DOI: 10.34057/PPj.2024.43.01.2023-10-8
Pelviperineology 2024;43(1):4-11

Molecular and *in silico* evidence: *DR4* gene rs20576 and rs6557634 variants are effective in the development of uterine leiomyoma

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Citation: Halıcı T, Akbaba E, Özdemir Ç, Dede B, Sezgin B, Edgünlü T. Molecular and *in silico* evidence: *DR4* gene rs20576 and rs6557634 variants are effective in the development of uterine leiomyoma. Pelviperineology 2024;43(1):4-11

ABSTRACT

Objectives: Leiomyomas are the most common benign tumours in women of reproductive age. In this study, we aimed to investigate the role of death receptor 4 (*DR4*) gene polymorphisms in the pathogenesis of leiomyoma.

Materials and Methods: In our study, 76 patients were diagnosed with leiomyoma and 81 patients without leiomyoma as healthy controls. The polymerase chain reaction-restriction fragment length polymorphism method was used to identify DR4 polymorphisms. We also determined the protein function and stability of rs20576 and rs6557634 variants by *in silico* approaches.

Results: There was a difference in the distribution of the genotypes of *DR4* gene rs20576 and rs6557634 polymorphisms between leiomyoma patients and health control groups (p=0.014 and p=0.039, respectively). In addition, the distribution of C alelle frequency of rs20576 and G allelle frequency of rs6557634 were significantly higher in leiomyoma patients (p=0.018 and p=0.029, respectively) and the A allele of both rs20576 and rs6557634 has had a protective effect against leiomyoma. According to *in silico* analysis results, rs20576 and rs6557634 have deleterious effects on protein function and structure.

Conclusion: The present results showed the association of gene variants of DR4 with leiomyomas. In addition, *DR4* gene rs20576 and rs6557634 polymorphisms effectively decrease protein stability and function. Further studies may be done on various polymorphisms belonging to DR4 receptors.

Keywords: Apoptosis; death receptor 4; gene polymophism; leiomyoma

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Received: 15 November 2023 **Accepted:** 21 November 2023

INTRODUCTION

Leiomyomas are monoclonal benign tumours originating from the smooth muscle cells of the uterus. Although most of the leiomyomas are asymptomatic in women of reproductive age, their incidence reaches 70% by the age of 50.[1] It is the most common cause of hysterectomy. Race, age, family history, parity, hypertension (Ht) and diabetes mellitus (DM) are associated with the risk of developing leiomyoma.[1-4] Genetic factors are associated with leiomyoma development as well. Clonal chromosomal rearrangements, including translocations, duplications and deletions in chromosomes 6, 7, 12, and 14 have been shown to be present in 40-50% of leiomyomas. Mediator complex subunit 12, fumarate hydratase, *HMGA1* and *HMGA2* of gene mutations are involved in the pathogenesis of leiomyoma.[5]

Apoptosis is programmed cell death that occurs in normal and pathological tissues. There is increasing evidence that genetic, environmental and hormonal factors impair apoptotic pathways in leiomyoma development.[6] It has been reported that the regulation of many genes related to apoptosis is disrupted in the development of leiomyoma. Disruption of apoptosis and proliferation in cells is thought to be an important step in the pathogenesis of leiomyomas.[7] There are many studies on apoptotic pathways and tumour formation in the literature.[8-10] The death receptor family is a member of the tumor necrosis receptor superfamily. It has 8 known members. DR4 is known as TRAILR1 or APO-2.[7] It has also been shown in the literature that *DR4* receptor gene polymorphisms, which play a role in the extrinsic pathway of apoptosis, are associated with tumour formation.

Loss of functionality of the apoptosis process at any stage may result in tumour formation. In our literature review, we found that the rs20576 and rs6557634 variants of the *DR4* receptor gene, which plays a role in the extrinsic pathway of apoptosis, have been studied in other diseases. Still, no study has yet been conducted to examine their relationship with leiomyomas. With this study, we aim to contribute to the literature to elucidate leiomyoma's etiopathogenesis by examining the effects of *DR4* gene rs20576 and rs6557634 variants on leiomyoma formation.

