HEE Gene Polymorphism And Its Association With Anemia

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Abstract- *Control of iron homeostasis is fundamental for solid focal sensory system work: iron lack is related with psychological impedance, yet iron over-burden is thought to advance neurodegenerative sicknesses. Explicit hereditary markers have been recently distinguished that impact dimensions of transferrin, the protein that vehicles iron all through the body, in the blood and cerebrum. HFE genotypes was fundamentally unique between Healthy Control bunch when contrasted with infection gathering (χ2=9.958, P=0.0069). HC bunch demonstrated an expansion of high normal (freak) genotype 'GG' when contrasted with Patients of iron lacking weak (1.0% versus 3.0%). Essentially, normal (wild sort) genotype 'CC' was available in altogether higher recurrence in HC when contrasted with iron insufficient pallid patients gathering (87.0% vs.76 .0%). Minor allele 'G' was observed to be in fundamentally higher recurrence in ailment bunch when contrasted with HC gathering while wild sort allele 'C' was available at altogether lower recurrence in the ailment gathering (χ2 = 10.36,, P= 0.0013). Minor allele 'G' have demonstrated a chances proportion of 2.109 which shows its defensive affiliation. Carriage rate of allele 'G' was higher in HC group whereas carriage rate of allele 'A' was higher in HC group (χ^2 =0.03697, , P=0.8475) but the values were not significant although carriage of 'G' allele shows an odds ratio of 1.057 indicates the protective role of this less common allele in our population.*

The pattern of genotype and allele distribution in disease and control group suggested a significant association of HFE genotype 'CC' and major allele 'C' carriage (p =0.0021; OR= 0.4537, 95% CI 0.2743-0.7505 and p=0.0015; OR=0.4742, 95% CI 0.2987-0.7529 respectively) in Susceptibility to iron deficiency anaemia and also shows the protective effect of HFE (H63D) genotype GG and minor 'G' allele carriage (p =0.02420; OR= 2.818, 95% CI 0.5103-15.56, and p=0.0015; OR=2.109, 95% CI 1.328 – 3.348 respectively). Odds ratio of minor allele 'G' carriage is 1.057 which did suggest any association of 'G' allele carriage with disease susceptibility.

Keywords- HFE gene, SNP: H63D, Genotype, Allele frequency, Anemia.

I. INTRODUCTION

Anaemia is a global public health problem affecting both developing and developed countries with major consequences for human health as well as social and economic development. It occurs at all stages of the life cycle, but is more prevalent in pregnant women and young children. Anemia can characterize as a qualitative or a quantitative deficiency of iron, a molecule inside the red blood cells (RBCs) **[1].**There are three major classes of anemia. The three main classes of anemia include excessive blood loss (acutely such as haemorrhage or chronically through low-volume loss), excessive blood cell destruction (haemolysis) or deficient red blood cell production (ineffective haematopoiesis). Iron deficiency anemia is the most common type of anemia overall in the world and has many reasons. Red Blood Cells often appear hypochromic and microcytic when observed with a microscope. Iron deficiency, the end result of a long period of negative iron balance, develops in three phases: depletion of stores, deficiency of erythropoiesis, and anemia **[1-2].**

Iron deficiency anemia (IDA) is the most common micronutrient disorder in the world (table 1), negatively affecting the health and socio-economic status of millions of men, women, and children. According to the World Health Organization (WHO), IDA constitutes a significant public health problem requiring immediate attention from governments, researchers and healthcare practitioners **[3].**Iron deficiency (ID) is inherently associated with poverty, and is thus particularly prevalent in the developing world where the problem is often exacerbated by limited access to appropriate healthcare and treatment. Iron deficiency and IDA result from a long term negative iron balance, culminating in decreased or exhausted iron stores. Iron, a component of every living cell, is intrinsically involved in numerous biochemical reactions in the body and is associated with oxygen transport and storage, energy production, DNA synthesis, and electron transport. Although the etiology of IDA is multifaceted, it generally results when iron demands are not met by iron absorption for any number of reasons. Individuals with IDA may have inadequate intake of iron due to poor quantity and/or quality of diet, impaired absorption or transport of iron, or chronic blood loss due to secondary disease. Consequences of IDA are

