



RESEARCH

Comparison of gene expressions in individuals with G6PD Mediterranean mutation and hemolytic anemia

G6PD Akdeniz mutasyonu olan hemolitik anemili bireylerde gen ekspresyonlarının karşılaştırılması

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Abstract

Purpose: In our study, we aimed to clarify the relationship between Glucose-6-phosphate dehydrogenase (G6PD) enzyme kinetics and mRNA expression levels of the G6PD gene in Gd-Med patients with and without hemolytic anemia.

Materials and Methods: The study group consisted of 30 cases with Gd-Med mutation and 30 cases with enzyme activity levels in the reference range. G6PD activity was determined by the Beutler method. G6PD enzyme was partially purified with DE-52 anion exchange resin, and its kinetic parameters were studied. Gd-Med mutation was genotyped by *Mbo*II enzyme digest and sequence analysis. The expression level of the G6PD gene was calculated according to the $2^{-\Delta\Delta Ct}$ formula.

Results: In our study, a significant difference was found between the K_{mNADP^+} and K_{mG6P} values of the cases with Gd-Med mutation and the control group. There was no significant difference between K_{mNADP^+} and K_{mG6P} values in Gd-Med mutated patients with and without hemolytic anemia. Gene expression results of 18 patients without hemolytic anemia were significantly higher than 12 patients with hemolytic anemia. In addition, there was a significant difference between these variables and the control group.

Conclusion: It might be a possible explanation that the substrate binding site of the enzyme in cases with Gd-Med mutation may have undergone post-transcriptional or post-translational modifications, and therefore gene expression might be changed. As a further study, the decrease in gene expressions of patients with hemolytic anemia with Gd-Med mutation can be clarified by evaluating the promoter side of the gene.

Keywords: Enzyme kinetics, gene expression, glucose-6-phosphate dehydrogenase, hemolytic anemia.

Öz

Amaç: Çalışmamızda, Gd-Akdeniz’li bireylerde hemolitik anemisi olan ve olmayan olguların Glukoz-6-fosfat dehidrogenaz (G6PD) enzimi kinetiği ve G6PD geninin mRNA ekspresyon seviyeleri arasındaki ilişkinin irdelenerek bu duruma açıklık getirmeyi hedefledik.

Gereç ve Yöntem: Çalışma grubu, Gd-Akdeniz mutasyonu bulunan 30 olgu ve enzim aktivite düzeyi referans aralığında olan 30 olgudan oluşmaktadır. G6PD aktivitesi Beutler yöntemi ile tayin edilmiştir. G6PD enzimi DE-52 anyon değiştirici reçine ile kısmi olarak saflaştırılarak kinetik özellikleri çalışılmıştır. *Mbo*II enzim kesimi ve sekans analizi ile Gd-Akdeniz mutasyonu genotiplendirilmiştir. G6PD geninin ekspresyon seviyesi $2^{-\Delta\Delta Ct}$ formülüne göre hesaplanmıştır.

Bulgular: Çalışmamızda Gd-Akdeniz mutasyonu bulunan olguların K_{mNADP^+} ve K_{mG6P} değerleri ile G6PD enzim aktivitesi referans değerlerde olan olgular arasında anlamlı düzeyde fark bulundu. Hemolitik anemisi olan ve olmayan Gd-Akdeniz mutasyonuna sahip olguların ise K_{mNADP^+} ve K_{mG6P} değerleri arasında anlamlı fark bulunamadı. Hemolitik anemisi olmayan 18 hastanın gen ekspresyon sonuçları, hemolitik anemisi olan 12 hastaya göre anlamlı derecede yüksek olduğu görüldü. Ayrıca enzim aktivite düzeyi referans aralığında olan olgularla da bu değişkenler karşılaştırıldığında yine anlamlı farklılık olduğu belirlendi.

Sonuç: Gd-Med mutasyonu olan olgularda enzimin substrat bağlanma bölgesinin post-transkripsiyonel veya post-translasyonel modifikasyonlara uğramış olması ve bu nedenle gen ekspresyonunun değişmesi olası bir açıklama olabilir. İleri çalışma olarak Gd-Akdeniz mutasyonuna sahip hemolitik anemisi bulunan olguların gen ekspresyonlarındaki azalmayı geninin promotör bölgesi değerlendirilerek açıklık getirilebilir.

