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GENETIC ANALYSIS OF HEMOCHROMATOSIS AND ITS IMPACT ON LIVER FUNCTION IN THE POPULATION OF LAHORE, PAKISTAN

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Abstract

Hemochromatosis is an autosomal recessive iron overload disorder. Hemochromatosis occurs due to the failure of the Hepcidin response to the liver. The high level of iron in the plasma stores in the various organs and destroys the organs. It is a genetic disease. Hemochromatosis in which the HFE gene is involved is called primary hereditary hemochromatosis. The mutation in the HFE gene takes place at the p.C282Y. The methodology used in this research was organic DNA Extraction, Gel Electrophoresis, Tetra ARMS PCR, and DNA Sanger's sequencing. The milder form of the disease, juvenile hemochromatosis (type II) is associated with mutations in either a new gene recently described as hemojuvelin 2 or antimicrobial peptide Hepcidin. Non-HFE hemochromatosis involves HJV, HAMP, TRF2, and SLC40A1 genes. 3, 4 in people with hemochromatosis, the hepcidin produced by hemojevuline, 2 and transferrin receptor 2 (TfR2) is decreased due to mutations in HFE. Typically, its expression is induced by high blood iron. Indeed, TfR2 is expressed in hepatocytes and has the 6/7 mutational pattern seen in hemochromatosis type III. This study investigates the genetic basis of hemochromatosis in the Pakistani population, focusing on the TFR2 gene variant rs7385804. It identifies a significant association between the C allele and disease susceptibility, with demographic data revealing a higher prevalence in males and onset in the mid-40s. While the study highlights the importance of genetic screening, there is a research gap in understanding the interaction between genetic and environmental factors. Future research should focus on larger, ethnically diverse samples and the development of personalized therapies. These findings could improve

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early diagnosis, intervention, and management of hemochromatosis, enhancing patient outcomes and quality of life. Moreover, more epidemiological work needs to be done to understand hemochromatosis's occurrence and its consequences on liver health across different populations.

Index Terms: Hemojuveline, Hepatocytes, Hemochromatosis, Hepcidin, Ferroprotein.

I. INTRODUCTION

Hemochromatosis is a genetic disorder characterized by iron overloads in the body.(Bardou-Jacquet et al.) The over-accumulation of dietary iron takes place in different organs like the liver, pancreas, and heart, which if not controlled can result in severe liver complications, diabetes as well as heart complications (Kanwar and Kowdley) The key controller of iron absorption in the liver is Hepcidin. When its level is going to decrease it causes iron overload. The HFE gene is located on chromosome 6, and its mutations disrupt the normal function of the HFE protein, leading to increased intestinal absorption of iron.(Fernandes et al.) Normally, the HFE protein interacts with the transferrin receptor to regulate the amount of iron absorbed from the diet. However, this interaction is impaired in individuals with the C282Y mutation, resulting in excessive iron absorption and subsequent deposition in various organs, including the liver, heart, and pancreas. Over time, this iron overload can lead to serious complications such as liver cirrhosis, diabetes, and heart disease.(Costa et al.) The penetrance of HH, or the proportion of individuals with the mutation who develop clinical symptoms, is variable. Not all individuals with the C282Y mutation will develop iron overload or related complications, indicating the involvement of other genetic, environmental, or lifestyle factors in the disease expression. In addition to HFE mutations, other genes such as HJV, HAMP, and TFR2 have been implicated in non-HFE-related forms of hereditary hemochromatosis, though these are much rarer.(Katsarou et al.)

Hereditary hemochromatosis or primary hemochromatosis also termed HFE hemochromatosis results from the mutation in the HFE gene. HFE gene also known as human homeostatic iron regulator protein an activator of Hepcidin.(Finlayson) Hemochromatosis occurs in the body due to the results of pathogenetic variants in different genes which increases iron body stores and also a heavy amount of iron stores in the different organs of the body. The 2 most crucial advancements in the field of this disease have taken place in the past few decades. The first one is the identification of the HFE gene and the second one is the discovery of Hepcidin. Both of these have resulted in the manifestation and clear viewpoint of the understanding of iron metabolism in the body.(Moretti et al.) In primary hemochromatosis, there are 2 types of disease type 1 and type 2 also called juvenile. The type 1 population has mutated genes which include the people of Europe within the age of 40-60 years. In women, the loss of blood during the menstruation cycle and loss of iron during pregnancy are the late manifestations of hemochromatosis.(Barton, Edwards and Acton)

There is a strong susceptibility to this disease also found in those patients with heavy alcohol consumption. The high amount intake of alcohol increases iron overload and there is a chance of the developing of liver cancer. Juvenile or type 2 occurs at an early age or

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in childhood. It causes a decrease in sex hormone production. Secondary hemochromatosis is developed when a patient has taken a high amount of iron supplements, received a transfusion of blood, and also due to anemia. This iron overload also takes place due to the mix-up of the increased iron absorption and the recurrent iron transfusion.(Geller and de Campos) The alcoholic liver is also the cause of secondary iron overload, the increased level of the iron in liver due to alcohol has been studied in a recent study. Alcohol results in increased iron absorption is expression of Hepcidin takes place due to high intake of alcohol(Pietrangelo "Hemochromatosis: An Endocrine Liver Disease"). Iron overload in the liver is also the cause of hepatocellular carcinoma and hepatocellular carcinoma is the most dangerous malignancy of all over the world. It is increasing day by day and is most common all over the world. In HCC end organ damage of an individual takes place. When hemochromatosis remains undiagnosed at later stages this disease is the cause of liver cirrhosis, fibrosis, and hyperpigmentation and it is also referred to as bronze diabetes.(Gochee et al.) The main mutations in HFE hemochromatosis are C282Y (Cysteine to Tyrosine substitution at amino acid 282) and H63D (Histidine to Aspartic acid substitution at amino acid 63). H63D is often referred to as a high iron gene HFE mutation.(Peesapati et al.)

