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Impact of Multidrug Resistance Gene 1 (MDR1) C3435T Polymorphism on Chronic Myeloid Leukemia Response to Tyrosine Kinase Inhibitors

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ABSTRACT

Background: Single nucleotide polymorphisms (SNPs) of multiple drug resistance (MDR1) gene are associated with altered P-glycoprotein (p-gp) activity and contribute to resistance to tyrosine kinase inhibitors (TKIs) in chronic myeloid leukemia (CML).

Objectives: We aimed to demonstrate the association between MDR1 gene C3435T polymorphism and molecular response in newly diagnosed chronic phase (CP) CML patients to standard dose upfront imatinib and nilotinib therapy.

Patients and Methods: MDR1 C3435T was genotyped using polymerase chain reaction Restriction Fragment Length Polymorphisms (PCR-RFLP) at diagnosis. BCR-ABL1 transcripts level was measured by Real Time Quantitative polymerase chain reaction (RQ-PCR) at diagnosis then every 3 months.

Results: This study included 74 Philadelphia (Ph') positive CP-CML patients; 38 males and 36 females. Median age at diagnosis was 38 years (18-78). Median BCR-ABL1 level was 101%. Forty patients received imatinib (54%) while 34 received nilotinib (46%). Optimal response at 12 month was 35% in the imatinib arm versus 80% in the nilotinib arm (p=0.001). The frequency of MDR1 SNP C3435T was 46%, 32% and 22% for CC, TT and CT genotypes, respectively. Optimal response at month 12 differed significantly between imatinib and nilotinib among patients with MDR1 3435TT genotype (11% versus 83%, respectively, p=0.002) while less significant difference was found between the two drugs in CC and CT genotypes (35% vs. 75% and 60% vs. 83%, respectively, p=0.042 & p=0.588).

Conclusion: MDR1 3435TT may be used as an additional criterion for initiating nilotinib instead of imatinib as front line therapy for CP-CML patients. We demonstrated the usefulness of MDR1 SNP polymorphism in the identification of CML patients who may or may not respond optimally to imatinib.

Key Words: *CML – SNP – MDR1 gene – C3435T – Molecular response.*

INTRODUCTION

Despite of striking efficacy of tyrosine kinase inhibitors (TKIs) in treatment of chronic phase chronic myeloid leukemia (CP-CML), resistance develops over time in many patients **[1]**. One of the limitations in CML treatment is the development of multidrug resistance (MDR1), a well-known mechanism responsible for drug resistance by over expression of ABCB1 transporter genes **[2]**. This gene encodes P-glycoprotein (P-gp) that transports anti-leukemia drugs out of the cell. MDR1 product is an ATP-driven efflux pump contributing to the pharmacokinetics of drugs that are P-gp substrates. Imatinib and nilotinib have been reported as substrates of P-gp-mediated efflux **[3-5]**.

MDR1 gene polymorphisms alter the expression level of P-gp and consequently result in drug resistance. Variations in the MDR1 gene product can directly affect the therapeutic effectiveness **[6]**. The up-regulation of drug transporter (ABCB1) is one of the specific causes of resistance to TKIs. SNPs in ABCB1 gene have the potential to alter protein function and also influence the efficiency of absorption or elimination of BCR-ABL inhibitors **[7]**.

More than 50 single nucleotide polymorphisms (SNPs) have been identified, so far, in MDR1 gene by use of polymerase chain reaction Restriction Fragment Length Polymorphism

(PCR-RFLP). These SNPs affect the expression and function of the P-gp in many ways. MDR1 C3435T is associated with altered P-gp activity and is considered one of the most important MDR1 gene polymorphisms as it was demonstrated to be the main functional polymorphism affecting mRNA stability **[8-10]**. The polymorphism at exon 26 C > T at 3435 position is a synonymous polymorphism which contributes to the change in substrate selectivity of P-gp, without any significant change in protein expression levels **[9]**.

Our main objective was to investigate the possible influence and association between MDR1 gene polymorphism C3435T and molecular response to newly diagnosed CP-CML Egyptian patients receiving upfront standard doses of either imatinib or nilotinib.

