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MDR1 Haplotypes and G2677T/A Polymorphism Predict Imatinib Response in Tunisian Patients with Chronic Myeloid Leukemia

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Abstract

Background: The role of human multidrug resistance gene (*MDR1*) SNPs in the interindividual variability of imatinib mesylate (IM) response has received considerable attention. We aimed to study the association between SNPs of the *MDR1* gene (C1236T, G2677T/A, C3435T) and IM response in chronic myeloid leukemia (CML) patients. ***Method***: A retrospective case-control study was conducted on 48 patients with CML undergoing IM therapy. All patients were genotyped using PCR-RFLP method. ***Results***: The genotype and allele frequencies of C1236T and C3435T were not significantly different between CML patients responders and non-responders to IM ($p > 0.05$). The frequencies of 2677T allele and 2677TT genotype were significantly increased in CML patients IM responders which as compared with IM non-responders (50% vs 26.9%, $p = 0.013$ and 27.3% vs 3.8%, $p = 0.029$ respectively). Whereas the 2677AA genotype and CAC haplotype were found only in CML patients IM non-responders (15.4%). ***Conclusion***: Pretreatment genotyping of G2677A/T appears to be useful for predicting IM resistance, which may allow the best choice of drug treatment for CML patients.

Keywords

Chronic Myeloid Leukemia, Imatinib Mesylate, P-Glycoprotein, Multi Drug Resistance, G2677T/A, Haplotype

1. Introduction

Imatinib mesylate (IM), a selective tyrosine kinase inhibitor (TKI) of the BCR-ABL activity, has been approved as a standard of care for chronic myeloid leukemia (CML) therapy [1] [2]. Despite IM's excellent therapeutic efficacy, a significant proportion of patients with CML can develop resistance against this drug (primary refractoriness or secondary) and are at a greater risk of disease progression [3] [4]. Consequently, treatment options for these patients have improved by second-generation TKI (Dasatinib and Nilotinib), which are more potent and selective inhibitors of the BCR-ABL tyrosine kinase activity than IM [5]. The decision-continuing therapy or switching TKI is compounded by the rising cost of Dasatinib and Nilotinib in predominantly self-payment systems as exists in Tunisia. So, knowledge of the biomarkers predicting the answer to IM therapy would help perform therapy decisions. Moreover, identifying the IM-resistant patients and early therapy switching will help to improve progression-free survival (PFS) [6] [7] [8] [9].

IM resistance is a multifactorial phenomenon in CML patients [10]. Some mechanisms can be affecting the systemic levels or intracellular concentrations of IM, such as oxidative stress, amplification of the *bcr-abl* gene, kinase domain point mutations of the *bcr-abl* gene, overexpression of P-glycoprotein (P-gp), or genetic variability in terms of single nucleotide polymorphisms (SNPs) of the multidrug resistance 1 (*MDR1*) gene in tumor cells [11] [12] [13] [14].

Genetic variations (SNPs) of *MDR1* have been defined as potential factors in interpatient variability to treatment response [15]. *MDR1* is a highly polymorphic gene with at least 50 SNPs. Some of these SNPs have been extensively studied in CML disease: two silent SNPs C1236T (rs1128503), and C3435T (rs1045642) in the exon 12 and 26, respectively; and one missense SNP G2677T/A (rs2032582) located in exon 21 [14] [16]-[26]. However, although various attempts to explain the impact of the *MDR1* variants on IM efficacy in patients with CML, the outcomes remain contradictor rather than conclusive. Some studies have found a potential correlation between C1236, C3435T, and G2677T/A SNPs and IM response in CML patients [14] [16]-[21], while other studies failed to find this correlation [22] [23] [24] [25]. Moreover, the role of SNPs in the *MDR1* gene on clinical evolution in CML patients is not clear [27]. Hence, the present study was performed to study the *MDR1* gene SNPs (C1236T, G2677T/A, and C3435T) in CML patients to understand 1) their associations with the acquisition of IM resistance and 2) their influences on clinical-CML evolution.

