ORIGINAL ARTICLE

Relationship Between TMPRSS6 Polymorphisms and Serum Iron Status in the Treatment of Iron Deficiency Anemia in Subclinical Hypothyroidism: A Pilot Study

Subklinik Hipotiroidizmde Demir Eksikliği Anemisinin Tedavisinde TMPRSS6 Polimorfizmleri ile Serum Demir Durumu Arasındaki İlişki: Pilot Çalışma

Nurten Bahtiyar[1] Birsen Aydemir[2] Deniz Ahmet Cinemre[3]

Güneş Cihan Cinemre[3] Cengiz Karacaer[4] Leyla Sevinç Afşar[5]

Fatma Behice Serinkan[6]

1- Department of Biophysics, Cerrahpaşa Faculty of Medicine, İstanbul University-Cerrahpaşa, İstanbul, Türkiye
2- Department of Biophysics, Faculty of Medicine, Sakarya University, Sakarya, Türkiye
3- University of Pittsburgh Kenneth P. Dietrich School of Arts and Sciences, Pittsburgh, PA, USA
4- Department of Internal Medicine, Faculty of Medicine, Sakarya University, Sakarya, Türkiye
5- Department of Institute of Medical Biochemistry, Institute of Health Sciences, Sakarya University, Sakarya, Türkiye
6- Department of Medical Biochemistry, Faculty of Medicine, Sakarya University, Sakarya, Türkiye

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Correspondence:
Birsen Aydemir, Department of Biophysics, Faculty of Medicine, Sakarya University, Sakarya, Türkiye.

E mail: baydemir@sakarya.edu.tr

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ABSTRACT

Objective: *This study aims to investigate the impact of three SNPs (rs4820268, rs2235321, rs855791) in the TMPRSS6 gene on iron metabolism in patients with subclinical hypothyroidism and iron deficiency anemia (IDA), as well as to evaluate the effects of iron-only and iron-thyroxine combination treatments on individuals with heterozygous and mutant genotypes.*

Material and Method: *Ninety-five patients with subclinical hypothyroidism and IDA, alongside 30 healthy controls, participated in the study. Participants were grouped as follows: Control (N=30), Hypothyroidism (N=30), Hypothyroidism IDA (N=30), and Hypothyroidism IDA+Thyroxine (N=35). TMPRSS6 rs4820268 and rs855791 polymorphisms were analyzed using TaqMan SNP Genotyping Assays and qPCR, while rs2235321 was genotyped using Allele-Specific PCR (ASPCR). Complete blood count, iron levels, and total iron-binding capacity (TIBC) were measured with an automated analyzer, while ferritin levels were analyzed using immunoassay.*

Results: *No significant differences were found in the genotype and allele distributions of TMPRSS6 polymorphisms (rs4820268, rs2235321, rs855791) between patients and controls. For rs4820268 and rs855791 heterozygous and mutant genotypes, ferritin levels were lower in the Hypothyroidism and Hypothyroidism IDA groups compared to controls, while TIBC was higher in the Hypothyroidism group. Ferritin was elevated and TIBC decreased in the Hypothyroidism IDA+Thyroxine group compared to Hypothyroidism and Hypothyroidism IDA groups. In terms of the rs2235321 polymorphism, iron and ferritin levels were higher in the Hypothyroidism+IDA+Thyroxine group than in the other hypothyroidism groups, but TIBC was lower.*

Conclusion: *No significant variation was observed in the genotype and allele frequencies of TMPRSS6 polymorphisms between healthy individuals and those with hypothyroidism. Nevertheless, considering the relationship between hypothyroidism, iron metabolism, and treatment response, particularly in patients receiving combined therapy, treatment-related changes in TIBC and ferritin levels were observed.*

Keywords: *Subclinical Hypothyroidism, Iron Deficiency Anemia, TMPRSS6 Gene, SNP.*

ÖZET

Amaç: *Bu çalışma, TMPRSS6 genindeki üç tek nükleotid polimorfizminin (SNP) (rs4820268, rs2235321, rs855791) subklinik hipotiroidizm ve demir eksikliği anemisi (DEA) olan hastalarda demir metabolizması üzerindeki etkilerini incelemeyi ve heterozigot ve mutant genotiplere sahip bireylerde yalnızca demir ve demir-tiroksin kombinasyon tedavilerinin etkilerini değerlendirmeyi amaçlamaktadır.*

