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Paraoxonase Gene Polymorphism Analysis in Pediatric Diabetic Patients

Pınar Kanlıoğlu [1], Selahaddin Tekeş [2], Ruken Yıldırım [3], Diclehan Oral [2], Yusuf Kenan Haspolat [4]

1 Pediatrics Cardiology Department, Batman Training and Research Hospital, Batman, Türkiye

2 Medical Biology and Genetics Department, Dicle Medical School, Diyarbakir, Türkiye

3 Pediatric Endocrinology Department, Selahaddin Eyyubi State Hospital, Diyarbakir, Türkiye

4 Pediatrics Department, Dicle Medical School, Diyarbakir, Türkiye

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Abstract

Objective: Identifying gene polymorphisms linked to childhood-onset diabetes mellitus (DM), a multifactorial disease, will illuminate its genetic underpinnings. This study aimed to explore the association between childhood DM and the polymorphisms Paraoxonase1 (PON1) 55 and PON1 192.

Method: A total of 129 children who applied to our Child Health and Diseases Endocrinology outpatient clinic participated in this prospective research. The study included 75 children with diabetes, aged 3–15 years (Patient Group-PG), and 54 healthy children (Control Group-CG) as a control. Genotyping of PON1 55 and PON1 192 polymorphisms was performed in study patients using Polymerase Chain Reaction (PCR) and RFLP methods following DNA isolation.

Results: Of 54 CG, 21 (38.9%) displayed the homozygous normal (LL) PON1 55 genotypes. The PG contained 25 (33.3%) homozygous normal patients out of 75. In the CG, 25 participants (46.3%) and 39 participants in the PG (52.0%) had the heterozygous (LM) genotype. The homozygous mutant (MM) genotype was observed in 8 (14.8%) of CG and 11 (14.7%) of PG. The CG showed 67 L alleles, representing 62.0% of the total alleles. On the other hand, the number was 89 (59.3%) in the PG. The two groups showed no statistically significant differences in gene polymorphisms, allele frequencies, and M and R allele distribution.

Conclusion: No significant relationship was observed between PON1 55 and PON1 192 gene polymorphisms and DM in our study.

Keywords: Diabetes mellitus, DNA Extraction, Paraoxonase Gene, Polymerase Chain Reaction, Polymorphism

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Correspondence / Yazışma Adresi: *Pınar Kanlıoğlu, Pediatrics Cardiology Department, Batman Training and Research Hospital, Batman, Türkiye e-mail: kanlioglupinar@gmail.com*

Pediatrik Diyabetik Hastalarda Paraoksonaz Gen Polimorfizm Analizi

Öz

Amaç: Çok faktörlü bir hastalık olan çocukluk çağı başlangıçlı diyabet (DM) ile ilişkili gen polimorfizmlerinin belirlenmesi, hastalığın genetik temellerini aydınlatacaktır. Bu çalışma, çocukluk çağı DM ile Paraoksonaz 1 (PON1) 55 ve PON1 192 polimorfizmleri arasındaki ilişkiyi araştırmayı amaçlamaktadır.

Yöntemler: Bu prospektif araştırmaya Çocuk Sağlığı ve Hastalıkları Endokrinoloji polikliniğimize başvuran toplam 129 çocuk katılmıştır. Çalışmaya 3-15 yaş arası 75 diyabetli çocuk (Hasta Grubu-HG) ve kontrol grubu olarak 54 sağlıklı çocuk (Kontrol Grubu-KG) dahil edilmiştir. Çalışma hastalarında DNA izolasyonunu takiben Polimeraz Zincir Reaksiyonu (PCR) ve RFLP yöntemleri kullanılarak PON1 55 ve PON1 192 polimorfizmlerinin genotiplendirmesi gerçekleştirildi.

Sonuçlar: 54 KG'nin 21'i (%38,9) homozigot normal (LL) PON1 55 genotipini gösterdi. PG, 75 hastanın 25'ini (%33,3) homozigot normal hastadan oluşturdu. KG'de 25 katılımcı (%46,3) ve HG'de 39 katılımcı (%52,0) heterozigot (LM) genotipe sahipti. Homozigot mutant (MM) genotipi, KG'nin 8'inde (%14,8) ve HG'nin 11'inde (%14,7) gözlendi. KG, toplam alellerin %62,0'sini temsil eden 67 L aleli gösterdi. HG'de ise bu sayı 89 (%59,3) idi. İki grup arasında gen polimorfizmleri, alel frekansları ve M ve R alel dağılımı açısından istatistiksel olarak anlamlı bir fark görülmedi.