2. Materials and Methods

A retrospective case-control study on 48 Tunisian patients with CML was conducted from the Hematology Department of Hedi Chaker University Hospital from June 2015 to January 2016. The diagnosis and classification of patients were based on the European Leukemia Network (ELN 2016) criteria [6]. Our study was approved by the Ethics People's Protection Committee (PPC) (0015/

2016) of the South of Tunisia. All participants provided informed consent.

Pregnant women, patients suffering from any other hematological illnesses, with previous treatment for CML, in accelerated or blastic phase or Philadelphia positive (Ph+) or acute lymphoid leukemia (ALL), with bcr-ab gene mutations related to IM resistance were excluded.

Demographic and clinical variables at diagnosis (Sokal score, treatment protocol (TKI used, and therapy failure)) were collected retrospectively from the medical files of the patients.

Patients were followed-up from the date of CML diagnosis and the initiation of IM treatment for overall survival (OS) and progression-free survival (PFS). The follow-up period is 3-year.

Sokal score was calculated to prognosticate the CML patients at diagnosis [28]. Patient risk was categorized as low (score < 0.8), intermediate (score 0.8 - 1.2), and high (score > 1.2).

2.1. Definitions and Approaches for the Evaluation of Response to Imatinib

One year later of IM treatment, the response to IM was defined by the ratio of the *bcr-abl* gene to the *abl* gene [6]. Patients were classified into two groups: patients who failed to achieve major molecular response (MMR) if the *bcr-abl* gene ratio > 0.1% (IM non-responders) and those who achieved MMR if the ratio ≤ 0.1% (IM responders). Resistance was distinguished as either primary (fail to achieve a response) or secondary (loss of response).

A complete cytogenetic response (CCyR) is obtained if the bcr-abl gene ratio < 1% six months from the initiation of IM treatment [29].

A complete hematologic response (CHR) is obtained with normal lab levels laboratory tests three months from the initiation of IM therapy.

2.2. DNA Extraction

Blood sampling was collected from each patient in a tube comprising ethylenediaminetetraacetic acid (EDTA). Genomic DNA from peripheral leukocytes was isolated using the routine salting-out procedure and stored at −80˚C until analyzed [30]. The quality and concentration of DNA in all samples were measured by the OD260/OD280 absorbance ratio using nanodrop 2000 (Thermo Scientific).

2.3. Detection of MDR1 Polymorphism

Genotyping of C1236T, C3435T, and G2677T/A SNPs of *MDR1* gene was performed using a PCR-RFLP method. PCR was performed in 25 µl total volume with 500 ng of genomic DNA, 12.5 pmol of each primer (Takara Bio USA, Inc.), and 2X Premix EmeraldAmp GT PCR Master Mix (Takara Bio, USA, Inc). PCR cycles were affected by a thermal cycler Applied Biosystems™ MiniAmp™ Plus (Thermo Ficher Scientific, USA). After restriction digest, the DNA fragments

were separated by 4% agarose gel electrophoresis. Then, imaging was performed by Gel Doc TM EZ Imager (Bio-Rad) (Figure 1).

One representative of each of the SNP genotypes was confirmed by sequence analysis (Figure 2).