Anahtar kelimeler: Enzim kinetiği, gen ekspresyonu, glukoz-6-fosfat dehidrogenaz, hemolitik anemi

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INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD, EC: 1.1.1.49, β -D-Glucose-6-Phosphate, NADP oxidoreductase) is the first and rate-limiting enzyme of the pentose phosphate pathway¹. The pentose phosphate pathway (PPP) primary function is to provide the organism with NADPH and ribose phosphates^{1,2}. This vital enzyme is defined as an 'ubiquitous' enzyme, which means that it is present in every organism³. G6PD deficiency is the most common inherited enzyme disease affecting more than 500 million people worldwide⁴.

G6PD was first discovered in horse erythrocytes in 1931 by Warburg and Christian⁵. According to the World Health Organization G6PD Deficiency Working Group (WHO-G6PD) criteria (biochemical, kinetic, clinical), over 400 G6PD variants have been identified^{6,7}. According to the screening studies conducted in the Çukurova region, the rate of G6PD deficiency was reported to be 8.2%⁸.

The G6PD gene is localized in the 28th band (Xq28) of the long arm of the human X chromosome and consists of 13 exons and 12 introns^{3,9}. The size of G6PD enzyme is 515 amino acids^{8,10}. Its mRNA is 20.114 base pairs (bp) long¹¹.

The G6PD Mediterranean (Gd-Med) variant is the most common variant of Mediterranean littoral regions¹². The Gd-Med mutation occurs with the C→T conversion of the 563rd nucleotide of exon 6 in the G6PD gene^{4,13}. In the Gd-Med variant, the enzyme activity is considerably reduced (<10) and can cause severe hemolytic anemia^{14,15}. Enzyme deficiency leads to hemolytic anemia at various levels^{2,14}. Erythrocyte enzyme defects emerged with the first definition of G6PD enzyme deficiency as the cause of hemolytic anemia^{9,16}. G6PD enzyme deficiency is one of world's most common clinically important enzyme defects, causing hemolytic anemia^{12,17}. This is manifested by a decrease in G6PD enzyme activity and a change in kinetic parameters^{14,18}.

It has been observed that some of the patients with Gd-Med mutation are affected when exposed to oxidative agents, whereas some patients are not¹⁸. Examining the expression of the G6PD gene at the mRNA level may clarify this exciting situation. In this study, we aimed to clarify the relationship between

hemolytic anemia and mRNA levels in patients with Gd-Med mutation.

MATERIALS AND METHODS

Sample collection and activity determination

The procedures to be performed were explained to the participants in detail, and informed consent was obtained. The study was initiated with the decision of the Çukurova University Ethics Committee (6.12.2012/14-5).

The cases who consulted our department to evaluate G6PD enzyme activity were enrolled in the study. The patients with low G6PD enzyme activity were accepted as the study group and divided into two sub-groups; with or without hemolytic anemia. The cases with G6PD enzyme activity between the reference values were taken as the control group. Any other enzyme defect causing hemolytic anemia, blood transfusion in the last three months, and chronic diseases requiring regular drug use were excluded from the study.

The study population was determined as 26 with G-power program by the effect size 0.8, $\alpha=0.05$, power $(1-\beta)=0.80$ at a confidence level of 95%. Fifty-three cases G6PD enzyme specific activity lower than the reference values (7-13 U/g Hb) or zero were collected, and 30 samples had shown the Gd-Med kinetics and mutation were included in the study group. It was evaluated whether the cases had hemolytic anemia or not. In addition, 30 samples whose enzyme specific activity level was within the reference range were studied as a control group. 10 ml of venous blood samples were collected into EDTA tubes, and G6PD enzyme activities were determined immediately by spectrophotometer (Shimadzu UV-260, Japan). Beutler method was used for enzyme specific activity determination^{2,12}.

Enzyme purification

G6PD enzyme was partially purified from DE-52 (Whatman, USA) ion exchange column¹⁹. 25 g of DE-52 anion exchange resin, which was swollen overnight in 5 mM sodium phosphate buffer, was washed with the buffer until its pH was 7.0. The prepared hemolysate and anion exchange resin were mixed at a ratio of 1:3 and mixed at +4°C overnight with a low-speed shaker. The supernatant was

removed by measuring the protein activity each time (reading its optical density at 280 nm). The resin washing process was continued with 5 mM sodium phosphate buffer until the protein activity was zero. The mixture of hemolysate and anion exchange resin was transferred to the column. The column was washed with 5 mM sodium phosphate buffer containing 0.25 M KCl. Elutions were collected, and protein concentrations were measured. The G6PD kinetics of the elution with the highest activity were studied by measuring the G6PD activities of the eluates with high protein concentration. The eluates were stored at -20°C until the kinetic parameters were performed²⁰.

