Evaluation of TFR2 rs7385804 polymorphism as a genetic marker for hemochromatosis in the pakistani population

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Abstract

Hemochromatosis is an autosomal recessive iron overload disorder. The TFR2 gene variant rs7385804 has been implicated in hemochromatosis, but its role in the Pakistani population remains unexplored. This study intended to examine the association of the TFR2 gene variant rs7385804 with hemochromatosis in Pakistani individuals. We employed a case-control study design, recruiting 200 hemochromatosis patients and 200 healthy controls from the Pakistani population. Deoxyribonucleic acid was isolated from blood samples using the phenol-chloroform method. Sanger sequencing and Tetra-ARMS PCR were used to identify the TFR2 genes and their variant rs7385804. Our results showed a significant association between the TFR2 gene variant rs7385804 and hemochromatosis in the Pakistani population ($p < 0.001$). The rate of the abnormal alleles was (35%) higher than that of the control (15%). Sanger sequencing confirmed the presence of the variant in 70% of patients, while Tetra-ARMS PCR showed 90% concordance with sequencing results. This study demonstrates the utility of Sanger sequencing and Tetra-ARMS PCR in detecting the TFR2 gene variant rs7385804 in the Pakistani population. Our findings suggest that this variant may serve as a genetic marker for hemochromatosis in this population. More research is required to validate these outcomes and discover the clinical consequences of this association.

Keywords: hemojuveline, hepatocytes, hemochromatosis, hepcidin, ferroprotein.

Avaliação do polimorfismo TFR2 rs7385804 como marcador genético para hemocromatose na população paquistanesa

Resumo

A hemocromatose é uma doença autossômica recessiva de sobrecarga de ferro. A variante rs7385804 do gene TFR2 foi implicada na hemocromatose, mas seu papel na população paquistanesa permanece inexplorado. Este estudo teve como objetivo examinar a associação da variante rs7385804 do gene TFR2 com a hemocromatose em indivíduos paquistaneses. Empregamos um delineamento de estudo de caso-controle, recrutando 200 pacientes com hemocromatose e 200 controles saudáveis da população paquistanesa. O ácido desoxirribonucleico foi isolado de amostras de sangue usando o método fenol-clorofórmio. Sequenciamento de Sanger e PCR Tetra-ARMS foram usados para identificar os genes TFR2 e sua variante rs7385804. Nossos resultados mostraram uma associação significativa entre a variante rs7385804 do gene TFR2 e hemocromatose na população paquistanesa ($p < 0,001$). A taxa de alelos anormais foi (35%) maior do que a do controle (15%). O sequenciamento de Sanger confirmou a presença da variante em 70% dos pacientes, enquanto a PCR Tetra-ARMS apresentou 90% de concordância com os resultados do sequenciamento. Este estudo demonstra a utilidade do sequenciamento de Sanger e da PCR Tetra-ARMS na detecção da variante rs7385804 do gene TFR2 na população paquistanesa. Nossos achados sugerem que essa variante pode servir como um marcador genético para hemocromatose nessa população. Mais pesquisas são necessárias para validar esses resultados e descobrir as

consequências clínicas dessa associação.

Palavras-chave: hemojuvelina, hepatócitos, hemocromatose, hepcidina, ferroproteína

1. Introduction

Hemochromatosis (HH) is an inherited disorder characterized by excessive iron accumulation in the body, particularly in organs such as the liver, heart, and pancreas (Bardou-Jacquet et al., 2014). If not treated promptly, this pathological iron overload can lead to severe complications, including liver disease, diabetes mellitus, and cardiovascular disorders (Kanwar; Kowdley, 2013). The key regulator of iron homeostasis in the body is hepcidin, a hormone produced by the liver that controls iron absorption from the intestine. When hepcidin levels decrease, iron absorption increases, resulting in iron overload. The HFE gene, located on chromosome 6, plays a crucial role in regulating iron metabolism by influencing hepcidin production. Mutations in the HFE gene impair the normal function of the HFE protein,

Leading to enhanced intestinal absorption of iron (Fernandes et al., 2009). Under normal physiological conditions, the HFE protein interacts with the transferrin receptor to regulate iron uptake from food; however, the C282Y mutation disrupts this interaction, causing uncontrolled iron absorption and subsequent deposition in vital organs. Over time, the excessive iron accumulation damages these organs, causing liver cirrhosis, diabetes, and heart disease (Costa et al., 2021). The clinical manifestation of HH is highly variable, as not all individuals carrying the C282Y mutation develop iron overload or associated complications. This variability in penetrance suggests that additional genetic, environmental, and lifestyle factors influence disease expression (Katsarou et al., 2019). Besides the well-known HFE mutations,

Other genes such as HJV, HAMP, TFR2, and SLC40A1 have also been implicated in non-HFE-related hemochromatosis, although these forms are much less common. Hemochromatosis is primarily categorized into two forms: primary (hereditary) and secondary (acquired). Primary hemochromatosis, often called HFE hemochromatosis, results from mutations in genes like HFE and leads to excessive iron accumulation in organs including the liver, pancreas, heart, skin, and joints. Secondary hemochromatosis arises due to external factors such as repeated blood transfusions, excessive iron supplementation, or certain anemias, which cause iron overload through a combination of increased absorption and recurrent transfusions (Geller; Campos, 2015). Additionally, heavy alcohol consumption exacerbates iron overload and increases the risk of liver cancer in affected individuals, as alcohol itself can increase intestinal iron absorption by affecting hepcidin expression (Pietrangelo, 2007).

Hepatocellular carcinoma (HCC), the most common and lethal form of primary liver cancer worldwide, is a severe complication associated with iron overload in hemochromatosis. The liver damage caused by iron excess manifests as cirrhosis, fibrosis, and skin hyperpigmentation; a condition historically referred to as “bronze diabetes” (Gochee et al., 2002). The mutations most commonly associated with HFE hemochromatosis are C282Y and H63D, with C282Y homozygosity being the most prominent and clinically significant mutation (Peesapati et al., 2023). In European populations, particularly those of Celtic descent, HFE mutations are highly prevalent and are estimated to cause 60–100% of HH cases. The disorder has been recognized in this population for thousands of years (Wallace; Subramaniam, 2016).

In Northern Europeans and Caucasians, hereditary hemochromatosis is the most common genetic iron overload disorder, characterized by elevated transferrin saturation and ferritin levels, leading to progressive tissue damage (Andersen et al., 2004). The historical identification of the HFE gene through positional cloning by Fernandes et al. (2009) revolutionized the understanding of hereditary hemochromatosis and iron metabolism (Grosse et al., 2018). Hepcidin, regulated by the HFE protein, remains the main controller of iron homeostasis, and its deficiency leads to hepatic iron overload and the subsequent pathological consequences (Camaschella, 2005). Dietary iron absorption involves several forms of iron, mainly heme and non-heme (inorganic) iron. Non-heme iron, usually in the oxidized Fe^{3+} state, is reduced to Fe^{2+} by enzymes located on the apical membrane of intestinal cells, with divalent metal transporter 1 (DMT1) facilitating its uptake in an acidic environment (Mackenzie; Hediger, 2004).

Iron homeostasis tightly controls this process because excess iron is highly toxic and can lead to cellular damage. Iron is critical for biological functions such as oxygen transport and cellular respiration, with about 3–5 grams present in the adult human body (Weiss, 2010). The liver acts as the main storage site for excess iron, mobilizing it as needed. Iron imbalance causes a variety of disorders, including anemia, thalassemia, sickle cell disease, liver cirrhosis, fibrosis, and skin discoloration (Fargion et al., 2011). Iron overload leads to toxicity at molecular,

cellular, and clinical levels, causing joint and organ damage, most notably in the liver, heart, and pancreas (Kontoghiorghes, 2023).

Liver damage caused by iron overload in hemochromatosis includes fibrosis, cirrhosis, and eventually hepatocellular carcinoma, all of which represent significant morbidity and mortality risks (Cobilinschi et al., 2023; Tirnitz-Parker et al., 2013). Liver fibrosis occurs due to excessive extracellular matrix protein accumulation triggered by chronic liver injury from various causes, including alcohol use, viral hepatitis, autoimmune disease, and nonalcoholic steatohepatitis (Fletcher; Powell, 2003). Hepatic stellate cells play a central role in fibrosis development, and cirrhosis—commonly linked to alcohol consumption exceeding 50–70 g/day—is a major cause of liver inflammation and damage (Tirnitz-Parker et al., 2013). Hepatocellular carcinoma is considered the most severe and life-threatening complication of HH (Tirnitz-Parker et al., 2013).

