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Research Paper

An Association study TRANSFERIN (TF) gene polymorphism with Iron Deficiency Anaemia

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ARTICLE DETAILS

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ABSTRACT

Iron-deficiency anemia (IDA) is the most common type of anemia, caused by inadequate iron availability for hemoglobin production due to the lack of dietary iron or insufficient uptake of iron. Transferrin (TF) exerts a crucial function in the maintenance of systematic iron homeostasis. The expression of the TF gene is controlled by transcriptional mechanism, although little is known about its influence on IDA. Hence, the aim of the current investigation was to determine the functional polymorphism (rs3811647) of TF gene in iron deficiency anemia. TF (Transferin) gene SNP rs3811647 create restriction site for *HinII.* The PCR products when digested by restriction enzyme and wild type allele 250 bp segment which were generated by PCR but the mutant allele shows 180 and 70 bp segments. The product sizes are Wild type homozygote, 250 bp; mutant homozygote, 180 and 70 bp; and heterozygote, 250, 160, and 70 bp respectively. Overall distribution of TF (Transferin) genotypes was significantly different in healthy control group as compared to disease group (χ^2=13.01, P=0.0015**). HC group showed an decrease frequency of mutant 'GG' genotype as compared to Patients of Anaemia (*1.11*% *vs.* 4.37%). An odds ratio of 0.4584 in Anemia group respectively for 'AA' genotype indicated a protective effect of this type genotype in our population whereas an odds ratio of 4.072 for Mutant GG Anaemia patients group respectively indicated a positive association of this wild type genotype with the disease, heterozygous is also significantly different but may be not protective because of odds ratio of 0.7094. allele 'A' was found to be in significantly low frequency in disease group as compared to HC group thus allele '*G*' was present in significantly low frequency in the healthy control group (χ^2 =12.07 P= 0.0005***). G allele shows an odds ratio of 0.5065 which indicates its protective association. The pattern of genotype and allele distribution in disease and control group suggested a significant association of TF (Transferin) gene SNP rs3811647 to Anaemia in our tribal population.

1. INTRODUCTION:

Iron homeostasis is essential for numerous physiological processes. However, excess of iron could form toxic free radicals, and it thus requires precise regulation. Because humans do not possess an active mechanism for iron excretion, this regulation is carried out mainly by modulating the uptake of iron from the diet by enterocytes and transfer of this iron to the systemic circulation **[1,5].** The key proteins that control this process also control the release of stored iron to plasma, to supply iron for erythropoiesis and other metabolic activities. Small deviations from correct iron levels are the ultimate cause of several disorders, of which iron deficiency anaemia (IDA) is the most prevalent. Indeed, it is considered a pandemic according to the WHO, which classifies women in fertile age as the most at-risk group. Moreover, iron deficiency is associated with other disorders. Iron balance is essential for all cell life. Iron homeostatic mechanisms evolved to avoid iron excess and the generation of harmful reactive oxygen species by reutilizing body iron and limiting its uptake from the environment. The inevitable other side of the coin is the easy development of iron deficiency **[2,4].** Iron deficiency is the depletion of total-body iron, especially of macrophage and hepatocyte iron stores. Because the largest amount of iron is consumed for hemoglobin (Hb) synthesis to produce 200 billion erythrocytes daily, anemia is the more evident sign of iron

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deficiency, and iron deficiency anemia is often considered synonymous with iron deficiency **[3].** However, iron deficiency is a broader condition that often precedes the onset of anemia or indicates deficiency in organs/tissues other than those involved in erythropoiesis, such as skeletal muscles an he heart, the latter highly iron dependent for myoglobin and energy production to sustain mechanical contraction **[1-5].**