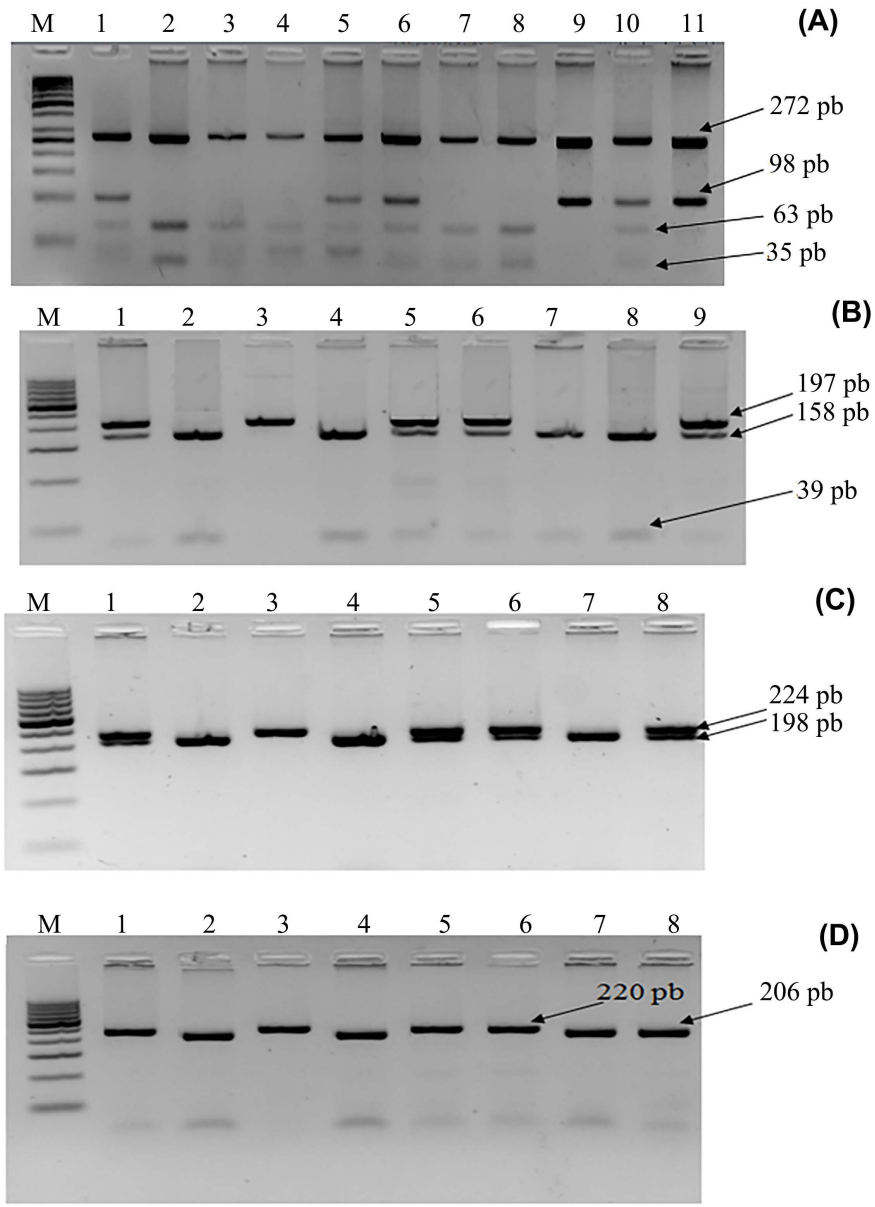

Figure 1. Determination of C1236T, C3435T, and G2677T/A genotypes of MDR1 polymorphisms by gel electrophoresis after PCR-RFLP. (A) PCR amplification of locus C1236T digested by HaeIII. M = Molecular marker; lanes 1, 5, 6, and 9 = CT; lanes 2, 4, 7, and 8 = CC; lane 3 = TT. (B) PCR amplifications of locus C3435T digested by Sau3aI. M = Molecular marker; lanes 1, 5, 6, and 10 = CT; lanes 2, 3, 4, 7, and 8 = CC; lanes 9 and 11 = TT. (C) PCR amplifications of locus G2677T digested by BanI. M = Molecular marker; lanes 1, 5, 6, and 8 = GT; lanes 2, 4, and 7 = GG; lane 3 = TT. (D) PCR amplifications of locus G2677A digested by BsrI. M = Molecular marker; lanes 1, 3, 5, and 6 = GG; lanes 2, 4, 7, and 8 = AA. MDR1, multidrug resistance; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism.

