the forward one.21 µl of the master mix were transferred to each tube, and 4 µl of DNAs under test are transferred to each tube according to labeled number (1st tube was left without DNA).

Table 1: Some specifications of the primers used. (OD, Absorption units at 260 nm)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence(5’ to 3’)</th>
<th>pmol/OD</th>
<th>µg/OD</th>
<th>Backbone Molar.wt(Da)</th>
<th>Picomoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>T315I</td>
<td>CTAGGTCATGAAACTCAA Reverse 5540</td>
<td>30.2</td>
<td>5508</td>
<td>72947</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACTCCAGACTGCCACAGCAT Forward 5063</td>
<td>32.0</td>
<td>6335</td>
<td>89635</td>
<td></td>
</tr>
<tr>
<td>E255K</td>
<td>CACGCTCTGTGACTCTTReverse 6402</td>
<td>34.9</td>
<td>5371</td>
<td>154475</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CGCGACATCAATAAGGForward 5559</td>
<td>30.4</td>
<td>5493</td>
<td>104533</td>
<td></td>
</tr>
</tbody>
</table>

For PCR Amplification, tubes were put into the Techne TC-512 PCR machine and a program was made for the gene (T315I) as: 94ºC for 30 sec. for denaturation, 60ºC for 60 sec. for elongation, 72ºC for 80 sec. for extension. This was repeated for 50 cycles (about 3 hours), after which tubes were transferred out for the Gel electrophoresis.

Gel Electrophoresis:
1.5% agarose was prepared as described by Sambrook et al (Sambrook J, Russell D.W., 2001), by weighing 1.5 g of agarose powder and dissolve in 100 ml 1×TBE buffer. It was then mixed, microwaved for 1 minute and left to cool on the bench for 5 minutes down to about 60º C. 3 µl of Ethidium bromide (10 mg/ml) were added to the gel and swirl to mix. The electrophoresis tank was prepared and the comb was placed correctly then the gel was slowly poured. Air bubbles were avoided or pushed aside using disposable tips. The gel was left to set for about 30 minutes.
The comb and metal gel-formers were removed carefully to insure the safety of the wells, and then the 1×TBE running buffer was poured into the gel tank to submerge the gel 2 – 5 mm depth.

Samples and controls were prepared by mixing in separate tubes 4 µl of each sample with 2 µl of the loading dye. Each sample was then transferred to the well paying great attention on labeling and order. Lane 1 was used for the DNA ladder (marker) which was prepared by mixing 4 µl of the marker with 1 µl of the marker loading dye. After samples and controls were transferred to the wells, the lid was put on and the electric current was applied in a range of 25 – 45 Ampere. The gel was then left for about 30 min for the DNAs to migrate towards the positive pole.

**Visualization of PCR Product:**

This was done using Syngene UV Tran illuminator and Canon PC 1049 gel photography camera. The whole gel with its stand was taken out from the tank and only the gel was put on the Syngene UV Tran illuminator. The camera was put on the illuminator and both devices were operated. Photographs were captured and stored for further analysis. The second lane was usually a negative control; where a sample of no DNA was run. Remaining lanes were used for the samples. Product length expected for T315I mutant was 994 bp, and 798 bp for E255K mutant, so DNA Logical Ladder is suitable for sizing and quantifying double-stranded DNA from 100 bp to 10 kb. DNA Logic-Ladder was consisted of the following 15 DNA fragments: 100 bp, 200 bp, 300 bp, 400 bp, 600 bp, 800 bp, 1 kb, 1.5 kb, 2 kb, 3 kb, 4 kb, 5 kb, 6 kb, 8 kb, and 10 kb. A sample was considered positive if a sharp band appeared at the running way from the well to the positive pole. Double or triple bands at close loci were considered vague. When a sharp band was noticed; a quick comparison was made regarding the DNA ladder to identify the approximate length.

**Statistical Analysis:**

SPSS software was used.