There are 5 genes involved with various subtypes of hemochromatosis disorder. These genes are HFE, TFR1, HAMP, HJV and SLC40A1. 60-100% of cases of hemochromatosis in the European population are dominantly caused by the HFE gene. In the Celtic population thousands of years ago this disorder has been recognized. (Wallace and Subramaniam)There is increased Transferrin saturation and Ferritin levels in this disorder and it is also the cause of many diseases like liver cirrhosis, fibrosis, hepatocellular carcinoma, and bronze coloring of the skin. In Northern European people hereditary hemochromatosis is one of the most popular disorders (Andersen et al.)In the Caucasian population, genetic hemochromatosis is also the most prevalent disorder. Various mutations occur in different genes involved in hemochromatosis but the most common is the HFE gene mutation. Positional cloning is used by Feder and Colleagues for the identification of HFE-linked disorders. Hepcidin is regulated normally by HFE protein Hepcidin is the main controller of iron metabolism.(Grosse et al.)Von Recklinghausen was the first person in the 19th century who introduces the term “hemochromatosis”. Symptoms of hemochromatosis include skin pigmentation, cirrhosis, diabetes mellitus, and liver fibrosis. When a mutation occurs at the C282Y position of the HFE gene Hepcidin liver hormone is reduced and with the reduction of the Hepcidin level in the liver iron levels increase in the liver which causes hyperferritnemia and liver cirrhosis (Camaschella)

Mostly in the form of heme, inorganic, and Ferritin absorption of dietary iron is carried out. The dietary iron which is inorganic state mostly exists in the oxidized form Fe+3 and reduces to Fe+2 for intestinal uptake this process of reduction takes place in the intestinal cells of the apical membrane by the seductresses. Divalent metal transport is the key relevant that assisted in the transportation of Fe2+ in the apical membrane.(Babitt and Lin) All of this transportation takes place in an acidic environment In humus, the vital

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process occurs named as iron homeostasis its name work is to control the iron excess in the body which is very harmful for the body and destroys the cells Iron is very important for many processes in the body mainly it is required for biological functions like oxygen transport and cellulose respiration. 3-5 grams of iron is present in the adult human body for vital functions.(Weiss) Iron is stored in the liner which is in excessive amounts and mobilized in demand. Indigenous peoples, refugees, and immigrants from low or middle-income countries, and those people who cannot afford high-quality diets have more chances often anemia and iron deficiency development.(Wessling-Resnick)

There are many diseases associated with iron overload. Billions of people patients are affected worldwide due to the iron in balance. Iron overload is responsible for serious damage and fatalities. Anemia, Thalassemia, Sickle cell disease melodiousness, linear cirrhosis, fibrosis, and bronze skin disease are caused by the iron in balance in the body(Fargion, Valenti and Fracanzani). Iron load toxicity in medicine from molecular to Cellular Aspects to Clinical Implications. In Joints and various organs such as the liver, heart, and Pancreas excessive iron is stored which is very harmful to the organs and results in the dyes functions of the body organs(Kontoghiorghes). There are many fetal outcomes of hemochromatosis related to the liver. Some of these are liver cirrhosis, hepatocellular carcinoma, fibrosis, and bronze skin disease. The impact of hemochromatosis on the liver is a topic of interest because the liver is the most affected. By the hemochromatosis disorders. Liver hormone Hepcidin is going to be reduced which can cause hepatic iron overload (Cobilinschi et al.)

Hemochromatosis disorder related to the liver is the main cause of hepatic cancer. 6th most prevalent diagnosed cancer is liver cancer. Hepatic cancer mortality rate in China is the highest among all over the world. Hepatocellular carcinoma and Hepatic Cholangio Carcinoma (ICC) are primary liver cancer types. Hepatic Angiosarcoma (HA) is the 3rd most prevalent liver cancer, begins in endothelial cells of the blood of lymphatic vessels.(Paganoni, Lechel and Vujic Spasic) There are 2 types of hemochromatosis disorder the first one is primary and the 2nd one is secondary hemochromatosis. Primary hemochromatosis is also known as hereditary or HFE hemochromatosis. There is iron overload in different organs body like the liver, pancreas, heart, skin, and joints in primary hemochromatosis. The HFE gene is involved in hereditary hemochromatosis. A biological scientist first identified HFE gene mutation. The HFE gene has many different mutations including H63D, C282Y, and S65C. The most prominent is the C282Y Homozygous mutation (Shamas)

Secondary hemochromatosis is also known as nonhereditary or non-HFE hemochromatosis. 4 types of non-HFE hemochromatosis genes result in nonhereditary hemochromatosis. These are HJV, HAMP encoding Hepcidin, TFR2 transferrin receptor 2, and the solute carrier family 40 member 1 (SLC40A1) encoding the ferroportin 1. The allelic frequency of these genes ranges from 0.00007-0.0004. Hemochromatosis is directly involved in liver fibrosis, resulting in chronic liver damage with a large accumulation of extracellular matrix protein. Alcohol consumption, nonalcoholic

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steatohepatitis, viral hepatitis, autoimmune hepatitis, nonalcoholic fatty liver disease, and cholestasis liver disease lead to the development of liver fibrosis. There are many novel targets and signaling pathways including the following processes apoptosis, oxidative stress, epigenetics, inflammation, and receptor-generated mediators in liver fibrosis. (Fletcher and Powell)Alcohol intake in heavy volume approximately is the most common cause of liver Cirrhosis (>50-70 g/day) is the most common cause of liver inflammation and injury. Hepatic stealate cells are the key players which are involved in the development of liver cirrhosis. Hepatocellular carcinoma is considered the most dangerous and life-threatening complication related to hemochromatosis disease .(Tirnitz-Parker et al.)

Genomic studies have also identified population differences in the prevalence of HH-related mutations. The C282Y mutation is most commonly found in populations of European ancestry, with a carrier frequency of about 1 in 8 individuals.(Burke et al.) In contrast, it is rare in Asian and African populations. This mutation likely originated from a single ancestor in Northern Europe, with evidence suggesting a selective advantage related to iron metabolism in the historical context of iron-deficient diets. Advancements in genomic technologies have facilitated better understanding of the molecular basis of HH and have improved diagnostic accuracy through genetic testing.(Grosse et al.) These developments allow for early detection and management of the disease, potentially preventing the severe consequences of untreated iron overload.(Savatt et al.)

The highest prevalence of HH is observed in individuals of Celtic origin, with a carrier frequency of 1 in 10 and a homozygous prevalence of 1 in 200 among Northern Europeans. In the general European population, the prevalence of C282Y homozygosity ranges from 0.2% to 0.5%. In the United States, HH prevalence is similar to that in Europe, with a homozygous C282Y prevalence of about 0.5% among non-Hispanic whites. The condition is less common in African Americans, Hispanics, and Asian populations.(Katsarou et al.) Among individuals of European ancestry in Australia, the prevalence of HH is comparable to that in Europe, with a C282Y homozygosity rate of approximately 0.5%. HH is rare in Asian populations, with significantly lower carrier rates of the C282Y mutation. In Japan, China, and other East Asian countries, the prevalence of HH is negligible. HH is also rare in African populations.(Waalen, Nordestgaard and Beutler) The C282Y mutation is almost non-existent, and other genetic mutations associated with iron overload are more common in sub-Saharan Africa, though these mutations are not as strongly linked to HH. Data on HH in South American populations are limited, but the condition appears to be less prevalent than in European-descended populations. The prevalence of HH in the Middle East is low, with very few cases reported. The genetic mutations associated with HH are uncommon in this region.(Barton, Edwards and Acton)