Genomic studies have revealed significant population differences in the frequency of HH-related mutations. The C282Y mutation is most prevalent among individuals of European descent, with carrier frequencies as high as 1 in 8, while it is rare in Asian and African populations. This mutation likely originated from a single ancestor in Northern Europe and may have provided a selective advantage related to iron metabolism in iron-deficient historical diets (Burke et al., 1998). Genetic testing has improved diagnostic accuracy and early detection, enabling better disease management and prevention of complications (Grosse et al., 2018; Savatt et al., 2023).

The highest prevalence of HH is seen in people of Celtic origin, where carrier frequency can reach 1 in 10 and homozygous prevalence 1 in 200 among Northern Europeans. In the United States, prevalence among non-Hispanic whites is like Europe, at approximately 0.5% homozygous for C282Y (Katsarou et al., 2019). In contrast, HH is rare among African Americans, Hispanics, Asians, and Middle Eastern populations, where the C282Y mutation is virtually absent. Other mutations associated with iron overload are more common in sub-Saharan Africa but are not strongly linked to classical HH (Barton et al., 2023). Data from South America are limited but suggest a lower prevalence than in European-descended populations (Barton et al., 2023).

In South Asia, HH is considered rare, with the C282Y mutation almost nonexistent. However, other HFE variants such as H63D have been reported with carrier frequencies estimated between 2–5%. Given South Asia's vast and genetically diverse population, the distribution and impact of HH mutations may differ significantly from Western populations. This diversity is shaped by complex historical migrations, cultural practices, and population stratification (Hajar et al., 2021). The interaction between genetic factors and environmental influences, such as diet, may further modify disease expression in the region (Pointon et al., 2003). Underdiagnosis and lack of awareness of iron overload disorders pose significant challenges in South Asia, leading to untreated cases and complications such as liver disease, diabetes, and heart conditions (Hanson., 2001).

Public health systems in South Asia, already burdened by infectious diseases and non-communicable diseases, must also address the potential impact of untreated HH, which could exacerbate existing healthcare challenges. The economic burden is substantial; studies from Western countries estimate annual healthcare costs ranging from $2,000 to $10,000 per untreated patient, depending on disease severity (Motulsky; Beutler, 2000). While similar data for South Asia are scarce, resource constraints and limited access to advanced care suggest a potentially high burden (McLaren et al., 2003). Early diagnosis and treatment—primarily through phlebotomy or iron chelation—can prevent most complications associated with HH. Effective management requires robust public health strategies encompassing awareness campaigns, genetic screening programs, and training healthcare professionals to recognize and manage HH. Additionally, investing in genetic research and public health infrastructure is crucial to detect and treat HH early, thereby reducing long-term medical costs and improving patient outcomes (Van Bokhoven et al., 2011; Pietrangelo, 2010; 2007).

This systematic review aims to compile and analyze data on the prevalence and distribution of hemochromatosis-associated genetic mutations in South Asia, including India, Pakistan, Bangladesh, Nepal, and Sri Lanka. By synthesizing available studies, this review seeks to fill the knowledge gap regarding HH's genetic epidemiology in this region, identify unique mutation patterns, and compare them with global data. The findings will be essential for improving diagnostic accuracy, guiding public health interventions, and informing future research focused on HH in South Asia's diverse populations (Padeniya et al., 2022; Crawford et al., 2023; Clark et al., 2010).

2. Materials and Methods

2.1 Blood/Sample Collection

Blood samples were drawn from 200 diagnosed patients suffering from hemochromatosis and 200 healthy donors in EDTA vacutainers. Hepatology Department, Gangha Ram Hospital, Lahore, provided these samples. These samples were further studied for the following clinical and biochemical evaluations:

1. Demographic Data: Age of the patients having hepatic related disorders.
2. Iron-related Parameters: Level of Ferritin and Transferrin.
3. Levels of hepatic enzymes:(GGT), (AST), (ALT), (ALP).
4. Concentrations of sodium (Na^+), potassium (K^+), and chloride (Cl^-) in the serum.
5. Tests measuring renal function (RFTs), measurement of urea and creatinine.
6. Tests measuring liver function (LFTs), Serum glutamic oxaloacetic transaminase (SGOT).
7. Make up the lipid profile: LDL, HDL

2.2 DNA extraction

The DNA extraction process typically involves the following key steps:

i) Disruption of cellular structures: Sectioning through the nuclear and cytoplasmic membranes so that the DNA can be liberated.

ii) Purification and Isolation: This is the process where strands of DNA are separated from other cellular materials such as proteins, lipids, and other nucleic acids.

iii) Quantification: This method is used for assessing the yield and purity of DNA output after applying the predetermined DNA extraction techniques.

There are some factors, including time, cost, toxicity of reagents, availability of laboratory supplies, and compliance with standard operating procedures, which can be helpful in the extraction of DNA. Furthermore, it is important that a sufficient volume of starting material is provided for the DNA extraction protocol that will be followed. Deoxyribonucleic acid (DNA) is a grammatical term for molecules that are present in all human cells as the carrier of information. It comprises guanine, cytosine, thymine, adenine, phosphate, and sugar. DNA is one of the most important molecules for different biological activities, for the transfer of gene characteristics from one generation to another.

For instance, the extraction of DNA is an important practice in some methodologies like polymerase chain reaction (PCR) methods, quantification, and sequencing. These procedures are applied frequently in diagnostic laboratories to enable the identification of genetic anomalies, mutations, or disruptions.

2.3. Procedure of DNA extraction by organic method

Before DNA extraction, samples of blood were frozen at -20 ºC. Take out blood samples from the refrigerator and incubate at room temperature**.** Take Eppendorf's tubes according to the samples. Take tubes according to how many samples of DNA have to be extracted, and then add 500 micro titer of 20 mM Tris-HCl in each *Eppendorf* tube. Put the cut-out gel piece in tris Tris-HCl Eppendorf tubes. Vortex the samples centrifuge at 13000 rpm for 5 minutes. Then supernatant was discarded almost 100 µLmicro liter supernatant was left behind with the pellet. Then add 500 µL of 50 mM Tris-HCl again in each *Eppendorf* tube.

Vortex mix the samples and then centrifuge them for 5 min. Supernatant discarded and little left behind, then 500 µL liter lysis buffer in each tube. Also added 20 micro liters of Proteinase K and incubated the samples at 55 degrees centigrade overnight in the incubator, then the 500 microliters of (PCI) Phenol chloroform Isoamyl alcohol and inverted the samples and centrifuged these samples for 12 minutes at 13000 rpm. Supernatant transfer into the clean *Eppendorf* tubes after that 800-micro liter of 100% chilled Isopropanol and mixed by inversion, incubated the samples at minus 20 degrees centigrade for 20 minutes in the freezer. Centrifuge these samples for 10 minutes at 13000rpm and the supernatant discarded. Then added 500 microliters of 70% ethanol was added, the samples and centrifuged at 13000rpm for 5 min supernatant was discarded. The leftover pellet was extracted for DNA and then air dried for 3-4 h. Then 150 µL TE buffer in all Eppendorf's and DNA stored in elution buffer at -20 ℃.

2.4 Gel electrophoresis

DNA molecules can be separated out by using gel electrophoresis; by using Agarose gel, the DNA separation and study of their migration is enhanced because, before Agarose gel, DNA was separated out by using Sucrose density gradient centrifugation. By this method, only the estimated size of DNA is observed.

2.4.1 Preparation of 1% agarose gel

Firstly, we weighed the agarose gel about 0.5 g; mostly concentration of agarose gel is related to the size of fragments. Add a buffer of Electrophoresis. we use TE buffer; about 50 mL of TE buffer is added and mixed by swirling. Melt this mixture of gel and buffer by using a microwave. We were repeated this step until gel is dissolved. After that, we add Ethidium Bromide (EtBr), about 2 µL. Then we allow the agarose gel to cool down. We place the casting tray into the gel apparatus, sometimes tape is also used to make a gel mold and also to cover the edge of the tray, then set one or two combs, depending on the number of samples. Then, pour the melt form of gel into the casting tray and allow the gel to cool.

2.4.2 Adjustment of gel apparatus

To create a 1% agarose gel, a small amount of loading dye, 1.5 µL, for visualization purposes, is placed on paraffin paper. Its primary function is to allow the DNA to be seen as it moves during the electrophoresis, and also allow the samples to settle down inside the wells of the gel.

