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كلية العلوم للبنات

Ministry of Higher Education & Scientific Research
University of Babylon
College of Science for Women
Biology Department

Association between MDR1 polymorphism Incidence in CML Patients: Advances in Molecular and Clinical Anticancer Treatment Resistance

A Graduate Research Project

Submitted to the Council of the College of Science for Women and the Committee of the Undergraduate Studies of Babylon University in Partial Fulfillment of the Requirements for the Degree of B.Sc. of Biology/Microbiology

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(إِن يَنصُرْكُمُ اللَّهُ فَلَا غَالِبَ لَكُمْ ۖ وَإِن يَخْذُلْكُمْ فَمَن ذَا الَّذِي يَنصُرُكُم مِّن بَعْدِهِ ۗ وَعَلَى اللَّهِ فَلْيَتَوَكَّلِ الْمُؤْمِنُونَ)

صدق الله العلي العظيم

(سورة ال عمران اية ١٦٠)

الإهـــداء

من قال انا لها " نالها" وانا لها وان ابت رغما عنها اتيت بها.

الى الايادي الطاهرة التي ازالت من طريقي اشواك الفشل وكانوا الي السند والقوة الى من رسموا الي المستقبل بخطوط من الثقة والصب رسموا لي طريق مليء بالنجاحات

الى من كانوا الداعمين لي بالأوقات الصعبة.

اليكم عائلتي ...

اهدي فرحت تخرجي الى تلك الانسانة العظيمة التي كانت لي الاب والام والسند التي كانت تنتظر ان تقر عيناها برؤيتي في هكذا يوم.

امي وغاليتي الثانية جدتي ...

اما بعد..

ولا ننسى كل اساتذة والمشرفين على مسيرتي العلمية من أستاذ واستاذة والدكاترة المحترمين نشكر جهودكم المبتذلة لنا طوال تلك السنين.

اهدي هذا العمل القيم

إلى الوالدين الكريمين حفظهما الله

وإلى كل أفراد أسرتي

والى روح جدي وجدتي رحمهما الله

والى كل من ساهم في تلقيني ولو بحرف في حياتي الدراسية

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Elaf

وزارة التعليم العالي والبحث العلمي
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العلاقة بين تعدد الأشكال MDR1 لدى مرضى سرطان الدم النخاعي المزمن: التقدم في مقاومة العلاج الجزيئي والسريري المضاد للسرطان

بحث تخرج

مقدم الى مجلس كلية العلوم للبنات ولجنة الدراسات الجامعية الاولية / جامعة بابل كجزء من متطلبات شهادة البكالوريوس في علوم الحياة / علم الأحياء المجهرية

الطالبة

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Association between MDR1 polymorphism (G2677T) Chromosome Incidence in CML Patients: Advances in Molecular and Clinical Anticancer Treatment Resistance.

Abstract

MDR1 polymorphism G2677T affects Ph chromosomal frequency in habitual myeloid leukemia (CML) cases. The MDR1 G2677T polymorphism's counteraccusations on habitual myeloid leukemia (CML) may lead to new medicine resistance treatments for cancer. Its main ideal was to measure the frequency of ABCB1 (MDR1, P-glycoprotein) polymorphisms in habitual myeloid leukemia cases. Using PCR- RFLP, 99 CML samples and 60 controls were examined for the MDR1 gene(G2677T) polymorphism. According to genotype distribution, CML cases were more likely to have the TT genotype (49.2vs.33.3 in the control group). This study genotyped 73 CML cases and 12 healthy controls for the G2677T polymorphism. Control and CML allele frequentness and genotype distributions were anatomized. A gender-specific connection between the G2677T polymorphism and CML vulnerability was also delved. Results indicate a substantial connection($p<0.002$) between G2677T polymorphism and CML threat. CML cases had advanced TT genotypes than controls. G and T allele distributions were analogous when the two sets of data were compared. A coitus-specific connection($p<0.005$) was set up between the G2677T polymorphism and CML vulnerability. manly TT carriers had a advanced CML threat than ladies. The G2677T gene polymorphism, which affects men and women else, may contribute to heritable CML vulnerability, according to our findings. Eventually, the G2677T gene polymorphism is related with advanced threat of habitual myeloid leukemia (CML), emphasizing the need for farther exploration to establish the molecular causes and remedial counteraccusations.