Transferrin (Tf) is an iron-binding plasma protein that delivers iron to cells via the transferrin receptor pathway. A molecule of Tf can bind two atoms of ferric iron with high affinity. Iron chelation by transferrin serves three main purposes: to maintain ferric iron in a soluble form under physiologic conditions; to facilitate regulated iron transport and cellular uptake, and to maintain ferric iron in a redox-inert state, avoiding the generation of free radicals **[4,7].** Moreover, diferric Tf stimulates hepcidin expression, the central regulatory molecule of systemic iron homeostasis, through a TfR2/HFE mediated pathway. The expression of the Tf gene is controlled by transcriptional mechanisms and is tissue-specific **[6,9].** Many environmental factors are known to affect plasma Tf levels: in iron deficiency, the rate of Tf synthesis in the liver increases significantly, whereas inflammatory or immunologic stimuli may decrease the levels of circulating Tf. Recent studies observed increased Tf levels under hypoxia, a response that may facilitate iron supply for erythropoiesis. Nevertheless, little is known about the genetic factors that influence Tf levels in humans, although its expression pattern appears to show sexual dimorphism **[8].** Our research group recently published that only a few SNPs could explain a large percentage of the heritable variation of serum transferring levels; one of these loci is SNP rs3811647, located in intron 11 of the human transferrin gene (*Tf*), which is in agreement with other data from the bibliography. 10 Based on these studies, we hypothesised that SNP rs3811647 increases transferrin expression. Here we show that this SNP constitutes an intronic enhancer that modulates *Tf* expression in hepatoma cells **[6-9].**

The Transferrin *(Tf)* gene located on bovine chromosome 1q41-q46, where it consists of 7 exons and spans about 39 kb of genomic DNA. Many polymorphisms have been found in the bovine *Tf* gene. Transferrin is an iron-binding β-globulin plasma protein synthesized by the liver with a molecular weight about 80 kDa. It has two separate ironbinding sites, and each of them is capable of binding one atom of ferric iron **[11,14].** In human plasma, transferrin is normally at 2.0-3.2 mg/mL and is typically one-third saturated with iron. Transferrin may contribute to innate host defense against bacterial and fungal pathogens by limiting microbial access to iron. Transferrin also inhibits bacterial adhesion and has an iron-independent antifungal effect. In addition, in animals with diagnosed mastitis, the transferrin concentration in milk is higher than that in healthy animals. These results suggest a possible relationship between the *Tf* gene and mastitis in dairy cattle **[12].** It is useful to study the genetic variations of candidate genes and their associations with milk production and somatic cell count (SCC), which have a high genetic positive correlation with mastitis (with an estimated average coefficient of 0.7). The objective to this study was to identify the polymorphism transferrin gene (*Tf*) A14037G, C14081T SNPs and their association with the productive performance (some milk and growth parameters) of 165 Holstein-Frisian cows and their progeny **[10-13].**

2. MATERIALS AND METHODS:

2.1 Study Population:

Our tribal population consisted of 340 unrelated subjects comprising of 160 Aneamia patients and 180 ethnically matched controls of central Indian population were included in this study. In this tribal population, most of people economically weak are mainly living in Rewa Sidhi, Satna and Sahdol district.

2.2 Blood collection and plasma/serum separation:

Venous blood samples were obtained from the subjects after 12 hours of overnight fasting in vacutainers with and without appropriate anti-coagulants. Immediately, plasma and serum from the respective vacutainers were separated by centrifuging the tubes at 1000 rpm for 10 min. at 4˚C

2.3 Method for DNA isolation:

Genomic DNA was extracted from whole blood by the modification of salting out procedure described by Miller and coworkers (Miller *et al*. 1988). Frozen blood sample was thawed at room temperature. 0.5 ml. of whole blood sample was suspended in 1.0 ml. of lysis buffer (0.32 M Sucrose, 1 mM Mgcl2, 12 mM Tris and 1% Triton-X-100) in a 1.5 ml. microcentrifuge tubes. This mixture was mixed gently by inverting the tube upside down for 1 min. The mixture was than allowed to stand for 10 min. at room temperature to ensure proper lysis of cells. The mixture was centrifuged at 11,000 rpm for 5 min. at 4˚C to pellet the nuclei. The supernatant was discarded carefully in a jar containing disinfectant, as pellet formed is loosely adhered to the bottom of centrifuge tube. The pellet was resuspended in 0.2 ml. of lysis buffer and recentrifuge at 11,000 rpm for 5 min. The pellet was than dissolved in 0.2 ml. of deionized autoclaved water and mixed thoroughly on vortexer. The mixture was centrifuged at 14,000 rpm for 1 min. at 4˚C. Supernatant was discarded to gain an intact pellet. To the above pellet, 80 µl. of proteinase K buffer (0.375 M Nacl, 0.12 M EDTA, pH 8.0) and 10 µl. of 10% SDS (10% w/v SDS, pH 7.2) was added. Mixture was well frothed with the help of micro tip to allow proper lysis of pelleted nuclei. After digestion was complete, 100 µl. of saturated cold 5M Nacl was added and shaken vigorously for 15 sec. To the above mixture 0.2 ml. of deionized, autoclaved water and 0.4 ml. of phenol-chloroform (4:1 v/v) was added to remove most of the non nucleic acid organic molecules. Microcentrifuge tube was inverted upside down until the solution turned milky. Phases were separated by centrifuging the above mixture at 12,000 rpm for 10 min. at 4˚C. Aqueous (top) layer was saved and transferred in another microcentrifuge tube. Transferring of any interface layer was avoided. To the aqueous layer, 1 ml. chilled absolute ethanol was added and the tube was inverted several times until the DNA precipitated. DNA