MATERIALS AND METHODS

Study Cohort

Our study was conducted within the ethical principles regarding medical research on humans included in the Declaration of Helsinki. The study protocol was approved by Muğla Sıtkı Koçman University Faculty of Medicine Medical Ethics Committee (decision dated: 22.12.2021, numbered: 27/VIII) and all participants provided written informed consent. One hundred-sixty two women over the age of 18, who were not pregnant and had no known malignant disease, and who applied to Muğla Training and Research Hospital Obstetrics and Gynaecology Outpatient Clinic were included in the study. After transabdominal and transvaginal ultrasonographic evaluations of the patients, necessary information was given to a total of 162 women 81 women who were diagnosed with leiomyoma in the case group and 81 healthy women in the control group, and informed consent was obtained from the individuals. Of the patients in the leiomyoma group, 5 were excluded from the study due to the diagnosis of cancer, including 1 leiomyosarcoma, 2 adenocarcinoma *in situ*, 1 smooth muscle cell tumour of undetermined malignant potential, and 1 breast cancer. The study was continued with a total of 157 participants, including the case group consisting of 76 female patients with leiomyoma and the control group consisting of 81 healthy women without leiomyoma. 2 mL were separated from the blood taken routinely and taken into tubes containing 2% ethylenedimethyltetraacetic acid and included in the study.

Genotyping Determination

We isolated DNA from peripheral blood leukocytes with a Hibrigen Blood DNA Isolation Kit (MG-KDNA-02-250; Hibrigen Biotechnology R&D Industry and Trade Inc., Gebze, Kocaeli, Türkiye). By using polymerase chain reaction (PCR)- restriction fragment length polymorphism (RFLP) method, the rs20576 and rs6557634 SNPs in the *DR4* gene were identified. PCR was performed with a 25 µL volume of 100 ng DNA, 20 pmol of each primer, 1.5 mM $MgCl_2$, Thermo Scientific PCR MasterMix. Amplification was performed on an automated Thermal Cycler (Thermo, ABI). Fragment separation at 120 V for 40-50 minutes on a 3.5% agarose gel containing 0.5 mg/mL ethidium bromide was used to determine the RFLP products. For each gel lane, a 100-bp DNA ladder (Fermentas Vilnius, Lithuania) was used as a size reference. The gel was viewed using a gel electrophoresis visualizing system (Cleaver Scientific Ltd., Clear View UV Trans illuminator, Rugby, UK) under UV light. PCR and RFLP conditions are shown in Table 1.

Statistical Analysis

The sample size of the study was calculated as 153 people.[11] Descriptive statistics; numbers for categorical variables, mean and standard deviation values for continuous variables and percentages is used. It is planned to use Kolmogorov-Smirnov, Shapiro-Wilk tests for normality in univariate analyzes, Student's t-test in cases where two means with the parametric condition are compared and for non-parametric cases, it is planned to

use the Mann-Whitney U test. Pearson chi-square value for the parametric condition and Fisher's Exact test result for the non-paremetric condition were given for the categorical variables. In the analytical analyzes, $p<0.05$ was accepted as the significance limit. SPSS 23.0 package program was used for statistical analysis.

Prediction of Deleterious Missense SNPs

PolyPhen-2 (Polymorphism Phenotyping v2) (http://genetics.bwh.harvard.edu/pph2/) tool was used to show the deleterious effect of the missense variants (rs20576 and rs6557634) on the protein. The PolyPhen-2 predicts the possible impact of amino acid changes on the stability and functionality of human proteins using structural and comparative evolutionary considerations.[12]

Protein Stability Change Prediction

The change in protein stability of rs20576 and rs6557634 variants was analysed with the MUpro (http://mupro.proteomics.ics.uci.edu/) and I-Mutant tools (https://folding.biofold.org/i-mutant/i-mutant2.0.html). MUpro tool uses support vector machine and neural networks machine learning methods. If the confidence score was <0, the mutation has decreased protein stability. But if the confidence score was >0, the mutation has increased protein stability. I-Mutant2.0 is a tool that automatically forecasts changes in protein stability brought on by single-point mutations using support vector machines.[13,14]

Prediction of Gene-gene Interactions

DR4 gene of its association with other genes in order to predict was used, GeneMANIA (https://genemania.org/) (accessed on 27 August 2023). The prediction of gene-gene interaction predictor GeneMANIA is based on the basis of co-localization, pathways, protein domain similarity, co-expression and genetic and protein interaction.[15]

RESULTS

Associations of DR4 Variants with Uterine Leiomyomas

We included 157 participants in our study, including 76 case groups and 81 control groups. While 56.7% of the participants applied to our outpatient clinic due to any gynaecological symptoms, 43.3% applied for routine annual control. We obtained anamnesis information from the participants. Accordingly, we detected Ht in 26.1% and DM in 17.2% of them. The socio-demographic and disease characteristics of the research group are shown in Table 2.