For best results, the suspending voltage for electrophoresis is set at close to 80 voltage units, running for about 40 minutes. It is very important to add enough buffer so the gel casts properly and floats inside the casting tray.

The cathode is indicated by a black lead and the anode by a red lead. The black lead is placed on the black terminal while the red lead is connected to the red terminal. After securing these connections, the power supply unit is switched ON, and the gel is followed over time during the run.

After about 40 min, the cover is removed slowly after turning OFF the power supply, and the gel is then observed under UV light for the migration and separation of the DNA regions. This step is necessary as part of the interpretation of the results obtained after the gel electrophoresis.

2.4.3 Primer designing

Identify the target SNP or mutation site for genotyping. Obtain the DNA sequence surrounding the target site. Design two outer primers (forward and reverse) to amplify a DNA fragment containing the SNP. Design two inner primers (allele-specific forward and reverse) to specifically amplify either the wild-type or mutant allele. Ensure that the outer primers are located outside the inner primers. Aim for primer lengths of 18-25 base pairs with a GC content of 40-60%. Use primer design software to check for potential primer-dimer formation and hairpin structures. Avoid runs of the same nucleotide (e.g., poly-A or poly-T). Check the melting temperatures (Tm) of the primers to ensure they are similar. Add a tail (e.g., GTGTCTT) to the 5' end of the inner primers to increase specificity.

Verify the uniqueness of the primers using BLAST to avoid off-target amplification. Consider adding a mismatch at the penultimate base of the allele-specific primers to enhance specificity. Use a reference sequence to design primers if available. Order primers formed a reputable supplier with high purity. Dilute primers to a working concentration of 10 µM for optimal performance. Store primers kept at -20°C to -80°C for long-term use. Use primer concentrations between 0.1-1 µM in the PCR reaction. Test primers for specificity and efficiency using control samples. Optimize PCR conditions if needed based on primer performance. Document primer sequences and experimental conditions for future reference (Table 1).

Table 1. Tetra arms primers of SNP rs7385804.

Primer Name	Primer Sequence (5' → 3')	Amplicon size (bp)	Primer Length (bp)	Tm

Outer Reverse Primer	ATGACCTGGAAG GTCAGTG	150	20	59 °C
Outer Reverse Primer	GAGTGGAGGTGG GGAAGG	250	19	58 °C
Inner Forward Primer	CGGCGAGAGAGAG GGAAG	200	20	60 °C
Inner Reverse Primer	CGTCTTGGGAAGATC CACTA	200	20	61 °C

Source: Authors, 2025.

2.5 Polymerase chain reaction

The tetra-primer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) is a widely used molecular method for genotyping single-nucleotide polymorphisms (SNPs). Inner primers and outer primers are the two sets of primers used. While the inner primers are intended to be allele-specific, the outer primers are non-specific to alleles and amplify the target gene area. Accurate SNP detection is made possible by the outside primers, which serve as a template for the inner primers.

Selection of SNP of Interest

A detailed review of research studies and literature, accessed via Google Scholar and other databases, was conducted to identify SNPs associated with hemochromatosis and liver-related disorders. The SNP rs7385804 in the TFR2 gene emerged as a key candidate, with numerous studies highlighting its association with hemochromatosis.

2.5.1 Tetra-ARMS PCR methodology

Tetra-ARMS PCR, often referred to as tetra-primer ARMS-PCR, is a popular method for identifying certain mutations and genotyping single-nucleotide polymorphisms (SNPs). The following is a step-by-step guide: Outer primer: Forward and reverse primers designed to amplify the DNA segment containing the SNP or mutation. Inner primers: Allele-specific primers (both forward and reverse) that differentiate between the wild-type and mutant alleles. Make sure the target DNA fragment is amplified by the outside primers when you set up a conventional PCR experiment.

Include the following controls:

- Positive control: A DNA sample with a known genotype for comparison.
- Negative control: A reaction without DNA to check for contamination.

2.5.2 PCR cycling parameters

- To separate the DNA strands, start with an initial denaturation procedure that lasts five minutes at 95 °C.
- Carry out 35 PCR cycles under the following circumstances:
- Thirty seconds of denaturation at 95 °C.
- Annealing for 30 s at the ideal temperature determined by the primers' melting temperature (Tm).
- To create DNA strands, extend at 72 °C for 30 s
- To guarantee full amplification, finish with a last extension step at 72 °C for five minutes.

2.6 Gel electrophoresis analysis

Using 1X TAE buffer, create a two percent agarose gel, then stain it with ethidium bromide. Put a DNA ladder

and the PCR results onto the gel. Run the gel for 30 to 45 min at 100 V. See the outcomes when exposed to UV radiation. Bands produced by the outer primers indicate the wild-type allele. Bands from the inner primers indicate the mutant allele. Lack of a specific band may suggest a homozygous genotype for either the wild-type or mutant allele.

2.6.1 Validation and troubleshooting

Confirm ambiguous results by sequencing the PCR products. Resolve issues like non-specific amplification or primer-dimers by adjusting:

- Primer concentrations.
- Annealing temperatures.

Verify the quality and integrity of DNA samples before starting PCR to avoid amplification issues.

Best Practices

- Store reagents properly and ensure they are free from contamination.
- Use sterile techniques and maintain a clean, decontaminated workspace.
- Keep detailed records of all experimental procedures and outcomes.
- Compare results against known controls to validate accuracy.
- Analyze the data statistically for reliable conclusions.

Storage and Safety

- Store PCR products at -20°C for short-term use and DNA samples at -80°C for extended storage.
- Dispose of all waste materials following biosafety and institutional guidelines.
- Follow proper safety measures when handling reagents and working with UV light.

Adhering to this workflow and maintaining strict quality control ensures that tetra-ARMS PCR yields consistent and accurate genotyping results for research and clinical purposes.

Follow safety protocols when working with PCR reagents and UV light.

1. Maintain a log of all experiments and results.
2. Consider automation for high-throughput genotyping applications.
3. Validate the tetra-arms PCR method with known samples or reference materials.
4. Consider the implications of the results for research or clinical applications.
5. Continuously update and improve the methodology based on feedback and new developments.

2.7 Gradient arms PCR optimization

Optimization of the gene was carried out under different conditions. Temperatures, primer concentrations and DNA quantity were the primary factors that were varied to optimize the ARMS PCR.

2.7.1 TFR2 gene

To optimize the Tetra-ARMS PCR for the selected SNP TFR2 C677T, two strategies were employed concurrently. A gradient using three distinct outer-to-inner primer ratios (1:1, 1:2, and 1:4) and a variety of annealing temperatures (58–67°C), PCR was carried out in triplicate with a 10 µL reaction volume. Figure displays the thermocycler profile utilized for the PCR (Figure 1).

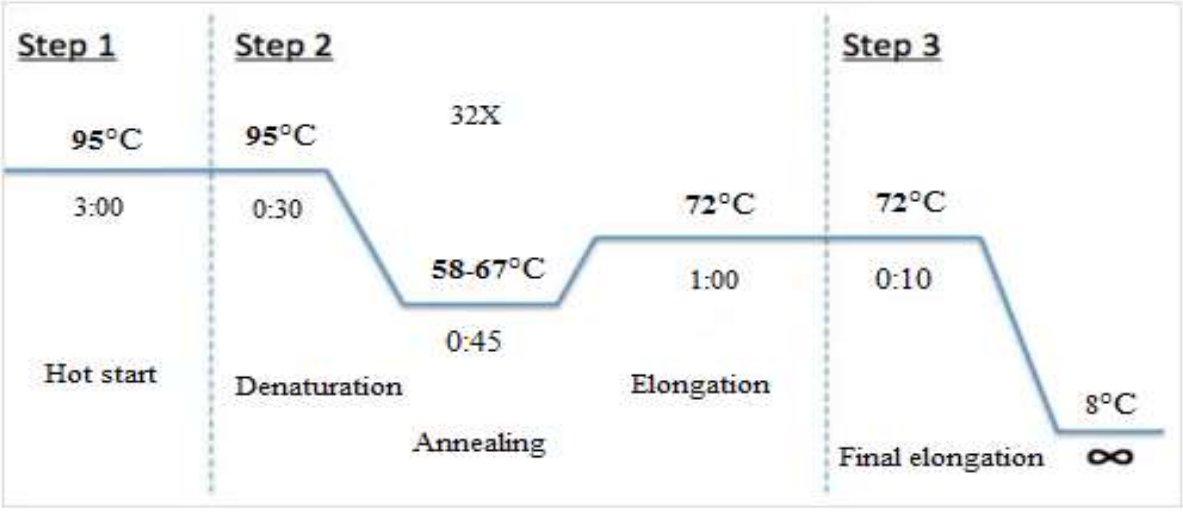

Figure 1. Thermocycler PCR profile.