precipitates like thread. This was centrifuged at 14,000 rpm for 4 min. at 4˚C to pellet the DNA thread. Supernatant was discarded. The pellet was washed twice with 1 ml. of 70% alcohol. The mixture was again centrifuged at 14,000 rpm for 1 min. 4˚C. Supernatant was discarded and pellet was air dried for 10-20 min. The pelleted DNA was rehydrated in 100-200 µl. of TE buffer pH 7.4 (10 mM Tris-HCL pH 7.4, 1mM EDTA, pH 8.0). DNA was allowed to dissolve overnight at 37˚C before quantization.

2.4 Determination of quality and quantity of isolated DNA:
The isolated DNA is to be used for PCR based study. Therefore, its suitability for PCR along with its size heterogeneity is among the most important criterion for purity. As a matter of general practice all DNA preparations were tested for quality and quantity measures, as described in the following paragraphs.

2.5 Quantitation by UV spectrophotometry:
The isolated genomic DNAs were then tested for purity by measuring their absorbance values at 230 nm, 260 nm, 280 nm and 300 nm using a UV visible spectrophotometer (Systronic, India). A DNA preparation was considered to be good if it had A 260 nm / A 280 nm ratio as approximately 1.8 and A 300 nm was 0.1 or lesser. The absorbance at 260 nm was used to calculate the amount of DNA, using the relationship that double stranded DNA at 50µg/ml concentration has an absorbance= 1.0 at 260 nm.

2.6 Agarose Gel Electrophoresis:
Gel electrophoresis of the genomic DNAs was carried out for qualitative estimation of samples prepared. A good DNA preparation appears as single band. A horizontal agarose slab gel electrophoresis apparatus (Bangalore Genei, Bangaore, India) was used. In brief, 4-5 µl of each genomic DNA was loaded on 0.8 agarose (0.8 % w/v, Sigma) containing ethidium bromide solution (0.5 µg/ml) and electrophoresis was done at 80 V in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). Lambda DNA *Eco*RI / *Hind* ШI double digest (Bangalore Genei, Bangalore, India) was used as molecular weight marker after completion of electrophoresis, the DNA bands were visualized and photographed using an UV transilluminator (312 nm) and gel documentation system (Vilber Lourmate, Cedex 1, France) respectively.

2.7 Polymorphism screening:
In general, the genomic DNA extracted from peripheral blood of healthy individuals and diseased individuals was subjected to PCR followed by restriction digestion and electrophoresis to genotype both the groups for relevant gene of interest. All the PCRs were carried out in a PTC 200 thermal cycler (MJ Research Inc. USA). PCR is a rapid, inexpensive and simple mean of producing relatively large copy number of DNA molecules from the small amounts of source DNA material, even when the source DNA is of relatively poor quality. Due to the extreme sensitivity, precautions were taken against contamination of the reaction mixture with the trace amounts of DNA, which could serve as an unwanted template. Appropriate negative control was included in each PCR run carried out for each gene, to monitor this contamination of PCR mix to avoid any false positive results. The negative control used for PCR contained whole PCR reaction mix except target DNA which was replaced by HPLC purified water free of RNase, DNase, and any contamination from any other source resembling the gene sequence.

