MEDICAL LABORATORY MOLECULAR METHODS FOR IMATINIB THERAPY EVALUATION IN CHRONIC MYELOID LEUKAEMIA PATIENTS AND OTHER PATIENTS WITH VARIANTS CHRONIC MYELOID LEUKEMIA

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ARTICLE SUMMARY

Chronic Myeloid Leukaemia (CML) is a clonal disorder of a pluripotent stem cell characterized by the Philadelphia (Ph) chromosome. The Ph is a shortened chromosome 22 resulting from a reciprocal translocation between the *BCR* gene on chromosome 22 and the *ABL* gene on chromosome 9 (Mondal et al., 2006). The resulting overactive ABL tyrosine kinase appears to be responsible for uncontrolled myeloid cell proliferation (Hughes et al., 2006). Imatinib, also called Gliveec, is the first and most commonly used tyrosine kinase inhibitor (TKI) that inhibits by binding through ATP (adenosine triphosphate) onto the *BCR-ABL* fusion protein of which when absent the kinase leads to uncontrolled cell proliferation. The level of *BCR-ABL* mRNA can be used as a sensitive marker for disease progression (Hoffbrand et al., 2001). In approximately 5% of CML cases, patients present with a variant Ph chromosome in which the Ph chromosome is derived through rearrangements other than the classic t(9;22) (Hughes, et al., 2006).

The aim of this study is to assess the response to therapy between classic chronic myeloid leukaemia patients and variant translocation chronic myeloid leukaemia patients. Methods for monitoring treatment response include conventional cytogenetic analysis, Fluorescence in situ hybridization (FISH) and Quantitative PCR (polymerase chain reaction). *BCR-ABL* transcript levels quantified by PCR will be used to assess the response of therapy, which in turn influences the choice of clinical management. The response in therapy will be monitored in patients with variant Ph translocations compared to patients with classical Ph translocation who have or have not responded to treatment.

KEY WORDS

CML; chronic myeloid leukaemia

Ph; Philadelphia chromosome

BCR/ABL; BCR; gene on chromosome 22, locus q11 and ABL; gene on chromosome 9, locus q34

TKI; tyrosine kinase inhibitor

DNA; deoxyribonucleic acid

cDNA; complementary DNA

RNA; ribonucleic acid

mRNA; messenger RNA

FISH; Fluorescence in situ hybridization

PCR; polymerase chain reaction

RQ-PCR; real time quantitative polymerase chain reaction RQ-PCR; real time qualitative polymerase chain reaction

Cytogenetics; technique for detecting chromosomal aberrations

LITERATURE REVIEW

Most classic chronic myeloid leukaemia (CML) patients receive imatinib as first-line therapy. For these patients serial analysis BCR-ABL blood levels by real time quantitative polymerase chain reaction (RQ-PCR) provides the most accurate and clinically relevant monitoring strategy. The major advantage of RQ-PCR is the ability to accurately and frequently monitor the decline in transcript level of over a 4-5 log range using peripheral blood (Talpaz et al., 2002). In CML the breakpoint commonly occurs at introns 13 and 14 of *c-bcr* and in introns 2 of *c-abl* (b2a2 and b3a2) (Mondal et al., 2006).This has been called the major breakpoint (M-bcr) and produces a 210 Kda (p210) fusion protein. The second common breakpoint was detected in acute lymphoblastic leukaemia (ALL). This breakpoint occurs at exon 1 of c-*bcr* and intron 2 of c-*abl (*e1a2), producing the minor breakpoint (m-bcr) Mondal et al., 2006). This breakpoint produces a 190Kda (p190) fusion protein. In rare cases some patients present with a variant translocation. The most important technique to be utilized will be RQ-PCR (quantitative PCR). RQ-PCR remains a technically procedure. In order to achieve a high level of reproducibility and the reporting data in a biologically relevant manner, every aspect of the RQ-PCR technique requires thorough validation and optimization (Bustin, 2002; Bustin et al., et al 2004; Ginzinger, 2002).

The most important factors for reliable data are: the use of good quality RNA; careful assay design to exclude amplification of pseudogenes contaminating DNA and polymorphism in primer of probe binding sites; equal amplification efficiency of DNA plasmids standards and cDNA; selection of appropriate control gene to compensate for variations in the RNA quality and the efficiency of the reverse transcription; and the inclusion and appropriate monitoring of quality control samples to detect unreliable results (Hughes et al., 2006; Van der Velden et al., 2003).

