ORIGINAL ARTICLE

Application of HRM Analysis in Detection of *PDGFRA* Exon 10 Polymorphism in CML Patients with Imatinib Resistance

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ABSTRACT

Introduction: Imatinib mesylate has been widely used as a standard treatment for chronic myeloid leukemia (CML). It acts as a selective competitive inhibitor of the BCR-ABL tyrosine kinase. Despite the excellent efficacy on CML treatment, some patients developed resistance to the treatment. Mutation in the *PDGFRA* may be one of the factors involved in the mechanism of resistance that affects the response to imatinib. The mutational status of *PDGFRA* is highly relevant for prognosis and treatment prediction in CML patients. Thus, this study is intended to establish and validate a High Resolution Melting (HRM) analysis for *PDGFRA* exon 10 c.1432 T>C polymorphism in CML patients. **Methods:** High resolution melting (HRM) analysis was used to identify the c.1432 T > C polymorphism in *PDGFRA* exon 10 (n =86; response = 43; resistance = 43). The results from HRM analysis were compared and validated with Sanger sequencing. The association between the polymorphism and treatment response was assessed by statistical analysis using binomial logistic regression analysis. **Results:** HRM analyses showed two different melt curves. One curve followed the shape of the reference, homozygous wild type (TT) and the other curve showed a different melting profile than the reference with the TC genotype (heterozygous variant). The results revealed that heterozygous variant (TC) genotype showed a high risk of acquiring resistance with an OR of 3.795; 95% CI: 1.502-9.591, with a statistically significant association, p = 0.005. HRM analysis also showed 100% sensitivity and specificity in the detection of *PDGFRA* exon 10. **Conclusion:** The HRM analysis of *PDGFRA* exon 10 c.1432 T>C was successfully established. The exon 10 c.1432 T>C polymorphism shows a higher risk for the development of resistance toward imatinib treatment.
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Keywords: Chronic myeloid leukaemia, *PDGFRA*, Imatinib mesylate, polymorphism, HRM

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INTRODUCTION

Chronic myeloid leukemia (CML) is a myeloproliferative disorder resulting from the malignant expansion of haematopoietic stem cells. In CML, the myeloid cells have undergone uncontrolled expansion of mature and maturing granulocytic cells without losing their capacity to differentiate. The incidence was about 1 to 2 cases per 100,000 adults and was classified as the most common chronic myeloproliferative disorder (1). CML commonly affects older people with a median age of about 65 years and is rarely diagnosed in children (2).

CML is distinguished by the presence of the Philadelphia (Ph) chromosome, a cytogenetic hallmark caused by the chromosomal translocation t(9;22) (q34; q11) (3). This translocation occurs at the breakpoint cluster region (bcr) of chromosome 22 and in the region of chromosome 9 of the c-abl oncogene. This Ph chromosome harbours the BCR-ABL fusion gene, which codes for a chimeric BCR-ABL protein, resulting in dysregulated tyrosine kinase activity. The latter has been indicated to be responsible for the altered phenotype of CML cells (3,4). About 95% of CML patients were observed with the presence of the Ph chromosome (4).

The presence of well-defined pathogenetic of CML has led to the development of imatinib, which is a tyrosine kinase inhibitor that can inhibit both ABL and BCR-ABL proteins. Since the BCR-ABL fusion protein is a constitutively activated tyrosine kinase that causes CML, imatinib mesylate has been one of the treatment options in treating newly diagnosed patients or patients who have failed previous treatment (4,5). The clinical use of imatinib has resulted in a significantly improved

prognosis and survival rate where 80 to 90% of patients in the early chronic phase have shown major cytogenetic responses to imatinib.

Imatinib, 2-phenyl-aminopyrimidine formerly known as STI571 (Gleevec, from Novartis Pharma, Basel, Switzerland), is a selective competitive inhibitor of tyrosine kinase of BCR-ABL, KIT and PDGFR (5). Currently, it is used as a frontline treatment for CML patients. Treatment with imatinib resulted in marked clinical, haematological, cytological and molecular improvement (5). Still, the emergence of resistance to imatinib treatment has turned into a significant clinical issue in CML treatment. Imatinib resistance could be a primary or secondary resistance. For further definition, primary resistance is when there is an inability to achieve any initial landmark response toward imatinib, whereas for those patients who achieve a good initial response and then subsequently lose any relevant response toward imatinib, it is referred to as a secondary response or acquired resistance (Figure 1).