We compared socio-demographic variables such as body mass index (BMI), age and parity of the participants between the case and control groups (Table 3). Accordingly, the mean age of the case group was 44.81±6.97 years and the mean age of the control group was 43.23±9.78 years. There was no statistically significant difference between the case and control groups in terms of age ($p=0.248$). The mean BMI of the case group was 26.27±3.18 and the mean BMI of the control group was 25.61±3.36. We compared the mean BMI of the case and control groups and we did not find a statistically significant difference ($p=0.550$). The mean parity number in the case group was 1.89±1.05 and the mean parity number in the control group was 1.05±0.87. We compared the mean parity numbers between the case and control groups and we did not find a statistically significant difference ($p=0.210$).

We examined the status of Ht disease in 157 people who participated in the study. While 22 people have Ht disease in the case group, in the control group 19 people. Accordingly, the incidence of Ht in patients with leiomyoma is 28.9%, while the incidence of Ht in the control group is 23.5%. We did not find a statistically significant difference between the case group

Table 1. PCR and RFLP conditions used for the polymorphisms of *DR4* gene

(a) PCR conditions used for the polymorphisms of *DR4* gene

Gene	Polymorphism	Primers	Temperature of annealing	Product size
DR4	rs20576	P1 P2	61 °C	201 bp
	rs6557634	P3 P4	59 °C	230 bp

(b) Restriction enzymes, digestion conditions and restriction fragment sizes

Gene	Polymorphism	Restriction enzyme	Digestion conditions	Restriction fragment sizes
DR4	rs20576	*Taq*I	37 °C, 3 h	C allele: 201 bp A allele: 110 bp, 91 bp
	rs6557634	*Bse*GI	37 °C, 3 h	G allele: 230 bp A allele: 160 bp, 70 bp

P1: 5'-ATCCCACCTGGCCAGCTTTCCA–3' P2: 5'-AGACAGGAGTCTCGGGCTGCT–3'; P3: 5' ATCCTCTGGGAACTCTGTGG–3' P4: 5'-TACCACTCCCACCTTCACTGC–3'; PCR: polymerase chain reaction; RFLP: restriction fragment length polymorphism

Table 2. Distribution of the research group according to socio-demographic and disease characteristics			
Variables		**Number (n)**	**Percentage (%)**
Research group (n=157)	**Case**	76	48.4
	Control	81	51.6
Symptom (n=157)	+	89	56.7
	-	68	43.3
Symptom type (n=89)	**Bleeding**	44	49.4
	Pain	19	21.3
	Other	26	29.2
Hypertension (n=157)	+	41	26.1
	-	116	73.9
Diabetes mellitus (n=157)	+	27	17.2
	-	130	82.8

Table 3. Evaluation of the principled consistency in the selection of the control group versus the case group in determining the risk posed by the factor in the study with some socio-demographic variables			
Variables	**Research group (n=157)**		***p****
	Case (n=76)	**Control (n=81)**	
	Mean ± SD	**Mean ± SD**	
Age	44.81±6.97	43.23±9.78	0.248
BMI	26.27±3.18	25.61±3.36	0.550
Parity	1.89±1.05	1.05±0.87	0.210
SD: standard deviation; BMI: body mass index			

and the control group in terms of the incidence of Ht (p>0.05) (Table 4). We examined the DM disease status of 157 people who participated in the study. While 17 people have DM disease in the case group, in the control group 10 people. Accordingly, while the incidence of DM was 22.4% in the case group, it was 12.3% in the control group, and we did not find a statistically significant difference between the case group and the control group in terms of DM frequency (p>0.05) (Table 4).

Genotype distributions of *DR4* gene rs20576 polymorphism were examined, we detected 48.7% CC, 50% CA and 1% AA genotype in the case group and 29.6% CC, 63% CA and 7.4% AA genotype in the control group. Considering the genotype frequencies, the rs20576 polymorphism was significant between the two groups (p=0.014) (Table 5). Genotype distributions of *DR4* gene rs6557634 polymorphism are examined, and we detected 60.5% GG, 23.7% GA and 15.8% AA genotype in the case group and 40.7% GG, 39.5% GA and 19.8% AA genotype in the control group. Considering the genotype frequencies, the rs6557634 polymorphism was significant between the two groups (p=0.039) (Table 5). rs20576 for the CC genotype and rs6557634 for the GG genotype was the risk genotype for uterine leiomyomas. Allele frequencies differed significantly between the two groups. In the patient group of rs20576, the C allele was higher than the control group, while the G allele of rs6557634 was higher than the control group (p=0.018 and p=0.029, respectively) (Table 5). However, we found that the increased frequency of A allele was protective in terms of the risk of myoma formation.