In the United States, Canada, and Australia, HH prevalence mirrors that of Northern Europe due to the large proportion of the population with European ancestry. Non-Hispanic whites have a homozygosity rate of approximately 0.5% for the C282Y mutation

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in Asian populations.(Jones et al.) Studies in China, Japan, and South Korea show a very low prevalence of the C282Y mutation, with the mutation almost absent in these populations.(Pointon et al.) Other genetic mutations associated with iron overload are more common but do not lead to the classic HH seen in European populations. HH is also rare in South Asia like India, Pakistan, and Bangladesh. The C282Y mutation is virtually nonexistent in these populations. Studies indicate that iron overload in South Asian populations is more likely to result from dietary factors or other non-genetic causes rather than from HH. HH is uncommon and Middle Eastern populations, where the C282Y mutation is rare. Iron overload conditions in these regions are more likely to be linked to other genetic factors or environmental influences than HH.(Crawford et al.) The prevalence of hereditary hemochromatosis significant variation across different ethnic groups and geographical regions. While the condition is most prevalent among individuals of Northern European descent, it is rare in Asian, South Asian, African, and Middle Eastern populations. This disparity is largely attributable to the varying frequencies of the C282Y mutation and other genetic variants across these populations.(Crownover and Covey)

Studying hereditary hemochromatosis (HH) in South Asia is crucial due to its underexplored prevalence and potential health impacts in this diverse region. While HH is more common in European populations, recent studies suggest that mutations related to iron overload could be present in South Asia, albeit at lower frequencies.(Panigrahi et al.) For instance, the C282Y mutation, the most common HH-associated mutation, is rare in South Asians, but the H63D variant has a higher occurrence rate, with carrier frequencies estimated at 2-5%.(Lok et al.) Given South Asia's large and genetically diverse population, understanding HH's local prevalence and genetic variations is essential for improving diagnostic approaches, public health planning, and personalized medical interventions. Additionally, iron metabolism disorders are underdiagnosed in this region, potentially leading to overlooked cases of iron overload that can result in severe complications like liver disease and diabetes, underscoring the need for focused research.(Hanson, Imperatore and Burke)

The genetic diversity in South Asia is one of the highest in the world, shaped by complex historical migrations, cultural practices, and population stratification. This diversity plays a significant role in the prevalence and expression of hereditary hemochromatosis (HH) genetic mutations within the region.(Hajar et al.) While the C282Y mutation, predominantly responsible for HH in European populations, is rare in South Asians, other variants such as H63D and S65C have been detected, though at lower frequencies. The heterogeneity of these mutations across different ethnic groups in South Asia reflects the region's intricate genetic landscape. The impact of this genetic diversity on HH prevalence is multifaceted. Given the varied genetic backgrounds, certain populations in South Asia may harbor unique mutations affecting iron metabolism, potentially leading to undiagnosed or misdiagnosed cases of HH.(Pointon et al.) Additionally, the interaction between these genetic factors and environmental influences, such as diet and public health conditions, could further modulate the expression of HH-related symptoms in the

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region. This genetic variation necessitates a tailored approach to genetic screening and diagnosis of HH in South Asia. Understanding the specific mutations and their distribution can improve early detection and intervention strategies, reducing the risk of severe complications associated with iron overload in affected individuals.(Adams et al.)

Public health systems in South Asia, which already face significant challenges due to a high burden of infectious diseases and non-communicable diseases (NCDs), must also account for the potential impact of undiagnosed or untreated HH. Without proper recognition and diagnosis, HH can lead to severe complications such as liver cirrhosis, hepatocellular carcinoma, diabetes, and heart disease.(Lok et al.) These conditions require extensive medical care, placing a substantial burden on healthcare systems. The economic implications of HH are significant. Studies from Western countries estimate that the annual healthcare cost per untreated HH patient can range from $2,000 to $10,000, depending on the severity of iron overload and related complications.(Motulsky and Beutler) While specific data for South Asia is limited, these costs are likely to be high, particularly in resource-constrained settings where advanced medical care is less accessible. If left unaddressed, HH could contribute to the growing burden of NCDs in the region, exacerbating existing healthcare challenges.(McLaren et al.)

Early diagnosis and treatment, primarily through phlebotomy or iron chelation therapy, can prevent most of the severe complications associated with HH. However, for these interventions to be effective, there must be a robust public health strategy that includes awareness campaigns, genetic screening programs, and the training of healthcare professionals to recognize and manage HH.(Van Bokhoven, van Deursen and Swinkels) Furthermore, resource allocation needs to consider the potential hidden burden of HH. Investing in genetic research and public health infrastructure to identify and manage HH could mitigate long-term healthcare costs and improve patient outcomes. Tailoring public health initiatives to the unique genetic landscape of South Asia is essential to address this emerging health concern and ensure that the healthcare system is prepared to manage the potentially significant burden of HH.(Pietrangelo "Hereditary Hemochromatosis: Pathogenesis, Diagnosis, and Treatment")

The primary objective of this systematic review is to compile and analyze data on the prevalence of hemochromatosis genetic mutations in South Asia. Hemochromatosis is a genetic disorder characterized by excessive iron accumulation in the body, which can lead to serious health complications if not managed properly.(Kowdley et al.) While hemochromatosis is well-documented in Western populations, there is limited and inconsistent data regarding its prevalence in South Asian populations. This review aims to address this gap by systematically evaluating and synthesizing existing research studies that report on the frequency and distribution of hemochromatosis-related genetic mutations (such as HFE gene mutations) within South Asian countries, including but not limited to India, Pakistan, Bangladesh, Nepal, and Sri Lanka. By aggregating data from various studies, this review will provide a comprehensive overview of the genetic landscape of hemochromatosis in the region.(Padeniya et al.) The findings are expected

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to enhance our understanding of the genetic epidemiology of hemochromatosis in South Asia, identify any region-specific genetic variants, and highlight potential discrepancies with data from other regions.(Crawford et al.) This information will be crucial for improving diagnostic strategies, developing targeted public health interventions, and guiding future research directions in South Asia. Ultimately, this systematic review aims to contribute to the broader goal of optimizing hemochromatosis management and improving patient outcomes in this diverse and populous region.(Clark, Britton and Powell)

II. MATERIAL AND METHOD

A. Blood/Sample Collection

Fresh blood sample of 30 confirmed patients of hemochromatosis and 10 Blood samples of Healthy individuals was taken in EDTA vacutainers from hepatology department of Gangha Ram Hospital Lahore, Blood was further proceeded for the assessment of following parameters mainly age of hepatic patients, ferritin level, transferrin, level of hepatic enzymes Alkaline phosphatase (ALP). Alanine transaminase (ALT). Aspartate transaminase (AST). Gamma- glutamyl transferase (GGT). Serum electrolytes including sodium(Na^+), Potassium (k^+) and Chlorine (Cl^-), Renal function test (RFT's) including urea, Creatinine, Liver Functions Test (LFT's) involving Serum Glutamic Oxaloacetic Transaminase (SGOT), Lipid Profile test involving High Density Lipoprotein (HDL), Low density Lipoprotein(LDL) .