Sonuç: Çalışmamızda PON1 55 ve PON1 192 gen polimorfizmleri ile DM arasında anlamlı bir ilişki gözlenmedi.

Anahtar Kelimeler: Diabetes mellitus, DNA Ekstraksiyonu, Paraoksonaz Geni, Polimeraz Zincir Reaksiyonu, Polimorfizm.

INTRODUCTION

Diabetes mellitus (DM), a chronic illness with diverse origins, is defined by elevated blood glucose and urinary glucose due to insulin deficiency or dysfunction[1,2]. Type 1 DM (T1DM) is the most prevalent form of diabetes among children and adolescents [2][2].

A glycoprotein enzyme, paraoxonase (PON), a calcium-dependent ester hydrolase, possesses both arylesterase (E.C. 3.1.1.2) and paraoxonase (E.C. 3.1.8.1) activity[3]. HUMPONA is the human gene responsible for paraoxonase. The paraoxonase gene family is located on the long arm of human chromosome 7, specifically in the q21.3-q22.1 region. PON1, PON2, and PON3 are the three genes that were in the PON family. A remarkable structural similarity is observed in these genes. The first and most studied member of the PON family is PON1[1].

T1DM and Type 2 DM (T2DM) are associated with lower PON1 activity, according to numerous studies[3,4]. The drop might result from reduced antioxidant capacity caused by elevated oxidative stress in those with DM. The impact of genotypes on activity is probably negligible. Studies show an association between PON1 55L and diabetic retinopathy, and a stronger link between PON1 192R and cardiovascular disease in diabetics 5. Decreased glucose tolerance, pancreatic damage, and increased insulin resistance are associated with the L55M polymorphism[3]. The reason for the reduced PON1 in DM is yet to be completely understood. Nevertheless, increased glucose concentration may be considered a contributing factor. High-density lipoprotein (HDL) experiences both PON inactivation and increased lipid peroxidation due to glycation. Oxidation resistance increases in glycated HDL. Even healthy people with high blood sugar have lower PON1 activity[1]. The link between hyperglycemia, oxidative stress, atherosclerosis, and the function of paraoxonase in DM is evident. The hyperglycemia, hyperinsulinemia, high free fatty acids, and dyslipidemia seen in DM may be a consequence of excess reactive oxygen species (ROS), and the low PON1 activity in diabetic retinopathy and hypertension patients may reflect increased lipid peroxidation

vulnerability[4-6]. The protective effect of HDL against low-density lipoprotein (LDL) oxidation and atherosclerosis is modulated by serum PON1 levels, which are influenced by both environmental and genetic factors[7].

Patients with T2DM showed a higher frequency of PON1 192 RR and PON1 55 homozygous normal (LL) genotypes[8]. Research indicates that low PON1 activity in serum is unrelated to genotypes in coronary heart disease-related conditions including DM, hypercholesterolemia, and renal impairment. Studies have demonstrated decreased PON1 binding to HDL and activity in DM patients relative to healthy individuals[9].

This study aimed to investigate the association between PON1 55 and 192 gene polymorphisms and T1DM in pediatric patients.

METHOD

Patient and Control Groups

A prospective research study enrolled 129 children from our institution's Child Health and Diseases Endocrinology outpatient clinic. The study included 75 DM children, aged 3-15 (Patient group-PG), and 54 healthy children as a control (Control Group-CG). All patients in the diabetes group were diagnosed with T1DM according to the ISPAD Clinical Practice Consensus Guidelines 10, based on clinical presentation, autoantibody positivity, and insulin requirement from diagnosis onward. All procedures were conducted in accordance with institutional and national ethical standards, and the 2008 Helsinki Declaration revision. The ethics committee (Approval No. 334, 05.08.2014) approved our study, and we obtained informed consent from all participants' legal representatives.