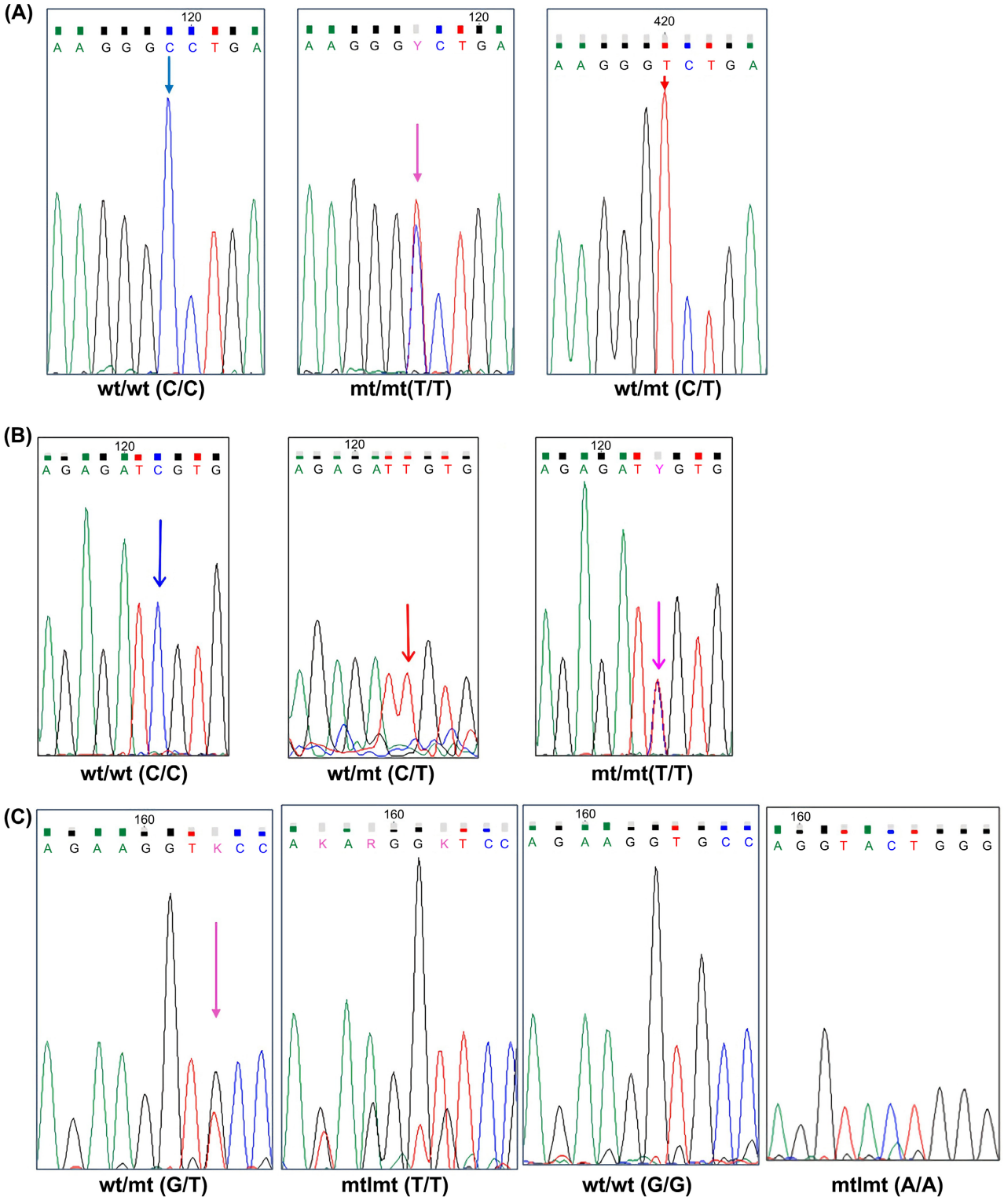

Figure 2. (A) Representative pyrograms for genotyping the C1236T SNP, illustrating an individual homozygous wild type (C/C), an individual homozygous mutant (T/T) and a heterozygous (C/T). (B) Representative pyrograms for genotyping the C3435T SNP, illustrating an individual homozygous wild type (C/C), a heterozygous (C/T), and an individual homozygous mutant (T/T). (C) Representative pyrograms for genotyping the G2677T/A SNP, illustrating a heterozygous (G/T), an individual homozygous wild type (G/G), and individuals homozygous mutant (T/T or A/A). The sequencing was performed on the forward strand. wt, wild type; mt, mutant.