B. DNA Extraction

On the back of every DNA extraction procedure following some points or basis are Some such as i) interruption of nuclear and cytoplasmic sheets ii) Refinements and separation of DNA from other different apparatus or components of cell like protein, lipid, protein and different nucleic acid iii) quantification after selecting a suitable method for DNA extraction .Different other factors should also notices such as time, cost, of whole procedure, latent toxicity profile, equipment of laboratory procedure and fully guideline idea ,mainly arrange required amount of samples for the following protocol of DNA Extraction.

Every living body in their cells consists of Deoxyribonucleic (DNA). DNA is a vital part of living organism because it contains necessary genetic information, make up with nitrogenous bases that is Guanine, Cytosine, Thymine and Adenine and Phosphate group and sugar group.

DNA extraction is very useful because extracted DNA is useful for other different biological manner techniques like PCR, Quantification, Sequencing useful for diagnostic lab for identification of genetic mutation, alternation and disturbance in genome.

DNA extraction is useful for identification of different genetic diseases, inherited which are possible to identify after DNA extraction

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C. Procedure of DNA Extraction by organic method

Before DNA extraction samples of blood were freeze at -20ºC. Take out blood samples from refrigerator and incubated at room temperature until it become thawed**.** Take Eppendorf's tubes according to the samples. Take tubes according to how many sample DNA have to be extracted after that added 500 micro titer of 20 mM tris HCL in each Eppendorf's tubes. Put cut out gel piece in tris HCL Eppendorf's tubes. Vortex the samples centrifuge at 13000rpm for 5 minutes. Then supernatant discarded almost 100 micro liter supernatant left behind with pellet. Then add 500 micro liters of 50mM Tris HCL again in each Eppendorf's tubes. Vortex mix again samples and then centrifuges samples for 5 minutes. Supernatant discarded and little left behind, then 500 micro liter lysis buffers in each tube. Also added 20 micro liters Proteinase K and incubated the samples at 55 degrees centigrade for overnight in the incubator, then the 500 microliters of (PCI) Phenol chloroform Isoamylalchol and invert mix the samples and centrifuge these samples for 12 minutes at 13000 rpm. Supernatant transfer into the clean Eppendorf's 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 supernatant discarded. Then added 500 microliters of 70% ethanol inverted mix the samples and centrifuge at 13000rpm for 5 minutes supernatant was discarded. The leftover pellet Extracted DNA and then air dried for 3-4 hours. Then 150 micro liter TE buffer in all Eppendorf's and DNA stored in elution buffer at **-**20℃.

D. 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.

Preparation of 1%Agarose Gel

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

Adjustment of Gel Apparatus

For 1% gel, we take 1.5 µl loading dye on the paraffin paper actually purpose of loading dye is to observe the migration of DNA fragments and it also helpful for the samples sink into the gel. Now, we adjust the voltage about 80. We set the timing of gel electrophoresis

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which is approximately 40mins for best results. Most important thing should be carefully using enough quantity of buffer that it fully covers the casting tray in the gel mold. Connect cathode (black lead) to the black electric point and Anode (red lead) to the red electric point and we fully adjust these points. Turn on the power supply and monitoring the running of gel. After running gel about 40mins, when electrophoresis is completed we turn off power supply and remove the cover. And then, check the migration of DNA fragments after expose under UV light.

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: Tetra arms primers of SNP rs7385804

Sr. No	Primer	Primer Sequence 5’ to 3’	Length of Primer	Tm	Amplicon size (bp)
1	Outer forward	AGTAGCTGGGAT CACAGGTGCC	22 bp	58	490
2	Outer reverse	AGCCTCCCAAGT AGCTGGGACT	22 bp	58	
3	Inner Forward	CCAAAATGCTGG GATTATAGGATT G	25 bp	58	C allele =252 G allele =248
4	Inner reverse	AGGCTGGGTGC GGTACGTG	19 bp	58	

E. PCR Polymerase chain Reaction (PCR)

The tetra-primer amplification refractory mutation system-polymerase chain (ARMS-PCR) reaction is genetic method to genotyping single nucleotide polymorphism (SNPs). In this technique two pairs of primers were used two outer and two inners. The outer primers are non- allele-specific primers that only amplify target gene sequence whereas

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inner primers are allele specific and outer primers serve as a template to the inner primers.

Selection of SNP of interest

To investigate interested SNP which is directly linked with hemochromatosis and liver related disorders, different researches performed by using Google scholar and Literature. The TFR2 gene with interesting SNP rs7385804. Many literatures reveal that SNP rs7385804 of TFR2 gene correlate with hemochromatosis.

Tetra Arms PCR

Tetra-arms PCR, also known as tetra-primer ARMS-PCR or tetra-primer amplification refractory mutation system PCR, is a technique used for genotyping single nucleotide polymorphism (SNPs) or detecting mutations. Here's a detailed methodology in 40 points.

Design four primers: two outer primers (forward and reverse) and two inner primers (allele- specific forward and reverse). The outer primers amplify a DNA fragment containing the SNP or mutation site. The inner primers are designed to be allele-specific, targeting either the wild- type or mutant allele. Perform a standard PCR reaction with the outer primers to amplify the target DNA fragment. Include a positive control (known DNA sample) and negative control (no DNA template) in the PCR reaction. Denature the DNA at 95°C for 5 minutes. Perform 35 cycles of PCR: denaturation at 95°C for 30 seconds, annealing at the primer-specific temperature for 30 seconds, and extension at 72°C for 30 seconds. Annealing temperature is determined by the Tm of the primers. After the final cycle, perform a final extension at 72°C for 5 minutes. Analyze the PCR products by gel electrophoresis.

Prepare a 2% agarose gel in 1X TAE buffer and add ethidium bromide for staining. Load the PCR products along with a DNA ladder onto the gel. Run the gel at 100V for 30-45 minutes. Visualize the DNA bands under UV light. The presence or absence of specific bands indicates the genotype of the sample. The wild-type allele will produce a band with the outer primers, while the mutant allele will produce a band with the inner primers. The absence of any band indicates a homozygous genotype (either wild-type or mutant). Validate the results by sequencing if needed. Troubleshoot any non-specific amplification or primer-dimers. Optimize PCR conditions if necessary, such as adjusting annealing temperature or primer concentrations. Ensure proper handling and storage of reagents to prevent contamination. Use sterile technique to avoid contamination of samples.