STUDY AIMS RESEARCH PROBLEM

Imatinib is the first and most commonly used tyrosine kinase inhibitor (TKI) developed that inhibits by binding to the ATP (adenosine triphosphate) site of the *BCR-ABL* fusion protein of which when absent the kinase leads to uncontrolled cell proliferation. The level of *BCR-ABL* mRNA can be used as a sensitive marker for disease progression (Hoffbrand et al., 2001). This therapy is widely used as a first line treatment for classic CML patients. There is widespread debate on the role of the inhibitor to control uncontrolled cell proliferation in variant CML as these patients are also given the same tyrosine kinase inhibitor. The effect of the inhibitor on variant translocation may not be the same as the classic CML.

SOLUTION TO PROBLEM

RQ-PCR (polymerase chain reaction), a highly sensitive molecular technique will be useful to measure the BCR-ABL levels of expression. This will make it possible to monitor a patient's response to TKI therapy. The patients' response to treatment will be monitored at 3-6 months intervals and this response will be compared between the variant Ph translocation and classical Ph patients who have or have not responded to treatment.

AIM

To assess the response of TKI therapy between classic chronic myeloid leukaemia patients and variant translocation chronic myeloid leukaemia patients.

OBJECTIVE

Quantification of BCR-ABL transcript levels by PCR will be used to assess the efficacy of therapy, which in turn influences the choice of clinical management.

IMATINIB MECHANISM OF ACTION

The chemotherapeutic agent, Imatinib, is a 2-phenyl amino pyrimidine derivative that acts as a specific inhibitor of a number of tyrosine kinase (TK) enzymes. It occupies the TK active site, leading to a decrease in activity. Imatinib is specific for the TK domain in Abl, and platelet-derived growth factor receptor (PDGF-R). As explained that in chronic myelogenous leukemia,

the Philadelphia chromosome leads to a fusion protein of abl with bcr (breakpoint cluster region), termed bcr-abl. As this is now a constitutively active tyrosine kinase, imatinib is used to decrease bcr-abl activity. The active sites of tyrosine kinases each have a binding site for ATP. The enzymatic activity catalyzed by a tyrosine kinase is the transfer of the terminal phosphate from ATP to tyrosine residues on its substrates, through tyrosine phosphorylation. Imatinib works by binding close to the ATP binding site of bcr-abl, locking it in a closed or self-inhibited conformation, and therefore inhibiting the enzyme activity of the protein. This fact explains why many BCR-ABL mutations can cause resistance to Imatinib by shifting its equilibrium toward the open or active conformation (Takimoto CH et al, 2008). Imatinib is quite selective for bcr-abl – it does also inhibit other targets mentioned, but no other known tyrosine kinases. Imatinib also inhibits the abl protein of non-cancer cells but cells normally have additional redundant tyrosine kinases which allow them to continue to function even if abl tyrosine kinase is inhibited. Some tumor cells, however, have a dependence on bcr-abl. According to Gambacorti-Passerini CB et al, the inhibition of the bcr-abl tyrosine kinase also stimulates its entry in to the nucleus, where it is unable to perform any of its normal anti-apoptopic functions. The Bcr-Abl pathway has many downstream pathways including the Ras/MapK pathway, which leads to increased proliferation due to increased growth factor-independent cell growth. This affects the cytoskeleton, which leads to increased cell motility and decreased adhesion. BCL-2 gene is responsible for keeping the mitochondria stable; this suppresses cell death by apoptosis and increases survival. (Weisberg E et al, 2007).

PHARMACOKINETICS

Imatinib is rapidly absorbed when given by mouth, and is highly bioavailable: 98% of an oral dose reaches the bloodstream. Metabolism of imatinib occurs in the liver and is mediated by several isozymes of the cytochrome P450 system, including CYP3A4 and, to a lesser extent, CYP1A2, CYP2D6, CYP2C9, and CYP2C19. The main metabolite, N-demethylated piperazine derivative, is also active. The major route of elimination is in the bile and feces; only a small portion of the drug is excreted in the urine. Most of imatinib is eliminated as metabolites; only 25% is eliminated unchanged. The half-lives of imatinib and its main metabolite are 18 and 40 hours, respectively. It blocks the activity of Abelson cytoplasmic tyrosine kinase (ABL), c-Kit and the platelet-derived growth factor receptor (PDGFR). As an inhibitor of PDGFR, imatinib mesylate appears to have utility in the treatment of a variety of dermatological diseases. Imatinib has been reported to be an effective treatment for FIP1L1-PDGFR alpha+ mast cell disease, hypereosinophilic syndrome, and dermatofibrosarcoma protuberans (Scheinfeld N, et al 2006).