2.4. Statistical Analysis

The statistical tests were performed by the SPSS program, version 20. Pearson chi-square test and Mann-Whitney U test were used to compare CML patients groups according to the type of data. Variables were expressed as mean (range) or frequency and percentage. $p \leq 0.05$ was considered statistically significant.

The OS and PFS were analyzed using the Kaplan-Meier test. The difference between Kaplan-Meier curves was determined by the log-rank test.

3. Results

3.1. Patients Characteristics

Our study consisted of a total of 48 patients with CML, of which 22 were classified as optimal responders to IM (9 males, 13 females, mean age = 49 years), and 26 were IM resistant (13 males, 13 females, mean age = 44.1 years). Clinical characteristics of both groups of patients are summarized in Table 1. The CML patients' responders and non-responders to IM had a similar distribution of sex and age ($p > 0.05$).

Table 1. Clinical characteristics of CML patients.

Characteristics	CML Patients		*p* value
	IM Responders (n = 22)	IM non-responders (n = 26)	
Sex Males/females	9/13	13/13	0.76
Age at diagnosis (y) mean (range)	49.0 (23 - 80)	44.1 (24 - 62)	0.22
Transcript type			
b2a2, N	5	11	0.18
b3a2, N	17	14	0.60
b2a2 + b3a3, N	0	1	0.45
Sokal score			
Low, N	7	11	0.32
Intermediate, N	8	10	0.83
High, N	7	5	0.79
Achievement of CCyR within 6 months, N	22	10	0.36
Treated with dasatinib, N	-	10	-
Treated with nilotinib, N	-	16	-
Primary failure of IM, N	-	20	-
Secondary failure of IM, N	-	6	-

Abbreviations: CML, Chronic myeloid leukemia; IM, imatinib; CCyR, Complete Cytogenetic Response; y, years.

3.2. Association of *MDR1* SNPs and Resistance to Imatinib

The genotype and allele frequencies for C1236T, C3435T, and G2677T/A and their association with IM response are listed in Table 2. The genotype distribution was consistent with the Hardy-Weinberg equilibrium ($p > 0.05$). Genotype and allele frequencies of C1236T and C3435T were not significantly different between the patient subgroups ($p > 0.05$) (Table 2). 2677T allele and 2677TT genotype frequencies were significantly higher in IM responders patients compared to those found in IM non-responders patients ($p = 0.013$ and $p = 0.029$, respectively) (Table 2). The homozygote variant 2677AA was found only in IM non-responders patients (15.4%) (Table 2).

The homozygote variant 2677AA was seen only but not significantly in patients with primary IM resistance (Table 3) and not achieving their CCyR (Table 3). The IM secondary failure patients not harbored 2677TT and 2677AA genotypes (Table 3). Furthermore, the patients treated with Dasatinib not harbored the 2677TT genotype (Table 3).

The three loci of *MDR1* (1236-2677-3435) were recognized in eight haplotypes (CGC, CGT, TGC, CTC, CTT, TTC, TTT, and CAC) (Table 4). The CAC

Table 2. Distribution of allele and genotype frequencies of MDR1 polymorphisms in CML patients.

Locus	Genotype	IM Responders (n = 22)	IM non-responders (n = 26)	*p* value
C1236T, n (%)	C	28 (63.6)	38 (73.1)	0.25
	T	16 (36.4)	14 (26.9)	
	CC	10 (45.5)	14 (53.8)	0.38
	CT	8 (36.4)	10 (38.5)	0.46
	TT	4 (18.1)	2 (7.7)	0.26
C3435T, n (%)	C	27 (61.4)	35 (67.2)	0.61
	T	17 (38.6)	17 (28.8)	
	CC	9 (41.0)	14 (53.8)	0.27
	CT	9 (41.0)	7 (27.0)	0.23
	TT	4 (18.0)	5 (19.2)	0.25
G2677T/A, n (%)	G	22 (50.0)	30 (57.7)	0.25
	T	22 (50.0)	14 (26.9)	0.013
	A	0 (0.0)	8 (15.4)	0.077
	GG	6 (27.3)	9 (34.6)	0.68
	GT	10 (45.5)	12 (46.2)	0.59
	GA	0 (0.0)	0 (0.0)	-
	TT	6 (27.3)	1 (3.8)	0.029
	AA	0 (0.0)	4 (15.4)	0.074

Values calculated by χ^2-test.