Kinetic study

The Lineweaver-Burk graph was used to measure the K_m values to evaluate the affinity of the G6PD enzyme against its substrates, NADP⁺ and G6P²². For this, the interval in which G6PD activities obtained during 5 min at different substrate concentrations increased linearly was detected. Velocity (V) and substrate (S) concentrations were converted to Lineweaver-Burk plots^{22,23}.

DNA extraction and molecular study

The DNAs of the samples were isolated in a DNA isolation device (Roche Magna Pure LC PG 0747, USA) and stored at -20°C until the molecular analyses. Gd-Med mutation, most common variant Çukurova region, was scanned in cases whose DNA was isolated²⁴. Amplification control of the G6PD gene was performed on a 1.5% agarose gel. Electrophoresis conditions were carried out at 150 mA current for 30 minutes²⁵. After running the gel, it was treated with ethidium bromide at a concentration of 0.5 $\mu\text{g}/\text{mL}$, which was prepared with distilled water. The washed gel was examined under UV light²⁶.

The amplified DNA products were examined for the presence of the Gd-Med mutation. The ΦX174 HaeIII Marker was used as a marker²⁷. After the desired region of the G6PD gene was amplified by PCR, the DNA product was cut with *Mbo*II, a restriction enzyme specific to the region for mutation²⁸. The solutions required for RFLP were incubated at 37°C overnight and then run in a 3% agarose gel, 150 mA current, for 70 minutes. Then the agarose gel was treated with ethidium bromide at a concentration of 0.5 $\mu\text{g}/\text{mL}$. The gel washed in

pure water was examined under UV light and photographed²⁹.

Sequence analysis

The cases with Gd-Med mutation after cutting with restriction enzyme were genotyped by sequencing analysis. The method based on the Sanger principle was used in an automated DNA sequence analyzer (ABI 3130 4-Capillary, USA)³⁰. For the amplification control, 1.5% agarose gel was poured and run at 150 mA current for 30 minutes. Then, the agarose gel was treated with ethidium bromide at a concentration of 0.5 $\mu\text{g}/\text{mL}$, and the gel washed in pure water was examined under UV light. The formed single-stranded DNA was cleaned with a cephalex column³¹. The samples were run by placing on the plate of the automatic sequence analyzer, and the reading of the gene sequence was performed with the sequence software program^{32,33}.

Gene expression analysis

The G6PD Gene Expression Kit (Roche, USA) was used for gene expression analysis. Beta Actin (β -Actin) was used as the reference gene. mRNAs were isolated from the subjects for gene expression. Firstly, mRNAs were obtained from leukocytes by lysing erythrocytes with "Red Blood Cell Lysis Buffer". In the second step, mRNA isolation was performed using the "High Pure" RNA Isolation Kit (Roche, USA). It was stored at -70°C until cDNA was obtained. In the next step, cDNAs were synthesized from mRNA with reverse transcriptase enzyme. cDNA was obtained with the "Transcriptor High Fidelity cDNA Synthesis" kit (Roche, USA). Gene expression of the G6PD enzyme was performed with the "RealTime ready Singe Assay" kit (Roche, USA). LightCycler 96-well plate was used for measurements. The plate was covered with a plate and centrifuged at 4.000 rpm for 4 minutes. The sample plate was loaded into RT-PCR (Light Cyler 480 Roche, USA)³⁴.

Statistical analysis

SPSS 20 program was used for statistical analysis. The distribution of the data was evaluated with the Shapiro-Wilk test. Repeated measures of ANOVA followed by Bonferroni post hoc were used for the evaluation for the differences between groups. The significance of the effects was presented using Gpower (Ver. 3.1.9.4). Effect sizes were calculated as

> 0.2 (small), > 0.5 (moderate), and > 0.8 (large). The results obtained were reported as mean value \pm standard deviation, and statistical significance was determined as $p < 0.05$.