The mechanisms of resistance can be either BCR-ABL dependent or BCR-ABL independent. The BCR-ABL dependent pathways may be involved in any tyrosine kinase domain mutation or overexpression of the BCR-ABL protein. However, in BCR-ABL independent pathways, the BCR-ABL tyrosine kinase was not only inhibited by imatinib but other alternative pathways may have become activated due to other key enzymes responsible for cell proliferation and immobilisation (5). Involvement of other tyrosine kinases that can also be inhibited by imatinib such as platelet derived growth factor receptor alpha (PDGFRA) can also be a potential determinant involved in imatinib resistance in CML patients (6).

PDGFRA is classified in one of the platelet-derived growth factor families and encodes for a cell surface type III-tyrosine kinase receptor (7). The full length of *PDGFRA* is encoded by a 6.4 kb transcript created from an upstream promoter of exon 1 (P1 promoter). It is located on chromosome 4 at 4q12 and has 23 exons and 1089 amino acid residues (7,8). The structure of the *PDGFRA* receptor is comprised of five immunoglobulin (Ig) extracellular domains, a juxtamembrane domain inside the cell membrane and two cytoplasmic tyrosine kinase domains (7, 9). *PDGFRA* receptor binds to its ligand (PDGF-A, -B, -C and –D) and induces receptor dimerization that leads to autophosphorylation of their cytoplasmic tyrosine residues. This activates intracellular kinase activity, thus initiating intracellular signalling (9). Upon binding, the receptor and its ligands will induce receptor dimerization that leads to autophosphorylation of cytoplasmic residues (7,9,10). This phosphorylation then initiates signalling pathways that control many significant cellular processes, such as cell proliferation and cell survival.

The first *PDGFRA* genetic alteration was reported by Todd and Gary Gilliland, in 1994 and was identified in chronic myelomonocytic leukemia (6,11). Later, many other *PDGFRA* mutations were described until now (12,13). Recent studies showed commonly mutated regions of *PDGFRA* were located in exons 10, 12, 14 and 18 of gastrointestinal stromal tumours (GISTs) that affect the response to imatinib treatment (13, 14, 15), which is a similar tyrosine kinase inhibitor used in CML. There is no data available on the association between *PDGFRA* and imatinib response in Malaysian CML patients. Thus, this study is the first genetic association study that aims to investigate the impact of *PDGFRA* exon 10 c.1432 T>C on imatinib response in Malaysian CML patients using a new developed technique, High Resolution Melting Analysis.

High Resolution Melting (HRM) analysis is a simple PCR-based technique for the detection of DNA sequence variation by measuring the changes in the melting of the DNA duplex (16). HRM analysis is a closed-tube assay