Table 3. Frequencies of G2677T/A genotypes and (1236-2677-3435) haplotypes of MDR1 gene in CML patients based on clinical patient's status.

		Patients with IM primary resistance (n = 20)	Patients with IM secondary resistance (n = 6)	Patients nilotinib-treated (n = 16)	Patients dasatinib-treated (n = 10)	patients achieved their CCyR (n = 10)	patients not achieved their CCyR (n = 16)
G2677T/A genotypes, n (%)	GG	6 (66.7)	3 (33.3)	6 (66.7)	3 (33.3)	4 (44.4)	5 (55.6)
	GT	9 (75)	3 (25)	7 (58.3)	5 (41.7)	5 (41.7)	7 (58.3)
	TT	1 (100)	0(0)	1 (100)	0(0)	1 (100)	0(0)
	AA	4 (100)	0 (0)	2 (50)	2 (50)	0 (0)	4 (100)
(1236-2677-3435) haplotypes of MDR1 gene, n (%)	CGC	11 (64.7)	6 (35.3)	10 (58.8)	7 (41.2)	8 (47)	9 (53)
	CGT	2 (100)	0 (0)	2 (100)	0(0)	2 (50)	2 (50)
	CTC	2 (100)	0 (0)	2 (100)	0(0)	0(0)	2 (100)
	CAC	5 (100)	0 (0)	2 (40)	3 (60)	0(0)	5 (100)

Abbreviations: IM, imatinib; CCyR, Complete Cytogenetic Response; MDR1, Multidrug Resistance 1.

Table 4. Distribution of haplotype frequency of MDR1 polymorphisms in CML patients.

Haplotypes of MDR1 gene			Haplotype frequency		*p* value
C1236T	G2677T/A	C3435T	IM Responders (n = 22)	IM non-Responders (n = 26)	
C	G	C	45.5	69.2	0.13
C	G	T	13.6	7.7	0.41
T	G	C	9.1	0	0.20
C	T	C	13.6	7.7	0.41
C	T	T	4.5	0	0.45
T	T	C	9.1	0	0.20
T	T	T	4.5	0	0.45
C	A	C	0	15.4	0.038

Values calculated by χ^2-test.

haplotype frequency was significantly higher in IM non-responders patients (15.4%) than in IM responders patients (0.0%) ($p = 0.038$) (Table 4). IM non-responders patients harbored just CGC, CGT, CTC, and CAC haplotypes (Table 4). Surprisingly, all patients with secondary resistance to IM carried the CGC haplotype (Table 3). Moreover, the CAC haplotype was detected only in CML patients with primary IM resistance (Table 3) and not achieving their CCyR (Table 3).

3.3. Influences of *MDR1* SNPs on Clinical-CML Evolution

Patients who had *MDR1* wild-type genotype showed significantly better OS compared to the variant genotype (C1236T; $p = 0.012$, C3435T; $p = 0.001$ and

G2677T/A; $p = 0.035$) (Figures 3(A)-(C), respectively).

Patients who had C3435T variant genotype showed significantly better PFS compared to the wild-type genotype ($p = 0.001$) (Figure 3(F)).

Patients who had G2677T/A wild-type genotype showed significantly better PFS compared to the variant genotype ($p = 0.018$) (Figure 3(G)).

4. Discussion

Imatinib was the first target of CML therapy. Despite the IM's excellent efficacy, nearly 35% - 40% of patients with CML have an inadequate response, being resistant to IM [3] [4]. The knowledge of early biomarkers predicting the answer to IM therapy would perform therapy decisions. Although several studies have been conducted in this area, the results remain inclusive. In most disease conditions, pharmacokinetics have been found to be a potential source of biomarkers and could account in part for inter-individual variation in drug response [18]. Thus, the *MDR1* gene, genetic variation could be a potential underlying mechanism of the sub-optimal response to IM. Of the numerous SNPs identified for *MDR1*, three variants had undergone extensive studies to determine their association with IMD therapy (C1236T, G2677T, and C3435T), with inconclusive findings. In the present study, we investigated whether three known SNPs in the *MDR1* gene (C1236T, G2677T/A, and C3435T) may have an impact on the therapy outcome and survival of CML patients treated with IM in the south of Tunisia.