DRUG INTERACTIONS

Substances influencing the activity of CYP3A4 change the plasma concentration of the drug. An example of a drug that increases imatinib activity and therefore side effects by blocking CYP3A4 is ketoconazole. Other drugs include itraconazole, clarithromycin, and grapefruit juice, among others. Simvastatin, ciclosporin, pimozide, warfarin, metoprolol, and possibly paracetamol

reduce plasma levels of levothyroxine via an unknown mechanism. As with other immunosuppressants, application of live vaccines is contraindicated because the microorganisms in the vaccine could multiply and infect the patient. Inactivated and toxoid vaccines do not hold this risk, but may not be effective under imatinib therapy (Klopp, T, et al 2010).

ADVERSE EFFECTS OF IMATINIB

The most frequently reported drug-related adverse events are nausea, vomiting, edema, and muscle cramps. Most events are mild to moderate, and only 2–5% of patients permanently discontinue therapy, most commonly because of rashes and elevations of transaminases (each in <1% of patients). Edema can manifest at any site, most commonly in the ankles and periorbital tissues. Severe fluid retention (pleural effusion, pericardial effusion, pulmonary edema, and ascites) is reported in 1–2% of patients taking imatinib. The probability of edema increases with higher imatinib doses and in persons >65 years old. Neutropenia and thrombocytopenia are consistent findings in all studies in leukemia patients, with a higher frequency at doses ≥750 mg. The occurrence of cytopenias also is dependent on the stage of CML, with a frequency of severe neutropenia and thrombocytopenia between two-and threefold higher in blast crisis and accelerated phase compared to chronic phase. In solid tumor patients, severe neutropenia has been reported in <5% of patients. Thrombocytopenia is much less common. Laurence, John and Keith (2006).

METHODOLOGY

A prospective-double cohort study will be performed using patients with the classical Ph translocation and the variant Ph positive patients. These cases will be confirmed by cytogenetic analysis. Samples would be identified from Patients visiting. The Johannesburg General Hospital oncology department and other samples sent from other hospitals to the Cytogenetics department for Ph chromosome analysis. The controls used in this study would be patients with the classical Ph translocation which have been divided into and bad responders to TKI therapy based on their BCR-ABL transcript levels. Data of these known patients will be obtained on the basis that all of them are on the same dosage of TKI (Imatinib oral dose of 400mg), not exposed priory to stem cell transplantation and the patients' compliance on the same therapy (Imatinib). A flow diagram of the study design is attached in Appendix 1.

CYTOGENETICS

Peripheral blood lymphocytes of patients will be cultured at 37^{o}C using RPMI 1640 reagent for 12 to 24 hours. Harvesting will be done by introducing the cultured into hypotonic solution and then fixative solution (3:1, methanol acetic acid respectively). Twenty cells in metaphases will be analyzed. This standard technique will be useful for the demonstration of karyopic abnormalities that may be additional to the classic t(9;22). This will give a clear distinction between the classic CML and those having an additional aberration thus variant CML.

FLUORESCENCE IN SITU HYBRIDIZATION

Analysis of BCR-ABL allows the examination of dividing cells in metaphases and non dividing cells in interphases by using fluorescently labelled probes to determine abnormalities. This technique is rapid and sensitive and allows more cells than cytogenetics (200-500 cells compared to 20 cells. It is reliable in assessing treatment responses (Chase et al., 1997).

PCR SPECIMEN PREPARATION

The concentration of starting material (that is RNA or ribonucleic acid) is of the utmost importance for RQ-PCR. This is because the same amount of starting material must be used in a RQ-PCR to make it semi quantitative. Samples which have yielded an RNA concentration below 100ng/µl, require concentration using the miVac DNA concentrator by GeneVac. Dilutions will be made on the RNA sample required to obtain a final amount of RNA in the sample of 1µl using SABAX water (see appendix 2 for sample and reaction sheet for cDNA (complementary DNA) synthesis for RQ-PCR).

COMPLEMENTARY DNA SYNTHESIS

cDNA will be synthesized using the enzyme MMLVRT kit from the company Invitrogen. cDNA is made from RNA so as to perform a PCR. mRNA (messenger RNA) is used as the starting material because this will be an indication of the levels of expression of the gene.

RQ-PCR RUN SETUP

RQ-PCR will be performed on the cDNA using Taqman mastermix and MGB probes and primers. These MGB probes are highly specific for the breakpoints of interest. The probes have a fluorescent marker and a fluorescent quencher. Upon replication the probe is displaced and the reporter is cleaved through the exonuclease activity of taq polymerase. This results in the reporter fluorescing which will then be measured by the real time machine. The *BCR-ABL* expression in both groups (classic CML and variant CML) will be evaluated and the response to tyrosine kinase inhibitor will be compared.