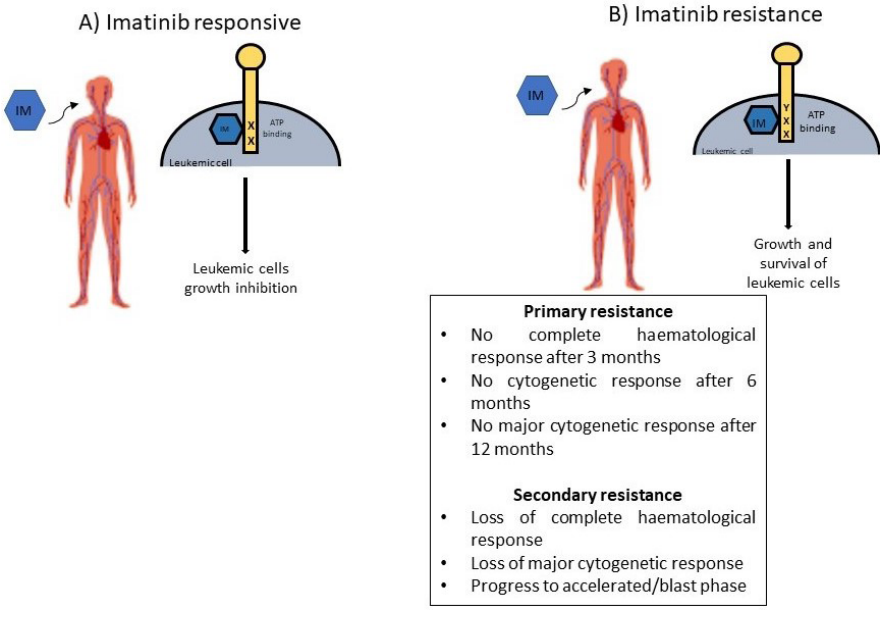

Figure 1: The imatinib responsive and resistance in CML patient. An illustration of the mechanism of imanitb in A) Imatinib responsive CML patient, in which imatinib bind to the tyrosine kinase receptor and inhbits cell proliferation and induces apoptosis lead to leukemic cell death B) Imatinib resistance CML patient with either a tyrosine kinase receptor mutation (BCR-ABL dependent mechanism) or any other mechanism of imatinib resistance (BCR-ABL independent mechanism) that resulted in active growth and survival of leukemic cells .

for assessing the presence of mutations without any post-PCR handling. In this analysis, the region of interest is first amplified by PCR in the presence of a fluorescence double stranded DNA (dsDNA)-binding dye, whereafter the product is gradually melted at a high temperature and emitted fluorescence is measured to generate a characteristics curve that is based on detecting small differences in PCR melting curves (16, 17). The duplex melting is monitored using intercalating dyes which bind to the dsDNA such as LCGreen and SYBR Green. This dye will fluoresce when bound but not after release on duplex melting. The amplicon melting base analysis has widely been used for both genotyping and mutation screening (18,19). Therefore, HRM analysis can be a suitable pre-screening method for mutation screening with a lower turn-around time and be highly sensitive and specific.

MATERIALS AND METHODS

Study subjects

This study was a comparative cross-sectional study approved by the Research and Ethics Committee of Universiti Sains Malaysia (JEPeM USM/JEPeM/15100445) and Ministry of Health, Malaysia (NMRR-16-2883-29440) complies with the Declaration of Helsinki.

The sample size was calculated using the previously estimated prevalence of *PDGFRA* in the response group (20), as well as the prevalence of *PDGFRA* variant in the resistance group (22). For this study, 86 CML patients (43 imatinib responsive and 43 imatinib resistant) were recruited from Hospital Universiti Sains Malaysia and Hospital Pulau Pinang based on the inclusion and exclusion criteria (Table I). Informed consent was obtained from all the patients before participating in this study.

The patients included were Ph chromosome-positive CML patients, treated with imatinib (400 mg daily) for at least 12 months. Following European LeukemiaNet recommendations (27) for the management of CML, the patients were grouped into imatinib responsive and imatinib resistant groups based on their hematologic, cytogenetic and molecular criteria. For imatinib responsive, the hematologic response was considered complete with a platelet count <450 x 109/L; white blood cell (WBC) count <10 x 109/L; differential without immature granulocytes <5 % basophils and nonpalpable spleen. While complete cytogenetic response was defined as 0% Ph+ metaphase, partial cytogenetic response was defined as 1–35% Ph+ metaphase, minor cytogenetic response was defined as 36–65% Ph+ metaphase, minimal cytogenetic response was defined as 66–95% Ph+ metaphase, and no cytogenetic response was defined as > 95% Ph+ metaphase.Molecular responses to responsive treatment were assessed according to the International Scale (IS). Those patients who did not achieve the above criteria were categorised under the imatinib resistant group.

Table I: Inclusion and exclusion criteria of study subjects

Inclusion criteria	Exclusion criteria
Imatinib resistant CML • Confirmed to have CML with Ph chromosome • Undergone imatinib treatment (400mg) for at least 12 months • Experience sign of primary or secondary resistance • Showed suboptimal response and failure to imatinib treatment	• CML patients with absence of Ph chromosome • CML patients who were treated with imatinib for less than 12 months • CML patients who are not treated with imatinib
Imatinib responsive CML • Confirmed to have CML with Ph chromosome • Undergone imatinib treatment (400mg) for at least 12 months • Showed suboptimal response to IM treatment	

DNA extraction

Three (3) ml of peripheral blood was collected from the subjects into EDTA-coated tubes. Genomic DNA was extracted using the GeneAll ExgeneTM Blood SV Mini Kit (GeneAll Biotechnology, Seoul, South Korea) according to the manufacturer's instructions.