devastating: inhibited growth, impaired cognitive development, poor mental and motor performance, reduced work capacity, and an overall decreased quality of life **[3-5].**
Iron Deficiency Anemia (IDA) was considered to be among the most important contributing factors to the global burden of disease. Iron deficiency is the most common single cause of anemia worldwide, accounting for about half of all anemia cases. It is more common in women than in men. Iron deficiency anemia is the most common form of anemia. Three stages of iron deficiency are (a) First stage characterized by decreased storage of iron without any other detectable abnormalities. (b) An intermediate stage of "latent iron deficiency", that is, iron stores are exhausted, but anemia has not occurred yet. Its recognition depends upon measurement of serum ferreting levels. The percentage saturation of transferring falls from a normal value of 30% to less than 15%. This stage is the most widely prevalent stage in India, and (c) The third stage is that of overt iron deficiency when there is a decrease in the concentration of circulating hemoglobin due to impaired hemoglobin synthesis **[6-8].**

Iron deficiency is one of the leading risk factors for disability and mortality worldwide, affecting both developing and developed countries with major consequences for human health as well as social and economic improvement. An estimated two billion people are affected, and menstruating women and children are populations at risk. **[9].** Iron deficiency anaemia is caused by a wide variety of factors that can be isolated, but more often coexist. It results from any situation in which dietary iron intake does not meet the body's demands. Physiological blood loss frequently contributes to the negative iron balance, but genetic factors also play a role. **[12].** It is well established that in a situation of iron-deficiency the supply of iron to transferrin is compromised, increasing the serum levels of the protein while transferrin saturation and total iron binding capacity are decreased; this leads to ferritin stores being progressively diminished. More recently, hepcidin has emerged as the central regulatory molecule of systemic iron homeostasis, inhibiting ferroportin which mediates iron export from hepatocytes, duodenal enterocytes and macrophages. Hepcidin mRNA is transcriptionally regulated through at least three pathways: interleukin 6 stimulates hepcidin expression through the STAT3 signaling pathway; bone morphogenetic proteins (BMP) increase hepcidin expression through the haemojuvelin/ BMP receptor/Smad 4 pathway; and transferrin stimulates hepcidin expression through a transferrin receptor 2 (TfR2)/HFE mediated pathway **[9-13].**

Mutations of several genes implicated in iron-overload have been widely studied. Haemochromatosis can be caused by mutations affecting any of the proteins that limit the entry of iron into the blood. In humans, mutations in HFE, TfR2, haemojuvelin and ferroportin genes can result in haemochromatosi **[15].** However, limited information is available about gene variants associated with iron deficiency anaemia. Atransferrinemia, due to rare mutations in the transferring gene (TF), leads to low or undetectable levels of the carrier protein. Mutations in the divalent metal transporter 1 gene have been found in patients with microcytic anaemia, low serum ferritin, and liver iron overload. Recent studies revealed that mutations in the matriptase gene (TMPRSS6) cause iron-refractory iron deficiency anaemia. Presence of the mutation G277S of the TF gene alone does not affect iron absorption in iron deficient women and it has been suggested that a combination of polymorphisms is involved in iron metabolism. The development of genome-wide association studies (GWAS) make it possible to associate genetic variations, such as single nucleotides polymorphisms (SNPs), with traits that could be related to a disease. **[14-16].**

II. MATERIALS & METHODS

Study population

The study population consisted of 430 unrelated subjects comprising of 180 iron deficiency anemic patients and 250 ethnically matched controls of central Indian population were included in this study. In this region Hindu, Muslim and some Sikh peoples are mainly living but most people belong to Hindu religion in Vindhyan region.

Inclusion and Exclusion criteria for Cases

Cases included consecutive patients who attended the District hospital Sidhi, Mishra nursing home Sidhi, D. K. Hospital Sidhi, Devam pathology Rewa, Goswami pathology Rewa , Ranbaxy pathology Regional collection centre Rewa, Chirayu hospital & research center Rewa. Iron deficiency anemia was diagnosed in accordance with World Health Organization (WHO Expert committee 2001) criteria. Adolescence, Man, non pregnant women, pregnant women, children under age of 18 years and any patients excluded from the study.