Key words:

ABCB1 (MDR1), RFLP, P-glycoprotein, *G2677T*, CML, Cancer

Introduction

About 15% of all adult leukemias are chronic myeloid leukemia (CML), a clonal myeloproliferative disorder1, 2. Its incidence ranges from 1.1 to 1.5 per 100,000 people. It typically manifested between the ages of 40 and 603,4,5.

There is a gene called ABCB1 that codes for a drug efflux transmembrane protein called P-glycoprotein (P-gp). It was able to release certain medications from the cells. Intestinal, hepatic, renal, CML stem cell, and circulating leukocyte ABCB1 expression has been documented in CML patients6,7.

There are almost one hundred SNPs in the coding areas of MDR14,8. Many disorders have been examined using the C3435T polymorphism, which is situated in exon 26. Although its prevalence varies by race, it is prevalent across all ethnic groups. This non-synonymous polymorphism in exon 21 (G2677T) is associated with the 3435C>T polymorphism. There is yet no obvious mechanism for how these SNPs regulate P-gp expression 9. While this may be the case, research has shown that haplotypes carrying the mutant alleles exhibit significant structural alterations, leading to altered binding site conformations and reduced P-gp activity in cell lines.10, 11.

The primary objective of this research was to determine how common the SNPs C3435T and G2677T are in the Multidrug Resistance 1 gene in patients with chronic myeloid leukemia (CML).

Materials and Methods

In this study, 90 patients with chronic myeloid leukemia (CML) who received treatment at Babylon Province's Merjan Hospital between Jan./2023 and Oct./2023 were included. Conventional clinical and laboratory data, supported by molecular analysis, led to the diagnosis of CML. with an age range (8-76) year. The research included sixty randomly selected healthy individuals from different Iraqi populations. After a comprehensive examination, blood and serum samples were collected from all patients and controls. Patients and controls were each given an EDTA tube to collect a blood sample. In accordance with the manufacturer's procedure, DNA was extracted from the study subjects' peripheral blood using Promega Wizard genomic kits. RFLP and conventional PCR, to identify mutant genes with the help of a single primer. As a lyophilized byproduct of many picomole fixations, the primer was

provided by the Bioneer (Korea) Organization. In order to prepare a 10µM concentration for use as a work primer, 10 pmol/µl of the lyophilized preliminary was resuspended in 90 µl of free DNase/RNase until the final concentration reached 10µM. The stock solution was then stored at -20°C.
In this study, the G2677T gene was amplified using specific primers. The forward primer sequence was 5′-TTTAGTTTGACTCACCTT CCCG–3′, while the reverse primer sequence was 5′-TGCAGGCTATAGGTTCCAGG–3′. The resulting product had a size of 224 base pairs. The experimental design and methodology followed for this gene amplification are detailed in the study by Sailaja et al. in 2010.

Study Subjects:

The practical side of the study was done in the period between "September 2015 until June 2016".One hundred and fifty samples were collected. Two enrolled groups of subjects were involved in this study.

Patients:

This study includes 90 patients with Chronic Myeloid Leukemia (CML) admitted to Marjan Hospital. Patients were included (38 males and 52 females), with an age range (8-76) years, they were diagnosed by specialist physicians and selected in the current study. Blood and serum samples were taken from every patient and control having thoroughly examined.

Healthy control group

Sixty of actual healthy persons from various Iraqi populations were arbitrarily involved in the study.

Materials:

Equipment:

The equipment which was used with their sources are shown in Table 1.

Table (1) Equipment are needed in this project.