Maintain a clean workspace and regularly decontaminate surfaces and equipment. Document all steps and results carefully for reproducibility. Compare results with known genotypes or controls. Consider using replicate samples for validation. Use appropriate statistical tests to analyze the data. Ensure the PCR machine is calibrated correctly for accurate temperature control. Check the integrity of DNA samples before starting the PCR reaction. Use high-quality DNA extraction methods to avoid PCR inhibitors. Consider using alternative genotyping methods for confirmation. Store PCR products at -

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20°C for short-term storage. Store DNA samples at -80°C for long-term storage. Dispose of PCR waste properly according to institutional guidelines.

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.

Gradient Arms PCR Optimization

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

TFR2 GENE

To optimize TERA-ARMS PCR of selected SNP *TFR2* C677T, two strategies were applied simultaneously. Gradient PCR was run in the triplicate of 10µL with three different mutual ratio of outer and inner primers (1:1, 1:2 and 1:4) at a range of annealing temperature (58-67°C). Thermocycler profile for PCR is shown in Figure 3.

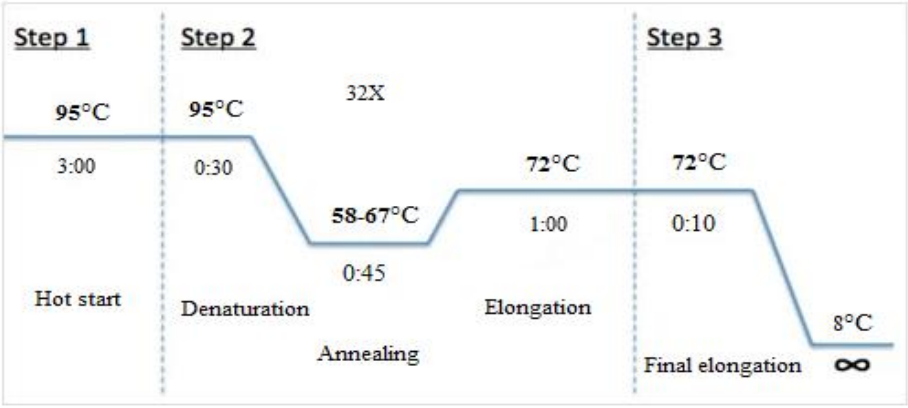

Figure 1: Thermocycler PCR profile

After optimization the best results with the gene was obtained under the following conditions

TFR2 GENE

All the DNA samples collected were processed for ARMS PCR using the optimized conditions got from previous step. Kyratec Super cycler thermal-cycler was used and the reaction components for 10 µL PCR is shown in Table 3.

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Table 2: Reagent Components for TETRA-ARMS PCR

Sr. no	Reagents	Quantity
1	Master mix (2X)	5 µL
2	Outer forward primer (0.2µM)	0.2 µL
3	Outer reverse primer (0.2µM)	0.2 µL
4	Inner forward primer (0.2µM)	0.2 µL
5	Inner reverse primer (0.2µM)	0.2 µL
6	Nuclease free water	3.1 µL
7	Taq DNA polymerase (5µM)	0.1 µL
8	Sample DNA (100ng)	1 µL
Total		**10µL**

Gel Electrophoresis for PCR Analysis

To authenticate the amplified SNPs amplicons of *TFR2*C677T, amplicons were checked on 3% agarose gel in 0.5X TBE buffer. The maximum capacity of the wells of the gel was filled with an overall amount of 4 uL of liquid. (2uL PCR product + 2uL Tracking dye). Ethidium bromide (EtBr) was used to access the bands under UV transilluminator. Size of the allele specific products were estimated through comparison with 1Kb DNA ladder (GeneRuler, Thermoscientific). Photograph of the gel was captured via the gel documentation system.

Validation of Tetra-Arms PCR Output Through Sanger Sequencing

For confirmation of the genotyping results of SNPs via TETRA-ARMS PCR, random samples, representative of all three genotypes of *TFR2* C677T (CC, CT and TT) were sequenced through sanger sequencing. For Sanger sequencing, amplification PCR was performed using outer forward and outer reverse primers only using similar optimal conditions of ARMS PCR with an annealing temperature of 60°C.

1.5% Agarose Gel Electrophoresis

To authenticate the amplified SNPs amplicons of *TFR2* C490T amplicons were checked on 1.5% agarose gel in 0.5X TBE buffer. The maximum capacity of the wells of the gel was filled with an overall amount of 4 uL of liquid. (2uL PCR product + 2uL Tracking dye). Ethidium bromide (EtBr) was used to access the bands under UV transilluminator. Size of the allele specific products were estimated through comparison with 1Kb DNA ladder (GeneRuler, Thermoscientific). Photograph of the gel was captured via the gel documentation system in the means of further analysis

Post Amplification Purification

Primers, dNTPs abundance, salts, primer dimers, are all in excess amount, so prior to sequencing PCR, the amplified product needs to be purified. These contaminants may prevent the PCR reaction from sequencing. However, they can be eradicated by a number of other possible purifying techniques. The High Prep ^{TM} kit system was used for purification of the PCR product. The prospect of optimum environment for reversible DNA adsorption on paramagnetic beads serves functioning as the PCR's framework cleanup

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procedure. The first three essential steps were binding, washing and elution operations. Add 1X beads solution according to PCR volume. For 15µL reaction volume add 15µL beads solution. Mix both by pipetting then short spin. Keep at room temperature but not on magnetic plates for 5 min. Then add 50µL absolute ethanol in that mixture and keep for 10 more min at room temperature. Place your samples now on magnetic plate for 3 min. While samples were on magnetic plate aspirate the supernatant through pipette carefully without disturbing settled magnetic beads. Add 50µL of 70% freshly prepared alcohol and remove it with pipette. Air dry beads plate with open cap of tube and placed on magnetic plate for 5 min till the residual ethanol evaporate. Do not over dry the beads. Now remove samples from magnetic plate and add 12µL of H_2O or TE for elution. Mix by pipetting. Place for 5 min on table and if need short spin the samples. After 3 min aspirate the supernatant, that was purified the sample now. Run gel with 2µL DNA to know the concentration.