Gereç ve Yöntem: *Çalışmaya subklinik hipotiroidizm ve DEA tanılı 95 hasta ile 30 sağlıklı kontrol dahil edilmiştir. Katılımcılar şu gruplara ayrılmıştır: Kontrol (N=30), Hipotiroidizm (N=30), Hipotiroidizm+DEA (N=30) ve Hipotiroidizm+DEA+Tiroksin (N=35). TMPRSS6 rs4820268 ve rs855791 polimorfizmleri TaqMan SNP Genotipleme Analizleri ve qPCR ile, rs2235321 polimorfizmi ise Alel-Spesifik PCR (ASPCR) yöntemi ile genotiplendirilmiştir. Tam kan sayımı, demir seviyeleri ve total demir bağlama kapasitesi (TDBK) otomatik analizör ile ölçülürken, ferritin seviyeleri immünoassay yöntemi ile analiz edilmiştir.*

Bulgular: *TMPRSS6 polimorfizmlerinin (rs4820268, rs2235321, rs855791) genotip ve alel dağılımlarında hasta ve kontrol grupları arasında anlamlı bir fark saptanmamıştır. Rs4820268 ve rs855791 heterozigot ve mutant genotipleri açısından incelendiğinde, Hipotiroidizm ve Hipotiroidizm+DEA gruplarında ferritin seviyeleri kontrol grubuna göre daha düşük, TDBK ise Hipotiroidizm grubunda daha yüksek bulunmuştur. Hipotiroidizm+DEA+Tiroksin grubunda ise ferritin seviyeleri artarken, TDBK düşmüştür. rs2235321 polimorfizmi açısından, Hipotiroidizm+DEA+Tiroksin grubunda demir ve ferritin seviyeleri diğer hipotiroidi gruplarına göre daha yüksekti, ancak TDBK daha düşüktü.*

Sonuç: *TMPRSS6 polimorfizmlerinin genotip ve alel frekanslarında sağlıklı bireyler ile hipotiroidi hastaları arasında anlamlı bir farklılık gözlenmemiştir. Bununla birlikte, hipotiroidizm, demir metabolizması ve tedavi yanıtı arasındaki ilişki dikkate alındığında, özellikle kombine tedavi alan hastalarda TDBK ve ferritin seviyelerinde tedaviye bağlı değişiklikler gözlemlenmiştir.*

Anahtar Kelimeler: *Subklinik Hipotiroidizm, Demir Eksikliği Anemisi, TMPRSS6 Geni, SNP*

INTRODUCTION

Thyroid hormones play a crucial role in normal development, metabolic balance, and the physiological functions of body tissues. Among hormonal disorders, hypothyroidism, characterized by insufficient secretion of thyroid hormones, represents the largest category. Subclinical hypothyroidism is a thyroid disorder in which thyroid-stimulating hormone (TSH) levels are elevated above normal, while free thyroid hormone levels remain within the normal range in the blood (1-3).

Subclinical hypothyroidism, a common clinical issue, has a prevalence of 4–10% in the general population and exceeds 20% in women over 60 years of age (4). In addition, the evaluation and therapy of subclinical thyroid dysfunction is a controversial issue, and there are different practices in the therapy of patients with TSH values of 4.5-10 mIU/liter in consensus panels conducted in different years (2,5,6).

The prevalence of anemia is high in individuals with hypothyroidism. Therefore, it is crucial to identify the points where iron metabolism intersects with thyroid hormone action and signaling pathways. Several factors are involved in iron metabolism, and several rare genetic markers with a significant impact on this process have been identified. Genome-wide association studies have pinpointed several single nucleotide polymorphisms (SNPs) that contribute to erythropoiesis and have a lesser impact on iron metabolism (7-9). Research has suggested that SNPs in the transferrin (TF), human hemochromatosis (HFE), transferrin receptor 2 (TFR2), and transmembrane protease, serine 6 (TMPRSS6) genes are genetic risk factors influencing iron homeostasis. Matriptase-2, encoded by the TMPRSS6 gene, is a serine protease that inhibits hepcidin expression by cleaving membrane-bound hemojuvelin (10).