Equipment	Company / Country
Autoclave	HIRAYAMA / USR
Electrophoresis power supply	pelex/ France
Eppendorf centrifuge	MIKRO 120 / Germany
Horizontal Electrophoresis unit	pelex/ France
Vortex	Griffin / England
Water bath	Techne DRI-Block /UK
Sensitive balance	Sartorius /Germany
Deep freeze(-80Cº)	Forma Scientific Inc/USA
Gel photo documentation system	American science and surplus/FRANCE
Incubator	Memmert GmbH/Germany
Microwave oven	American science and surplus/USA
Nanodrop device	Thermo Scientific Inc/USA
PCR device	Applied bio system/USR
Ultra violet light transiluminator	Ultra violet products Inc/UK
Micropipettes	Slamid/ United States
Refrigerator	Ishtar/ Iraq
Heparin Tube	Fluka/Switzer Land
Beakers	Volca/England
Tips (Different sizes)	Jippo / Japan
PCR Tubes	Eppendorf /Germany

Chemical:

The chemicals used and their sources are given below in table (2):

Table (2): chemicals used in the study

Chemical Material	Company / Country
Agarose	**promega / USA**
DNA ladder marker (100 &25 bp)	**Promega/USA**
DNA loading dye	**Promega/USA**
Red Safe	**Bio basic/USA**
Tris Borate EDTA buffer(10 x)	**Sigma/USA**
Ethanol 70%	**BDH/ England**
Primers	**Bioneer/Korea**
Isopropanol	**BDH / England**
PCR pre mix (master mix)	**Promega/USA**
Wizerd genomic DNA purification kit	**Promega/USA**
Nuclease-Free Water	**Promega/ USA**
Ban I Restriction enzyme	**Biolabs/England**

Methods:

Blood Sampling:

About five milliliters of venous blood were collected from each patient in the study. The blood was divided into two parts: one part (about two milliliters) was collected into EDTA containing tubes for genetic part. The second part of the blood was placed in gel tube for thirty minutes, then transferred to plain tube and serum was obtained by centrifugation at 3000 rpm for 15 min ; after that the serum collected and kept in the freezer (-20 ^{o}C) until it was used for the immune and viral assay.

Isolation of genomic DNA:

Genomic DNA was used for molecular study by sequestered from the fresh blood, which collected in tubes of anticoagulant EDTA and for frozen blood samples we recommended using protease K were applied using for DNA purification; Promega Wizard genomic kits. The isolation of DNA depended on the 5 stage procedure utilizing salting out techniques (Sambrook and Manianatis , 1989):

- Lysis of the RBCs in the Cell Lysis Solution.
- Lysis of the WBCs and their nuclei in the Nuclei Lysis Solution.
- The cellular proteins were then removed by a salt out precipitation step using the Protein Precipitation Solution.
- The genomic DNA was concentrated and desalted by Isopropanol precipitation.
- The genomic DNA was rehydrated using the DNA Rehydration Solution.

Isolation kit Components:

Components	Amount	Components	Amount
Cell Lysis Solution	500 ml	Protein Precipitation Solution	125 ml
Nuclei Lysis Solution	250 ml	DNA Rehydration Solution	100 ml

Electrophoresis of Agarose Gel:

Agarose gel electrophoresis was embraced to affirm the nearness and uprightness of the separated DNA after genomic DNA extraction in 1.5% agarose with 100 V for 10 min. (Sambrook and Maniatis , 1989).

Gel Electrophoresis Reagents:

- Powder of Agarose
- TBE Buffer with 1X concentration
- Loading dye
- Red Safe
- DNA Ladder Marker

Protocol of Gel Electrophoresis:

Tris Borate EDTA Buffer preparation (1X TBE)

This solution was prepared by adding 900 ml Distill water to 100 ml 10X TBE (Promega/ Germany) ,forming 1 liter of(1x) TBE buffer (Sambrook and Russel, 2001).