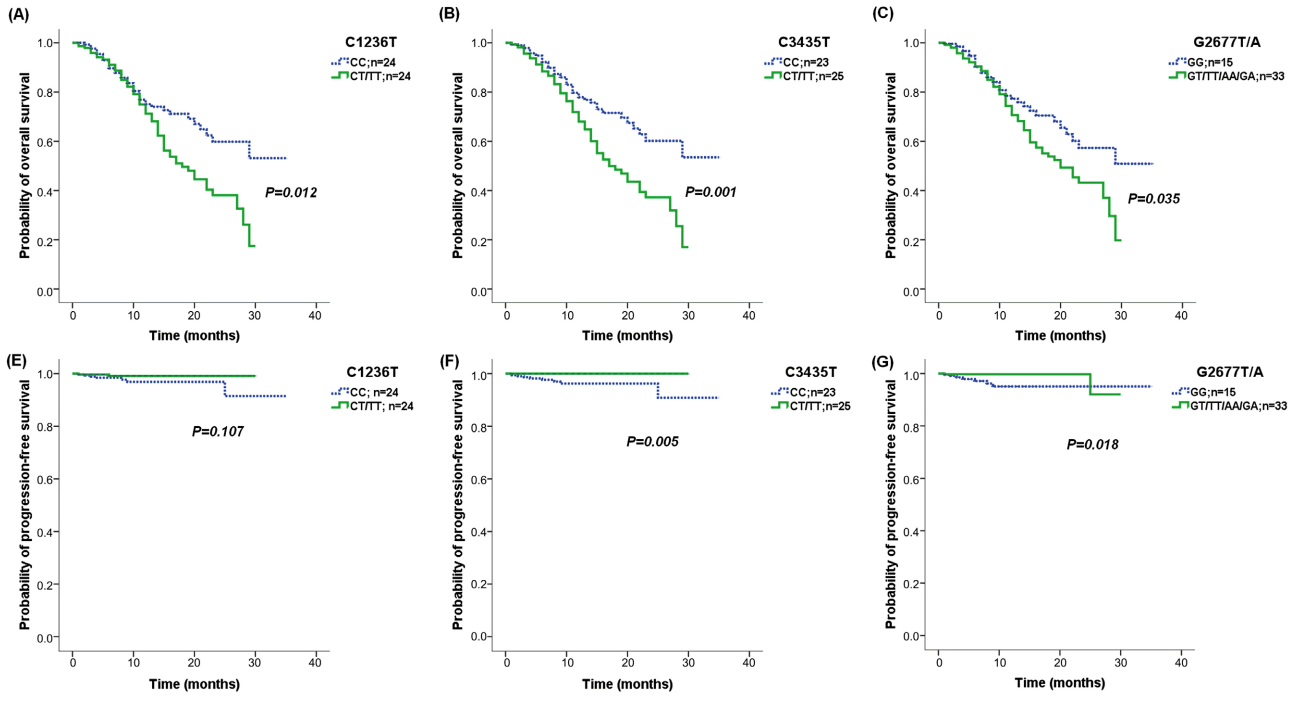

Figure 3. Kaplan-Meier survival analysis. Three-year overall survival according to C1236T (A), C3435T (B), and G2677T/A (C) Genotypes in CML patients. Three-year progression-free survival according to C1236T (E), C3435T (F), and G2677T/A (G) Genotypes in CML patients. CML, chronic myeloid leukemia.