Hepcidin is a key regulator of human iron homeostasis, controlling both dietary iron absorption and iron release by macrophages (11). Several studies have identified multiple TMPRSS6 SNPs that are associated with iron-refractory iron deficiency anemia (IRIDA) and iron deficiency anemia (IDA), as well as low iron and blood indices. These SNPs were classified into synonymous, missense, intron, 5'-UTR, and intergenic variants. The most frequently reported TMPRSS6 SNPs, rs855791 and rs4820268, have been linked to poor iron status biomarkers and low blood indices. Other TMPRSS6 SNPs, such as rs2235321, rs2235324, rs5756504, rs5756506, and rs1421312, were also associated with iron deficiency biomarkers (12-18). Most of the studies on TMPRSS6 SNPs affecting biochemical parameters have been conducted in Caucasian populations, with fewer studies in Asian populations (13-17). Several studies have found specific SNPs in TMPRSS6 to be associated with iron and hematological parameters (8,19,20). Batar et al. suggested in their study that variations in TMPRSS6 may not be a risk factor for IDA. However, they found that TMPRSS6 polymorphisms were associated with increases in various iron-related hematological parameters (21). Our study aims to explore the impact of three SNPs (rs4820268, rs2235321, and rs855791) within the TMPRSS6 gene on patients with subclinical hypothyroidism. Additionally, we seek to assess how iron-only and combined iron and thyroxine treatments influence iron metabolism in individuals with heterozygous and mutant genotypes.

MATERIALS AND METHODS

Case selection

The study included 95 patients diagnosed with newly identified IDA and subclinical hypothyroidism, referred to the Internal Medicine outpatient clinic at Sakarya University Training and Research Hospital, along with 30 healthy individuals. Participants were divided into four groups based on complete blood count, iron levels, iron-binding capacity, ferritin, TSH, and free thyroxine (fT4) values: a Control group (N=30), a Hypothyroidism group of untreated subclinical hypothyroid patients (N=30), a Hypothyroidism IDA group of subclinical hypothyroid patients with IDA receiving iron treatment (80 mg ferrous sulfate) (N=30), and a Hypothyroidism IDA + Thyroxine group of subclinical hypothyroid patients with IDA receiving both iron and thyroxine treatment (80 mg ferrous sulfate and 25 µg levothyroxine) (N=35). All participants gave informed consent, and the study protocol was approved by the Ethics Committee of Sakarya University Medical Faculty (29.09.2016-E.12812).

Sample collection

Blood samples were collected after an overnight fast, using tubes containing ethylenediaminetetra-acetic acid (EDTA) and tubes without anticoagulants (Vacuette®, Z Trace Elements Serum Clot Activator; Greiner Bio-One GmbH, Austria). Whole blood in tubes without anticoagulants was allowed to clot and then centrifuged at 1500×g for 10 minutes to separate the serum. The samples were stored at -20 °C until further biochemical analysis. Complete blood count was performed using the Abbott Diagnostics Cell Dyn 3700 hematology analyzer (Abbott Diagnostics, IL, USA). Iron levels and total iron-binding capacity (TIBC) were measured using the Abbott Architect C16000 autoanalyzer (Abbott Diagnostics, IL, USA). TSH, fT4, and ferritin were analyzed using the Abbott ARCHITECT i2000SR immunoassay analyzer (Abbott Diagnostics, IL, USA).

DNA isolation and TMPRSS6 polymorphisms analysis

Genomic DNA was isolated from peripheral blood using a DNA extraction kit (Jena Bioscience GmbH, Jena, Germany) according to the manufacturer's instructions. The concentration and purity of the DNA samples were determined using a NanoDrop spectrophotometer, which measured the 260/280 nm optical density ratio. TaqMan® SNP Genotyping Assays (Applied Biosystems, Life Technologies, USA) and qPCR ProbesMaster (Jena Bioscience, Germany) were used to investigate the polymorphisms of rs4820268 and rs855791 related to the TMPRSS6 gene, as indicated in Table 1. According to the qPCR ProbesMaster protocol, each reaction was prepared as follows: 10 µL of qPCR Probes Master, 1 µL of PrimerProbMix, 7 µL of PCR-grade water, and 2 µL

Table 1: Context sequences of the investigated TMPRSS6 polymorphisms (rs4820268 and rs855791)

SNP ID	Context sequence [VIC/FAM]	
rs855791	GCGTGGCGTCACCTGGTAGCGATAG CCTCGCTGCACAGGTCCTGTGGGAT	[A/G]
rs4820268	CCTACCTTCCTGGCACTGCTCTTC TCGCTGCCGTTGAGACAATCAGGCT	[A/G]

Table 2: TMPRSS6 (rs2235321) genetic PCR mixture (final volume 25 µl)

	Stock solution in molarity	Working solution of molarity	Final molarity
PCR Buffer	10X	—	1X
Primers C, A, Common	100 µM	10 µM	0.4 µM
dNTPs	100 mM	2 mM	0.2 mM
Taq Polymerase	5U/µM	—	1 U
DNA	—	—	~50ng

Table 3: Bands formed after electrophoresis of TMPRSS6 (rs2235321) gene

Amplification PCR Product	Normal homozygous (GG)		Heterozygous (AG)		Mutant homozygous (AA)	
	C tube	A tube	C tube	A tube	C tube	A tube
TMPRSS6 (rs2235321) (122 bp)	—		—	—		—

of DNA on ice. PCR amplification was performed using a Bio-Rad CFX device (Bio-Rad Laboratories, Irvine, CA, USA) with Bio-Rad CFX Manager Software. Amplification was performed on a Bio-Rad CFX device with an initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. SNP genotypes were determined from fluorescence data using allelic discrimination plots and amplification curves.