Sequencing PCR

The PCR sequencing process makes use of the purified amplification product. The chain termination method is the principal for this sort of PCR reaction of sequencing. There two separate reactions were set for every amplicon using reverse and forward primers in order to double confirm the allele present in that sample. For the reverse primer generated sequence, prior to analysis, the results were transformed into a forward complementary arrangement. For sequencing PCR reaction in addition to primer and template, the BigDye Terminator Kit v 3.1 (Applied Biosystem) was used that contained reaction enzymes, buffers, normal dNTPs and four kinds of dye-labeled dideoxynucleotides (ddNTPs) in a stochiometric ratio. Four dye labeled ddNTPs will give different color fluorescence in the Genetic analyzer when they will be activated through an argan laser. ddATP-dR6G express green color, ddCTP-dTAMRA blue, ddTTP-dR110 red and ddGTP-dROX will give black color flourescence. DNA template that need to be used in reaction was estimated by the formula i.e. Q=1/50 ng, where Q=quantity of DNA and 1=DNA template length. There is a list of the reagents used in PCR in Table 5.

Table 3: Reagents of Sequencing PCR Reaction Mixture

Sr. no	Reagents	Volume µL
1	Sequencing buffer (5X)	1.5µL
2	Big dye (0.5X)	1µL
3	Reverse of Forward primer (5µM)	1µL
4	Template DNA	2µL
5	PCR water	4.5µL
Total volume		**10µL**

Statistical Analysis

Allelic and genotypic analysis of TETRA-ARMS data was performed using the SHEsis online version (http://analysis.bio-x.cn/myAnalysis.php). SNPstats (https://www.snpstats.net/start.htm) an online SNP analysis software was used to develop genetic models of association. A 95% confidence level was used for all statistical

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tests. The epidemiological analysis of the analyzed patients with reference to TFTs, age and gender were calculated by SPSS Statistics 21.0 (https://www.ibm.com/products/spss-statistics) using two tailed $\chi 2$. P-value <0.05 and <0.01 were taken as statistically significant.

III. RESULTS

The present research investigated any association of polymorphisms in TFR2 gene variants in the development of hemochromatosis disorder. Also, to find out any relationship of sex, age, and ratio TFR2 with liver patients. The epidemiological data and blood samples were collected from the Sir Ganga Ram Hospital, Lahore Punjab, Pakistan. Hemochromatosis disorders become common nowadays and mostly occur in countries that have a high amount of ferritin. The 30 samples were collected from infected individuals with different genders in different age groups.

A. DNA extraction by Organic Method

The blood cells' DNA was extracted using a modified organic method. The concentrations of DNA were quantified and measured by using the nanodrop. A variable range of results obtained, ranged from 23.8-1289.8 ng/µL. The integrity of DNA was measured on 1% agarose gel by comparing it with standard DNA. Figure 4.1 shows the results obtained from nanodrop. shows the results obtained from nanodrop. DNA was successfully extracted from 30 case samples and 10 control samples.

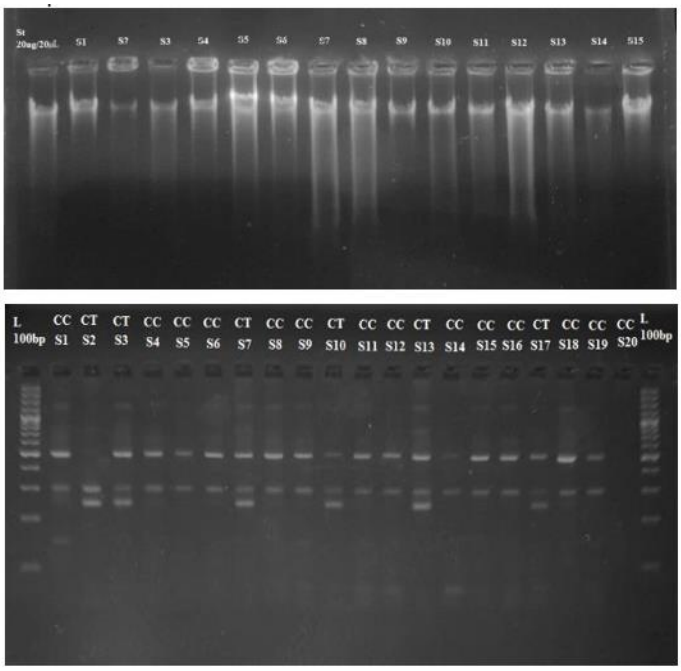

Figure 2: Genomic DNA on 1% agarose; Well No.1 contain standard DNA samples (St) 20ng/20µL, Well No. 2-16 contain DNA samples (S1-S15)

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Figure 3: DNA quantification of blood samples from nanodrop

B. Tetra Arms PCR Optimization

For optimization of TETRA-ARMS PCR, various conditions of annealing temperatures and primer concentrations were applied and both variants were successfully amplified with different optimal conditions as follows.

TFR2 PCR Optimization

The TFR2 gene variant was optimized by Gradient PCR through allele-specific primers. Amplicons of PCR were loaded in the wells of 2% agarose gel to confirm their presence and compared with a DNA ladder of 100bp.

The amplicon sizes for *TFR2* were 490bp for a whole fragment, 248bp for the C allele, and 298bp for the G reference allele. Annealing temperature 60°C using a 1:1 ratio of outer and inner primers generated better results than other conditions.

Figure 7 shows the results run on agarose gel for gradient PCR optimizations of the TFR2 variant. Further, all 162 samples of patients and 114 samples of controls were processed via these TETRA-ARMS PCR conditions run on these conditions Figure 4.3

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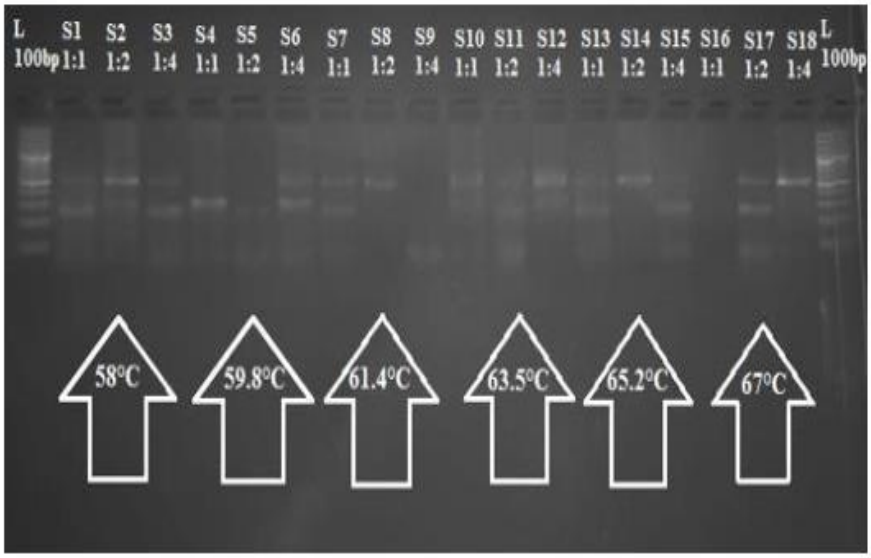