RESULTS

Kinetic study

Enzyme activity levels were determined by quantitative G6PD enzyme activity assay. The enzyme activities of the subjects were between 0-4.6 U/g Hb (reference range 7-13 U/g Hb)⁸, $\bar{x} \pm SD$; 1.62 ± 1.27 . The partially purified G6PD enzyme samples' kinetic properties were determined. According to the K_{mNADP^+} and K_{mG6P} values obtained from the Lineweaver-Burk curve, K_{mNADP^+} was found to be 1.1 – 2.4 μM ($\bar{x} \pm SD$; $1.2 \pm 0.4 \mu M$),

K_{mG6P} was between 21.8 – 37 μM ($\bar{x} \pm SD$; $23.4 \pm 3.2 \mu M$).

Molecular study

Amplification results for Gd-Med mutation in individuals with G6PD enzyme deficiency were displayed by 2% agarose gel electrophoresis. The amplicon size of the amplification performed to detect the Gd-Med mutation in exon 6 was 547 base pairs (bp) long (Figure 1).

The *Mbo*II restriction enzyme fraction of 547 bp amplicons which were formed for the presence of the Gd-Med mutation, was visualized by 3% agarose electrophoresis. Figure 1 represents the amplification result of 10 cases.

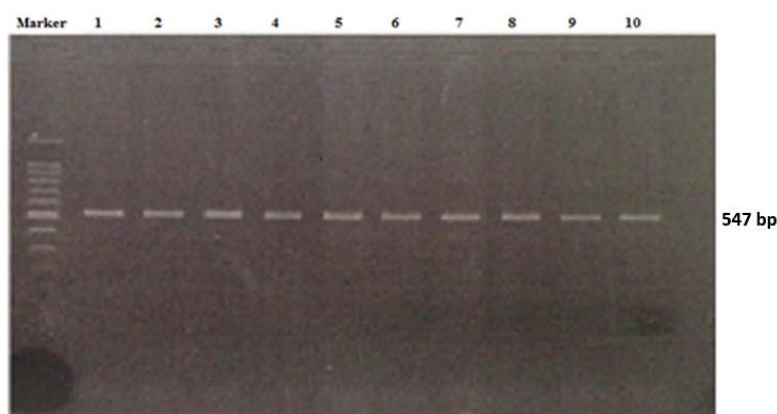


Figure 1. The image of G6PD Gene in 2% agarose gel electrophoresis before enzyme digestion. '100 Base-Pair' Ladder was used as a marker.

As the amplicons were cut by *Mbo*II restriction enzyme, Gd-Med mutation was determined (21 sample were hemizygous, 8 samples were heterozygous) (Figure 2 represents 20 of 30 cases). Even the sample does not contain the C→T change, which represents Gd-Med mutation, after the *Mbo*II restriction enzyme cutting process, the appearance of the amplified sample in the gel would be in the form of two bands, 377 and 119 bp. On the other hand, if

the individual had a hemizygous or homozygous genotype with 563 C→T mutation, the appearance in the amplified gel would be in the form of 277, 119, 100 bp (See Figure 2, column 1, 3, 5, 7, 8, 10, 12, 15, 17, 18, 19, 20). If the sample had heterozygous mutation (one X gene normal, another X gene mutant), the gel contains four bands, 377, 277, 119 and 100 bp (See Figure 2, column 2, 4, 6, 9, 11, 13, 14, 16).

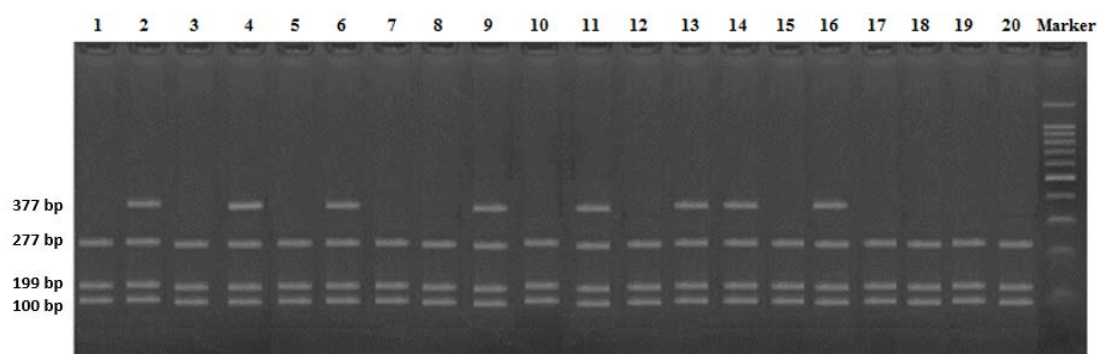


Figure 2. Appearance in 3% agarose gel after cut with *Mbo*II Restriction Endonuclease enzyme. '100 Base-Pair' Ladder was used as a marker.