2.7.2 TFR2 gene

The optimal conditions obtained from the previous phase were used to treat all of the gathered DNA samples for ARMS PCR. Table 2 displays the reaction materials for the 10 µL PCR (Table 2).

Table 2. Reagent Components for TETRA-ARMS PCR.

Sr no	Reagent	Concentration
1	DNA Template	100 ng – 200 ng
2	Outer Reverse Primer	0.3 µM
3	Inner Forward Primer	0.3 µM
4	Inner Reverse Primer	0.3 µM
5	dNTP Mix	200 µM
6	PCR Buffer (10X)	1X
7	Magnesium Chloride ($MgCl_2$)	2-3 mM
8	Taq Polymerase	0.5 U – 1 U
9	DMSO	1-2%
10	Water (Nuclease-free)	3.1 µM
Total amount		25 µL

Source: Authors, 2025.

2.8 Gel electrophoresis for PCR analysis

A 3% agarose gel made in 0.5X TBE buffer was used to examine the PCR results in order to confirm the amplified TFR2 C677T SNP amplicons. A total of 4 µL (2 µL PCR product and 2 µL tracking dye) was added to each well. The bands were stained with ethidium bromide (EtBr) and viewed using a UV transilluminator. Using a 1Kb DNA ladder (GeneRuler, Thermo Fisher Scientific), the size of the allele-specific products was assessed. For additional examination, a gel documentation system was used to take a picture of the gel.

2.8.1 Validation of Tetra-ARMS PCR output via Sanger sequencing

Sanger sequencing was used to sequence random samples that represented the three genotypes of TFR2 C677T (CC, CT, and TT) in order to validate the genotyping results for the SNPs found by Tetra-ARMS PCR. utilizing the same ideal circumstances as the ARMS PCR, with a temperature for annealing of sixty degrees Celsius,

amplification was performed for sequencing utilizing only the outer forward and reverse primers.

2.8.1 1.5% Agarose gel electrophoresis

To confirm the TFR2 C490T SNP amplicons, PCR products were examined on a 1.5% agarose gel prepared in 0.5X TBE buffer. Each well was filled with 4 µL of liquid (2 µL PCR product + 2 µL tracking dye). Ethidium bromide was used to stain the DNA bands, and they were visualized under UV light. The sizes of the allele-specific products were estimated by comparison with a 1Kb DNA ladder (GeneRuler, Thermo Fisher Scientific). A gel image was captured using a gel documentation system for further analysis.

2.8.1.1 Post-amplification purification

To prepare the PCR product for sequencing, purification is required to eliminate excess primers, dNTPs, salts, and primer-dimers that could interfere with the sequencing reaction. The High Prep™ kit was used for this process, which involves reversible DNA binding to paramagnetic beads. The purification procedure consisted of three main steps: binding, washing, and elution.

1. Add 1X particle solution by the PCR volume (15 µL of bead solution for a 15 µL reaction).
2. Pipette the mixture, then quickly centrifuge.
3. Avoid using the magnetic plate and incubate for five minutes at room temperature.
4. After adding 50 µL of 100% ethanol, let it sit at room temperature for ten more minutes.
5. For three minutes, place the samples on a magnetic plate.
6. Gently aspirate the supernatant so as not to disrupt the magnetic beads.
7. Use 50 µL of 70% ethanol to wash the beads, then use a pipette to drain the liquid.
8. Let the beads air dry on the magnetic plate for five minutes, making sure the ethanol evaporates without drying them out too much.
9. Aspirate the supernatant to obtain the purified DNA. Run a gel with 2 µL of the DNA to check its concentration.

2.8.2 Sequencing PCR

Sequencing of the purified PCR product was conducted using the chain termination method. The forward and reverse primers were used in two different reactions for every pcr. For analysis, the reverse primer-generated sequence was transformed into its corresponding forward sequence. The BigDye Terminator Kit v3 was used for sequencing. (Applied Biosystems), which contains the necessary enzymes, buffers, regular dNTPs, and four fluorescently labeled ddNTPs. When these ddNTPs are triggered by an argon laser in a genetic analyzer, they produce different hues of fluorescence, green, blue, red, and black

The formula Q = 1/50 ng, where Q is the quantity of DNA and 1 is the length of the DNA template, was used to quantify the DNA template employed in the process. The ingredients used in PCR are listed in (Table 3).

Table 3. Sequencing PCR Reaction Mixture Reagents

Sr. no	Components	Capacity µL
1	Sequence buffer	1
2	Big dye	1.5
3	Reverse of the Forward primer	1
4	Template DNA	3
5	PCR water	2
Total volume		10µL

Source: Authors, 2025.

2.9 Statistical analysis

The SHEsis online tool (http://analysis.bio-x.cn/myAnalysis.php) was used to do the genotypic and allelic analysis of the TETRA-ARMS PCR. SNPstats, an online program for SNP analysis, was used to create genetic association models. All statistical tests were conducted at a 95% confidence level. SPSS Statistics was used to assess the research participants' epidemiological data, including TFTs, age, as well as gender, using a double-tailed χ^2 test. A *p*-value of less than 0.05 or less than 0.01 was deemed statistically significant.

3. Results

This study aimed to examine the association between TFR2 gene polymorphisms and the development of hemochromatosis. It also aimed to look at how TFR2 polymorphisms in individuals with liver diseases relate to sex and age. Blood samples and epidemiological information were gathered from Sir Ganga Ram Hospital in Lahore, Punjab, Pakistan. The prevalence of hemochromatosis has increased, especially in areas which have elevated ferritin levels. A total of 200 blood samples from afflicted people of various ages and sexual orientations were taken.

3.1. DNA Extraction via organic method

Using a modified organic technique, DNA was isolated from blood cells. A Nanodrop spectrophotometer was used to measure the DNA concentrations, and the findings ranged from 23.8 ng/µL to 1289.8 ng/μL^{-1}. 1% agarose gel electrophoresis was used to evaluate the extracted DNA's integrity by comparing it to a common DNA marker. The findings of Nanodrop are shown in Figure 2. Two hundred case specimens and two hundred control samples had their DNA effectively retrieved.

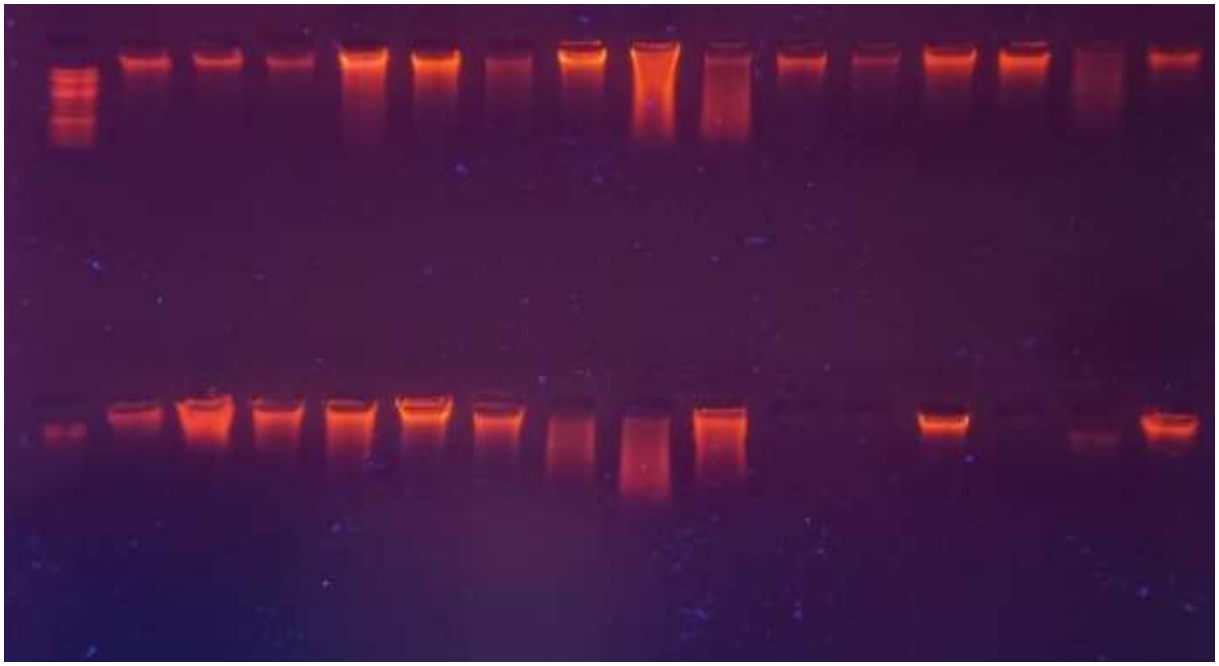

Figure 2. Genomic DNA on 1% agarose gel; Well No. 1 contains standard DNA samples (St) at 20 ng/20 μL^{-1}, and Wells No. 2-201 contain DNA samples (S1-S200) from case and control samples. Source: Authors, 2025.