Subsequently restriction enzyme digestion was performed by incubating the double stranded DNA with appropriate amount of restriction enzyme, in its respective buffer as recommended by the supplier and at optimal temperature for that specific enzyme. A typical digestion includes one unit of enzyme per microgram of starting DNA. One enzyme unit is usually defined as the amount of enzyme needed to completely digest one microgram of double stranded DNA in one hour at the appropriate temperature. Their biochemical activity of the restriction enzyme is the hydrolysis of phosphodiester backbone at specific sites in a DNA sequence. Precaution was taken to avoid star activity of restriction enzymes. When DNA is digested with certain restriction enzymes under non-standard conditions, cleavage can occur at sites different from the normal recognition sequence. Such aberrant cutting is called “star activity” which can be due to high pH (>8.0) or low ionic strength, extremely high concentration of enzyme (>100 U/µg of DNA) and presence of organic solvents in the reaction (e.g. ethanol, DMSO). The PCR and restriction digestion conditions were optimized for specific locus of relevant segment of the gene to be studied. The PCR products as well as the digested products were separated on either agarose gel or polyacrylamide gel depending on their size. Gels were stained with ethidium bromide solution (0.5 µg/ml) and subsequently visualized and photographed under UV transilluminator.

2.8 Detection of TF (Transferin) SNP Rs3811647 Polymorphism:
The TF (Transferrin) SNP rs3811647 is a single nucleotide polymorphism in the Transferrin (TF) gene located in intron 11 of the gene. This intronic SNP is linked to variations in transferrin expression and serum levels, with the 'A' allele (more common in some populations) associated with increased transferrin expression and potentially higher total iron binding capacity (TIBC). Primer sequences oligonucleotide sequence (primers) was designed to amplify the gene wild type gene is lack of restriction site for *HinII* enzyme but mutant allele contains a restriction site.

2.9 PCR Primer: The oligonucleotides sequences (primers) used were those described by Z Isam ***(Isam Z, et. al. 2018).***

Forward primer- 5`- CAAGGACCTCTGGACCTCCCTTTGC-3`
Reverse primer- 5`- GACCAAGCCCTGCACAGTGCCCAAG-3`

2.10 PCR Mix:

The PCR was carried out in a final volume of 25 µl, containing 100 ng of genomic DNA(4-5 µl), 2.5 µl of 10X *Taq* polymerase buffer (10 mM Tris HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.005% Tween-20, 0.005% NP-40; final concentration 1X; Genetix Biotech Asia Pvt. Ltd.,India), 1 µl of 10 mM dNTPs (Banglore Genei, Bangalore, India), 1 µl of 25 pmol/µl of forward and reverse primers specific for and 1 µl of unit of 1U/ µl Red Taq DNA polymerase (Bangalore genei).

2.11 PCR Thermal Program:

After an initial denaturation of 5 min at 94˚C, the samples were subjected to 35 cycles at 94˚C for 1 min, at 55˚C for 40 s, and 72˚C for 40 s, with a final extension of 10 min at 72˚C in a thermal cycler. A 100bp ladder with amplified product has been run under 1 % agarose gel electrophoresis. 238bp product will be generated after PCR.

2.12 Restriction Digestion:

Restriction Digestion The 238-bp product was digested with AvaII enzyme (New England Biolabs, overly, MA) for 16 h at 37˚C. The PCR products when digested by restriction enzyme and wild type allele 250bp segment which were generated by PCR but the mutant allele shows 180 and 70 bp segments. The product sizes are Wild type homozygote, 250 bp; mutant homozygote, 180 and 70 bp; and heterozygote, 250, 180, and 70 bp respectively. Samples will analyzed by electrophoresis using 2.5% agarose gels to analyze the genotype pattern of the gene.

3. RESULTS:

3.1 Detection of Genetic Polymorphism in TF (Transferin) rs3811647 Gene:

TF (**Transferin**) gene SNP **rs3811647** create restriction site for *HinII.* The PCR products when digested by restriction enzyme and wild type allele 250 bp segment which were generated by PCR but the mutant allele shows 180 and 70 bp segments. The product sizes are Wild type homozygote, 250 bp; mutant homozygote, 180 and 70 bp; and heterozygote, 250, 160, and 70 bp respectively.