Questionnaire

A specially designed questionnaire was prepared [see Appendices].The questionnaire was divided into four main areas covering; demographic data, clinical health examination, knowledge, attitude and symptoms of iron deficient anemic patients. For verifying the consistency random samples were distributed and reused for this purpose. Modifications were also made based on the recommendations of the supervisors

and other specialists in the field concerning validity of the questionnaire.

Anthropometric and Biochemical Measurements

Anthropometry:

Height and Weight were measured in light clothes and without shoes in standing position as per standard guidelines. Body Mass Index (BMI) was calculated as weight in kilograms divided by height in meters squared.

Blood collection and plasma/serum separation:

Venous blood samples were obtained from the subjects after vacutainers with and without appropriate anti-coagulants. Immediately, Serum and plasma were obtained after centrifugation at 1000 g for 15 minutes and stored at -80°C.

Biochemical Analysis

Total red blood cells, white blood cells, haemoglobin, haematocrit, and mean corpuscular volume (MCV), mean concentration of haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), were determined by **complete blood cells counting methods** following standard laboratory techniques using the Symex NE 9100 automated haematology analyser (Symex, Kobe, Japan).

Serum iron, serum ferritin and serum transferrin were determined by the Modular Analytics Serum Work Area analyser (Roche, Basel, Switzerland). Transferrin saturation (%) was calculated as follows: **serum iron (µmol/L)/TIBC (µmol/L) × 100, where TIBC is total iron binding capacity, calculated as 25.1 × transferrin (g/L).** The protocol and principle of biochemical analysis has already been discussed in review section in diagnosis chapter.

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. micro centrifuge tubes. This mixture was mixed gently by inverting the tube upside down for 1 min. The mixture was then 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) were added to remove most of the non nucleic acid organic molecules. Micro -centrifuge 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 micro-centrifuge 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.

Determination of quality and quantity of isolated DNA:

The isolated DNA is to be used fossr 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.

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.

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, Bangalore, 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 solutions (0.5 µg/ml) and electrophoresis was done at 50 Volt in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). Lambda DNA Mbo1double digest (Bangalore Genei, Bangalore, India) was used as molecular weight marker after completion of electrophoresis, the DNA bands were visualized and photographed using a gel documentation system (Vilber Lourmate, Cedex 1, France) respectively.

Polymorphism screening

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 Biored 2000 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 (0.5µg/ml) and subsequently visualized and photographed under a gel documentation system UV transilluminator.

Detection of H63D Single Nucleotide Polymorphism via PCR-RFLP

The polymorphism of H63D gene has been amplified by PCR. This polymorphism is a functional polymorphism causing change of Amino acid from lysine to glutamine. Primer sequences oligonucleotide sequence (primers) were designed to amplify the gene wild type gene is lack of restriction site for **MboI** enzyme but mutant allele contains a restriction site.

5'…G A T C…3'
3'…C T A G…5'

Primer sequence used to amplify the H63D gene is given as follows. This primers were early used by (Abate N *et al*. 2003)-

H63D-Forward; 5'- ACATGGTTAAGGCCTGTTGC-3',
H63D-Reverse; 5'- GCCACATCTGGCTTGAAATT-3'.
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).

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 61˚C for 40 sec., and 72˚C for 40 sec., with a final extension of 10

min at 72˚C in a thermal cycler. A 100bp ladder with amplified product has been run under 3 % Agarose gel electrophoresis. 202bp product will be generated after PCR.

Restriction Digestion

Restriction Digestion The 202-bp product was digested with Mbo1 enzyme (Fermentas) for 1 to 16 hours at 37˚C. The wild-type genotype pcr product 202 bp was not digested, whereas The RFLP product (147+59) represent for homozygous (CC) , the mutated homozygous genotype (GG) was cut as a (99+59+48) represent the RFLP product for homozygous (GG). The heterozygous genotype (GC) RFLP was represented as 4 fragments of (147+99+59+48) bp. Samples were analyzed by electrophoresis using 3% Agarose gels to analyze the genotype pattern of the gene.