PATIENTS AND METHODS

Study population: The study included 74 CP-CML patients referred to the Clinical Hematology Unit, Kasr Al-Ainy Hospital, Cairo University in the period February 2012 to March 2013. Patients were followed for 12 months to evaluate their response to TKI therapy and its association with MDR1 C3435T polymorphism. The study was carried out according to the declaration of Helsinki and approved by the Institution Review Board. All patients provided written informed consents.

CP CML was defined according to WHO criteria **[11]**. Exclusion criteria were: Age <18 years, pregnant females, Ph' negative CML and patients previously treated with interferon-α. All patients were submitted at diagnosis to detailed medical history recording, complete physical examination, bone marrow aspirate (BMA), karyotype, complete blood count (CBC) with examination of peripheral blood film, estimation of Sokal and Hasford risk scores in addition to quantitative measurement of BCR-ABL1 transcripts by Real Time Quantitative polymerase chain reaction (RQ-PCR) at diagnosis and every 3 months interval. MDR1 gene C3435T was genotyped using PCR restriction fragment Length polymorphisms (PCR-RFLP). Major molecular response (MMR) was defined as a 3 log reduction of BCR-ABL1 transcripts level, corresponding to ≤0.1% on international scale (IS). Response to treatment was evaluated according to the 2013 European Leukemia Net (ELN) criteria **[12]**. All patients received either imatinib 400mg od or Nilotinib 300mg bid, orally.

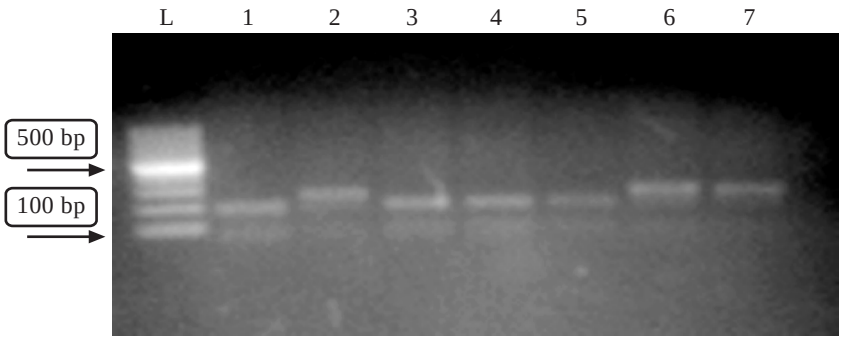
BCR-ABL1 transcripts measurement: Total RNA extraction was carried out from 5ml fresh peripheral blood leucocytes according to the initial silica extraction method using QlAmp RNA blood minikit (Qiagen, Hielden, Germany). For cDNA synthesis, 1μg total RNA from each sample was used to synthesize first-strand cDNA with random primers in a 20ul mix using MMLV reverse transcription enzyme (40U/ul) and RNasi Stop RNAses inhibitor (4U/ul) (RT-kit Plus, Nanogen). RT cycles consisted of 25ºC at 10min (primer annealing), 37ºC at 45min (reverse transcription step) and 93ºC at 3min (final denaturation).

BCR-ABL1 transcripts level was determined using RQ-PCR detection system (Ecco Illumina, USA). The ABL1 control gene and the BCR-ABL1 target gene were measured using Universal PCR master mix (Real Time alert QPCR, Nanogen) and specific primers and hydrolysis probes (Philadelphia p210 and Abl QPCR Amplimix and Ampliprobes). PCR mix (25ul) for ABL1 housekeeping gene and BCR-ABL1 target gene consisted of: 12.5ul Amplimaster (Tris HCl, MgCl2, dNTPs, Rox, Uracil–N–glycosidase, Taq DNA polymerase), 1.25ul amplimix (oligonucleotide primers), 1.25ul Ampliprobe (oligonucleotide fluorescent probe), 5ul distilled water and 5ul cDNA samples and standards. Quantitations were made against a set of 4 plasmid based p210 and ABL1 standards (Philadelphia p210 QPCR standards, Nanogen) of known concentrations (10^5, 10^4, 10^3 and 10^2 copies) used as positive control in each run as well as to generate a standard curve for the amplification assay. Cyclic conditions consisted of UNG decontamination 50ºC for 2min, initial denaturation 95ºC for 10min and 45 cycles of amplification at 95ºC for 15sec and annealing/extension at 60ºC for 1min. A set of reference RNA (4 samples) (Nanogen) with high and low BCR-ABL1 and ABL1 levels were processed in the same way as patient's samples and was used to convert results into International Scale (IS). Calculated conversion factor was 1.1 in our laboratory. Any result with ABL Ct >29 or undetermined was considered invalid.