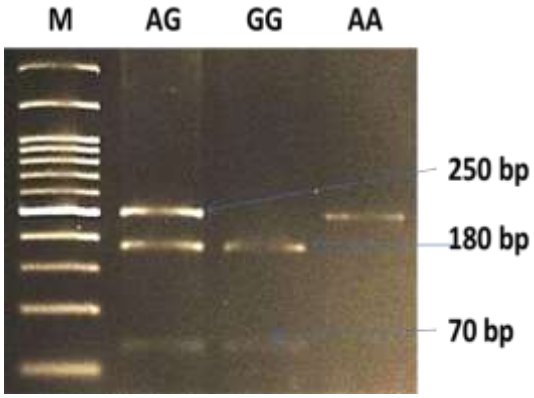


Fig.-1: Representative gel picture of TF (Transferin) polymorphism. Lane M represents 50 bp molecular marker, Lane AA Wild type genotype, Lane AG heterozygous genotype and Lane GG variant genotype.

The distribution of polymorphic genotype was strongly under HWE. The observed genotype frequencies, allele frequencies and carriage rates for TF (Transferin) SNP rs3811647 polymorphism are depicted in table 4.6 and table 4.7 and graph no. 4.4, 4.5, 4.6. Overall distribution of TF (Transferin) genotypes was significantly different in healthy control group as compared to disease group (χ^2=13.01, P=0.0015**). HC group showed an decrease frequency of mutant 'GG' genotype as compared to Patients of Anaemia (*1.11*% *vs.* 4.37%). Similarly, wild type '*AA*' genotype was present in significantly high frequency in HC as compared to Anaemia patients group (72.22% *vs.* 54.37%). An odds ratio of 0.4584 in Anaemia group respectively for 'AA' genotype indicated a protective effect of this type genotype in our population whereas an odds ratio of 4.072 for Mutant GG Anaemia patients group respectively indicated a positive association of this wild type genotype with the disease, heterozygous is also significantly different but may be not protective because of odds ratio of 0.7094.

Overall allele 'A' was found to be in significantly low frequency in disease group as compared to HC group thus allele '*G*' was present in significantly low frequency in the healthy control group (χ^2 =12.07 P= 0.0005***). Overall G allele shows an odds ratio of 0.5065 which indicates its protective association. Carriage rate of allele 'A' was high in HC group whereas carriage rate of allele 'G' was high in disease group (χ^2 =6.180 P=0.0129*) but the values were not significant. .The pattern of genotype and allele distribution in disease and control group suggested a significant association of TF (Transferin) gene SNP rs3811647 to Anaemia in our tribal population.

Table 1. Frequency distribution and association of Genotype, allele frequency and carriage rate of TF gene polymorphism in population of Vindhyan region using Chi Square Test

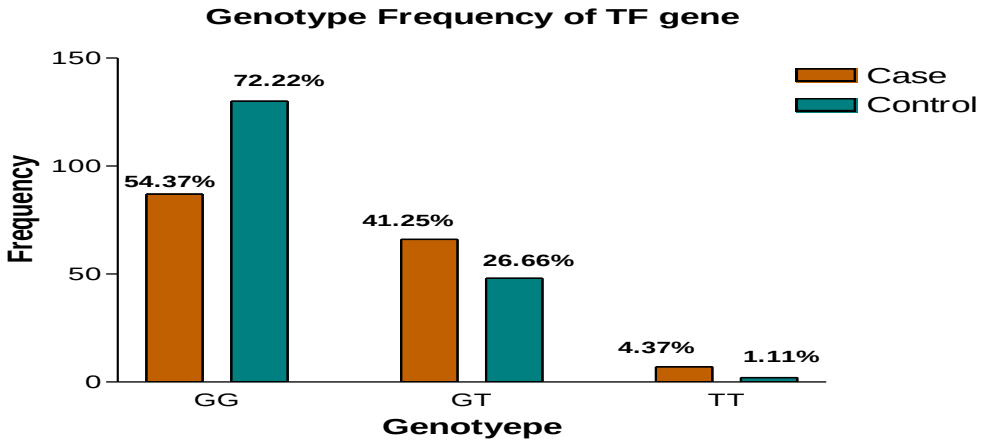
TF GENE	CASE N= 160		CONTROL N=180		CHI SQUARE VALUE χ^2 (P Value)
	N	%	N	%	
Genotype					
AA	87	54.37	130	72.22	
AG	66	41.25	48	26.66	13.01 (0.0015**)
GG	7	4.37	2	1.11	
Allele	240	75.00	308	85.55	
A	80	25.00	52	14.44	12.07 (0.0005***)
G					
Carriage Rate	153	67.69	178	78.07	
A	73	32.30	50	21.92	6.180 (0.0129*)
G					

(- denotes the level of significant association between case and control.)*
(N – Number of individuals in study group.)
(% - Genotype allele frequency and carriage rate expressed in percentage.)