In order to find the genotype that made the difference, we evaluated the genotypes in the case and control groups in the four-eyed table with pairwise comparisons (Table 6). Accordingly, when the difference between the mean percentages of CC and CA genotypes of rs20576 polymorphism in the case and control groups is considered; we found the mean percentage of CC genotype in the case group (49.3%) to be statistically significantly higher than in the control group (32%) (p=0.031). The risk of leiomyoma in those with the CC genotype was 2.069 times higher than the control group. Considering the difference between the mean percentages of CC and AA genotypes of rs20576 polymorphism in the leiomyoma and control groups; we found the mean percentage of CC genotype (97.4%) in the case group to be statistically significantly higher than in the control group (80%) (p=0.038). The risk of leiomyoma in those with the CC genotype was 9.250 times higher than in the control group. Considering the difference between the average percentages of

Table 4. Evaluation of the relationship of the research group between the presence of Ht and DM and the presence of leiomyoma

Variables						*p**
		Case		Control		
		Number	Percentage (%)	Number	Percentage (%)	
Ht (n=157)	+	22	28.9	19	23.5	0.434
	-	54	71.1	62	76.5	
DM (n=157)	+	17	22.4	10	12.3	0.096
	-	59	77.6	71	87.7	

* Pearson chi-square; DM: diabetes mellitus

Table 5. Distributions of DR4 genotypes and allele frequencies in between case and control groups

	Case n (%)	Control n (%)	χ^2 *p*-value	OR (95% CI)
Genotype rs20576				
CC	37 (48.7)	24 (29.6)	**0.014**	
CA	38 (50)	51 (63)		
AA	1 (1.3)	6 (7.4)		
Allele rs20576				Reference
A	40 (26.3)	63 (38.9)	**0.018**	0.561 [0.347-0.962]
C	112 (73.7)	99 (61.1)		1.304 [1.056-1.609]
Genotype rs6557634				
GG	46 (60.5)	33 (40.7)	**0.039**	
GA	18 (23.7)	32 (39.5)		
AA	12 (15.8)	16 (19.8)		
Allele rs6557634				Reference
A	42 (27.6)	64 (39.3)	**0.029**	0.591 [0.367-0.949]
G	110 (72.4)	99 (60.1)		1.275 [1.033-1.573]

OR: odds ratio; CI: confidence interval

CA and AA genotypes of rs20576 polymorphism in the case and control groups; the mean percentage of CA genotype in the case group (97.4%) was not statistically significant compared to the control group (89.5%) (p=0.235).

To find the genotype that made the difference, we evaluated the rs6557634 genotypes in the case and control groups in the four-eyed table with pairwise comparisons (Table 6). Accordingly, when the difference between the mean percentages of GG and GA genotypes of rs6557634 polymorphism in the case and control groups is examined; we found the mean percentage of GG genotype (71.9%) in the case group to be statistically significantly higher than in the control group (50.8%) (p=0.014). The risk of leiomyoma in those with GG genotype was 2.478 times higher than the control group. Considering the difference between the mean percentages of GG and AA genotypes of rs6557634 polymorphism in the case and control groups; we did not find the mean percentage of GG genotype (79.3%) in the case group to be statistically significant compared to the control group (67.4%) (p=0.161). Considering the difference between the mean percentages of GA and AA genotypes of rs6557634 polymorphism in the case and control groups; we did not find the mean percentage of GA genotype (60%) in the case group to be statistically significant compared to the control group (66.7%) (p=0.550).

Prediction of Deleterious Missense SNPs

The deleterious effects of rs6557634 (H141R) and rs20576 (E228A) were demonstrated *in silico*. As a result, deleterious effects were detected in the Polyphen-2 tool. The expected confidence score is for rs6557634 and rs20576 variant, respectively, 0.824 and 0.712.

Protein Stability Change Prediction

The protein stability was decreased in both the MUpro tool and the I-Mutant 2.0 tool (Table 7). rs6557634 and rs20576 can have a serious effect on the phenotype.