Preparation of agarose gel:

1- The amount of 1 X TBE (100 ml) was taken in a beaker
2- Agarose powder (1.5 gm) was added to the buffer
3- The solution was heated to boiling using a microwave oven for 2 min.
4-Red Safe (1 µl) of (10mg/ml) was added to the agarose solution.
5- The agarose was stirred in order to be mix and avoid making bubbles.
6- The solution was left to cool down at 50 – 60 ^{o}C.

DNA Loading & Electrophoresis:

DNA (3 µl) was mixed with (2 µl) loading dye. The samples loaded carefully into the individual wells of the gel, and then electrical power was turned on at 70 volt for 1 hour, afterwards the DNA moved from cathode (-) to anode (+) poles. The Red Safe stained bands in the gel were visualized using UV. Transilluminator at 350 nm and photographed.

PCR Technique:

In this study two types of PCR were used include RFLP and conventional PCR, to detect mutation genes by using nine primers

as shown in the following table. The primers were supplied by Bioneer (Korea) Organization as a lyophilized result of various picomols fixations. Lyophilized preliminary was disintegrated in a free DNase/RNase water to give a final concentration of 100 pmol/µl and kept as a stock in -20°C, to prepare 10µM concentration as work primer re suspended 10 pmol/µl in 90 µl of free DNase/RNase to reach a final concentration 10µM.

Table (3) Sequence of primers

GENE	Primer		bp	Ref
G2677T	F	5′- TTTAGTTTGACTCACCTT CCCG – 3′	224	Sailaja *et al.*, 2010
	R	5′- TGCAGGCTATAGGTTCCAGG – 3′		

A master premix of Bioneer was used, with components in table (4)

Table (4) Master Mix components

Item	Concentration
DNA Taq polymerase	1 U/ µl
Each: dNTP (dATP, dCTP, dGTP, dTTP)	250 Mm
Tris-HCl (pH 9.0)	10 mM
KCl	30 mM
MgCl2	1.5 mM

PCR protocol

A successful PCR program depends on the reaction conditions including reagents, temperature and the prevention of contamination. Previous study indicates that PCR is sensitive to reaction condition and that the optimization of these conditions is necessary to reach the highest specificity and product yield (Williams *et al.*, 1990). Standard amplification conditions were applied in PCR with primer sequences.

The annealing temperature in which primers hybridize to complementary sequences on the template DNA is perhaps the most critical in PCR programming. The annealing temperature for PCR primers is based on melting temperature (Tm) calculations. Tm is the temperature at which half of DNA strands are denatured. A Tm calculation for PCR primers is based on guanine and cytosine (G+C) content. The annealing temperature is usually below the Tm in 2-12 °C (Newton and Graham, 1997).

The primers concentration represents the optimal concentration. A decrease in the primer concentration leads to weak PCR product while an increase could result in the formation of primer dimer artifact, leading to misinterpretation of results (Saiki *et al.*, 1988). The PCR based techniques do not require highly purified DNA preparations as it works well with partially purified DNA samples (Edward *et al.*, 1991; McPherson and Moller, 2001). However, DNA extraction may contain inhibitory compounds, like detergents used in cell lysis and protein denaturation in addition to other inhibitory compounds that could interfere with PCR leading to a reaction failure (McPherson and Moller, 2001).

Detection of G2677T gene

The method of RFLP-PCR used by Sailaja *et al.*, (2010) was adapted for the analysis of the BanI SNP. The gradient condition of G2677T is explained in the following table. The PCR reaction mixture for gradient consisted of 5µl template DNA, 5µl master mix, 5µl forward and 5µl reverse primer in 20 µl of total reaction volume.

Table (5) gradient condition for G2677T

Step	Temperature C°	Time/min.	Cycles
Initial denaturation	94	5.	1
Denaturation	94	0.5	35
Annealing Zones	54-56-58-60-62-64	0.5	35
Extension	72	0.45	35
Final extension	72	10	1
Storage	4	∞	

After the determination of optimum annealing temperature by selecting the clearest band which is 58 C°, PCR mixture was 5µl DNA,5µl master mix, 1.5 forward and reverse primer, PCR conditions were performed as in the following tables.