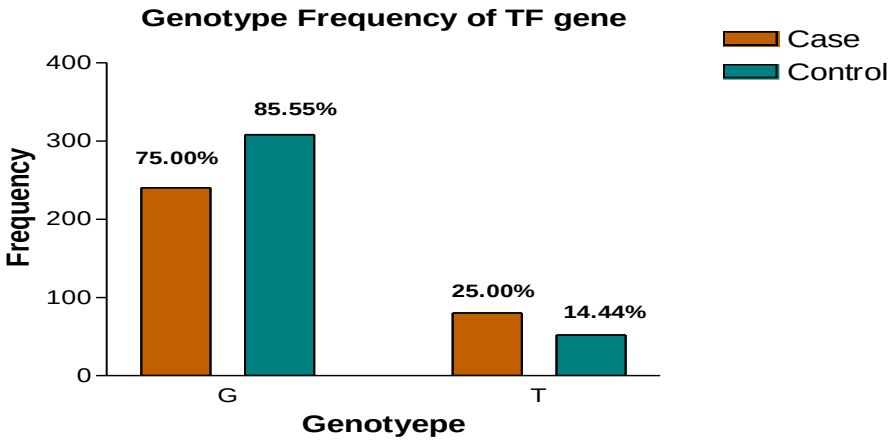
Table 2. Fisher Exact Test values of TF gene polymorphism

TF GENE	CASE N= 160		CONTROL N=180		P Value	Odds Ratio (95% confidence interval)
	N	%	N	%		
Genotype						
AA	87	54.37	130	72.22	0.0007***	0.4584 (0.2920 to 0.7195)
AG	66	41.25	48	26.66	0.0057**	1.931 (1.223 to 3.048)
GG	7	4.37	2	1.11	0.0894ns	4.072 (0.8332 to 19.90)
Allele	240	75.00	308	85.55		0.5065 (0.3437 to 0.7465)
A	80	25.00	52	14.44	0.0006***	1.974 (1.340 to 2.910)
G						
Carriage Rate	153	67.69	178	78.07		0.5887 (0.3869 to 0.8958)
A	73	32.30	50	21.92	0.0151*	1.699 (1.116 to 2.585)
G						

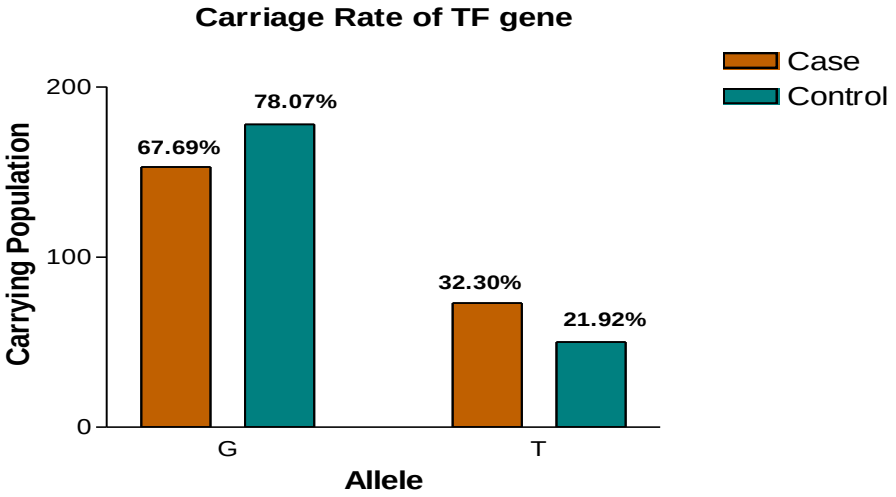
(- denotes the level of significant association between case and control.)*
(N – Number of individuals in study group.)
(% - Genotype allele frequency and carriage rate expressed in percentage.)