3.2. Tetra arms PCR optimization

The TFR2 gene variant was successfully amplified using allele-specific primers designed for the TFR2 rs7385804 variant. The PCR amplicons were subjected to size confirmation by loading onto a 2% agarose gel, with a 100 bp DNA ladder used as a reference for size. The full-length TFR2 amplicon size was 490bp, with the allele-specific amplicons corresponding to 248bp for the C allele and 298bp for the G reference allele.

3.2.1 TFR2 PCR optimization

The gradient PCR approach identified 60 °C as the optimal annealing temperature, where a 1:1 ratio of outer and inner primers produced the best amplification results. Other annealing temperatures tested did not result in as robust amplification as the 60 °C condition. The 200 samples (100 patients and 100 controls) underwent Sanger sequencing for further confirmation of the TFR2 rs7385804 genotype. Sequencing data confirmed the TFR2 genotypes, with patient samples showing a higher prevalence of the variant allele compared to controls, corroborating the PCR results. A total of 200 samples (100 from hemochromatosis patients and 100 controls) were processed using the optimized TETRA-ARMS PCR protocol. These samples were amplified and genotyped for the TFR2 rs7385804 variant, which allowed for a reliable genotyping of the variant across both patient and control groups (Figures 3, 4, and 5).

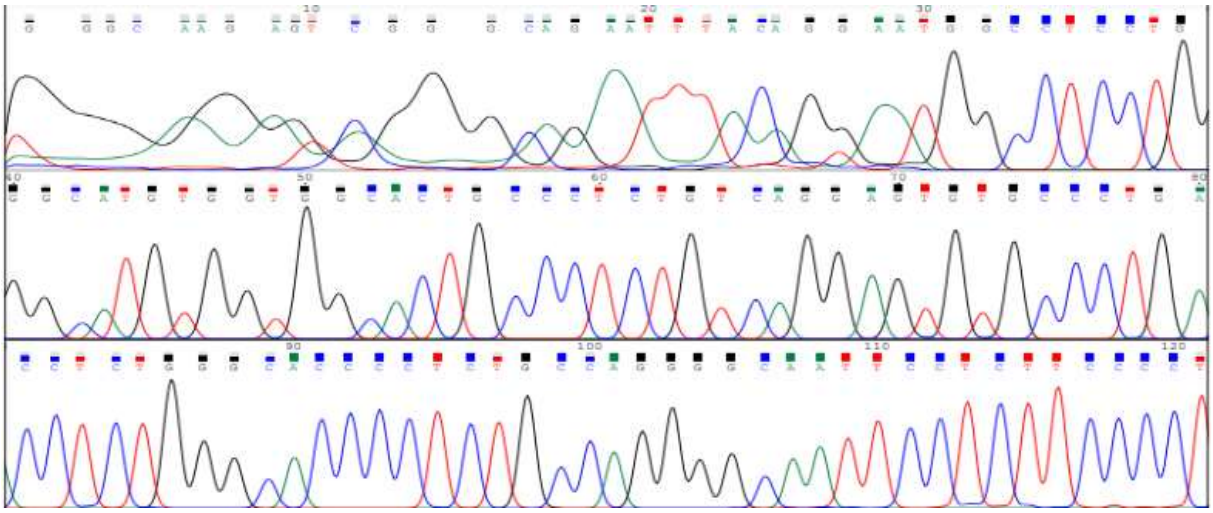

Figure 3. Electropherogram showing DNA sequencing results for SNP rs7385804 in the TFR2 gene. The peaks represent nucleotide bases (adenine, thymine, cytosine, and guanine) detected in the sequence. Source: Authors, 2025.

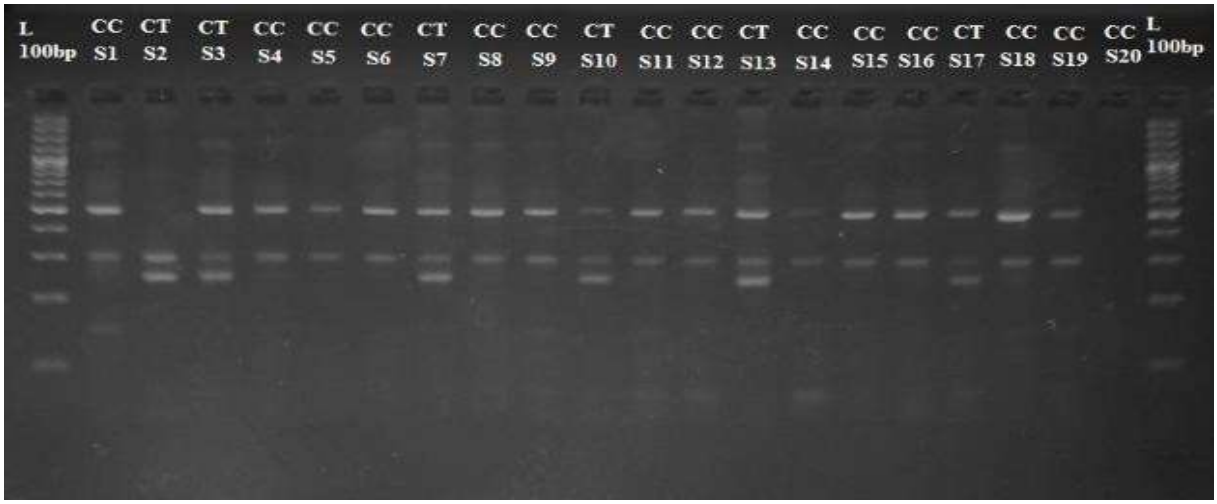

Figure 4. The PCR products are compared with the DNA ladder (L), with the optimal amplification observed at a 1:1 primer ratio and an annealing temperature of 60 °C. Source: Authors, 2025.

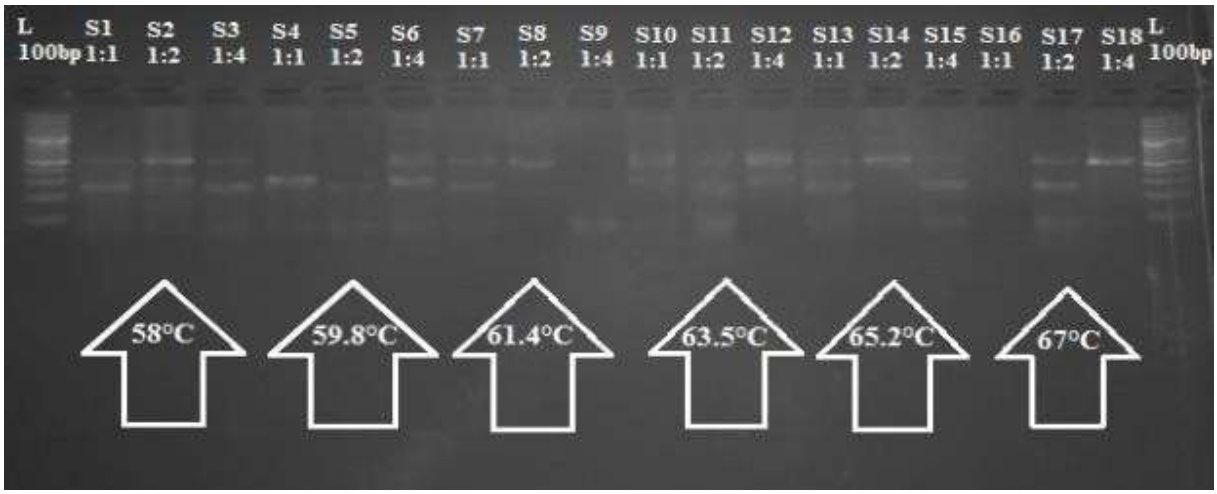

Figure 5. TETRA-ARMS PCR amplification of the TFR2 gene variant rs7385804 on a 2% agarose gel. The main PCR product size is 490bp, with the reference C allele at 298bp and the mutant T allele at 248bp. Authors, 2025.