MDR1 C3435T genotyping: Genomic DNA was extracted from peripheral blood according

to manufacturer instructions by salting out method using Gentra Puregene Blood kit (Qiagen, Hielden, Germany). The sequence of primers used for MDR1 exon 26 detection were: 5'-GCTGGTCCTGAAGTTGATCTGTGAAC-3' as forward and 5'ACATTAGGCAGTGAC-TCGATG AAGGCA-3' as reverse primer. The PCR reaction was performed in a final volume of 50ul including 1X buffer with 1.5mM MgCI2, 1µM of each primer, 200µM of each dNTP (Sigma), 2.5 units Hot start Taq DNA polymerase (5u/µL, Fermentas) and 200ng of genomic DNA. PCR amplification consisted of initial denaturation at 94ºC for 5min followed by 35 cycles of denaturation at 94ºC for 30sec, annealing at 61ºC for 30sec, and extension at 72ºC for 30sec. Final extension was performed at 72ºC for 4min. Amplified segments were analyzed by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and observed under ultraviolet light.

The PCR product (248 bp in size) was digested for 3h at 37ºC with 2U MboI restriction enzyme (Bioron). DNA fragments generated after restriction enzyme digestion were analyzed by electrophoresis on 2% agarose gel, stained with ethidium bromide and observed with an ultraviolet transilluminator (Fig. 1). The expected fragments sizes were: 238-bp fragment for TT genotype (T allele abolished restriction site), 172- and 60-bp fragments for the CC genotype, and 238-, 170- and 60-bp for the CT genotype **[6,13]**.



L: 100 bp ladder. Lanes 1, 3, 4, 5: CC genotype. Lane 2, 6, 7: CT genotype

Fig. (1): Electrophoresis of Mbol digested PCR product generated by amplified genomic DNA using MDR1 specific primers.

Statistical analysis:

All analyses were performed using the statistical package for social sciences (SPSS software version 21. Comparison between groups was performed using independent sample *t*-test or one way ANOVA for quantitative variables. Chi square and Fisher's exact tests were used for qualitative data. *p*-values <0.05 were considered statistically significant **[14]**.

RESULTS

Patients: This study included 74 Ph' positive CP-CML patients; 38 males (51%) and 36 female (49%). Median age at diagnosis was 38 years (18-78). According to Sokal risk scoring; 29/74 (39%) were high risk, 25/74 (34%) were intermediate and 20/74 (28%) were low risk group. On applying Hasford scoring system; 20/74 (37%) were high risk, 26/74 (35%) were intermediate and 20/74 (28%) were low risk. Both scores were correlated (p=0.001).

Treatment: 40 (54%) patient received imatinib while 34 (46%) received Nilotinib. Four patients on nilotinib were lost follow-up. One patient (1/43, 2%) stopped imatinib after 3 month due to grade 4 hematological toxicity and was shifted to 2nd generation TKIs. Three patients (3/30, 10%) stopped nilotinib after 6 months (1 due to persistent grade 2 hepatotoxicity; 2 due to grade 4 hematological toxicity). Two patients treated with imatinib (4.5%) progressed to blastic crisis 3 months after starting treatment while no patient progressed in the nilotinib arm.

BCR-ABL1: BCR-ABL1 transcripts level at diagnosis was 150.8%, ±140 with a range of 32-605 and a median of 101%; range between

25th percentile & 75th percentile was 55.5-208%. A significant higher optimal response rate was achieved in the nilotinib arm at month 12. Median BCR-ABL1 transcripts level was 0.1% at month 12 in the nilotinib arm versus 2% in the imatinib arm. Patient's response to both TKIs is shown in (Table 1). Kinetics of molecular responses is shown in (Table 2).