Table 6. Pairwise comparison of the rs20576 and rs6557634 genotypes to determine the group that makes the difference in the relationship between the genotype presence of leiomyoma and the evaluation of the risk of leiomyoma presence

Variables		Genotype				p*	OR [95% CI]
		Case	Control				
		Number	Percentage (%)	Number	Percentage (%)		
rs20576	CC	37	49.3	24	32.0	0.031*	2.069 [1.066-4.017]
	CA	38	50.7	51	68.0		
rs20576	CC	37	97.4	24	80.0	0.038**	9.250 [1.047-81.701]
	AA	1	2.6	6	20.0		
rs20576	CA	38	97.4	51	89.5	0.235**	4.471 [0.516-38.698]
	AA	1	2.6	6	10.5		
rs6557634	GG	46	71.9	33	50.8	0.014*	2.478 [1.194-5.144]
	GA	18	28.1	32	49.2		
rs6557634	GG	46	79.3	33	67.3	0.161*	1.859 [0.777-4.445]
	AA	12	20.7	16	32.7		
rs6557634	GA	18	60.0	32	66.7	0.550*	0.750 [0.291-1.930]
	AA	12	40.0	16	33.3		

*: Pearson chi-square; **: Fisher's Exact test; OR: odds ratio; CI: confidence interval

Table 7. Effect of variants in protein stability

SNP ID	Aminoacide change	I-Mutant 2.0	RI (kcal/mol)	MUpro	ddG
rs6557634	H141R	Decrease	5	Decrease	-0.67550955
rs20576	E228A	Decrease	9	Decrease	-1.4814493

ddG: delta-delta G; RI: reliability index

Prediction of Gene-gene Interactions

Our findings revealed that DR4 (TNFRSF10A) is physical interaction with 20 genes (*DAP3, CASP8, ARAP1, TNSF10, FADD, TNFRSF10C, MOAP1, TNFRSF10B, TNFRSF10D, TRADD, RASSF1, CFLAR, RIPK1, CASP10, TRAP1, MAO3K1, LRRC47, CPVL, UBL4A* and *NDUFA5*) co-expressed with 19 genes (*DAP3, CASP8, ARAP1, TNSF10, FADD, TNFRSF10C, MOAP1, TNFRSF10B, TNFRSF10D, TRADD, RASSF1, CFLAR, RIPK1, CASP10, TRAP1, MAO3K1, LRRC47, UBL4A* and *NDUFA5*), shared a domain with 11 genes (*CASP8, ARAP1, FADD, TNFRSF10C, TNFRSF10B, TNFRSF10D, TRADD, RASSF1, CFLAR, RIPK1* and *CASP10*) (Figure 1). A total of 408 connections were determined.

DISCUSSION

Leiomyomas are a common pathology in women of reproductive age and its etiopathogenesis has not been fully elucidated yet. We believe that any pathology that may affect apoptosis mechanisms may trigger monoclonal proliferation in myometrial cells and therefore may play a role in leiomyoma etiopathogenesis. Although single nucleotide polymorphisms do not directly cause disease, they can affect the formation of the disease and the responses to the treatments to be applied. We see that the DR4 receptor, one of the TRAIL receptors in the apoptosis extrinsic pathway, is frequently studied in the literature in terms of its association with diseases. In our study, we found that CC and GG genotype variants, respectively, of homozygous genotypes of *DR4* gene rs20576 and rs6557634 polymorphisms, were statistically significantly higher in patients with leiomyomas. Among the allele frequencies of *DR4* gene rs20576 and rs6557634 polymorphisms, we detected statistically significantly higher frequencies of C and G alleles in myoma patients, respectively, and we also found that the increase in A allele frequencies in both rs20756 and rs6557634 polymorphisms was statistically significantly protective in terms of the risk of leiomyoma formation. In addition, we determined that the *DR4* gene