The concentrations and purity of the genomic DNA were then measured using a spectrophotometer using Infinite® M200 NanoQuant (Tecan Trading AG, Switzerland). The concentration and purity of the DNA were determined by the absorbance ratio at 260 nm and 280 nm. The good quality of DNA was between 1.8 and 2.0 ratios. The range of the DNA concentration was between 50 ng/µl – 100 ng/µl.

Mutational/SNP analysis of exon 10

Mutational/SNP analysis of c.1432 T>C was performed by using HRM analysis which evaluates the melting temperature of the amplicon produced by PCR reaction. 20 ng of DNA was amplified using specific forward and reverse primer sequences for *PDGFRA* exon 10 c.1432 T>C (rs35597368) (For 5′-GAAACTTCCTGGACTATTTTGG-3′ and Rev 5′-CTCCACGGTACTCCTGTCTC-3′). The reaction was carried out in a total volume of 10 µl of LuminarisTM Color HRM Master Mix (ThermoFisher Scientific, Lithuania, Europe) with an initial denaturation at 95 °C for 1 minute, followed by annealing at 57 °C for 30 seconds and extension at 72 °C for 30 seconds. The HRM analysis then started with the heteroduplex formation at a temperature of 95 °C to 60 °C for 30 seconds. The wild-type DNA was also included in each run as a control along with the tested samples. All samples were tested in duplicate. The HRM assay was performed using a Thermo Scientific Pikoreal 96 Real-Time PCR machine (ThermoFisher Scientific, Vantaa, Finland).

After normalisation of the melting curve, samples with changes in DNA sequences discriminated clearly

from those with wild-type DNA. The HRM assay was validated by DNA sequencing for selected samples that exhibit different HRM profiles from the wild-type curve.

Direct sequencing

Sanger sequencing was run for a total of 28 CML samples that had different HRM profiles to confirm the genotype. The PCR reaction was first carried out using extended forward and reverse primers for *PDGFRA* exon 10 c.1432 T>C (For 5'- GGCCCTATACTTAGGCCCTTT -3' and Rev 5'- GTGAGTTCCTCAACAGTCAGGA -3' respectively) at annealing 56 oC. The size of the PCR product was 247 bp. PCR products were then purified using the GeneAll® ExgeneTM Blood SV mini DNA purification kit (GeneAll Biotechnologies Co, Ltd. South Korea) according to the manufacturer's protocol. The kit contained a PB buffer, a NW buffer, an EB buffer and a spin column and a spin column.

The PCR products were sent to First BASE Laboratories (Kuala Lumpur, Malaysia) for sequencing services. Samples were sent in 1.5 mL microcentrifuge tube and sealed with parafilm to prevent leakage together with the 10 µM of primer pairs. The results were downloaded from the FirstBase website and were analysed with the reference sequences for each exon using Bioedit Version 7.2.5.0 software.

Statistical analysis

The statistical analysis was performed using SPSS VERSION 21.0 (SPSS Inc, IL, USA). Genotypes and allele frequencies of *PDGFRA* exon 10 c.1432 T>C were calculated and compared between imatinib responsive and imatinib resistant CML patients using the chi-square test (χ^2). Statistical significance was determined as $p<0.05$. The odd ratios (ORs) and 95 % confidence intervals (CI) were calculated to determine the risk association of the genotype with imatinib susceptibility risk. The OR value greater than 1 was considered a susceptibility risk, while the OR value less than 1 was considered a protective risk. The sensitivity and specificity of HRM were measured using the formula (23).