Sample Collection & Process

Following a 12-hour fast, the study group provided at least 2ml of blood which were drawn into ethylenediaminetetraacetic acid (EDTA) tubes, then frozen at -20°C for later DNA extraction. This study did not use serum samples exhibiting hemolysis. HDL, LDL, total cholesterol, and glucose levels were determined from blood samples taken after a 12-hour fast, collected in biochemistry tubes with anticoagulant gel, and processed by centrifugation.

Genomic DNA was extracted from the blood samples of patients and controls using the standard sodium perchlorate method[11].

PON1 Leu55Met (L55M) Polymorphism PCR Stage

For PON1 L55M polymorphism, PCR was performed by adding five µl of genomic DNA (100 ng), one µl (10 pmol/µl) of each primer (F and R) (5' GAA GAG TGA TGT ATA GCC CCA G 3' and reverse 5' TTT AAT CCA GAG CTA ATG AAA GCC 3') (0.2 µM), five µl Taq Buffer (10 X), three µl MgCl2 (25 mM), four µl dNTP (2.5 mM), and 0.5 µl Taq DNA Polymerase enzyme (5 u/µl) to make a total volume of 50 µl. Amplification of the PON1 L55M polymorphism via PCR utilized a defined thermocycling program with appropriate primers. Thirty PCR cycles were performed after an initial 5-minute denaturation at 95°C; each cycle consisted of 94°C denaturation (1 min), 55°C hybridization (45 sec), and 72°C extension (1 min). A 5-minute, 72°C extension was added as the final step. This optimized protocol is adapted from the existing literature[12]. A 1% agarose gel electrophoresis at 90V for 45 minutes verified the 170 bp amplicon using a 12 µl sample (10 µl PCR product + 2 µl loading dye). A Techne PHC3 (USA) thermal cycler was used to perform PCR amplification.

PON1 Gln192Arg (Q192R) Polymorphism PCR Step

For PON1 Q192R polymorphism, PCR was performed by adding 7.5 µl of genomic DNA (150 ng), 1.5 µl of each primer (F and R) (10 pmol/µl) (forward 5'TAT

TGTTGCTGTGGGACCTGA G 3' and reverse 5' CAC GCT AAA CCC AAA TAC ATC TC 3' for PON 192 polymorphism) (0.3 µM), five µl Taq Buffer (10 X), two µl MgCl2 (25 mM), five µl dNTP (2.5 mM), and one µl Taq DNA Polymerase enzyme (5u/µl) to make a total volume of 50 µl with PCR grade water. The following thermocycler program was used for PCR amplification of the PON1 Q192R polymorphism using primers; initial denaturation at 96°C for 10 minutes, followed by denaturation at 95°C for 1 minute, hybridization at 58°C for 1 minute, extension at 72°C for 2 minutes (35 cycles), and final extension at 72°C for 10 minutes were optimized by rearrangement from the reference source[13]. The expected 99 bp amplicons were confirmed by electrophoresis of 10 µl PCR product with 2 µl loading buffer on a 1% agarose gel at 90 V for 45 minutes.

Cutting of PCR Products with Restriction Enzymes

Specific restriction enzymes were used to analyze PCR products for the presence of the relevant polymorphism.

PON1 L55M Polymorphism Region Enzyme Digestion

Digestion of a 15 µl PCR product was performed using 0.5 µl (5 units) of Nla III (10 u/µl) restriction enzyme, 2 µl 10X NE Buffer 4, and PCR grade water to 25 µl, incubated at 37 °C for 8 hours and 65 °C for 20 minutes (1 cycle). A 30 µl mixture of enzyme digest (25 µl) and loading buffer (5 µl) was subjected to 3% agarose gel electrophoresis (90V, 1 hr), alongside 100 bp Fermentas marker, and visualized with ultraviolet (UV). The homozygous mutant (MM) and heterozygous (LM) polymorphisms produced different digestion fragment sizes: 126 and 44 bp for MM, and 170, 126, and 44 bp for LM. PCR products from individuals LL remained undigested[12].