Genotyping of the TMPRSS6 (rs2235321) polymorphism was conducted using Allele-Specific PCR (ASPCR), a sensitive and specific method for detecting single nucleotide changes. This technique relies on primers binding to the mutation site, with amplification occurring only if the mutation is present. PCR mixture information of rs2235321 is shown in Table 2. The primers used were: Common primer: 5'-ATCCTTTCTCCCTCCTCTCT-3', Primer C (normal allele): 5'-AGCGAGGTCTATCGCTTT-3', Primer A (mutant allele): 5'-AGCGAGGTCTATCGCTTC-3'. Each sample was tested in two PCR tubes: one with Primer C and the common primer, and the other with Primer A and the common primer. Genotypes were determined as follows: GG (normal homozygous): Amplification in C tube only, AA (mutant homozygous): Amplification in A tube only, AG (heterozygous): Amplification in both tubes. PCR conditions included an initial denaturation at 95°C for 5 minutes, followed by 45 cycles of 95°C for 30 seconds, 60°C for 1 minute, and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. The amplified products (122 bp) were separated on a 3% agarose gel, stained with ethidium bromide, and visualized under UV light for genotype determination. The bands formed after electrophoresis of the TMPRSS6 (rs2235321) gene are presented in Table 3.

Statistical Analysis

Statistical analyses were performed using SPSS Statistics 21.0 software. The Hardy-Weinberg equilibrium was tested using Chi-square analysis. Genotype and allele frequencies were compared between the patient and control groups using Chi-square analysis. The odds ratio (OR) and 95% confidence intervals (CIs) were calculated to assess the effects of differences in allelic and genotype distributions. Comparisons of other parameters were made using the unpaired Student's t-test (for normally distributed variables) or the Mann-Whitney U test (for non-normally distributed variables). A P-value of < 0.05 was considered statistically significant.

RESULTS

The clinical characteristics are summarized in Table 4. Age and gender distributions were similar across all groups. Serum TSH levels were significantly higher in the Hypothyroidism and Hypothyroidism IDA groups compared to the control group ($P < 0.001$ for both). TSH levels were lower in the

Table 4: Comparison of some biochemical parameters between patients with subclinical hypothyroidism and control subjects

Parameters	Control (N=30)	Hypotyroidism (N=30)	Hypotyroidism IDA (N=30)	Hypotyroidism IDA+Thyroxyne (N=35)
TSH (mIU/L)	2.07±1.29	11.04±11.31a***	10.00±10.90b***	3.93± 1.77d**, e**
fT4 (pmol/L)	12.44±1.23	10.84±3.06	10.98±1.99	12.60± 1.95d*,e*
Erythrocyte (M/µL)	4.76±0.47	4.53±0.53a*	4.51±0.51	4.56±0.55d***,e*
Hct (%)	40.08±4.23	36.52±4.61	36.48±4.40	37.95±5.02
Hb (g/dL)	13.49±1.53	12.02±1.65a*	11.98±1.55b*	12.72±1.84
MCV (fL)	84.38±6.16	80.98±7.84	80.69±6.14	83.01±7.40
MCH (pg)	28.38±2.38	26.72±2.90	26.00±2.63b*	27.64±3.16
MCHC (g/dL)	33.62±0.59	32.89±1.09a*	32.45±1.03b**	32.82±1.47c***
RDW (%)	16.09±1.49	17.89±2.04	17.84±2.21b**	16.78±2.29c*
Iron (µg/dL)	79.10±35.08	58.82±29.19	54.47±20.73	80.43±32.22e*
TIBC (µg/dL)	304.80±102.60	383.50±77.54a***	351.60±93.38b***	282.10±58.83d**,e*
Ferritin (ng/mL)	43.07±39.96	11.01±8.51a***	13.81±7.11b***	34.71± 18.43d**,e**