III. RESULTS

Biochemical and clinical findings:

The descriptive data and comparison of biochemical parameters of anaemic patients versus controls are presented in Table. Biochemical test performed in the blood sample for following clinical parameters and the findings was tabulated. Statistical analysis was done by using student's t test and p value obtained suggests the level of significant changes here. The descriptive data and comparison of biochemical parameters of Iron deficiency anaemic patients versus controls are presented in Table. As expected the anaemic patients had higher levels of serum transferrin (P<0.0001) and Ferritin (P<0.0001) and Hemoglobin count (P<0.0001) compared to that of control subjects. Nominal difference was also observed for haemoglobin (P=0.0462), complete blood cells count (P=0.0024), serum profile (serum iron, ferritin, total iron binding capacity, % transferrin saturation), (P=0.0447) was not significantly different between two groups and all the clinical test results are tabulated in table 5 (B). **(See Table 5B)**

Table No. 5(A)
Comparison of anthropometric parameters of IDA patients and controls

Characteristics	Cases	Controls	P-value
n(Men/Women)	180(96/84)	250(146/104)	
Age(years)	52.5±12.5	53.0±14.2	0.7100
Height(m)	160.50±13.40	162.2± 12.000	0.1815

(*denotes level of significant change between case and control)

Table No. 5(B)
Comparison of Biochemical and clinical findings of iron deficient patients and controls

	Complete blood count						
	Hb (gm/dl)	WBC per/mm3	RBC	MCV	MCH	MCHC	PLT
case(n=180) Mean1	8.163			46.650	21.701	23.556	4.260
Control(n=210) mean2	14.289			61.356	27.894	32.431	4.487
S.D.	4.331			10.398	4.379	6.275	0.160

	Serum iron	T I B C	FERRITIN	%Saturation
Case(n=180) Mean1	15.018	446.004	22.397	17.342
Control(n=210) Mean2	22.89	370.265	56.754	36.201
S.D.	5.565	53.555	24.294	13.335

(* denotes the level of significant change between case and control)

Detection of Genetic Polymorphism in HFE Gene:

HFE polymorphism was analyzed by PCR-RFLP Method. PCR amplification with specific primers gave 202-bp product which was digested with Mbo1 enzyme (fermentas) for 16 h. at 37˚C. The wild-type genotype (CC) was digested and cut as a three fragments (99 +59 + 48 bp), whereas the mutated homozygous genotype (GG) was cut as a doublet of (147+59) bp. The heterozygous genotype (CG) was represented as 4 fragments of (147+99+59+48) bp as depicted in figure no.3.

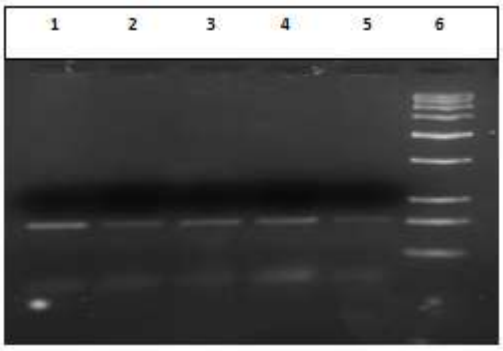


Figure No.2: Agarose Gel Electrophoresis PCR Amplification [L1-L5, PCR Product (202 bp), and L1 Ladder (100bp)]

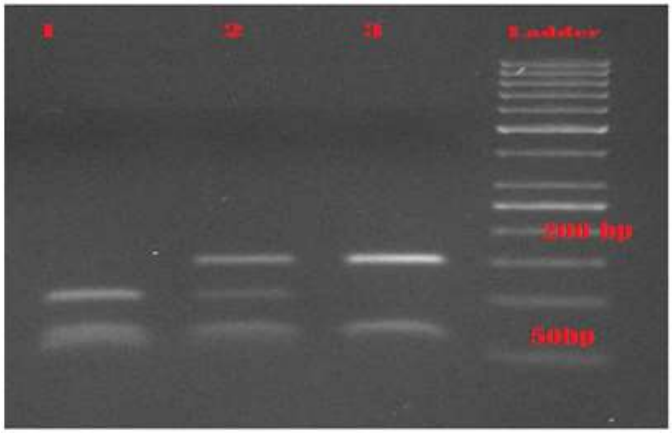


Figure No. 3;Agarose Gel Electrophoresis for HFE SNP (H63D) RFLP Product (Lane L represents 50 bp DNA ladder, Lane 1 (99 +59 + 48 bp) represents RFLP Product for wild type homozygous CC, Lane 2 (147+ 99 +59 + 48 bp) represent RFLP Product for heterozygous CG, Lane 3 (147+ 59 bp)represents RFLP Product for mutant homozygous GG.)