RESULTS

A total of 86 peripheral blood samples of CML patients were recruited in this study. The patients' characteristics including gender, age at time of diagnosis, ethnicity and response to the imatinib treatment are shown in Table II. There were 43 imatinib-responsive CML patients and 43 imatinib-resistant CML patients. Out of the 86 CML patients, 44 were female (18 CML responsive and 26 CML resistant) and 42 were male (25 CML responsive and 17 CML resistant). The age ranges for the response and resistant groups were from 24 – 72 years and 20 – 76 years respectively. The mean age of 45 and 43 years old for both study groups. All the CML patients were recruited from three major ethnic groups in Malaysia which are Malay, Chinese and Indian. In the response group, 20 were Malay, 18 were Chinese and 5 were Indian. whereas 30 Malay, 6 Chinese, and 7 Indians were CML resistant. The ratio of male to female CML patients recruited was 1:1.

Table II: CML patient's demographic data

	Imatinib responsive n = 43	Imatinib resistant n = 43
Gender, n (%)		
Female	18 (41.9)	26 (60.5)
Male	25 (58.1)	17 (39.5)
Age range	24 – 72	20 – 76
Mean age ± SD	44.56 ± 13.6	42.79 ± 13.7
Ethnicity, n (%)		
Malay	20 (46.5)	30 (69.8)
Chinese	18 (41.2)	6 (14)
Indian	5 (11.6)	7 (16.3)

CML: Chronic myeloid leukemia, n: number of samples

HRM analyses of exon 10 c.1432 T>C of *PDGFRA* with an amplicon size of 81 bp were successfully discriminate the c.1432 T>C wild type (TT) by those with the heterozygous variant (TC) as shown by the diagram below (Figure 2). The different melt curves were discriminated by the different in the melting temperature of the variant sequence. The results were consistent with the Sanger sequencing (Figure 3).

In this study we examined 53 cases (61.6 %) with a wild-type (TT) genotype and 33 cases (38.4 %) with a heterozygous variant (TC) in exon 10 of the *PDGFRA* gene. No homozygous variant (CC) genotype was found. The frequency of exon 10 c.1432 T>C was significantly higher ($p<0.005$) in the imatinib resistant group (53.5 %) compared to the responsive group. The allelic frequency (C allele) of exon 10 polymorphism was higher (26.74 %) in imatinib resistant compared to imatinib responsive although the difference was not significant. Table III shows the genotype and allele frequency of exon 10 c.1432 T > C. As for the risk association analysis, a significant ($p<0.05$) association between polymorphism and the risk of developing resistance to imatinib treatment was found (Table III). Table IV summarises the *PDGFRA* exon 10 status of 28 CML samples obtained by

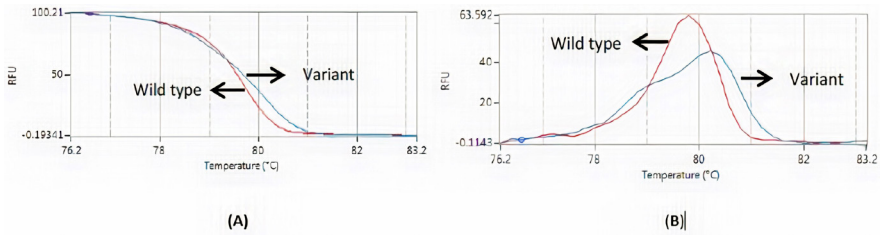

Figure 2: *PDGFRA* exon 10 heterozygous variant c.1432 T>C. The exon 10 c.1432 T>C that was differentiated from the wild type reference by different melting profile represented by (A) normalizes melt curves and (B) HRM graph.