**Results:**

For the purpose of this study 50 CML patients attending Radiation and isotope centre, whom confirmed to be positive for Philadelphia chromosome, were recruited. The number of male patients was 32 (64%), while the number of females was 18 (36%). Ages of the studied patients ranged from 2.5 to 70 years. The mean age of the population was 39.65 years. The mean age of males was 38.14 years; and that of females was 42.33 years. Duration of the disease was extracted from the structured questionnaire. It covers the period from date of first complain up to date of sample collection. The majority of the examined patients had a disease duration of less than one year: 25 patients (50% of the population), while only 4 patients (8%) had a duration of more than five years. Out of the 50 patients examined, Hydroxyurea was the starting treatment in 39 (78%) cases, before the commencement of Gleevec. Eleven patients (22%) received no treatment at the time of sample collection. Table 2 shows the distribution of the population according to Gleevec Status. The six patients who found to harbor a BCR-ABL mutant gene; could be divided into three categories according to Gleevec status. One group received Gleevec after Hydroxyurea; those were two patients (33.3%). The second group received only Gleevec, and those were also two patients (33.3%). The third group received no treatment until the time of sampling (33.3%). Table 2 below.

<table>
<thead>
<tr>
<th>Category</th>
<th>Number</th>
<th>Percentage of the six</th>
</tr>
</thead>
<tbody>
<tr>
<td>On Gleevec after Hydroxyurea</td>
<td>2</td>
<td>33.3%</td>
</tr>
<tr>
<td>Received only Gleevec</td>
<td>2</td>
<td>33.3%</td>
</tr>
<tr>
<td>No treatment*</td>
<td>2</td>
<td>33.3%</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>100%</td>
</tr>
</tbody>
</table>

*Until the time of sample collection

Out of the 50 patients examined, 5 patients (10%) were found to harbour the mutant allele at codon 315 of the BCR-ABL gene (315I). Forty five patients (90%) had the wild gene (T315I). Regarding mutation analysis at codon 255 of the BCR-ABL gene, only one patient (2%) was found to harbour the mutant allele (255K). No patient was found to harbour both mutations. (Figure 1).

In investigating the relationship between patients’ characteristics and the mutations, no statistically significant relationship was found between the occurrence of mutation(s) and the gender, age, ethnic group, duration of the disease, history of treatment, TWBCs and platelets count.
Frequencies of BCR-ABL T315I and E255K mutations plotted against wild type BCR-ABL.

Fig. 1: Frequencies of BCR-ABL T315I and E255K mutations plotted against wild type BCR-ABL.

Fig. 2: A gel electrophoresis photograph showing some of the samples run. Lane 1 (from left) is the DNA 100 bp marker. Lane 2 is the negative control. Lane 3 is the positive control. Lanes 4, 5, 6, 7, 8 were samples No. 600, 584, 539, 602, 593 respectively. (Samples 6, 7 were considered positive).

Discussion:
Mutations in the kinase domain (KD) of Bcr-Abl are the most prevalent mechanism of acquired imatinib resistance in patients with chronic myeloid leukemia. Although the majority of CML patients treated with Imatinib show significant - at least - hematologic responses, resistance to imatinib is still a problem mainly in patients in the accelerated or blast crisis phases of the disease. This was the first report of Imatinib mutations detection in Sudan. The small sample size (50) of the study may minimize reliable judges the study can offer, but many reasons interfere to force the researcher to stick in this number; the most obvious was the financial aid.

There seems to be a clear variation in frequencies of mutations in the ABL component of the BCR-ABL gene. T315I in this study was found 10% (5 patients), and this may be considered almost the same; when compared to results obtained by Willis and colleagues (Willis S.G., et al. 2005); who used cDNA sequencing and allele-specific oligonucleotide–polymerase chain reaction (ASO-PCR) assay in imatinib-naive CML patients. They detected T315I in 8 patients out of 66 (12.1%). Roche (Roche-Lestienne C. et al, 2003), screened for T315I using reverse transcription polymerase chain reaction restriction fragment length polymorphism (RT-PCR-RFLP) technique, and ASO-PCR . T315I was 12.5% (3 patients. However; Results obtained here regarding T315I was considered high if compared to results obtained by Hochhaus (Hochhaus A., S. Kreil, 2003); who detected T315I mutant to be only 1.0% using RT-PCR in 300 patients with BCR-ABL+ CML. Taking account of the relatively large sample of Hochhaus comparing to this study, the reason for such a variation may be the higher sensitivity of the ASO-PCR method of mutations screening, over the RT-PCR method.
E255K in this study was found 2% (one patient), and this could be considered somewhat the same as results of Sorel (Sorel N. et al, 2005); who detected E255K to be 1.69% in 59 imatinib-resistant CML patients using a screening method based on double-gradient-denaturing-gradient gel electrophoresis (DG-DGGE), which is generally performed on the ABL sequence by a single-step PCR. Results obtained here could be considered high comparing with results obtained by Willis and colleagues (Willis S.G., et al 2005); who detected zero E255K mutation out of their 66 patients using cDNA sequencing and allele-specific oligonucleotide–polymerase chain reaction (ASO-PCR).