PON1 Q192R Polymorphism Region Enzyme Digestion

To achieve a final volume of 25 µl, 15 µl of PCR product was combined with 1 µl of Alw I restriction enzyme (5 u/µl), 2 µl of 10X NE Buffer 4, and PCR grade water. A 3% agarose gel, containing a 100 bp DNA ladder (Fermentas), was used to electrophorese a mixture of 25 µl enzyme digest and 5 µl loading buffer at 90 V for an hour. The RR allele yielded 66-bp and 33-bp fragments upon digestion analysis, while the QR allele produced 66-bp, 33-bp, and 99-bp fragments. No digestion of the QQ (wild type) allele occurred[13]. Figures 1 and 2 present the enzyme digestion results for the studied polymorphisms.

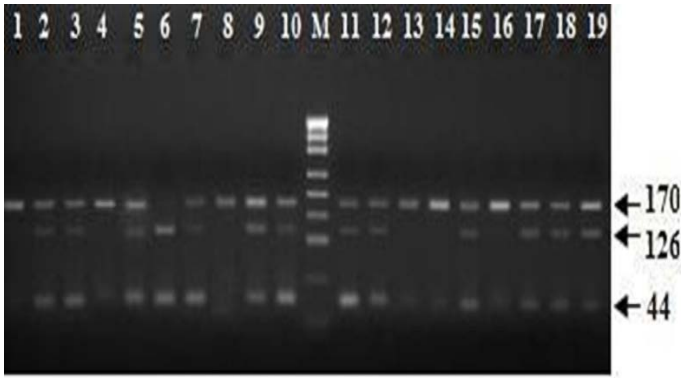

Figure 1: PCR-RFLP enzyme digestion results for PON1 L55M.

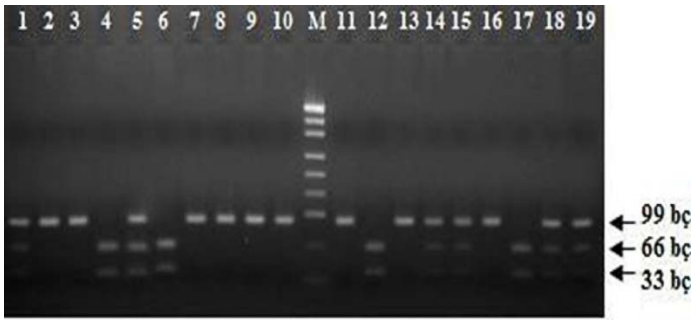

Figure 2: PCR-RFLP enzyme digestion results for PON1 Q192R.

Statistical Analysis

Patient data from the study were analyzed via the IBM Statistical Package for the Social Sciences (SPSS) for Windows 26.0 (IBM Corp., Armonk, NY) package program. Categorical data used frequencies and percentages in descriptive statistics; continuous data used means and

standard deviations. Categorical variables were compared using a Pearson chi-squared test. A chi-square analysis verified the genotype distribution's Hardy-Weinberg equilibrium. Analysis of categorical data employed chi-square tests; numerical data were analyzed with t-tests. Statistical significance was achieved with p-values less than 0.05.

RESULTS

A total of 129 children who applied to our institution participated in this research. A total of 75 patients (35 female, 40 male; 46.7% female, 53.3% male) constituted the PG, while the CG comprised 54 healthy individuals (28 female, 26 male; 51.9% female, 48.1% male). The mean age was 10.0±3.4 years for PG and 9.0±3.2 years for CG. PG and CG did not differ significantly in terms of sex and mean age.

Genotype and allele frequencies of PON1 55 and PON1 192 gene polymorphisms from PG and CG are in Tables 1 and 2. Among the 54 CG, 21 (38.9%) were LL for the PON1 55 genotype, whereas 25 (33.3%) of the 75 PG were. There was no statistically significant variation between the groups. In the CG, 25 participants (46.3%) and 39 participants in the PG (52.0%) had the LM genotype with no statistically significant difference. The CG had 8 (14.8%) MM subjects, while the PG had 11 (14.7%), a difference not statistically significant.