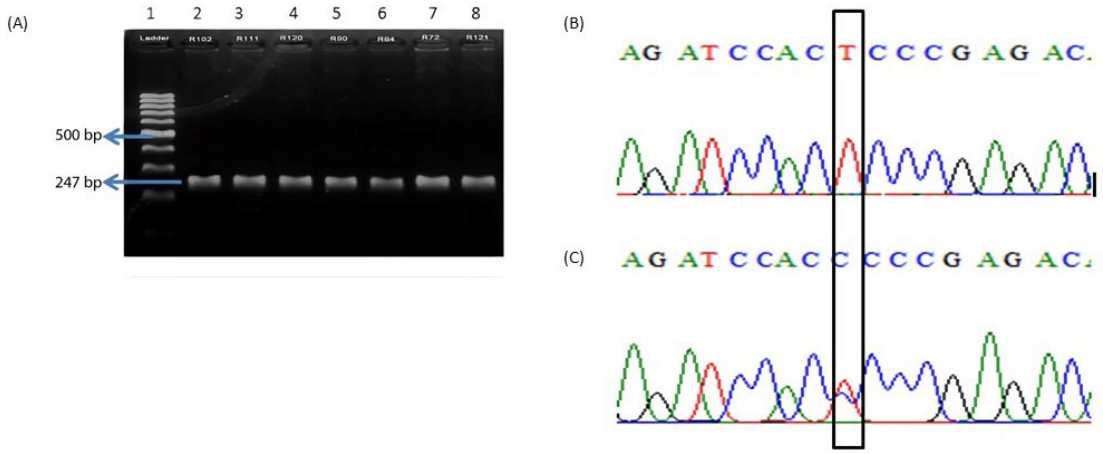

Figure 3: Direct DNA sequencing of *PDGFRA* exon 10 c.1432 T>C. (A) Gel electrophoresis from selected sample after HRM analysis. *Lane 1*: 100 bp ladder. *Lane 2* to 8: 247 bp of PCR products. Diagram showing part of the electropherogram for PDGFRA exon 10 c.1432 T>C (B) homozygous wild type (C) heterozygous variants.

Table III: Genotype, allele frequencies and risk association of exon 10 c.1432 T>C in CML patients

SNPs	Genotype	Frequencies (%)		P-value	OR (95% CI)	P-value
		Imatinib responsive n= 43	Imatinib resistance n= 43			
Exon 10	Homozygous wild type (TT)	33 (76.7)	20 (46.5)	**0.004**	1.00	
c.1432 T>C	Heterozygous variant (TC)	10 (23.3)	23 (53.5)		3.795 (1.502-9.591)	**0.005**
	Homozygous variant (CC)	0 (0.0)	0 (0.0)		-	-
	Allele:					
	T	76 (88.37)	63 (73.26)	**0.012**		
	C	10 (11.63)	23 (26.74)			

Bold value indicate statistical significance which is $p < 0.05$

Table IV: PDGFRA exon 10 polymorphism obtained by HRM analysis versus Sanger sequencing

		Sanger Sequencing			Total
		Wild type	Variant	Not evaluable	
HRM	Wild type	10	0	0	10
	Variant	0	18	0	18
Total		10	18	0	28

Sensitivity = 100 %
Specificity = 100 %

HRM analysis and Sanger sequencing.

DISCUSSION

Imatinib mesylate acts as a selective inhibitor of tyrosine kinase. Imatinib competitively binds to the adenosine triphosphate (ATP) binding site of the tyrosine kinase proteins. The binding then inhibits the transfer of terminal phosphate to the tyrosine residues, thus inhibiting the downstream enzymatic activity. While imatinib showed highly effective treatment for CML patients, a minority of patients failed or showed suboptimal response to imatinib. The patterns of resistance have evolved and focus not only on the mutations in the BCR-ABL gene, but also may be mediated by other mechanisms independent of BCR-ABL.

Imatinib is a strong *PDGFRA* inhibitor and imatinib sensitivity has been described both for BCR-ABL and in *PDGFRA* mutants. Several reports on gastrointestinal stromal tumours (GISTs) have identified the association of activating mutations in *PDGFRA* with imatinib resistance (24,25). *PDGFRA* mutations have also been documented to show a link to imatinib sensitivity for about 4.7% of GISTs (13). Till date, this is the first study reporting on the association of the *PDGFRA* exon 10 c.1432 T>C polymorphism with imatinib response in Malaysian CML patients.

This polymorphism has also been described in studies involving malignant peripheral nerve sheath tumours (MPNST) and gliomas (26,27). The polymorphism resulted in the change of codon 478 from serine to proline which has been shown to reveal no constitutive phosphorylation and gain-of-function polymorphism (27). Our results on the exon 10 c.1432 T>C polymorphism association study however, showed that the variant genotype (TC) was significantly correlated with a higher risk of developing resistance to imatinib treatment. In contrast, Holtkamp et al. found a responding MPNST to imatinib treatment in a previous study (27). In the present study on GIST, *PDGFRA* exon 10 mutants were found to be sensitive and respond well

to imatinib treatment (13). However, we postulated that there might be different mechanisms that take part in the resistance of imatinib in CML patients and also different in the study population as compared to the previous study.