*Data are presented as mean ± SD. IDA, Iron deficiency anemia, Hct, Hematocrit, Hb, Hemoglobin, MCV, Mean corpuscular volume, MCH, Mean corpuscular haemoglobin, MCHC, Mean corpuscular hemoglobin concentration, RDW, Red cell distribution width, TIBC, Total iron binding capacity, TSH, Thyroid stimulating hormone, fT4, Free thyroxine, aControl vs. Hypotyroidism, bControl vs. Hypotyroidism IDA, cControl vs. Hypotyroidism IDA+Thyroxyne, dHypotyroidism vs. Hypotyroidism IDA+Thyroxyne, eHypotyroidism IDA vs. Hypotyroidism IDA+Thyroxyne, *P<0.05, **P<0.01, ***P<0.001.*

Hypothyroidism IDA + Thyroxine group compared to both the Hypothyroidism and Hypothyroidism IDA groups ($P < 0.01$ for both). fT4 levels were elevated in the Hypothyroidism IDA + Thyroxine group compared to the Hypothyroidism and Hypothyroidism IDA groups ($P < 0.05$ for both). No significant differences in fT4 levels were found among the other groups ($P > 0.05$ for all) (Table 4).

When evaluating biochemical parameters, erythrocyte count, hemoglobin (Hb), mean corpuscular hemoglobin concentration (MCHC), and ferritin levels were lower, while TIBC was higher in the Hypothyroidism group compared to the Control group ($P < 0.05$, $P < 0.05$, $P < 0.05$, $P < 0.001$, and $P < 0.001$, respectively). In the Hypothyroidism IDA group, Hb, mean corpuscular hemoglobin (MCH), MCHC, and ferritin levels were lower, while red cell distribution width (RDW) and TIBC were higher compared to the control group ($P < 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.001$, $P < 0.01$, and $P < 0.001$, respectively). In the Hypothyroidism IDA + Thyroxine group, MCHC was lower, and RDW was higher compared to the control group ($P < 0.001$ and $P < 0.05$, respectively). No significant differences were observed in other parameters ($P > 0.05$).

In the comparison between the Hypothyroidism and Hypothyroidism IDA+Thyroxine groups, erythrocyte count and ferritin were higher in the Hypothyroidism IDA+Thyroxine group, while TIBC was lower ($P < 0.001$, $P < 0.01$, and $P < 0.01$, respectively). In the comparison between the Hypothyroidism IDA and Hypothyroidism IDA+Thyroxine groups, erythrocyte count, iron, and ferritin were higher in the Hypothyroidism IDA+Thyroxine group, while TIBC was lower ($P < 0.05$, $P < 0.05$, $P < 0.01$, and $P < 0.05$, respectively). No significant differences were observed in other parameters ($P > 0.05$).

We investigated the polymorphisms located in the TMPRSS6 gene (rs4820268, rs2235321, and rs855791), and the genotype frequencies for both the patient and control groups are presented in Table 5. Statistical analysis of the allele frequencies and genotype distributions of the rs4820268, rs2235321, and rs855791 SNPs revealed no significant differences. To evaluate whether these gene polymorphisms (rs4820268, rs2235321, and rs855791) influenced iron, TIBC, and ferritin levels, these parameters were compared between the patient groups and the control group for homozygous mutant and heterozygous genotypes (Table 6).

Initially, iron, TIBC, and ferritin levels for the rs4820268 polymorphism were compared between homozygous mutant and heterozygous genotype carriers (Table 6). Ferritin levels were found to be lower in the Hypothyroidism and Hypothyroidism IDA groups compared to the Control group ($P < 0.01$ and $P < 0.05$, respectively). Ferritin levels were also lower in the Hypothyroidism group compared to the Hypothyroidism IDA + Thyroxine group ($P < 0.05$). Furthermore, TIBC levels were higher in the Hypothyroidism group compared to the Control group ($P < 0.05$), and lower in the Hypothyroidism IDA + Thyroxine group compared to both the Hypothyroidism and Hypothyroidism IDA groups ($P < 0.001$ and $P < 0.05$, respectively).