Table I: PON1 55 polymorphism genotype and allele frequency

PON1 55 Genotyping	Control Group (n=54) n (%)	Patient Group (n=75) n (%)	P-value	X^2
LL	21 (38.9)	25 (33.3)	0.516	0.422
LM	25 (46.3)	39 (52.0)	0.523	0.409
MM	8 (14.8)	11 (14.7)	0.981	0.001
Alleles				
L	67 (62.0)	89 (59.3)	0.661	0.192
M	41 (38.0)	61 (40.7)	0.661	0.192

LL: Homozygous normal. LM: Heterozygous. MM: Homozygous mutant. X2: Chi-Square Analysis

Table II: PON1 192 polymorphism genotype and allele frequency

PON1 192 Genotyping	Control Group (n=54) n (%)	Patient Group (n=75)n(%)	P-value	X^2
QQ	26 (48.0)	33 (44.0)	0.641	0.218
QR	20 (37.0)	36 (48.0)	0.215	1.536
RR	8 (14.8)	6 (8.0)	0.220	1.507
Alleles				
Q	72 (66.7)	102 (69.0)	0.822	0.051
R	36 (33.3)	48 (32.0)	0.822	0.051

QQ: Homozygous normal. QR: Heterozygous. RR: Homozygous mutant. X2: Chi-Square Analysis

The CG showed 67 L alleles, a frequency of 62.0%. In comparison, 89 patients (59.3%) were observed in the PG; this difference was not statistically significant. CG and PG showed similar M allele frequencies (38.0% vs 40.7%), a difference not statistically significant. Of the CGs, 26 of 54 (48%) had the QQ PON1 192 genotype; this was similar to the PG, where 33 of 75 (44%) showed this genotype (p>0.05). Heterozygosity (QR) was observed in 20 (37.0%) of the CG and 36 (48.0%) of the PG, with no statistically significant difference between the groups. Homozygous mutant (RR) was observed in 8 (14.8%) of the CG and 6 (8.0%) of the PG, a difference that was not statistically significant.

The CG showed a Q allele frequency of 66.7% (72 Q alleles). Conversely, the PG exhibited a rate of 102 (69.0%), revealing no statistically significant variation. CG and PG showed similar R allele frequencies (33.3% vs 32.0%), with no statistical significance.

In the PG (glycated hemoglobin [HbA1c] ≤ 6.5%, n=14), genotype analysis for PON1 55 showed LL in 9 (64.3%), LM in 4 (28.6%), and MM in 1 (7.1%) patient. Among 61 patients with HbA1c ≥6.6%, 16 (26.2%) were LL, 35 (57.4%) LM, and 10 (16.4%) MM. Groups with different HbA1c levels had a statistically significant difference (p=0.024) in their PON1 55 gene polymorphism (Table 3).

Table III: Relationship between PON1 55 and PON1 192 gene polymorphisms and HbA1c levels

	HbA_{1C} ≤6.5% (n=14) n (%)	HbA_{1C} ≥6.6% (n=61) n (%)	P-value	X^2
PON1 55			0.024	7.429
LL	9 (64.3)	16 (26.2)		
LM	4 (28.6)	35 (57.4)		
MM	1 (7.1)	10 (16.4)		
PON1 192			0.120	4.243
QQ	5 (35.7)	28 (45.9)		
QR	6 (42.9)	30 (49.2)		
RR	3 (21.4)	3 (4.9)		

LL: Homozygous normal. LM: Heterozygous. MM: Homozygous mutant. QQ: Homozygous normal. QR: Heterozygous. RR: Homozygous mutant. X2: Chi-Square Analysis

DISCUSSION

Our study focused exclusively on pediatric T1DM patients, whereas most existing literature addresses PON1 polymorphisms in adults with T2DM. In T2DM, decreased PON1 activity and specific genotypes, such as PON1 192R and PON1 55L, have been repeatedly linked to cardiovascular and microvascular complications[4,5]. However, in T1DM, especially in the pediatric age group, evidence is limited and inconsistent[7,9,14]. Akgün et al.[14] reported that PON activity was significantly lower in children and adolescents with T1DM compared to healthy controls, but broader genetic associations remain unclear. The absence of significant associations in our cohort may reflect fundamental differences in disease mechanisms: T1DM is primarily autoimmune-mediated, whereas T2DM is strongly influenced by metabolic and environmental risk factors[2,14].

PON, a glycoprotein, is a calcium-dependent ester hydrolase exhibiting both arylesterase (E.C. 3.1.1.2) and PON (E.C. 3.1.8.1) activities. The three types are PON1, PON2, and PON3[1]. Among these, PON1 was the first discovered and has been studied the most The involvement of PON1 in HDL structure has been reported[15]. The polymorphisms PON1 55 and PON1 192 are the most frequently investigated. These polymorphisms are linked to various pathophysiological conditions[1,5,8].