Overall distribution of HFE genotypes was significantly different between Healthy Control group as compared to disease group (χ^2=9.958, P=0.0069). HC group showed an increase of high common (mutant) genotype 'GG' as compared to Patients of iron deficient anaemic (1.0% *vs. 3.0*%). Similarly, common (wild type) genotype '*CC*' was present in significantly higher frequency in HC as compared to iron deficient anaemic patients group (87.0% *vs.76* .0%). An odds ratio of 2.818 for common (rare) genotype 'GG' indicated a strong protective effect of this mutant type genotype in our population whereas an odds ratio of 0.4537 of iron deficient anaemic patients group respectively indicated a positive association of common type genotype 'AA' with the disease, heterozygous Genotype 'AG' have shown an odds ratio of 2.095 which also suggest its protective role.

Minor allele 'G' was found to be in significantly higher frequency in disease group as compared to HC group whereas wild type allele 'C*'* was present at significantly lower frequency in the disease group (χ^2 = 10.36,, P= 0.0013). Minor allele 'G' have shown an odds ratio of 2.109 which indicates its protective association. Carriage rate of allele 'G' was higher in HC group whereas carriage rate of allele 'A' was higher in HC group (χ^2 =0.03697, , P=0.8475) but the values were not significant although carriage of 'G' allele shows an odds ratio of 1.057 indicates the protective role of this less common allele in our population. The pattern of genotype and allele distribution in disease and control group suggested a significant association of HFE genotype 'CC' and major allele 'C' carriage (p =0.0021; OR= 0.4537, 95% CI 0.2743-0.7505 and p=0.0015; OR=0.4742, 95% CI 0.2987-0.7529 respectively) in Susceptibility to iron deficiency anaemia and also shows the protective effect of HFE (H63D) genotype GG and minor 'G' allele carriage (p =0.02420; OR= 2.818, 95% CI 0.5103- 15.56, and p=0.0015; OR=2.109, 95% CI 1.328 – 3.348 respectively).

Odds ratio of minor allele 'G' carriage is 1.057 which did suggest any association of 'G' allele carriage with disease susceptibility. The pattern of genotype distribution, allele frequency and carriage rate in disease and control group suggests HFE is significantly associated with iron deficiency Anemia in our population.

Table No. 7

Frequency distribution and association of Genotype, allele frequency and carriage rate of HFE polymorphism in population of Central India using Chi Square Test.

HFE gene	CASE N= 180		CONTROL N=250		CHI SQUARE VALUE χ^2 (P Value)
	N	%	N	%	
Genotype					
CC	136	76.00	218	87.0	9.958 (0.0069)
CG	40	21.0	30	12.0	
GG	4	3.0	2	1.0	
Allele					
C	312	86.5	466	93.00	10.36 (0.0013)
G	48	13.5	34	7.00	
Carriage Rate					
C	176	97.00	248	99.00	0.03697 (0.8475)
G	24	24.00	32	13.00	

N – Number of individuals in study group

%- Genotype allele frequency and carriage rate expressed in percentage

* denotes the level of significant association between case and control.

Table No. 8

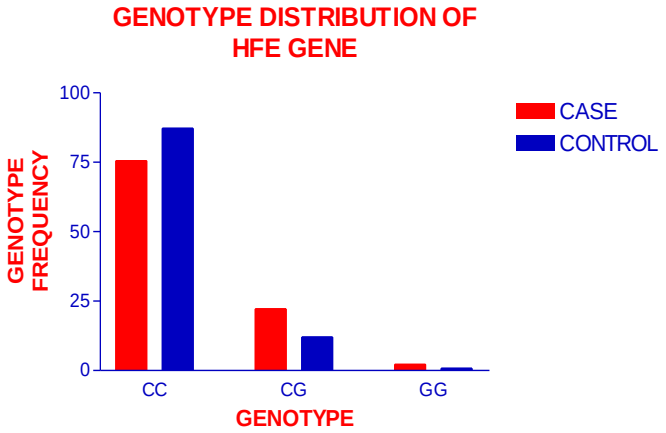
Fisher Exact Test values of HFE polymorphism