A nucleotide change in the *PDGFRA* exon 10 region (coding for the extracellular domain of the *PDGFRA* tyrosine kinase receptor) may modulate ligand-binding and dimerization of the receptor (7, 10, 12). Imatinib usually occupies the ATP-binding site of the *PDGFRA* receptor and blocks the binding of ATP which in turn prevents substrate phosphorylation and inhibits the downstream signalling pathway (28). Thus, the presence of nucleotide changes or mutations on the *PDGFRA* structure may reduce the binding ability of imatinib, resulting in less inhibition of tyrosine kinase activity and perhaps leading to resistance. Furthermore, a finding by Kimchi-Sarfaty et al., showed that a silent polymorphism can affect protein folding that later alters substrate specificity and protein function (29). This mechanism might influence the response to imatinib therapy in CML patients.

In addition, the HRM technique used for the genotyping of exon 10 c.1432 T>C polymorphism has successfully distinguished the polymorphism from the wild type. This HRM technique is based on the evaluation of the melting temperature of an amplicon produced by a simple PCR reaction. The technology distinguished nucleic acid based on their dissociation behaviour (melting analysis). A heterozygous exon 10 polymorphism was detected based on the change in melting curve shape. Exon 10 polymorphism of the *PDGFRA* gene can be directly assessed solely through HRM analysis because wild type DNA (control) was co-examined in every run. All samples with the presence of other mutations or polymorphisms analysed in this study will show a different melting curve profile from the curve of exon 10 c.1432 T>C, thus allowing direct identification. With this aspect, the HRM analysis provides results faster than Sanger sequencing.

This technique has also been shown to have a higher detection rate of heterozygous variants with about 100% sensitivity and specificity (19, 30, 31). HRM and Sanger sequencing were used to look for polymorphisms in exon 10 of the *PDGFRA* gene in 28 CML samples. All samples that were evaluated by HRM were also evaluable by Sanger sequencing. The two methods showed complete concordance. Tindall et al., found 100% specificity of HRM in distinguishing heterozygous from wild type (17). This finding was in parallel with the current study that can clearly distinguish exon 10 heterozygous mutation/polymorphism from wild-type samples where each heterozygous followed a unique melting path that transformed the shape of the melting curve. In addition, Farrar et al., also showed that overall sensitivity and specificity for detection of heterozygous variants was 96.9% and 97.1% respectively (32). Therefore, the reliability of the established HRM analysis of exon 10 c.1432 T>C could be clearly confirmed.

The present study suggests HRM analysis as a pre-screening method that require less skill, are fast, economical, and suitable to be adapted in clinical practise to detect hot spot mutations. However, it also has certain limitations (33). The presence of unknown polymorphisms in the gene may interfere with result analysis. The assay also unable to detect polymorphisms or mutations of the whole exon or gene. In this study, designed amplicon with less than 150 bp were used so that the wild -type and heterozygous curves was no too small to be distinguished.

CONCLUSION

Genetic variation in *PDGFRA* exon 10 may modulate the evolution of resistance to imatinib treatment in Malaysian CML patients. This present study gave an overview of the distribution of *PDGFRA* exon 10 polymorphisms in CML patients and their association with the imatinib response. The established HRM analysis could also be a promising screening tool for mutations and polymorphisms of the *PDGFRA* gene in CML patients who have undergone imatinib mesylate treatment. HRM analysis is currently being considered as the best method for mutation and polymorphism scanning because when implemented correctly it can reduce the need for sequencing by 95 to 99 %. In addition, this discovery clearly highlights the relevance of molecular studies on *PDGFRA* in monitoring the response to targeted therapies in CML to make the best treatment decisions for the patients in order to maximise treatment outcome. However, analysis of other regions of the *PDGFRA* gene will be needed with a larger sample size to significantly conclude the association between *PDGFRA* gene variants and imatinib resistance.

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