Table (1): Response of CP-CML patients to treatment with TKIs.

Parameter	Imatinib		Nilotinib		*p* value
	N	%	N	%	
Response at 3 months:					
Optimal	14/40	35	17/30	56	
Warning	24/40	60	12/30	41	0.196
Failure	2/40	5	1/30	3	
Response at 6 months:					
Optimal	14/37	38	18/30	60	
Warning	7/37	19	6/30	20	0.109
Failure	16/37	43	6/30	20	
Response at 12 months:					
Optimal	13/37	35	24/30	80	
Warning	5/37	13	4/30	13	<0.001
Failure	19/37	52	2/30	7	

CP-CML: Chronic phase-chronic myeloid leukemia.

Table (2): Kinetics of molecular response of CP-CML patients to treatment with TKIs.

Time of testing	Drug	No.	Median (%)	Range	*p* value
BCR-ABL1% M3	Imatinib	40	21.5	0.0-560.0	0.040
	Nilotinib	30	8.0	0.0-382.0	
BCR-ABL1% M6	Imatinib	37	6.7	0.0-287.0	0.070
	Nilotinib	30	1.0	0.0-225.0	
BCR-ABL1% M9	Imatinib	37	3.0	0.0-110.0	0.006
	Nilotinib	30	0.3	0.0-55.0	
BCR-ABL1% M12	Imatinib	37	2.0	0.0-157.0	0.001
	Nilotinib	30	0.1	0.0-18.0	

CP-CML: Chronic phase-chronic myeloid leukemia.
M: Month.

MDR1 C3435T genotypes: 74 CML patients were genotyped for MDR1 C3435T allele in addition to 72 normal controls. MDR1 CC genotype was the most frequent among patients and normal controls (Table 3).

Optimal response at month 12 was significantly higher in nilotinib arm among patients carrying the TT genotype, however, the difference was less but still significant in CC genotype and no difference was found between both TKIs in the CT genotype group. Difference in response achieved between the 2 drugs in relation to individual MDR1 C3435Tgenotypes is shown in (Table 4).

Table (3): MDR1 C3435T genotype and allele frequency distribution in CML patients and controls.

	Patients (n=74)	Controls (n=72)	*p* value
C3435T genotype (n, %):			
CC	34 (46%)	44 (61%)	
CT	16 (22%)	12 (17%)	
TT	24 (32%)	16 (22%)	0.180
Allelic frequencies (%):			
C allele	57%	68%	
T allele	43%	32%	0.230

CC : Homozygous 3435C genotype.
CT : Heterozygous C 3435T genotype.
TT : Homozygous 3435T genotype.
C : 3435C allele.
T : 3435 T allele

Table (4): Impact of C3435T genotypes on the response of CP-CML patients to imatinib and nilotinib.

Time point	C3435T genotypes						*p*1-value	*p*2-value	*p*3-value
	Imatinib			Nilotinib					
	TT No. (%)	CT No. (%)	CC No. (%)	TT No. (%)	CT No. (%)	CC No. (%)			
Month 3:									
Optimal	2 (17%)	5 (62%)	7 (35%)	6 (60%)	5 (63%)	6 (50%)			
Warning	9 (75%)	3 (37%)	12 (60%)	4 (40%)	3 (37%)	5 (42%)	0.074	1.00	0.473
Failure	1 (8%)	0 (0%)	1 (5%)	0 (0%)	0 (0%)	1 (8%)			
Month 6:									
Optimal	1 (10%)	5 (62%)	8 (42%)	5 (50%)	5 (71%)	8 (61%)			
Warning	4 (40%)	0 (0%)	3 (16%)	3 (30%)	1 (14%)	2 (15%)	0.129	1.00	0.497
Failure	5 (50%)	3 (37%)	8 (42%)	2 (20%)	1 (14%)	3 (23%)			
Month 12:									
Optimal	1 (11%)	6 (60%)	6 (35%)	10 (83%)	5 (83%)	9 (75%)			
Warning	2 (33%)	1 (10%)	2 (12%)	1 (8%)	1 (17%)	2 (17%)	0.002	0.59	0.042
Failure	6 (55%)	3 (30%)	9 (53%)	1 (8%)	0 (0%)	1 (8%)			

CP-CML: Chronic phase-chronic myeloid leukemia. *p*1-value for TT. *p*2-value for CT. *p*3-value for CC.