INTERPRETATION

An amplification plot and a standard curve will be generated by RQ-PCR of BCR-ABL. From this the levels of BCR-ABL will then be ascertained. These levels of BCR-ABL will then be plotted on a graph of the percentage of the BCR-ABL expression to that of time on treatment.

STATISTICAL ANALYSIS

The data of the paired groups will then be analysed using t-test together with the amplification plot and standard curve to determine its statistical significance. Due to the scarcity of patients

presenting with a variant translocation the sample size will be small however the information derived from this study will have a significant impact on these patients' treatment.

ETHICAL CONSIDERATIONS

1.1 Consent will be obtained from subjects and a consent form will be filled by participants.

1.2 For a control, whole blood will be used from a healthy donor for the application of the study, thus ethical clearance will be required from The Swaziland Ethics Committee.

1.3 Blood will be drawn by qualified phlebotomist (the local clinic Nurse or a registered Biomedical Technologist) and will be disposed of according to safety standards while respecting the integrity of the subjects.

1.4 The procedure and analysis will be done according to the standard operations procedure and with a South African National Accreditation Systems accredited laboratory.

1.5 The left over blood will be autoclaved and thereafter discarded in special medical waste containers.

TIME AND COST BUDGET

Molecular techniques are very expensive. The test is charged based on the cost of the reagent per month and dosage dependant, for example 400mg/day therefore an increase in dosage will increase the price. For costs of running the tests kindly refer to appendix 4.

FINANCIAL IMPLICATIONS

Molecular techniques are very expensive to purchase as they utilize many different kits which are imported from abroad. The treatment (dosage) outways the actual cost of running the test.

PHARMACOVIGILANCE

Pharmacovigilance refers to the study of the safety of marketed drugs examined under the practical conditions of clinical use in large communities. Since this study will possibly reveal that there is no association in TKI therapy response between classic chronic myeloid leukaemia patients and variant translocation chronic myeloid leukaemia patients, the risks posed by Imatinib to subjects will be minimized by regulating and managing dosage administration vs side effects to subjects.

POSSIBLE OUTCOMES

This study will possibly reveal that there is no association in TKI therapy response between classic chronic myeloid leukaemia patients and variant translocation chronic myeloid leukaemia patients.

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APPENDICES

APPENDIX 1

Study design: A prospective double cohort study

Ph positive patient (straight t(9;22) Patient positive for variant translocation

- Conventional Cytogenetics and FISH (Fluorescent *in situ* Hybridization)
- Quantitative PCR (RQ-PCR) at 3 months intervals

Evaluate and compare the response to tyrosine kinase inhibitor by measuring the level of *BCR-ABL* expression in both groups. Good and bad straight CML will be used as controls.

APPENDIX 2

Sample and reaction sheet for cDNA synthesis for RQ-PCR

Individual performing test:			**Date:**
Description:	Lot number:	Description:	Lot number:
Random primers:		M-MLV	
dNTP mix		RNAse uot	

Samples

Sample (lab no.)	[]ng/µl	Vol RNA to obtain 1 ng/µl	Vol distilled water

• **Incubate at 70◦C for 10 minutes, cool on ice.**

	1×	×
MMLV-RT (200U/µl)	**0.5 µl**	

RT buffer (5×)	**5 µl**	
dNTP mix (1mN)	**2.5 µl**	
DTT(10Mm)	**2.5 µl**	
Random primers(25 µm/µl)	**2 µl**	
RNAse OUT ribonuclease inhibitor (40U/µl)	**0.5 µl**	
Distilled water	**2 µl**	
Total volume of master mix	**15**	

APPENDIX 3

A	**Negative control**		**RT-RNA from positive presentation sample**		**Negative control**		**RT-RNA from positive presentation sample**				**IE6**	**IE5**
B											**IE5**	**IE4**
C											**IE3**	**IE3**
D											**IE2**	**NTC**
E											**IE1**	
F											**NTC**	
G												
H												

BCR/ABL GUS BLANK GUS PLASMIDPLASMID

APPENDIX 4

Test requested	**Price per sample**	**×20 samples**
cytogenetics	**R1300**	**R26000.00**
Fluorescence in situ hybridization	**R1700.00**	**R34000.00**
RQ-PCR	**R2149.00**	**R42980.00**

Where R stands for South African Rands