Table (6) PCR condition for G2677T

Step	Temperature C°	Time/min.	Cycles
Initial denaturation	94	5	1
Denaturation	94	0.5	35
Annealing	58	0.5	35
Extension	72	0.45	35
Final extension	72	10	1
Storage	4	∞	

A volume of 10 µl of the amplicon was processed with 10 U Ban I (Biolabs) with restriction buffer (5 µl) which provided with the enzyme in a total volume of 20 µl. Then it placed in a water bath at 37°C and for 2 hours. The recognition site for the Ban I:
5'…G▼GYRCC…3'
3'…CCRYG▼G…5'
Genomic source for Ban I is *Paenibacillus thiaminolyticus*

Digested products were electrophoresed on 2.5% agarose gel gel using 1X TBE buffer system at 70V for 30 min and 100V for 30 min. , then they were analyzed in a gel documentation system.

The size of reaction products was 26 bp and 198 bp for 2677G allele where as 2677T was identified by the single fragment of 224 bp. (Sailaja *et al*., 2010).

G2677T gene detection

For the analysis of the *BanI* SNP, the RFLP-PCR method originally utilized by Sailaja et al. (2010) was modified. According to the paragraph below, the gradient condition of *G2677T* is described. In 20 µl of total reaction volume, there was 5µl of template DNA, 5µl of master mix, 5µl of forward primer, and 5µl of reverse primer in the PCR reaction mixture for gradient.

The polymerase chain reaction (PCR) protocol consisted of several steps. Initially, an initial denaturation step was performed at 94°C for 5 minutes to prepare the DNA template. Subsequently, a denaturation step at 94°C for 0.5 minutes was carried out for 35 cycles to separate the DNA strands. The annealing step involved a temperature gradient, starting from 54°C and increasing to 64°C in increments, each lasting 0.5 minutes. This step was repeated for 35 cycles. The extension step, conducted at 72°C for 0.45 minutes, facilitated the synthesis of complementary DNA strands. A final extension step at 72°C for 10 minutes was performed to complete the PCR process. The final step involved storing the samples at 4°C indefinitely. This PCR protocol aimed to amplify specific DNA sequences through a defined series of temperature and time intervals.

Following the selection of the most visible band at 58°C as the optimal annealing temperature, the following conditions were used to conduct the PCR: 5µl DNA, 5µl master mix, 1.5 forward and reverse primers.

The PCR procedure for G2677T mutation consisted of several essential steps. At first, a denaturation step was carried out at a temperature of 94°C for a duration of 5 minutes, which created the best conditions for the following amplification process. Subsequently, a denaturation process was carried out at a temperature of 94°C for a duration of 0.5 minutes. This phase was specifically intended to separate the DNA strands and commence the amplification cycles. The annealing process took place at a temperature of 58°C for a duration of 0.5 minutes. This allowed the primers to attach to the target DNA, which in turn facilitated the creation of complementary strands during 35 cycles. The extension phase, carried out at a temperature of 72°C for a duration of 0.45 minutes, facilitated the process of DNA synthesis by the DNA polymerase enzyme. To assure the completion of any partially synthesized DNA strands, a last extension step was performed at a temperature of 72°C for a duration of 10 minutes. Ultimately, the samples were forever preserved at a temperature of 4°C. The objective of this PCR process was to duplicate targeted DNA sequences by carefully regulating temperature and time, hence facilitating effective and precise amplification.

The enzyme was provided in a total volume of 20 µl by treating 10 µl of the amplicon with 10 U of Ban I (Biolabs) in 5 µl of restriction buffer. Subsequently, it underwent a two-hour immersion in a water bath maintained at a temperature of 37 degrees Celsius. The location where the Ban is acknowledged:

5'…G▼GYRCC…3'

3'…CCRYG▼G…5'

The *Bacillus thiaminolyticus* genome is the one that encodes *Ban I*. After digestion, the products were subjected to electrophoresis on a 2.5% agarose gel using a 1X TBE buffer system.