Figure 4: TETRA-ARMS PCR optimization run on 2% agarose for TFR2; Bands are shown in lanes(S1-S18) having primer concentrations 1:1, 1:2, 1:4 respectively at different temperatures and compared with Ladder(L)

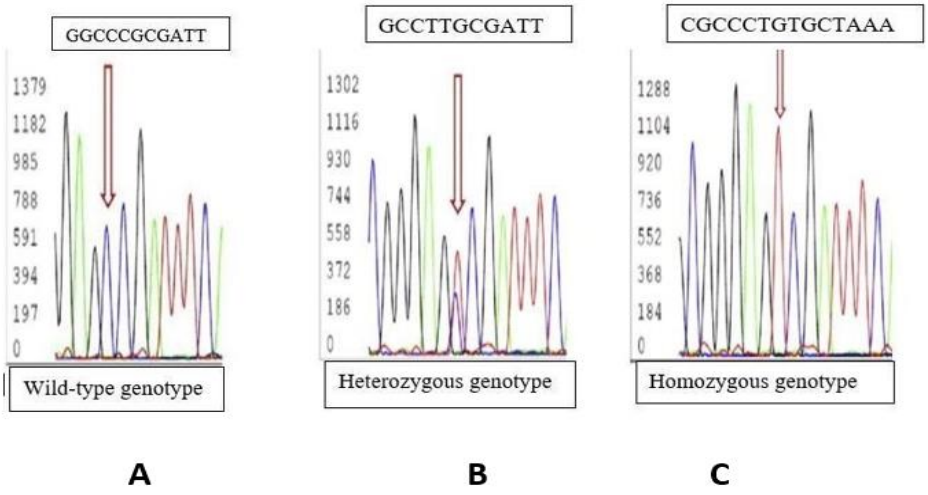

Figure 5: TETRA-ARMS PCR main product size 490bp, reference C allele 298bp and mutant T allele 248bp of TFR2 for study sample in 2% agarose; Bands are shown in lanes(S1- S10) (hemochromatosis and S11-S20 hemochromatosis) and compared with Ladder(L)

Figure 5*:* Chromatogram showing Sanger's sequencing results of TFR2 variant with random samples; (A) TFR2 wild type GG genotype, (B) TFR2 heterozygous CG genotype, (C) TFR2 homozygous CC genotype

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Validation of Tetra-Arms generated alleles through sanger sequencing

For confirmation and validation of the SNPs detected by ARMS PCR of *TRF2* variant, random nine samples representative of all three genotypes (CC, CG and GG) were sequenced. Figure 4 the amplicons of some of the samples, amplified using outer primers and purified prior to sequencing PCR. TFR2 variant ranging from 0.5-8ng/µL. show the selected SNP's sequencing electropherogram (rs7385804) where exact position is highlighted. The X-axis presents the genotypes / alleles and the Y-axis represents the peak's height expressed by means of relative fluorescence unit (rfu).

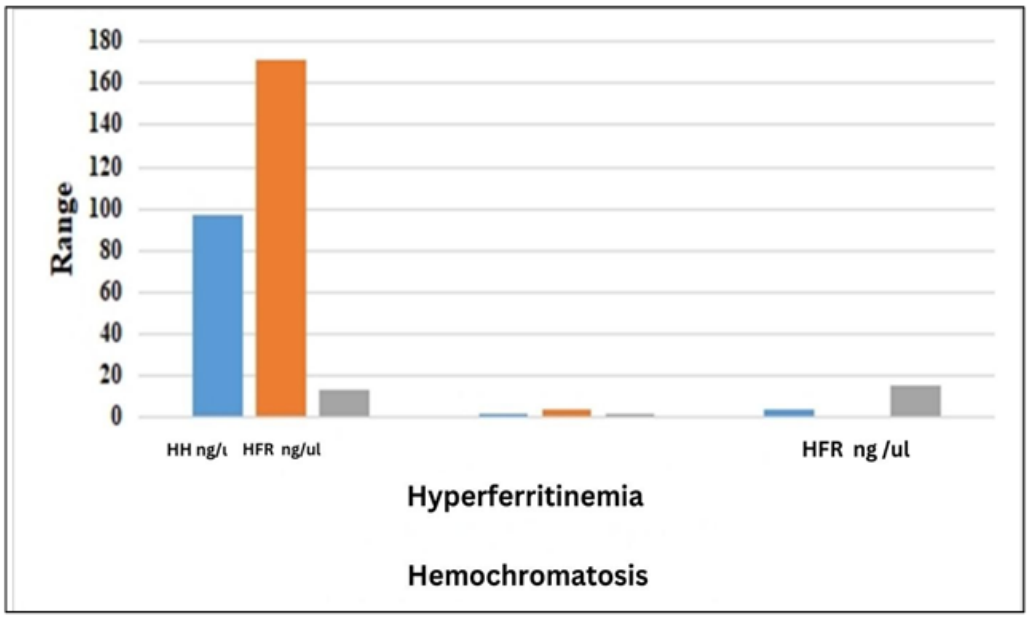

Figure 6: Frequencies of TFR2 in case and control

Statistical analysis in Case and control sample

Using SHEsis and SNP stats software, the allele and gene frequencies of 114 controls and 162 case samples were estimated. A 95% confidence level was used for all statistical tests. The data were also analyzed by using two-tailed χ2 and TFTs, age, and gender were calculated by using SPSS. Statistical significance was defined as a P-value of <0.05 and >0.01.

Alleles and genotypes frequencies of Rs7385804 (Tfr2 C677g) and Rs1805087 (Tfr2 A2756g)

SHEsis and SNPstats were also used to compute the genotype and allele frequency of rs7385804 *TFR2* C677G and. the genotypic model of the genotypes rs7385804 depicted in Table 4.2 and Table 4.3 showed allelic model using SHEsis software.