Next, the patient and control groups with homozygous mutant and heterozygous genotypes of the rs2235321 polymorphism were compared (Table 6). It was found that iron levels were higher in the Hypothyroidism IDA + Thyroxine group compared to the Hypothyroidism and Hypothyroidism IDA

Table 5: Distribution of genotype and allele frequencies of rs4820268, rs2235321 and rs855791 SNPs in healthy controls and subjects with subclinical hypothyroidism (untreated, treated with IDA or and IDA+Thyroxine)

Genotype	Control	Hypotyroidism n (%)	Hypotyroidism IDA n (%)	Hypotyroidism IDA+Thyroxyne n (%)
rs4820268 Genotypes				
GG	12 (41)	10 (42)	3 (16)	8 (27)
AG	12 (41)	11 (46)	11 (58)	15 (50)
AA	5 (18)	3 (12)	5 (26)	7 (23)
Allele frequency				
G	36 (62)	31 (65)	17 (45)	31 (52)
A	22 (38)	17 (35)	21 (55)	29 (48)
rs2235321 Genotypes				
AA	2 (10)	3 (14)	2 (11)	2 (6)
AG	13 (65)	18 (82)	15 (83)	29 (91)
GG	5 (25)	1 (4)	1 (6)	1 (3)
Allele frequency				
A	17 (43)	24 (55)	19 (53)	33 (52)
G	23 (57)	20 (45)	17 (47)	31 (48)
rs855791 Genotypes				
AA	10 (34)	10 (40)	1 (5)	5 (16)
AG	18 (62)	14 (56)	18 (90)	26 (81)
GG	1 (4)	1 (4)	1 (5)	1 (3)
Allele frequency				
A	38 (66)	34 (68)	20 (50)	36 (56)
G	20 (34)	16 (32)	20 (50)	28 (44)

Table 6: Effect of TMPRSS6 gene polymorphisms (rs4820268, rs2235321, and rs855791) on iron levels, TIBC, and ferritin in individuals with heterozygous and mutant genotypes

	Control AG+AA	Hypotyroidism AG+AA	Hypotyroidism IDA AG+AA	Hypotyroidism IDA+Thyroxyne AG+AA
rs4820268	**(n=17)**	**(n=14)**	**(n=14)**	**(n=22)**
Iron (µg/dL)	76.82±28.13	58.60±30.76	59.63±19.07	82.48±33.11
TIBC (µg/dL)	310.80±78.84	383.60±81.88a*	365.50±95.91	284.20±53.72c***,d*
Ferritin (ng/mL)	41.46±41.58	11.61±8.78a**	13.98±7.32b*	33.00±16.88c*
rs2235321	**(n=18)**	**(n=19)**	**(n=16)**	**(n=30)**
Iron (µg/dL)	76.65±26.60	49.44±11.02	55.80±21.61	83.04±31.54c**,d*
TIBC (µg/dL)	326.50±74.39	381.40±62.44	353.90±98.36	281.20±57.74c***,d**
Ferritin (ng/mL)	33.38±32.10	10.59±8.74a**	13.76±7.49b*	35.12±18.98c***,d**
rs855791	**(n=19)**	**(n=15)**	**(n=19)**	**(n=27)**
Iron (µg/dL)	78.60±35.30	58.82±29.19	54.47±20.73	83.14±33.94
TIBC (µg/dL)	319.30±89.68	384.00±79.64a*	351.60±93.38	279.60±60.65c***,d*
Ferritin (ng/mL)	41.14±40.21	10.98±8.74a**	13.81±7.11b*	35.01±18.68c*

*Data are presented as mean ± SD. IDA, Iron deficiency anemia, TIBC, Total iron binding capacity, aControl vs. Hypotyroidism, bControl vs. Hypotyroidism IDA, cHypotyroidism vs. Hypotyroidism IDA+Thyroxyne, dHypotyroidism IDA vs. Hypotyroidism IDA+Thyroxyne, *P<0.05, **P<0.01, ***P<0.001.*

groups ($P < 0.01$ and $P < 0.05$, respectively). TIBC levels were lower in the Hypothyroidism IDA + Thyroxine group compared to the Hypothyroidism and Hypothyroidism IDA groups ($P < 0.001$ and $P < 0.01$, respectively). Additionally, ferritin levels were lower in the Hypothyroidism and Hypothyroidism IDA groups compared to the Control group ($P < 0.01$ and $P < 0.05$, respectively), and higher in the Hypothyroidism IDA + Thyroxine group compared to both the Hypothyroidism and Hypothyroidism IDA groups ($P < 0.001$ and $P < 0.01$, respectively).