The gel was electrophoresed at 70V for 30 minutes and 100V for 30 minutes. Subsequently, they were examined using a gel documentation system. The reaction products for the 2677G allele were 26 bp and 198 bp in size, while the 2677T allele was detected by a single 224 bp fragment. 12, 13.

Evaluation using statistical methods

Statistical Package for the Social Sciences (SPSS) 15.0 was used to examine the eventuality information. To determine whether there was a statistically significant link between genotype and CML

presence and prognosis, a chi-square test was computed. The two-tailed p-values were determined with a significance level of $p<0.05$.

Results & Discussion

The study found that patients with chronic myeloid leukemia (CML) had a higher prevalence (40.2%) of an extended repetition of the 2677 TT genotype, while controls (33.3%) did not demonstrate this. This is depicted in the image. The G2677T polymorphism did not exhibit a statistically significant correlation with gender. The 2677TT genotype was found in 49.2% of healthy persons, whereas the GT genotype was found in 58.3%.

Individual variability might result from various lifestyles and varying degrees of exposure to different risk factors. The numerical values are 1, 4, 15, and 16. Ethnicity can also be of importance. Studies on lung cancer (18,19) indicate that a decrease in the ability to transport naturally existing carcinogens may be associated with decreased expression of P-gp, hence increasing the risk of developing CML (17, 18).

A strong correlation was observed between the G2677T polymorphism and changes in plasma levels of P-gp 11,20, either in an increased or decreased manner. Individuals possessing a 2677 TT genotype demonstrated a notable decrease in the expression of P-gp operator RNA (12,21) compared to the overall population with a 2677 GG genotype.

Contrary to what was expected, particular pharmacokinetic investigations have shown that the presence of the 2677T mutant allele has a contrary effect, leading to increased transportation compared to the 2677G allele. Nevertheless, Tanabe (2001) demonstrated that there is no significant reversal of P-gp expression in the placenta in relation to the G2677T polymorphism 22,23,24. The observed variances can be due to the near proximity of different amino acids at position 893, which can lead to varying consequences depending on their arrangement.

Tables 7 and images 1 and 2 illustrate the repetitive nature of the MDR1 gene (G2677T) polymorphism.

Table (7) displays the frequency of genotypes for Ban I enzyme polymorphisms of the G2677T gene in patients with chronic myeloid leukemia (CML) and the control group.

Genotype of G2677T	Healthy		CML Patients		p value
	No	%	No	%	
GG	1	8.3	11	18.0	0.2
GT	7	58.3	20	32.8	
TT	4	33.3	30	49.2	
Allele frequency					
G	9	38	42	34	0.77
T	15	63	80	66	