Globally, our results showed that the genotypes and allele frequencies of C1236T and C3435T differ between CML responder patients and non-respondents to IM, but not significantly. Furthermore, we found that the G2677T/A SNP plays a role in the IM response within the recessive model. As was confirmed by the significantly increased frequencies of 2677T allele and 2677TT variant in CML patients IM responders than IM non-responders, and by the presence of variant 2677AA only in CML patients with primary IM resistance and not achieving their CCyR. Interestingly, we found also that CCyR was observed only in patients with GG, GT, and TT genotypes at 2677 positions. In addition, we noticed that the IM secondary failure patients did not harbor the 2677TT. Given all these findings, we suggest that the 2677AA variant could be an independent risk factor for resistance, while the 2677TT variant could be a protective factor resistance to IM. This ascertainment was consistent with a study reported by Ni *et al.* (2011), which found that IM optimal response was significantly associated with the 2677TT variant [21]. Likewise, an Egyptian study showed that carriers of the 2677GT genotype had a higher CCyR rate compared with carriers of the 2677TT/AT/AA/AG/GG genotype [18]. However, a multi-centric study revealed that the incidence of cytogenetic response was higher among patients with CML with the genotype *MDR1* 2677 GA/AT/AA [24]. Furthermore, Sailaja *et al.* (2010) showed high frequencies of 2677GG and GT genotypes in patients with a failure of cytogenetic response [31].

The association between of the *MDR1* gene (C1236T, G2677T/A, and C3435T SNPs) and IM resistance acquisition in CML patients was extensively studied and meta-analyzed [14] [25] [26]. A recent meta-analysis (2021) concluded that *MDR1* SNPs genotypes are not significantly related to the IM response in patients with CML [26], which is contrary to some previous studies that showed genetic variations were associated with the increasing risk of resistance to IM in CML patients [17] [24]. These heterogeneous results illustrate a potential role of ethnicity in the genetic context [32].

With regard to the impact of *MDR1* SNP (C1236T, G2677T/A, and C3435T) on responses to second-generation TKI, our study did not identify an association between *MDR1* genotypes and alternative treatments (switched to Nilotinib or Dasatinib) in CML patients. Dessilly *et al.* (2016) suggest that C1236T, G2677T/A, and C3435T SNPs had a significant effect on anti-proliferative activity and intracellular levels of IM, but not on nilotinib, dasatinib, and ponatinib [33].

Some studies have shown that haplotype *MDR1*, rather than individual SNPs, is a better predictor of IM response, but to date, the findings are contradictory [16] [20] [24] [34]. In our study, the three loci of *MDR1* (1236-2677-3435) were recognized in eight haplotypes: CGC, CGT, TGC, CTC, CTT, TTC, TTT, and CAC. Worthy of mention that the CAC haplotype has been observed exclusively in CML patients with primary IM resistance and not achieving their CCyR. Also, IM non-responders patients harbored just CGC, CGT, CTC, and CAC haplotypes. Our finding is consistent with a Tunisian study reported by Ben Hassine *et al.* (2017), which found that the CAC haplotype has been seen only in IM

non-responders patients [20]. However, Lardo *et al.* (2015) have revealed that CAC haplotype has been detected only in one CML patient who reached an MMR after 12 months of IM treatment [34]. Au *et al.* (2014) found that the wild-type haplotype (CGC) was associated with resistance to IM [24], similar to that reported in the Caucasian population [16].

The influence of C1236T, G2677T/A, and C3435T genotypes on the clinical evolution of CML patients is not yet clear. Our results demonstrated that the likelihood of the OS was significantly improved in patients who had *MDR1* wild-type genotype (1236CC, 3435CC, and 2677GG) and that the PFS probability was significantly better in patients who had 3435CT/TT or 2677GG genotypes. Bharathi M *et al.* (2020) reported that CML patients with variant of the *MDR1* genotype (1236CT/TT, 3435CT/TT, or 2677GT/TT) had significantly better PFS than the wild type genotype [27].

In conclusion, C1236T and C3435T SNPs from the *MDR1* gene were found not to be directly associated with the IM response in CML patients. G2677T/A SNP of *MDR1* gene might be a suitable predictor for IM response in Tunisian CML patients if the study results could be replicated in larger studies of the Tunisian CML population. The specific effect of C1236T, C3435T, and G2677T/A genotypes on the clinical evolution of CML patients is still not known fully, and future detailed studies are essential to further confirm the conclusions of our study.

Conflicts of Interest

The authors declared no potential conflicts of interest.

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