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Table 4: Genotypic frequency of TFR2 in cases and control

Sr. no	Genotype	Frequency		p-value
		Control	Case	
1	C/C (homozygous wild)	81 (0.711)	96 (0.593)	
2	C/G(heterozygous)	24 (0.229)	63 (0.389)	
3	C/C (homozygous mutant)	0 (0.000)	3 (0.019)	0.006594

Table 5*:* Allelic frequency of TFR2 in case and control

Sr. no	Allele	Frequency		p-value	Odd Ratio (CI)
		Control	Case		
1	C	186 (0.816)	255 (0.787)	0.003325	0.476858 (0.288808~0.787354)
2	G	24 (0.114)	69(0.213)		

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 Table

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

Sr. no	Model	Geno type	Control	Case	p-Value	OR (95% CI)	AIC
1	Co-dominant	C/C	81 (77.1%)	96 (59.3%)	0.0035	1.00	352.6
		C/T	24 (22.9%)	63 (38.9%)		2.21 (1.27-3.86)	
		T/T	0 (0%)	3 (1.8%)		NA (0.00-NA)	
2	Dominant	C/C	81 (77.1%)	96 (59.3%)	0.0022	1.00	352.5
		C/T - T/T	24 (22.9%)	66 (40.7%)		2.32 (1.33-4.03)	
	Recessive	C/C – C/T	105 (100%)	159 (98.2%)	0.082	1.00	358.9
		T/T	0 (0%)	3 (1.8%)		NA (0.00-NA)	
4	Over-dominant	C/C – T/T	81 (77.1%)	99 (61.1%)	0.0056	1.00	354.2
		C/T	24 (22.9%)	63 (38.9%)		2.15 (1.23-3.74)	
5	Log additive	---	---	---	0.0013	2.34 (1.37-4.01)	351.5

Table 7: Association between Hemochromatosis and Hyperferritnemia

Sr. No	Groups	HH (ng/dL)	HFR (ng/mL)	HH (uIU/mL)
1	Control	(97 ± 14)	(1.7 ± 0.6)	(4.1 ± 0.13)
2	Hemochromatosis	(171 ± 16)	(4.37 ± 0.23)	(0.28 ± 0.002)
3	Hyperferritinemia	(13.2 ± 3.0)	(0.87 ± 0.03)	(15.2 ± 3.8)

Discussion

Hemochromatosis is a complex genetic disorder characterized by excessive iron absorption, leading to iron overload, particularly in the liver. This study focuses on the genetic basis of hemochromatosis, specifically investigating the association of a

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polymorphism in the TFR2 gene (rs7385804, C490G) with the development of the disorder. Additionally, it explores the relationship between sex, age, and TFR2 ratio in liver patients.

The study's findings reveal a significant association between the TFR2 gene variant rs7385804 and hemochromatosis. The genotype frequencies in the case group were 59.3% for C/C, 38.9% for C/G, and 1.8% for G/G, compared to 77.1%, 22.9%, and 0% in the control group. The allelic frequency of the C allele was slightly lower in cases (78.7%) compared to controls (81.6%), while the G allele frequency was higher in cases (21.3%) than in controls (18.4%). This suggests that the C allele of rs7385804 may be a risk factor for developing hemochromatosis.

The study also uncovered demographic patterns among hemochromatosis patients. A significantly higher prevalence of hemochromatosis was found in males (85%) compared to females (15%), indicating that sex-specific factors are crucial in the diagnosis and management of the disorder. The mean age of hemochromatosis patients was 44.82 years, suggesting that the disorder tends to manifest later in life. Additionally, the TFR2 ratio, calculated as the ratio of TFR2 genotype frequencies to the total number of patients, was 0.593, indicating a potential marker for genetic predisposition to hemochromatosis.

This research is particularly relevant in the context of the Pakistani population, where the frequency of hemochromatosis is high. The TFR2 gene has been extensively studied for its role in various diseases, including hemochromatosis and hyperferritinemia. Variations in this gene can affect serum ferritin metabolism, crucial for DNA synthesis, methylation, and genomic stability. The study confirms the association between the TFR2 gene variant rs7385804 and hemochromatosis using TETRA-ARMS PCR and Sanger sequencing, highlighting the potential for designing cost-effective screening tests for these gene variants.

The study analyzed 162 hemochromatosis patients and 114 healthy controls, with genotype frequencies and statistical analysis performed using SNPstat and SHEsis software. The results indicated that the TFR2 variant's minor/risk allele (T) had a higher frequency in cases than in controls, projecting an association between this allele and hemochromatosis susceptibility. The dominant inheritance model was identified as the best fit for predicting disease risk, with the presence of the "C" allele increasing the relative risk for hemochromatosis in the studied population.

Interestingly, while the G allele of the TFR2 variant showed higher frequency in cases, the association with disease susceptibility was insignificant, suggesting variability in genetic risk factors. This finding underscores the importance of considering multiple genetic and environmental factors in understanding hemochromatosis risk.

The study also explored the relationship between TFR2, age, and gender with hemochromatosis and hyperferritinemia. It found increased TFR2 levels in hemochromatosis patients and a higher prevalence of hemochromatosis among females

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for both conditions. The mean age for hemochromatosis was 46.87 years, and for hyperferritinemia, it was 44.82 years.

The variations in hemochromatosis types, ethnicity, and lifestyle factors, such as consanguineous marriages in Pakistani society, may contribute to the differing risk patterns observed. The study suggests that genetic polymorphism studies should be population-specific to yield accurate results. A larger sample size and further research across different ethnic groups are recommended to validate these findings and explore the correlation between TFR2 and HFE gene polymorphisms with hemochromatosis risk more comprehensively. This research lays the groundwork for future studies that could lead to the development of targeted screening and management strategies for hemochromatosis in at-risk populations.

IV. CONCLUSION

In conclusion, our study provides valuable insights into the genetic basis and demographic associations of hemochromatosis, focusing on the TFR2 gene variant rs7385804. We found a significant association between the C allele of rs7385804 and the development of hemochromatosis, highlighting the importance of genetic screening in at-risk populations.

Additionally, our study revealed intriguing demographic patterns, with a higher prevalence of hemochromatosis in males and a mean age of onset in the mid-40s. The theoretical importance of our study lies in advancing the understanding of the genetic basis of hemochromatosis, particularly the role of the TFR2 gene variant rs7385804. This knowledge can aid in the development of targeted therapies and personalized treatment strategies for individuals with hemochromatosis.

The practical importance of our study is evident in its implications for clinical practice. The identification of the C allele of rs7385804 as a risk factor for hemochromatosis suggests that genetic screening for this variant may help identify individuals at higher risk of developing the disorder. This can lead to early diagnosis and intervention, potentially preventing or delaying the onset of iron overload and its associated complications, such as liver dysfunction and hepatocellular carcinoma.

Future perspectives on the effects of hemochromatosis on the liver include advancing our understanding of the molecular mechanisms underlying iron overload, developing targeted therapies to prevent or treat hepatic complications, and exploring non-invasive methods for early detection and monitoring of liver damage.

Additionally, there is a growing interest in personalized medicine approaches to tailor treatment strategies based on individual genetic profiles and disease progression patterns. Continued research in these areas holds promise for improving outcomes and quality of life for individuals with hemochromatosis.

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