Finally, the iron, TIBC, and ferritin levels of homozygous mutant and heterozygous genotypes in the rs855791 polymorphism were compared (Table 6). Iron levels were found to be higher in the Hypothyroidism IDA + Thyroxine group compared to the Hypothyroidism IDA group ($P < 0.05$). TIBC levels were higher in the Hypothyroidism group compared to the Control group ($P < 0.05$), and lower in the Hypothyroidism IDA + Thyroxine group compared to both the Hypothyroidism and Hypothyroidism IDA groups ($P < 0.001$ and $P < 0.01$, respectively). Moreover, ferritin levels were lower in the Hypothyroidism and Hypothyroidism IDA groups compared to the Control group ($P < 0.001$ and $P < 0.01$, respectively), and higher in the Hypothyroidism IDA + Thyroxine group compared to both the Hypothyroidism and Hypothyroidism IDA groups ($P < 0.01$ for both).

DISCUSSION

In this study, the three most common SNPs (rs4820268, rs2235321, and rs855791) in the TMPRSS6 gene were investigated in patients with subclinical hypothyroidism and IDA, and the effects of iron-only or combined iron and thyroxine treatments on certain biochemical parameters were evaluated. Although no statistically significant differences were detected among the selected SNPs between the groups, notable changes in iron, TIBC, and ferritin levels were observed in association with the heterozygous and mutant variants of rs4820268, rs2235321, and rs855791 in the treatment of anemia in subclinical hypothyroidism. To the best of our knowledge, this is the first study to examine the status of rs4820268, rs2235321, and rs855791 SNPs in the TMPRSS6 gene in patients with subclinical hypothyroidism and IDA.

Thyroid hormone plays a vital role in metabolism regulation. Thyroid disorders due to iodine deficiency and IDA are major global health concerns. While IDA and thyroid hormone deficiency are treatable (22), subclinical hypothyroidism treatment remains debated (23). Studies suggest IDA treatment in subclinical hypothyroidism is more effective with thyroid hormone supplementation (3, 24), and genetic factors influence iron metabolism (25). TMPRSS6 variants are linked to serum iron levels and hematological parameters in various diseases and healthy individuals (19, 26-30). Genome-wide studies in European and Asian populations identified TMPRSS6 and TF gene variants affecting iron status. A 2009 study on European and South Asian individuals found rs855791 (V736A) as the most associated SNP, with the A allele linked to lower Hb levels via hepcidin regulation (8). In Australian individuals, rs855791 correlated with reduced transferrin saturation, serum iron, Hb levels, and mean corpuscular volume (MCV) in both adolescents and adults (19). Jallow et al. examined the effects of TMPRSS6 and TF SNPs on iron status in 1,316 healthy Gambians from the Keneba Biobank. TMPRSS6 SNPs (rs2235321, rs855791, rs4820268, rs2235324, rs2413450, rs5756506) and TF SNPs (rs3811647, rs1799852) were assessed for iron biomarkers, with some SNPs linked to ferritin, hepcidin, Hb, transferrin, unsaturated iron-binding capacity (UIBC), and transferrin saturation, though their contribution to population variance was minimal (29). Delbini et al. sequenced the TMPRSS6 gene in 16 IRIDA patients, identifying 27 polymorphisms. Eight SNPs and four haplotypes were significantly associated with IRIDA, including rs855791, rs2235320, rs4820268, rs11704654, and rs2543519. These nonsynonymous variants were linked to altered amino acid sequences, leading to dysregulated hepcidin expression and changes in MT-2 catalytic activity (20). Elmahdy et al. found rs855791 and rs4820268 significantly associated with reduced Hb, MCV, MCH, ferritin, and iron levels, along with increased TIBC in IDA and IRIDA patients (31). Another study analyzing multiple TMPRSS6 SNPs in IDA patients found no direct impact on IDA but noted their influence on iron metabolism parameters (21). In our study, no significant differences in TMPRSS6 polymorphisms (rs4820268, rs2235321,

rs855791) were observed between subclinical hypothyroidism and control groups. However, in patients, heterozygous and mutant variants were associated with decreased ferritin and increased TIBC levels.

Poggiali et al. studied the response to iron therapy in relation to TMPRSS6 polymorphisms and found significant differences in the frequencies of common TMPRSS6 polymorphisms, including V736A, SNP-120, SNP-113, F5F, P33P, K253E, S361S, Y418Y, D521D, D15accc, and Y739Y, between persistent IDA patients (with poor oral iron therapy response) and the control group. They also observed that heterozygous and homozygous rs855791 genotypes were associated with lower serum iron, transferrin saturation, Hb, and MCV levels in IDA patients (32). Shinta et al. investigated the effects of dietary iron on anemia and iron deficiency in young children. Data from 121 Indonesian children aged 6–17 months were analyzed. The minor alleles of TMPRSS6 rs855791 (A) and rs4820268 (G) were found to reduce serum ferritin levels by 4.50 g/L and 5.00 g/L, respectively. However, no significant association was observed between these SNPs and soluble transferrin receptor (sTfR) or hemoglobin (Hb) concentrations (13). In our study, no significant differences were found in rs4820268, rs2235321, and rs855791 SNPs between subclinical hypothyroid patients receiving iron therapy and healthy individuals. However, in the patient group, ferritin levels were lower in heterozygous and mutant variants of these SNPs compared to controls. In patients receiving both iron and thyroxine therapy, iron and ferritin levels increased, and TIBC decreased, compared to those receiving only iron therapy. These findings suggest that specific TMPRSS6 SNPs (rs4820268, rs2235321, and rs855791) may influence iron metabolism in subclinical hypothyroid patients undergoing combined therapy.