Sequence analysis

Sequence analysis was performed in 30 cases in order to confirm the Gd-Med mutation results obtained by

enzyme cutting process. Figure 3 shows the G6PD sequence analysis of the heterozygous C→T change in the 563rd nucleotide of the 6th exon.

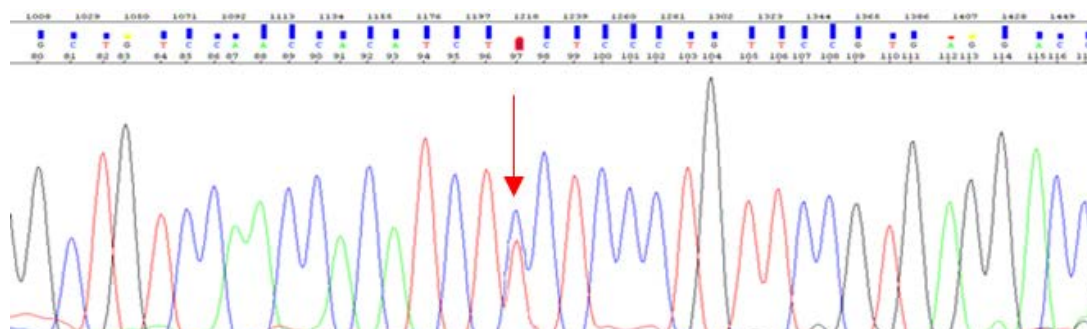


Figure 3. DNA sequence analysis image of the Gd-Med mutation. The red arrow indicates the C→T change in the 563rd nucleotide.

Gene expression

The G6PD mRNA expressions of 30 samples with Gd-Med mutation and 30 samples with enzyme activity at reference values were studied. G6PD mRNA expression of Gd-Med patients (Figure 4) and patients with normal enzyme activity (Figure 5) is observed by RT-PCR.

β-Actin was used as the reference gene in the study. The expression image of the reference gene is given in Figure 6.

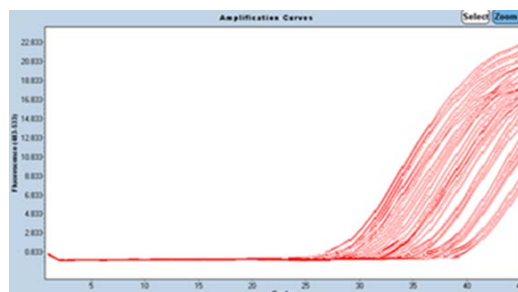


Figure 4. RT-PCR image of the mRNA expression of the G6PD Gene in cases with Gd-Med.

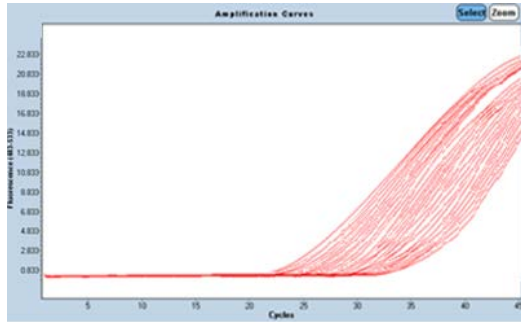


Figure 5. RT-PCR image of the mRNA Expression of the G6PD Gene in cases with G6PD enzyme activity at reference values.

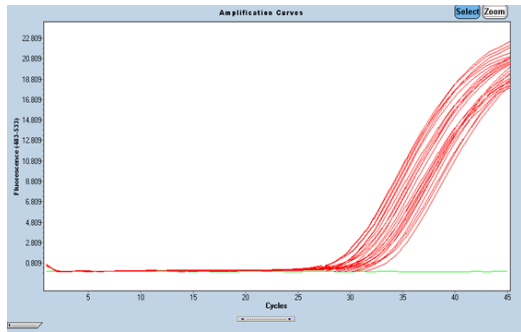


Figure 6. Expression image of the β -Actin.

The mRNA expression levels of the cases were calculated according to the $2^{-\Delta\Delta Ct}$ formula³⁵. The kinetic data and expression results are shown in Table 1.