Graph.-1: Genotype Frequency of TF gene



Graph 2: Allele Frequency of TF gene



Graph 3: Carriage rate of TF gene

4. DISCUSSION:

Iron (Fe) is an essential metal ion for living beings; although it is the fourth most abundant mineral in the earth's crust, it is the most prevalent nutritional deficiency worldwide. It participates in a variety of vital physiological processes such as oxygen transportation, energy production in the brain by cytochrome oxidase, enzymatic cofactor in the synthesis of neurotransmitters and myelin **[14,17].** The main consequence of iron deficiency is the generation of anaemia which allows us to estimate its prevalence in a given population indirectly by red blood cell counting. However, this approach has the limitation of including other aetiologies. The worldwide prevalence of iron deficiency is approximately 30%, resulting in close to 2 billion people with anaemia of this cause. In developing countries, the prevalence of anaemia among pregnant women and children under two years exceeds 50% **[6,15].**. In Chile, the epidemiological anaemia data is scarce and sectored, with values ranging from 5.1% in women up to 36% in infants of low socioeconomic status **[13,16].** The impact of iron deficiency occurs not only in the hematopoietic system and is more evident in the early stages of life affecting preschool children who suffer from behavioral and affective disorders, increased infection susceptibility, and pregnant women having increased risk of preterm delivery, low birth weight, and death in the newborn (NB). Children, especially premature NB children from mothers with iron deficiency, adolescent girls, and women of childbearing age represent the most vulnerable population for this deficiency because of their increased demand and/or physiological loss of Fe **[14-17].**

There is no epidemiological data available of iron deficiency in the elderly, but they are expected to have a higher prevalence of anaemia than in the general population, since longevity is associated with a variety of physiological dysfunctions, chronic and inflammatory diseases, and occasionally inadequate diet that lower reserves and availability of Fe. Clinical manifestations of anaemia in the elderly add to changes in sensory organs, increasing the risk of falls, with a decline in mobility and loss of autonomy **[18].** that results in an increase in health expenses. When facing a patient with iron deficiency anaemia (IDA), the hematimetric and ferrokinetic classical standards can be altered by concomitant anaemia of chronic disease (ACD) secondary to infectious, neoplastic, or inflammatory diseases [12–15]. ACD is the consequence of the production of pro-inflammatory cytokines (IL1, 6, TNFα) **[19].** and some anti-inflammatory cytokines (IL-10) which induce the reticuloendothelial system to store Fe limiting its availability for erythropoiesis, decrease the half-life of erythrocytes, inhibit the production of erythropoietin (Epo) and decrease the sensitivity of erythroid precursors to Epo. Thus, ACD by itself results in hypoferremia and hyperferritinemia thereby complicating etiological diagnosis of patients with simultaneous IDA. Moreover, normal physiological levels of serum iron are difficult to establish in a population due to its circadian rhythm [18], technical limitations of the method, and frequent indication of ferrous salts **[18-20].**

In Fe deficiency, the decreased serum iron concentration leads to an increase in total capacity of iron binding (TIBC) and a decreased saturation of the iron transporter transferring (Tsat). Ferritin (Ferr) and transferrin (Tf) have the disadvantage of being acute phase reactants with limited value in the differential diagnosis of ACD from IDA. The above considerations justify efforts to design a highly sensitive and specific test to detect iron deficiency, ideally before the development of anaemia **[21].** Staining of the iron deposits in bone marrow (BM) remains as the gold standard, but it is an invasive technique. We assessed the use of soluble transferrin receptor (sTfR), present in the serum that can be easily quantified by conventional techniques and presents great potential for the distinction between IDA and ACD highly necessary for the therapeutic treatment in the elderly. Its' concentration rises when there is marked lack of intracellular iron as the cell increases the number of receptors on its membrane **[22].** These parameters can eliminate the need of using BM aspirate to diagnose iron deficiency in some cases. However, it must be remembered that the sTfR commonly is ubiquitously expressed at low levels. Its expression can be elevated, in a variety of human cancers. In addition to its role in iron metabolism it has been suggested that sTfR may play a role in cellular signaling and proliferation stimuli **[21-22].**