El-Lebedy D et al. 4 found statistically significantly higher PON1 55 LL, LM, and MM genotype frequencies (p =0.009) in 68 Egyptian patients versus a CG of 50. The frequency of the PON1 55 LL genotype was statistically significantly greater (p = 0.002) in 100 DM patients with adult cardiac complications than in a CG of 100[5]. DM patients with microvascular complications in a study showed a statistically significantly higher frequency of the PON1 55 LL genotype than the control group (p = 0.001)[5]. Despite analysis, DM patients without complications showed no statistically significant difference from CG in PON1 55 gene polymorphism[5]. No statistically significant variation in PON1 55 LL, LM, and MM genotype frequency was observed between PG and CG in the current study. The results demonstrate a closer relationship between PON gene polymorphisms and DM complications compared to the disease itself.

A statistically significant difference (p = 0.02) in PON1 192 QQ, QR, and RR genotype frequencies was observed between PG and CG controls in the El-Lebedy D et al.[4] study, with higher frequencies in the PG. The study of Helaly et al.[5] showed a greater prevalence of PON1 192 polymorphism's QQ and QR genotypes among DM patients with complications versus the CG. PON1 192 QQ, QR, and RR genotype frequencies were similar in DM patients without complications and the CG (p>0.05) 5. Regarding PON1 192 genotype frequencies (QQ, QR, RR), our study found no statistically significant difference between PG and CG. The insignificant difference statistically hints at a possibly stronger connection between PON gene polymorphisms and complications. More studies of pediatric T1DM patients are necessary for improved comparative analysis.

A study 4 showed significantly higher (p = 0.03) L, M, Q, and R allele frequencies of PON1 55and

PON1 192 gene polymorphisms among PG compared to CG. The study by Helaly et al.[5] revealed no statistically significant difference. Likewise, the present study revealed no statistically significant variations in allele frequencies between the CG and PG.

Zhou et al. investigated the relationship between PON1 lactonase activity, its genetic variations, and gestational DM (GDM) in newborns[16]. The study included 362 neonates born to mothers with GDM and 302 control neonates. Increased lactonase activity and PON1 levels were observed in neonates of mothers with GDM, researchers reported[16]. A novel founding from their research was the elevated level and lactonase activity of PON1 in neonates born to mothers with GDM. In addition, they reported that PON1 polymorphisms were influenced by the -108C/T and/or 192Q/R variants and lipoprotein metabolism issues[16].

Adult studies complement the data already presented on neonates, children, and adolescents. Grzegorzewska et al.[6] found an association between the PON1 rs705379 TT genotype and T1DM nephropathy among those on hemodialysis. Higher atherosclerosis risk is linked to the rs854560 T allele; the T alleles of both PON1 SNVs result in decreased serum PON1 activity from lower expression. Preventing T1DM nephropathy and atherosclerosis may involve targeting increased diminished PON1 activity[6]. The research by Nawaka et al.[8] focused on PON1 activity and genetic variations in individuals with T2DM and comorbid chronic kidney disease (CKD). The PON1 L55M and Q192R polymorphisms were found to affect PON1 activity. Conversely, the PON1 L55M and Q192R polymorphisms may prove unsuitable genetic biomarkers for CKD in type 2 DM. Yari et al.[17] reported, via a case-control study, that rs2891168 and rs662 gene variants may increase T2DM and coronary artery disease risk (CAD).

To the best of our knowledge, this study is the initial investigation into the connection between paraoxonase gene polymorphism and pediatric DM. Other studies in this field are related to the adult age group. Research on adults primarily investigates PON1 activity, rather than PON gene polymorphism.

CONCLUSION

This dataset shows no significant relationship between DM and polymorphisms in the PON1 55 and PON1 192 genes; however, to enhance the accuracy of assessing this association in pediatric DM patients, a more substantial number of patient and control samples is recommended.

Ethics Committee Approval: All procedures were conducted in accordance with institutional and national ethical standards, and the 2008 Helsinki Declaration revision. The ethics committee (Approval No. 334, 05.08.2014) approved our study, and we obtained informed consent from all participants' legal representatives.

Conflict of Interest: The authors declared no conflicts of interest.

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