A significant difference was found between the Km_{NADP^+} and Km_{G6P} values of the cases with Gd-Med mutations and those with G6PD enzyme activity at reference values ($p < 0.01$). There was no significant difference between Km_{NADP^+} and Km_{G6P} values in patients with and without hemolytic anemia with Gd-Med mutation. As the gene expressions of the patients with Gd-Med mutation was compared in existence of hemolytic anemia, there was a significant difference between these groups ($p < 0.05$). In addition, the gene expression of the individuals who had Gd-Med mutation but G6PD enzyme activity in the reference range were compared in the existence of hemolytic anemia, there was a significant difference between these groups ($p < 0.01$).

Table 1. Kinetic data and expression results of the cases.

Cases	n	Km_{G6P} ($\bar{x} \pm SD$)	Km_{NADP^+} ($\bar{x} \pm SD$)	$2^{-\Delta\Delta Ct}$ ($\bar{x} \pm SD$)
Cases with Gd-Med mutation	30	$23.4 \pm 3.2 \mu M^*$	$1.2 \pm 0.4 \mu M^\ddagger$	$0.59 \pm 0.3^\ddagger$
Cases with hemolytic anemia	12	$22.5 \pm 2.8 \mu M$	$1.1 \pm 0.2 \mu M$	$0.28 \pm 0.4^\S$
Cases without hemolytic anemia	18	$23.1 \pm 2.3 \mu M$	$1.1 \pm 0.8 \mu M$	0.52 ± 0.6
Cases with normal G6PD enzyme activity	30	$60.82 \pm 7.4 \mu M$	$3.6 \pm 1.67 \mu M$	2.12 ± 2.06

*: A significant difference was found between the Km_{G6P} values of the cases with and without Gd-Med mutation. $p < 0.01$

†: A significant difference was found between the Km_{NADP^+} values of the cases with and without Gd-Med mutation. $p < 0.01$

‡: A significant difference was found between the $2^{-\Delta\Delta Ct}$ values of the cases with and without the Gd-Med mutation. $p < 0.01$

§: A significant difference was found between Gd-Med patients with hemolytic anemia and those without. $p < 0.05$

(Km : Michaelis constant, SD: Standard deviation)

DISCUSSION

Glucose-6-phosphate dehydrogenase enzyme deficiency is the most common enzyme deficiency in the world³⁶. The frequency of G6PD enzyme deficiency varies according to geographical regions, and its incidence is higher in Mediterranean countries³⁷. The incidence of G6PD enzyme deficiency is 0.5% in Turkey and 8.2% in the Çukurova region. Over 140 mutations of this vitally important G6PD enzyme were identified³⁸. Gd-Med is the most detailed and most studied variant so far. Molecular studies have shown that the Gd-Med

variant is common in Çukurova and Antalya regions. In our study, 30 of 58 cases with Gd-Med mutation were included in the experiment. We determined that the Gd-Med variant was the most common variant in our study with a frequency of 56.6%. In a study carried out to detect the Mediterranean mutation in Syria, the frequency of Gd-Med was found at a rate of 83%³⁹. In a study to determine the prevalence of mutations causing G6PD deficiency in Indian populations, the incidence of Gd-Med mutation was determined as 14.1%. The authors showed that the most common variant in the Indian population was Gd-Orissa with 56.5%⁴⁰. As a result of molecular

genotyping of G6PD mutations in the Brazilian Amazon, the frequency of Gd-Med was 41%⁴¹.

The Gd-Med mutation, which has such a high regional distribution, causes jaundice in newborns and increases sensitivity to certain drugs. In addition, broad beans and infections can provoke hemolytic anemia. Hemolytic anemia is most common health problem in the Mediterranean mutation subgroup^{4,42}. Angelo et al. presented a shortened red blood cell (RBC) circulation life (55 days) and increased glutathione (GSH) levels were observed in mice with the Gd-Med mutation⁴³. Awaida et al. determined that 32% of patients with Gd-Med mutation in Southern Jordan showed acute hemolysis. In a study of Palestinians in the Gaza Strip, they reported that more than 80% of children presenting with hemolytic anemia had G6PD deficiency, and 34% of them had the Gd-Med variant¹⁰. Twelve (40%) of the 30 patients who had hemolytic anemia with Gd-Med mutation were enrolled in our study.