The Associations between genetic variants in the hepcidin regulation pathway and iron status have been reported in previous studies. Most of these studies were conducted in populations of European descent and relatively few studies have been conducted in Chinese populations. Studies evaluated associations between single-nucleotide polymorphisms (SNPs) in the hepcidin regulation pathway, serum ferritin (SF) and soluble transferrin receptor (sTfR) in Chinese adolescents. Carriers of the G/G genotype of rs4820268 exhibited significantly lower SF levels than A allele carriers did (p=0.047). For sTfR, rs1880669 in *TF*, rs4901474 in *BMP4*, and rs7536827 in *HJV* were significantly associated with an sTfR ≥4.4 mg/L status **[23].** However, in general linear model analysis, after adjustment for age, sex, and location, only rs1880669 exhibited a stable association with higher sTfR levels (p=0.032). Iron-deficiency anemia (IDA) is the most common type of anemia, caused by inadequate iron availability for hemoglobin production due to the lack of dietary iron or insufficient uptake of iron. Transferrin (TF) exerts a crucial function in the maintenance of systematic iron homeostasis. The expression of the TF gene is controlled by transcriptional mechanism, although little is known about its influence on IDA. Hence, the aim of the current investigation was to determine the functional polymorphism (rs3811647) of TF gene in iron deficiency anemia. Another work on transferrin gene polymorphism have similar methodology. Iron deficiency has been found to be linked to sleep disorders **[24].** Both genetic and environmental factors are risk factors for skewed iron metabolism, thus sleep disruptions in autism spectrum disorders (ASD). Caucasian study was to assess the prevalence of single nucleotide polymorphisms (SNPs) within transferrin gene (TF) rs1049296 C>T, rs3811647 G>A, transferring receptor gene (TFR) rs7385804 A>C, and hepcidin antimicrobial peptide gene (HAMP) rs10421768 A>G in Polish individuals with ASD and their impact on sleep pattern **[25].** There were 61 Caucasian participants with ASD and 57 non-ASD controls enrolled. Genotypes were determined by real-time PCR using TaqMan SNP assays. The Athens Insomnia Scale (AIS) was used to identify sleep disruptions. All analyzed SNPs were not found to be linked to insomnia. The investigated polymorphisms are not predictors of sleep disorders in the analyzed cohort of individuals with ASD **[23-25].**

Our statistical outcome from polymorphic study of TF (Transferin) gene revealed TF (Transferin) gene SNP rs3811647 create restriction site for *HinII.* The PCR products when digested by restriction enzyme and wild type allele 250 bp segment which were generated by PCR but the mutant allele shows 180 and 70 bp segments. The product sizes are Wild type homozygote, 250 bp; mutant homozygote, 180 and 70 bp; and heterozygote, 250, 160, and 70 bp respectively (Depicted in figure no. 4.2.) The distribution of polymorphic genotype was strongly under HWE. The observed genotype frequencies, allele frequencies and carriage rates for TF (Transferin) SNP rs3811647 polymorphism. Overall distribution of TF (Transferin) genotypes was significantly different in healthy control group as compared to disease group (χ^2=13.01, P=0.0015**). HC group showed an decrease frequency of mutant 'GG' genotype as compared to Patients of Anaemia (*1.11*% *vs.* 4.37%). Similarly, wild type '*AA*' genotype was present in significantly high frequency in HC as compared to Anaemia patients group (72.22% *vs.* 54.37%). An odds ratio of 0.4584 in Anaemia group respectively for 'AA' genotype indicated a protective effect of this type genotype in our population whereas an odds ratio of 4.072 for Mutant GG Anaemia patients group respectively indicated a positive association of this wild type genotype with the disease, heterozygous is also significantly different but may be not protective because of odds ratio of 0.7094. Overall allele 'A' was found to be in significantly low frequency in disease group as compared to HC group thus allele '*G*' was present in significantly low frequency in the healthy control group (χ^2 =12.07 P= 0.0005***). Overall G allele shows an odds ratio of 0.5065 which indicates its protective association. Carriage rate of allele 'A' was high in HC group whereas carriage rate of allele 'G' was high in disease group (χ^2 =6.180 P=0.0129*) but the values were not significant. The pattern of genotype and allele distribution in disease and control group suggested a significant association of TF (Transferin) gene SNP rs3811647 to Anaemia in our tribal population **.**

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