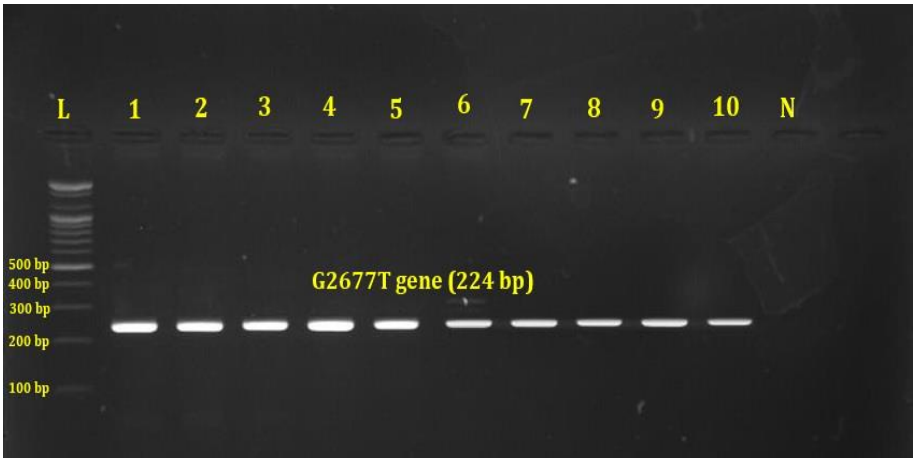

Figure 1. shows the electrophoresis of the G2677T gene's PCR products. Lane L contains the 100 bp DNA ladder, which is embedded on a 5% NuSieve® 3:1 agarose gel in 1X TBE buffer with 0.5 µl of Red Safe . G2677T was exposed in lanes 1-10. The PCR product control was negative, denoted by N.

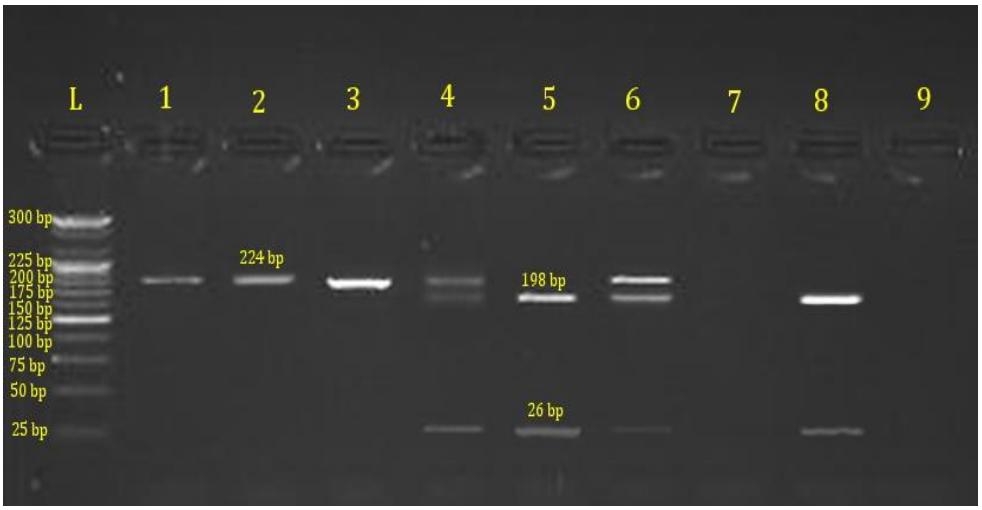

Figure 2. the G2677T gene's RFLP-PCR products were electrophoresed. A 25 bp DNA ladder, 5% NuSieve® 3:1 agarose gel in 1X TBE buffer with 0.5 µl of Red Safe are all contained in the L lane. The 2677G Ban I enzyme has two pieces, one of 198 base pairs and the other of 26 base pairs, whereas the 26 was found with a single 224 base pair fragment (1, 2 and 3). Four and six lanes were homozygous for the TT genotype, five and eight lanes were heterozygous for the TG genotype, and seven lanes were homozygous for the GG genotype. After enzyme digestion, the PCR product in lane 9 remained negative, serving as a control.

Table 8. Examine the link between the G2677T polymorphism in chronic myeloid leukemia (CML) and the control group using a table.

Gene polymorphism	NO. of Control	Control		NO. of CML	CML		P-Value
		No	%		**No**	%	
G2677T	**60**	**12**	**20.0**	**90**	**61**	**67.8**	**<0.002**

Table (9) Examine the correlation between the G2677T gene variant and sex.

Gene target	Sex/Control				Total		Sex/CML				Total		P-Value
	Male		**Female**				**Male**		**Female**				
	No	%	**No**	%	**No**	%	**No**	%	**No**	%	**No**	%	**<0.005**
G2677T	**4**	**2.6**	**8**	**5.1**	**12**	**7.7**	**26**	**5.1**	**35**	**6.9**	**61**	**12.1**	

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Conflicts of Interest:

The authors assert that they have no conflict of interest.

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