It is remarkable that the same irritant agent does not have the same effect in individuals with the Gd-Med mutation, and the cases may have a heterogeneous clinic^{24,44}. The clinical differences of the cases with the Gd-Med mutation have not been attributed to any reason so far. We designed our study to explain this interesting situation from different aspect. In this study, it was found that the K_m values of cases without Gd-Med mutation are significantly higher than the cases with Gd-Med mutation.

Two important limitations of this study could be addressed in future research. The first limitation is the small sample size. Increasing the number of cases with low G6PD enzyme activity and hemolytic anemia could reflect a larger population. Another limitation is that we focused only on the Gd-Med mutation. Other G6PD mutations that cause hemolytic anemia should also be evaluated. It was shown that the K_{mNADP^+} and K_{mG6P} values, which are indicators of the enzyme's affinity for its natural substrates, NADP and G6P, decreased significantly in Gd-Med cases^{9,23}. Mahmoud Sirdah et al. showed that the Arg72 and Ser73 hydrogen bonding interactions, which are considered to be the key interaction point for the binding affinity of the NADP⁺ substrate to the G6PD enzyme, are lost in the Gd-Med mutation⁴⁵. The Gd-Med mutation is thought to affect the binding affinity of the enzyme to its natural substrates, leading to the development of clinical symptoms associated with G6PD deficiency^{23,45}.

In our study, which focuses on the topic of how the affinity of the G6PD enzyme to its substrates is reflected in gene expression in patients with hemolytic anemia, the G6PD gene expressions of the patients with and without hemolytic anemia were compared with one of the enzyme's natural substrates, the K_m values, and a significant relationship was determined between the groups. In addition, the kinetic studies on cases with Gd-Med mutation in the literature are in accordance with our results. In our knowledge, our study is the first research that investigate the gene expressions and kinetic parameters of the Gd-Med cases with hemolytic anemia.

In our study, there was a significant difference between the gene expression levels of the cases with the Gd-Med mutation and the cases with the G6PD enzyme activity in the reference range. In addition, the $2^{-\Delta\Delta C_t}$ values of the Gd-Med mutated hemolytic anemia patients' gene expression levels were significantly reduced compared to the patients without hemolytic anemia. The decrease in the expression of the gene also affects the function of the protein, indirectly reflecting the enzyme deficiency.

Batetta et al. declared that G6PD enzyme deficiency patients with leukemia had higher levels of G6PD gene expression compared to non-leukemic patients⁴⁶. These results suggest that G6PD activation, regulated by cellular requirements, may be an important cellular event that occurs in response to cell proliferation. Although the results of Batetta et al. seems as the opposite of our study, we can speculate that it can be assumed that they represent a cell strategy to compensate for very low enzyme activity in order to allow G6PD-deficient cells to proliferate.

In the literature, it was shown that up-regulation of the hypolactasia-related lactase-phlorizin hydrolase (LPH) gene changed the tolerance to dietary lactose and decreased the gene expression level⁴⁷. In our study, the gene expressions of the Gd-Med mutated patients with hemolytic anemia decreased similar to the LPH gene expression.

The kinetic and molecular results suggest that the G6PD enzyme acquired different characteristics related to the post-transcriptional or post-translational modifications. In studies to examine the relationship of the promoter region of the G6PD gene with enzyme deficiency, it was determined that hypermethylation of the promoter region is

associated with enzyme deficiency. It has been reported that hypermethylation of the promoter region of the G6PD gene is significantly increased in enzyme-deficient individuals^{48,49}. It has been shown that mutations in the promoter region of the G6PD gene do not change the level of G6PD enzyme activity and the expression level of the G6PD gene^{50,51}. In the light of all this information, further studies should evaluate the methylation of the promoter region of the G6PD gene in cases with Gd-Med mutations and evaluate whether epigenetic factors in the promoter region cause hemolytic anemia. These studies will help to prevent hemolytic anemia due to Gd-Med.

Author Contributions: Concept/Design : AT; Data acquisition: BG; Data analysis and interpretation: BG, AT; Drafting manuscript: BG; Critical revision of manuscript: AT; Final approval and accountability: BG, AT; Technical or material support: AT; Supervision: AT; Securing funding (if available): n/a.

Ethical Approval: For this study, ethical approval was obtained from the Ethics Committee of Çukurova University Faculty of Medicine Non-Interventional Clinical Research dated 06.122.2012 and numbered 14/5.

Peer-review: Externally peer-reviewed.

Conflict of Interest: Authors declared no conflict of interest.

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