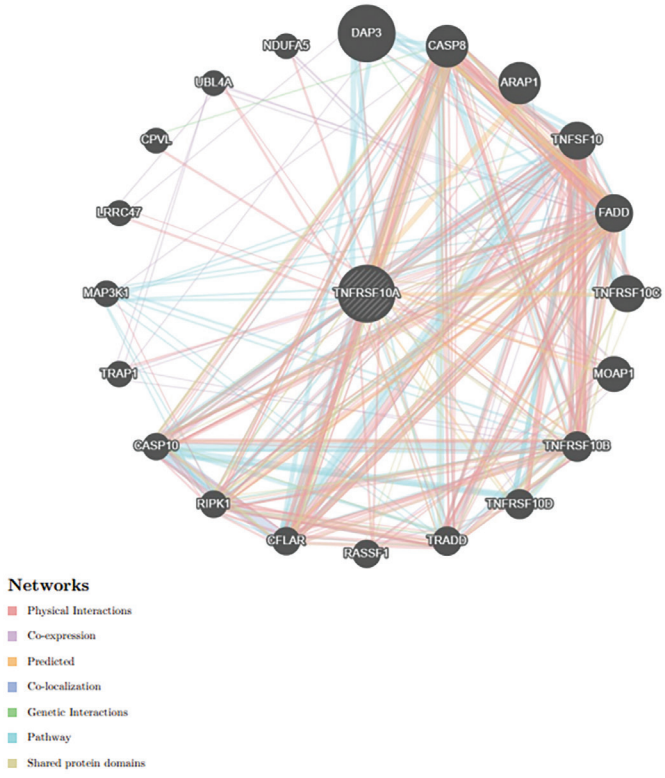

Figure 1. Gene-gene interaction network of DR4

rs20756 and rs6557634 variants significantly reduced protein stability and function.

Previous studies investigating the association of *DR4* gene variants with different diseases support our molecular results. Edgünlü et al.[11] found that the *DR4* gene rs6557634 polymorphism has a protective affect on Alzheimer's disease. In a study that investigated the association of *DR4* gene variants with prostate cancer risk in a North Indian population, it was found that rs6557634 polymorphism AA genotype and A allele, rs4871857 polymorphism GG genotype and G allele increased the risk.[16] Rai et al.[17] investigated the *DR4* gene polymorphisms rs20576 and rs6557634 in their study, in which they investigate the relationship of complex interactions of ADRB3 variation with other candidate gene variants with gallbladder cancer. In their study, they found that heterozygous genotypes of *rs20576* and *rs6557634* gene polymorphisms increased the risk of gallbladder cancer.[17] Körner et al.[18], in their study on the relationship between *DR4* gene rs20575 and rs20576 polymorphisms in hepatitis C virus (HCV) infected patients, and the risk of hepatocellular carcinoma (HCC) stated that genotype distribution were not different between healthy controls and HCV-positive patients without HCC. However, they found the frequencies of rs20575 and rs20576 to be statistically significantly high in patients with HCC. They associated the risk of HCC with the allele of rs20575 and the homozygous AA genotype of rs20576 and determined the coexistance of the two as indenpendent risk factors in the development of HCC. In adition, they found the HCV viral load to be statistically significantly higher in patients carrying the allele of rs20575 and the homozygous AA genotype of rs20576 simultaneously.[18] Kim et al.[19] in their study investigating the relationship between polymorphisms of TRAIL, TRAIL receptors and osteoprotegerin genes, and endometriosis, stated that *DR4* gene rs20575 and rs2230229 polymorphisms were not observed, and that there was no statistically significant difference in the genotype distributions of TRAIL, DR4, DR5 and OPG and the allele frequency distributions of single or combined polymorphisms between the groups with endometriosis disease and those without endometriosis disease.

CONCLUSION

In our study, we investigated the role of allele and genotype frequencies of *DR4* gene rs20576 and rs6557634 variants in leiomyoma etiopathogenesis and we believe that these variants may have a role in the development of leiomyomas. In addition, our *in silico* analyses showed that rs20576 and rs6557634 variants may play a role in the pathogenesis of various diseases, especially leiomyomas. Our study is the first to investigate the relationship between *DR4* gene rs20576 and rs6557634 variants and leiomyoma etiopathogenesis. The relationship of DR4 with leiomyomas may be manifested more precisely if polymorphisms of the *DR4* gene, gen expression, epigenetic mechanisms, and TRAIL protein levels in serum are analysed and evaluated together in a larger group of patients.

ETHICS

Ethics Committee Approval: The study protocol was approved by Muğla Sıtkı Koçman University Faculty of Medicine Medical Ethics Committee (decision dated: 22.12.2021, numbered: 27/VIII).

Informed Consent: All participants provided written informed consent.

Contributions

Concept: T.H., E.A., T.E.; Design: T.H., E.A., B.S., T.E.; Data Collection or Processing: T.H., Ç.Ö., B.D., T.E.; Analysis or Interpretation: T.H., E.A., Ç.Ö., B.D., B.S., T.E.; Literature Search: T.H., E.A., Ç.Ö.; Writing: T.H., E.A., B.D., B.S.

DISCLOSURES

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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