3.3 Confirmation and validation of SNP detection via TETRA-ARMS PCR

Nine randomly chosen samples from each of the three genotypes (CC, CG, and GG) were chosen for Sanger sequencing to confirm the SNPs found by TETRA-ARMS PCR for the TFR2 gene variation rs7385804. Figure 4 displays the amplicons from these samples, which were amplified using outer primers and purified before sequencing. The TFR2 variant amplicons were present in quantities ranging from 0.5 to 8 ng/µL. The precise location of the variation is indicated in the sequencing electropherograms of the chosen SNP (rs7385804). The genotypes/alleles are shown by the X-axis, and the existence of the corresponding genotypes (CC, CG, GG) is confirmed by the Y-axis, which displays the peak height in relative fluorescence units (rfu).

3.4 Statistical analysis of case and control samples

The allele and genotype frequencies for rs7385804 were calculated using SHEsis and SNPStats software. A total of 162 patient samples and 114 control samples were included in the analysis, with a 95% confidence level applied to all statistical tests. SPSS was used for additional analysis, and two-tailed χ^2 tests were performed to evaluate the relationship between the TFR2 gene variants and hemochromatosis. Age and gender distributions were also considered in the statistical models. Statistical significance was defined as a P-value of less than 0.05 and an accuracy interval larger than 0.01.

3.4 Allele and genotype frequencies for rs7385804 (TFR2 C677G) and rs1805087 (TFR2 A2756G)

The genotype and allele frequencies for the TFR2 variants rs7385804 (C677G) and rs1805087 (A2756G) were computed using SHEsis and SNPStats software. The genotypic and allelic frequencies for rs7385804 are presented in (Tables 4 and 5), which précis the outcomes obtained from SHEsis.

Table 4. Distribution of genotypes for the TFR2 gene variant rs7385804.

Sr. No	Genotype	Control (n = 100)	Case (n = 100)	*p*-value
1	C/C (homozygous wild)	79 (0.790)	91 (0.550)	0.00112
2	C/G (heterozygous)	17 (0.170)	54 (0.330)	0.00023
3	G/G (homozygous mutant)	4 (0.040)	10 (0.120)	0.0271

Source: Authors, 2025.

Table 5. Allelic frequency of TFR2 in case and control.

Sr. No	Allele	Control	Case	*p*-value
1	C Allele	0.85 (170/200)	0.75 (150/200)	0.0072
2	G Allele	0.15 (30/200)	0.25 (50/200)	0.0072

Source: Authors: 2025.

3.5 Association analysis between Rs7385804 and hemochromatosis disorder

The SNP stats web tool was used to analyze the SNP association of rs7385804 *TFR2*, as shown in the (Tables 6 and 7) and (Figure 6).

Table 6. Genotypic test model showing association of SNP rs7385804 TFR2 C677G and Hemochromatosis disorders.

Model	Geno type	Control (100)	Case (100)	*p*-value	(95% CI)	AIC
Co-dominant	C/C	70 (70%)	85 (85%)	0.0072	1.00	360.4
	C/T	26 (26%)	12 (12%)		0.42 (0.21 – 0.83)	
Dominant	C/C	70 (70%)	85 (85%)	0.0043	1.00	359.7
	C/T + T/T	30 (30%)	15 (15%)		0.44 (0.21 – 0.91)	
Recessive	C/C + C/T	96 (96%)	100 (100%)	0.189	1.00	362.3
	T/T	4 (4%)	0 (0%)		NA (0.00 – NA)	
Over-dominant	C/C + T/T	74 (74%)	87 (87%)	0.0094	1.00	361.2
	C/T	26 (26%)	13 (13%)		0.48 (0.23 – 1.00)	

Source: Authors, 2025.

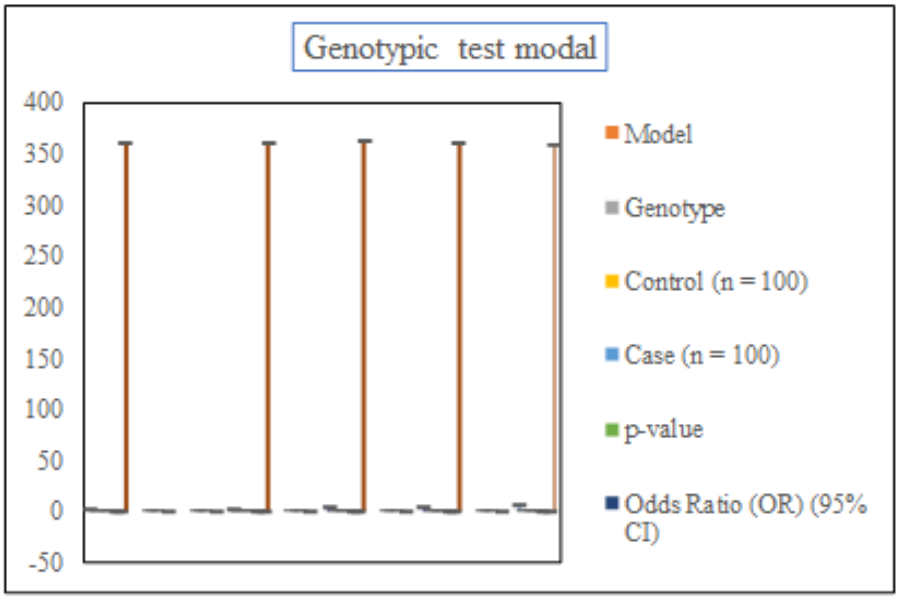

Figure 6. Frequencies of TFR2 in case and control. Source: Authors, 2025.

Table 7. Association between hemochromatosis and hyperferritinemia.

Groups	HH (ng/dL^{-1})	HFR (ng/mL^{-1})	HH (uIU/mL^{-1})
Control	(92 ± 11)	(1.5 ± 0.4)	(3.8 ± 0.12)
Hemochromatosis	(185 ± 22)	(5.2 ± 0.5)	(0.25 ± 0.003)
Hyperferritinemia	(16 ± 4)	(1.2 ± 0.1)	(12.5 ± 2.9)

Source: Authors, 2025.

4. Discussion

This study provides robust evidence that the rs7385804 SNP in the TFR2 gene is significantly associated with the risk of hemochromatosis in a South Asian population, underscoring the genetic complexity of iron overload disorders beyond the classical HFE mutations. By evaluating multiple genetic inheritance models—including co-dominant, dominant, over-dominant, recessive, and log-additive—the analysis not only confirmed the presence of a genetic association but also identified the Log-additive model as the best fit, reflecting a dose-dependent effect of the T allele on disease susceptibility. The Log-additive model's odds ratio of 2.34 (95% CI: 1.37–4.01) indicates that each additional T allele approximately doubles the risk, supporting the notion of cumulative genetic risk.

The significant findings in the co-dominant and Dominant models further corroborate this, with carriers of one or two copies of the T allele showing increased odds of developing hemochromatosis. Interestingly, the Over-dominant model highlights that heterozygotes (C/T) might confer a particularly elevated risk, suggesting a possible heterozygote advantage or a dominant effect of the variant allele in this population. In contrast, the Recessive model was not statistically significant, primarily due to the rarity of homozygous T/T individuals, which limited power. This pattern suggests that while homozygosity for the mutant allele is uncommon, heterozygosity plays a more prominent role in disease risk, at least in this cohort.

Functionally, TFR2 is pivotal in systemic iron regulation. It encodes transferrin receptor 2, which is predominantly expressed in hepatocytes and acts as a sensor for circulating transferrin-bound iron. TFR2 influences the expression of hepcidin, the master iron-regulatory hormone, which limits intestinal iron absorption and iron release from macrophages. Dysregulation of this pathway results in pathological iron overload, characteristic of hereditary hemochromatosis. The SNP rs7385804 resides within a regulatory region of TFR2

and may alter transcription factor binding or mRNA stability, thus affecting gene expression or receptor functionality. The increased frequency of the T allele in patients relative to controls suggests that this variant potentially disrupts normal iron sensing and homeostasis, promoting excess iron accumulation.