To the best of our knowledge, polymorphisms in the TMPRSS6 gene have not been studied in hypothyroidism, although they have been observed to contribute to iron deficiency and anemia in diseases such as celiac disease and diabetes. It is seen that the data obtained from these studies are also contradictory. A study on the rs855791 SNP and anemia in celiac disease found that the rs855791 variant was more prevalent in adults with celiac disease compared to a control group. However, no significant difference was observed between celiac patients with persistent IDA and those without IDA regarding the rs855791 polymorphism (33). Another study reported that the T allele of rs855791 was more frequent in celiac patients with persistent IDA and was associated with a lower response to oral iron supplementation (27). Liu et al. observed a trend toward a significant association between the T allele of rs855791 and an increased risk of gestational diabetes mellitus in pregnant Han Chinese women. They also found significant associations between rs855791 and rs4820268 SNPs with serum iron and transferrin saturation, suggesting a link between TMPRSS6 variants and gestational diabetes risk (34). Moremi et al. reported no significant difference in the frequency distributions of the TMPRSS6 c.2207C>T variant between multiple sclerosis patients and controls but found a significant difference in the risk of iron deficiency between homozygous T and C allele carriers, with lower ferritin levels in patients compared to controls (28). Similarly, Gan et al. found that both rs855791 (V736A) and rs4820268 (D521D) SNPs were significantly associated with ferritin, Hb levels, iron overload risk, and type 2 diabetes risk (26).

Several studies have shown that thyroid hormones stimulate red blood cell production (22-25). In hypothyroidism, erythrocyte lifespan is normal, but hypoproliferative erythropoiesis occurs. Various mechanisms are involved in the stimulation of erythropoiesis by thyroid hormones. One of these mechanisms is suggested to be related to erythropoietin, which increases metabolic rate and the resulting increase in oxygen demand. Although the data obtained are closely related to the improvement in iron variables, it has been suggested that stimulation of erythropoiesis by thyroid hormones is not the only mechanism, and that the effects of thyroid hormone on iron metabolism are quite complex. The TMPRSS6 gene, which encodes matriptase-2, plays a direct role in the regulation of dietary iron absorption and utilization. The TMPRSS6 SNP may be associated with an increased risk of iron-restricted erythropoiesis resulting from inadequate iron absorption from dietary sources. Although further functional studies are needed to elucidate the effect of TMPRSS6 polymorphisms on iron deficiency anemia developing in subclinical hypothyroidism, our findings suggest that combined homozygous and heterozygous genotypes in TMPRSS6 affect circulating iron, TIBC and ferritin levels. On the other hand, the distribution of genotypes of these polymorphisms is likely to be associated with various risk factors for the development of iron deficiency anemia. There were some limiting factors in our study. These limiting factors include the fact that our sample groups were collected from the same center and region at certain time intervals and the small number of patients in our groups in terms of follow-up.

CONCLUSIONS

In this study, hypothyroid patients receiving iron therapy were found to have lower ferritin and iron levels and higher TIBC levels compared to healthy individuals. In individuals receiving both iron and thyroid treatment, TIBC levels were found to decrease, while ferritin and iron levels increased. No significant differences were observed in the genotype and allele frequency distributions of TMPRSS6 rs4820268, rs2235321 and rs855791 between healthy and subclinical hypothyroid individuals. However, these polymorphisms affected treatment-related changes, especially in patients receiving combined therapy, where ferritin levels increased and TIBC decreased. These findings highlight the relationship between hypothyroidism, iron metabolism and response to treatment, but further studies in larger sample groups are needed to elucidate the underlying molecular mechanisms.

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

Ethics: The study protocol was approved by the Ethics Committee of the Medical Faculty at Sakarya University (29.09.2016-E.12812), and was conducted in accordance with the Declaration of Helsinki.

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