The variation in mutation detection between this study and other different studies may be due to technical or biological reasons. Therefore, it is possible that the frequency of positive results would increase with more sensitive assays covering more type of mutations. So when a patient shows a reasonable response to Imatinib; and shows no mutations in his/her Bcr-Abl kinase domain, this may not necessarily indicate an intact case of CML treatment. Some new mutations which are not yet discovered is another challenge, which highlights the need for screening or searching methods for them. Direct sequencing of the kinase domain is most commonly used but has limited sensitivity. (ASO-PCR) assays were considered the best in sensitivity by detecting mutations even after dilution in the 10,000-fold range.

This study did not observe significant relationships between patients, sex, age, ethnic group, disease duration, history of treatment, TWBCs and Platelets count; and the occurrence of T315I/E255K mutants. Willis and colleagues (Willis S.G., et al 2005); reported that disease phase, a low platelet count (compared to patients with normal platelets), age > 60 years and previous therapy with 6-Thioguanine (but not other cytotoxic drugs) were associated with mutation detection at p < 0.1.

Regarding the assumption that mutations can pre-exist the treatment with tyrosine inhibitors, the study found that two patients were not treated until the time of sample collection; and they were both positive for T315I mutant. This was in agreement with Roche et al (Roche-Lestienne C. et al, 2003); who stated that ABL mutations may pre-exist to Imatinib, which only appears to create a clonal selection of the minor population of cells carrying the mutation. They discussed that origins of those point mutations of the ABL gene are uncertain, but mutations may have been acquired during disease progression through associated genetic instability. Soverini et al (Soverini S., et al, 2007); agreed with the theory that mutations randomly arise as a consequence of a high genetic instability and very few of them will confer a growth advantage under the selective pressure of TKIs. Also this was in correspondence with the study of Kreuzer et al (Kreuzer K.A., et al 2003); who detected G255L mutation before commencement of Imatinib.

Mutations in the Abl KD occur spontaneously (or as a result of treatment with specific drugs; like 6-thioguanine, a known mutagenic agent throughout the course of the disease, but in many cases the mutated clone remains small. Specific clones with mutations that interfere with imatinib binding may expand when patients are treated with this drug, but other mutant clones may derive no selective advantage during imatinib treatment. It is possible that some mutant clones that expand and eventually constitute the bulk of the Ph-positive population enhance 'genomic instability' and thereby increase the risk of disease progression. However, it is equally possible that other clones detected at low level in chronic phase merely reflect the intrinsic instability of the Ph-positive cell population, which may well differ from patient to patient, but do not of themselves increase the risk of further leukemic transformation.

The study found no significant relationship (P >0.05) between mutations in patients who initially respond to Gleevec and then become resistant, and those who never taken Gleevec. As out of the six positives detected, four were on Gleevec after Hydroxyurea, while two positives received no treatment until the time of sample collection. The study reported two patients who had the T315I mutant gene; although they were clinically considered responsive. This can be discussed in many ways. Several mutations remain sensitive to the drug, as stated by Corbin et al (Corbin A.S., et al, 2003); who stated that while some Abl mutations lead to Imatinib resistance, many others are significantly, and some fully, inhibited. The role of many mutations in the emergence of resistance has not been established. There is an increasing need for biochemical and biologic characterization before a resistant phenotype can be ascribed to a mutant. To adequately interpret the results of kinase domain mutation analysis, the size of the mutant clone and the clinical context must be taken into account. Five patients of the population in this study were clinically resistant; (samples number 5, 8, 11, 46 and 124; a 43 years old male, 45 years old female, 25 years old male, 47 years old female and a 18 years old female respectively), and were in acceleration but they were negative for both T315I and E255K mutants. This may due to mechanisms of resistance other than KD mutations; or they may had other Imatinib insensitive mutations like Y253F or E255V. One patient (sample number 680; a 65 years old male), was clinically resistant and showed an accelerating CML, and he was having the E255K mutant. The study did not find more than one mutation in one patient.
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