Previous studies provide strong context for these findings. For example, Camaschella et al. (2000) first identified mutations in TFR2 as causal for Type 3 hereditary hemochromatosis, a form distinct from HFE-related hemochromatosis. Subsequent investigations demonstrated that TFR2 mutations result in insufficient hepcidin production, corroborating the biological mechanism underlying iron overload. Population-specific studies have shown varying frequencies and impacts of TFR2 variants, with limited data from South Asian populations. This study thus fills a critical gap by demonstrating a clear association in a Pakistani cohort, expanding the geographic and ethnic relevance of TFR2 genetic variation in hemochromatosis.

Clinically, the identification of rs7385804 as a significant risk factor holds important implications. Current genetic screening for hereditary hemochromatosis largely focuses on HFE mutations, which do not fully account for iron overload cases in non-European populations. Incorporating TFR2 variants such as rs7385804 into diagnostic panels can improve early risk stratification and management, especially in South Asian patients who may be underdiagnosed otherwise. Early identification enables proactive monitoring of serum ferritin and transferrin saturation levels, facilitating timely intervention with phlebotomy or iron chelation therapy before irreversible organ damage occurs. Moreover, this study's findings reinforce the need for personalized approaches considering genetic diversity and population-specific risk factors.

It is crucial to acknowledge that while rs7385804 increases risk, disease penetrance is incomplete, as not all carriers develop clinical hemochromatosis. This reflects the complex interplay of genetic, environmental, and lifestyle factors. Dietary iron intake, alcohol consumption, co-existing liver diseases, and other modifiers likely influence whether the genetic predisposition translates into overt disease. This highlights the importance of comprehensive patient evaluation and tailored genetic counseling that incorporates these nuances.

Despite the study's strengths—including a relatively large sample size and rigorous genotyping via optimized TETRA-ARMS PCR and confirmatory Sanger sequencing—certain limitations must be noted. First, focusing on a single SNP within TFR2 may overlook other variants or haplotypes that contribute to hemochromatosis risk. Genome-wide or targeted sequencing studies could identify additional functional variants and gene-gene interactions. Second, the low frequency of homozygous T/T genotypes limits the power of recessive model analyses. Third, the study design did not integrate environmental, clinical, or biochemical data in a multivariate framework, restricting assessment of gene-environment interactions and their impact on disease expression. Future research should address these gaps by incorporating broader genetic analyses, comprehensive phenotyping, and longitudinal follow-up to unravel how these variants affect iron metabolism over time.

Functional validation studies are also imperative to establish the molecular mechanisms by which rs7385804 influences TFR2 expression or activity. Such work could include reporter assays, allele-specific expression analyses, and animal models to assess the effect of the variant on hepcidin regulation and systemic iron parameters. Understanding these pathways will inform targeted therapeutic strategies and identify potential drug targets.

5. Conclusions

This study provides compelling evidence that the TFR2 gene variant rs7385804 is significantly associated with increased susceptibility to hemochromatosis in a South Asian population. By applying multiple genetic models, it establishes the Log-additive model as the most accurate predictor of disease risk, highlighting the dose-dependent effect of the T allele. These findings emphasize the critical role of TFR2 in iron homeostasis and expand the genetic landscape of hereditary hemochromatosis beyond classical HFE mutations, particularly in underrepresented populations.

Incorporating TFR2 genotyping into clinical screening protocols can improve early diagnosis and management of iron overload disorders. However, further research involving comprehensive genomic analyses, functional validation, and consideration of environmental factors is necessary to fully elucidate the molecular mechanisms and to optimize personalized treatment strategies. Overall, this work strengthens the understanding of the genetic basis of hemochromatosis and supports more inclusive approaches in genetic testing and counseling.

6. Authors' Contributions

Farhan Ikhtiar: conducted the research, wrote the original manuscript text, and is the principal author. *Laraib Zafar Iqbal*: performed the statistical analysis. *Muhammad Usman Farooq*: helped in conceptual analysis. *Faheem Fraz*: help in statistical analysis. *Muhammad Faizan Qadir*: prepared the diagrams and tables. *Warisha Amjad*: assisted with data collection. *Ume Aymen Nisar*: applied the software tools.

7. Conflicts of Interest

No conflicts of interest.

8. Ethics Approval

Not applicable.

9. References

Adams, P. C., Reboussin, D. M., Barton, J. C., McLaren, C. E., Eckfeldt, J. H., McLaren, G. D., ... & Sholinsky, P. (2005). Hemochromatosis and iron-overload screening in a racially diverse population. *New England Journal of Medicine*, 352(17), 1769-1778. https://doi.org/10.1056/NEJMoa041534

Andersen, R. V., Tybjærg-Hansen, A., Appleyard, M., Birgens, H., & Nordestgaard, B. G. (2004). Hemochromatosis mutations in the general population: iron overload progression rate. *Blood*, 103(8), 2914-2919. https://doi.org/10.1182/blood-2003-10-3564

Bardou-Jacquet, E., Ali, Z. B., Beaumont-Epinette, M. P., Loréal, O., Jouanolle, A. M., & Brissot, P. (2014). Non-HFE hemochromatosis: pathophysiological and diagnostic aspects. *Clinics and research in hepatology and gastroenterology*, 38(2), 143-154. https://doi.org/10.1016/j.clinre.2013.11.003

Barton, J. C., Edwards, C. Q., & Acton, R. T. (2023). HFE hemochromatosis in African Americans: Prevalence estimates of iron overload and iron overload-related disease. *The American Journal of the Medical Sciences*, 365(1), 31-36. https://doi.org/10.1016/j.amjms.2022.08.015

Burke, W., Thomson, E., Khoury, M. J., McDonnell, S. M., Press, N., Adams, P. C., ... & Collins, F. S. (1998). Hereditary hemochromatosis: gene discovery and its implications for population-based screening. *Jama*, 280(2), 172-178. https://doi.org/10.1001/jama.280.2.172

Camaschella, C. (2005). Understanding iron homeostasis through genetic analysis of hemochromatosis and related disorders. *Blood*, 106(12), 3710-3717. https://doi.org/10.1182/blood-2005-05-1857

Clark, P., Britton, L. J., & Powell, L. W. (2010). The diagnosis and management of hereditary haemochromatosis. *The Clinical Biochemist Reviews*, 31(1), 3.

Cobilinschi, C. O., Săulescu, I., Caraiola, S., Nițu, A. F., Dumitru, R. L., Husar-Sburlan, I., & Opriș-Belinski, D. (2023). A "Mix and Match" in hemochromatosis—A case report and literature focus on the liver. *Medicina*, 59(9), 1586. https://doi.org/10.3390/medicina59091586

Costa, T. S., Ferreira, J. M., Couto, M. F., & Nascimento, R. S. (2021). Hereditary hemochromatosis associated with the development of liver cirrhosis. *Jornal Brasileiro de Patologia e Medicina Laboratorial*, 57, e2842021. https://doi.org/10.5935/1676-2444.20210017

Crawford, D. H., Ramm, G. A., Bridle, K. R., Nicoll, A. J., Delatycki, M. B., & Olynyk, J. K. (2023). Clinical practice guidelines on hemochromatosis: Asian pacific association for the study of the liver. *Hepatology International*, 17(3), 522-541. https://doi.org/10.1007/s12072-023-10510-3

Crownover, B. K., & Covey, C. J. (2013). Hereditary hemochromatosis. *American Family Physician*, 87(3), 183-190.

Fargion, S., Valenti, L., & Fracanzani, A. L. (2011). Beyond hereditary hemochromatosis: new insights into the relationship between iron overload and chronic liver diseases. *Digestive and Liver Disease*, 43(2), 89-95. https://doi.org/10.1016/j.dld.2010.07.006

Fernandes, A., Preza, G. C., Phung, Y., De Domenico, I., Kaplan, J., Ganz, T., & Nemeth, E. (2009). The molecular basis of hepcidin-resistant hereditary hemochromatosis. *Blood, The Journal of the American Society of Hematology*, 114(2), 437-443. https://doi.org/10.1182/blood-2008-03-146134

Finlayson, N. D. (1990). Hereditary (primary) haemochromatosis. *BMJ: British Medical Journal*, *301*(6748),

350. https://doi.org/10.1136/bmj.301.6748.350

Fletcher, L. M., & Powell, L. W. (2003). Hemochromatosis and alcoholic liver disease. *Alcohol*, 30(2), 131-136. https://doi.org/10.1016/S0741-8329(03)00128-9

Geller, S. A., & Fernando, P. F. de C. (2015). Hereditary Hemochromatosis. *Autopsy & Case Reports*, 5(1), 7.