DISCUSSION

Resistance to BCR-ABL1 inhibitors is a pressing challenge in the treatment of CML. Although BCR-ABL1 point mutations and additional cytogenetic abnormalities are the main mechanisms of resistance, MDR1has been acknowledged among the mechanisms of resistance. The action of MDR1 is to reduce intracellular drug accumulation through Pgp-mediated efflux thus hampering the achievement of effective drug levels at the target site **[7]**. The possible importance of MDR1 SNPs has been recently appreciated.

The ENESTnd study, testing nilotinib 300mg twice daily versus imatinib 400mg once daily, reported a significantly higher rate of MMR after 1 year (50% vs. 27%) and 3 years (73% vs. 53%), and a significantly higher rate of MR 4.5 after 3 years (32% vs. 15%) in favor of nilotinib **[15]**. In this study, patient's response was assessed by RQ-PCR measurements of BCR-ABL1 mRNA transcripts level on IS. Early achievement of MMR on nilotinib included significantly higher rates of optimal responses at all-time points. According to ELN 2013, MMR rates at month 12 in our patients were higher in nilotinib than imatinib arm (80% vs 35%, $p<0.001$). Nilotinib was superior to imatinib in reduction of BCR-ABL1 transcripts level at month 3 (8% vs 21.5%, $p=0.040$), month 6 (1% vs 6.7% with $p=0.070$), month 9 (0.3% vs 3%, $p=0.006$) and month 12 (0.1% vs 2%, $p=0.001$). No patients on nilotinib progressed to accelerated phase or blastic crisis which is consistent with previous data that linked achievement of response to maintaining response and protection from progression **[16]**.

In this study, we observed a higher frequency of MDR1 3435TT genotype in CML patients than healthy controls. When the distribution of genotype frequency was analyzed as regards MMR at month 12, TT genotype patients had a significantly higher optimal response rate in nilotinib arm when compared to imatinib (83% vs 11%, $p=0.002$). Difference in response between both TKIs was less but still significant in CT ($p=0.042$) while no difference was observed in CC genotype ($p=0.588$). Patients with the least optimal response to imatinib at month 12 were those carrying the TT genotype. Interestingly, 2 of these patients transformed into acute leukemia. The lower response to imatinib therapy in our 3435 TT patients was almost similar to others who correlated higher frequency of TT genotype with failure to achieve hematological response **[17]**.

Results of this study differ from reports that showed decreased level of P-gp mRNA expression and the 3435 T allele **[18]** in addition to the lower activity of the T allele of the silent 3435C>T polymorphism **[9]**. Another study showed no difference regarding MMR rates among patients with CC, CT and TT genotypes **[19]**. However, in our patients' cohort, resistance to imatinib was proven to associate with the number of T alleles being more evident in the homozygous TT genotype. In consistence with other data **[10]**, our results show that there was a significantly higher resistance to imatinib in 3435TT carriers whereas CC and CT genotypes exhibit different prognosis. This may suggest that CML resistance may arise, among other causes, from an association with homozygous T allele.

Before starting TKI therapy, MDR1 gene polymorphism pattern testing in individual CML patients may be important in determining treatment strategy. Studies in CML patients showed different results regarding MDR1 SNP and it is obvious that racial differences can affect the frequency and effect on treatment of these polymorphisms **[18]**.

In conclusion, we demonstrated a significant correlation between MDR1 3435TT genotype and lower imatinib response in our CP-CML patients. We suggest that this may be used as an additional criterion for initiating nilotinib instead of imatinib as front line therapy for these patients. We need to expand this work on a larger scale population to prove the resistant effect of MDR1 3435TT genotype on imatinib and its therapeutic relevance for newly diagnosed CP-CML Egyptian patients.

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