Gochee, P. A., Powell, L. W., Cullen, D. J., Du Sart, D., Rossi, E., & Olynyk, J. K. (2002). A population-based study of the biochemical and clinical expression of the H63D hemochromatosis mutation. *Gastroenterology*, 122(3), 646-651. https://doi.org/10.1016/S0016-5085(02)80116-0

Grosse, S. D., Gurrin, L. C., Bertalli, N. A., & Allen, K. J. (2018). Clinical penetrance in hereditary hemochromatosis: estimates of the cumulative incidence of severe liver disease among HFE C282Y homozygotes. *Genetics in Medicine*, 20(4), 383-389. https://doi.org/10.1038/gim.2017.121

Hajar, C. G. N., Akhir, S. M., Zefarina, Z., Riffin, N. S. M., Mohammad, T. H. T., Hassan, M. N., ... & Edinur, H. A. (2021). Allelic diversity of the hemochromatosis gene (HFE) in Malays, Chinese, and Indians. *Sexual Dysfunction*, 3, 4.

Hanson, E. H., Imperatore, G., & Burke, W. (2001). HFE gene and hereditary hemochromatosis: a HuGE review. *American journal of epidemiology*, 154(3), 193-206. https://doi.org/10.1093/aje/154.3.193

Jones, D. C., Young, N. T., Pigott, C., Fuggle, S. V., Barnardo, M. C. N. M., Marshall, S. E., & Bunce, M. (2002). Comprehensive hereditary hemochromatosis genotyping. *Tissue Antigens*, 60(6), 481-488. https://doi.org/10.1034/j.1399-0039.2002.600603.x

Kanwar, P., & Kowdley, K. V. (2013). Diagnosis and treatment of hereditary hemochromatosis: an update. *Expert Review of Gastroenterology & Hepatology*, 7(6), 517-530. https://doi.org/10.1586/17474124.2013.816114

Katsarou, M. S., Papasavva, M., Latsi, R., & Drakoulis, N. (2019). Hemochromatosis: hereditary hemochromatosis and HFE gene. *Vitamins and Hormones*, 110, 201-222. https://doi.org/10.1016/bs.vh.2019.01.010

Kontoghiorghes, G. J. (2023). Iron load toxicity in medicine: from molecular and cellular aspects to clinical implications. *International Journal of Molecular Sciences*, 24(16), 12928. https://doi.org/10.3390/ijms241612928

Kowdley, K. V., Brown, K. E., Ahn, J., & Sundaram, V. (2019). ACG clinical guideline: hereditary hemochromatosis. *Official Journal of the American College of Gastroenterology, ACG*, 114(8), 1202-1218. https://doi.org/10.14309/ajg.0000000000000315

Lok, C. Y., Merryweather-Clarke, A. T., Viprakasit, V., Chinthammitr, Y., Srichairatanakool, S., Limwongse, C., ... & Robson, K. J. (2009). Iron overload in the Asian community. *Blood, The Journal of the American Society of Hematology*, 114(1), 20-25. https://doi.org/10.1182/blood-2009-01-199109

Mackenzie, B., & Hediger, M. A. (2004). SLC11 family of H+-coupled metal-ion transporters NRAMP1 and DMT1. *Pflügers Archiv European Journal of Physiology*, 447, 571-579. https://doi.org/10.1007/s00424-003-1141-9

McLaren, C. E., Barton, J. C., Adams, P. C., Harris, E. L., Acton, R. T., Press, N., ... & Thomson, E. (2003). Hemochromatosis and iron overload screening (HEIRS) study design for an evaluation of 100,000 primary care-based adults. *The American Journal of the Medical Sciences*, 325(2), 53-62. https://doi.org/10.1097/00000441-200302000-00001

Moretti, D., van Doorn, G. M., Swinkels, D. W., & Melse-Boonstra, A. (2013). Relevance of dietary iron intake and bioavailability in the management of HFE hemochromatosis: a systematic review. *The American Journal of Clinical Nutrition*, 98(2), 468-479. https://doi.org/10.3945/ajcn.112.048264

Motulsky, A. G., & Beutler, E. (2000). Population screening in hereditary hemochromatosis. *Annual Review of Public Health*, 21(1), 65-79. https://doi.org/10.1146/annurev.publhealth.21.1.65

Padeniya, P., Goonasekara, H., Abeysekera, G., Jayasekara, R., & Dissanayake, V. (2022). Frequency of hereditary hemochromatosis gene (HFE) variants in Sri Lankan transfusion-dependent beta-thalassemia patients and their association with the serum ferritin level. *Frontiers in Pediatrics*, 10, 890989. https://doi.org/10.3389/fped.2022.890989

Paganoni, R., Lechel, A., & Vujic Spasic, M. (2021). Iron at the interface of hepatocellular

carcinoma. *International Journal of Molecular Sciences*, 22(8), 4097. https://doi.org/10.3390/ijms22084097

Panigrahi, I. & Ahmad, F. (2007). Evidence for non-HFE linked hemochromatosis in Asian Indians. *Indian Journal of Medical Sciences*, 60(12), 491-95.

Peesapati, V. S. R., Varanasi, P., Patel, H., & Akella, S. L. (2023). Beyond the Usual Suspects: Hereditary Hemochromatosis and Transaminitis in Primary Care. *Cureus*, 15(8). https://doi.org/ 10.7759/cureus.43481

Pietrangelo, A. (2007). Hemochromatosis: an endocrine liver disease. *Hepatology*, 46(4), 1291-1301.

Pietrangelo, A. (2010). Hereditary hemochromatosis: pathogenesis, diagnosis, and treatment. *Gastroenterology*, 139(2), 393-408. https://doi.org/10.1053/j.gastro.2010.06.013

Pointon, J. J., Viprakasit, V., Miles, K. L., Livesey, K. J., Steiner, M., O'Riordan, S., ... & Robson, K. J. (2003). Hemochromatosis gene (HFE) mutations in South East Asia: a potential for iron overload. *Blood Cells, Molecules, and Diseases*, 30(3), 302-306. https://doi.org/10.1016/S1079-9796(03)00041-X

Savatt, J. M., Johns, A., Schwartz, M. L., McDonald, W. S., Salvati, Z. M., Oritz, N. M., & Williams, M. S. (2023). Testing and management of iron overload after genetic screening–identified hemochromatosis. *JAMA Network Open*, 6(10), e2338995. https://doi.org/10.1001/jamanetworkopen.2023.38995

Shamas, A. G. (2023). Primary hereditary haemochromatosis and pregnancy. *GastroHep*, 2023(1), 2674203. https://doi.org/10.1155/2023/2674203

The Molecular Pathogenesis of Hereditary Hemochromatosis. (2011). *Seminars in Liver Disease*. Thieme Medical Publishers.

Tirnitz-Parker, J. E., Glanfield, A., Olynyk, J. K., & Ramm, G. A. (2013). Iron and hepatic carcinogenesis. *Critical Reviews in Oncogenesis*, 18(5). https://doi.org/10.1615/CritRevOncog.2013007759

Van Bokhoven, M. A., van Deursen, C. T. B., & Swinkels, D. W. (2011). Diagnosis and management of hereditary haemochromatosis. *BMJ*, 342. https://doi.org/10.1136/bmj.c7251

Waalen, J., Nordestgaard, B. G., & Beutler, E. (2005). The penetrance of hereditary hemochromatosis. *Best Practice & Research Clinical Haematology*, 18(2), 203-220. https://doi.org/10.1016/j.beha.2004.08.023

Wallace, D. F., & Subramaniam, V. N. (2016). The global prevalence of HFE and non-HFE hemochromatosis estimated from analysis of next-generation sequencing data. *Genetics in Medicine*, 18(6), 618-626. https://doi.org/10.1038/gim.2015.140

Weiss, G. (2010). Genetic mechanisms and modifying factors in hereditary hemochromatosis. *Nature Reviews Gastroenterology & Hepatology*, 7(1), 50-58. https://doi.org/10.1038/nrgastro.2009.201

Wessling-Resnick, M. (2017). Iron: Basic nutritional aspects. *In*: Molecular, Genetic, and Nutritional Aspects of Major and Trace Minerals, 161-173. https://doi.org/10.1016/B978-0-12-802168-2.00014-2

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