HFE gene	CASE N= 180		CONTROL N=250		P Value	Odds Ratio (95% CI)
	n	%	n	%		
Genotype						
CC	136	76.00	248	87.00	0.0021	0.4537 (0.2743-0.7505)
CG	40	21.00	30	12.00	0.0054	2.095 (1.247-3.520)
GG	4	3.00	4	1.00	0.2420	2.818 (0.5103-15.56)
Allele						
C	312	86.5	466	93.00	0.0015	0.4742 (0.2987-0.7529)
G	48	13.5	34	7.00	0.0015	2.109 (1.328 – 3.348)
Carriage Rate						
C	176	97.00	248	99.00	0.8858	0.9462 (0.5386-1.662)
G	24	24.00	32	13.00	O.8858	1.057 (0.6015–1.857)

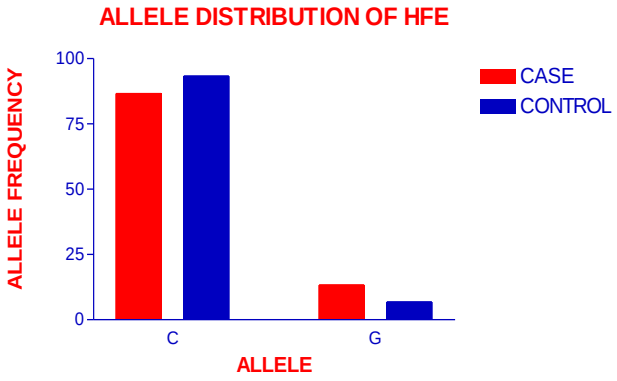
(* denotes the level of significant association between case and control)

N – Number of individuals in study group

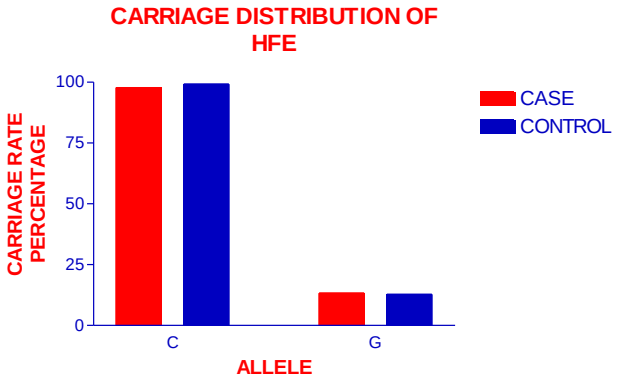
%- Genotype allele frequency and carriage rate expressed in percentage.



Graph No. 1 Genotype distribution of HFE



Graph No. 2 Allele Frequency Distribution of HFE



Graph No. 3 Carriage Rate Distribution of HFE

IV. DISCUSSION

Iron is a vital mineral in our body with many functions but primarily needed in the formation of haemoglobin. Iron deficiency results from an inadequate intake of foods rich in iron. Anaemia, on the other hand, is a disease condition due to decreased red blood cells for circulation to the body. Primary cause of anemia is iron deficiency. Iron deficiency is one of the leading risk factors for disability and mortality worldwide, affecting both developing and developed countries with major consequences for human health as well as social and economic improvement. An estimated two billion people are affected, and menstruating women and children are populations at risk **[17].**

Iron deficiency anaemia is caused by a wide variety of factors that can be isolated, but more often coexist. It results from any situation in which dietary iron intake does not meet the body's demands. Physiological blood loss frequently contributes to the negative iron balance, but genetic factors also play a role. Mutations of several genes implicated in iron-overload have been widely studied. Haemochromatosis can be caused by mutations affecting any of the proteins that limit the entry of iron into the blood. In humans, mutations in HFE, TfR2, haemojuvelin and ferroportin genes can result in haemochromatosis. However, limited information is available about gene variants associated with iron deficiency anaemia. **[18-19].**

The *HFE* gene is located at 6p21.3, approximately 4.6 megabases telomeric from *HLA-A*, and covers approximately 10 kilobases. Some polymorphisms in this gene has been reported and one polymorphism were included in this study. In the H63D mutation, a G replaces C at nucleotide 187 of the gene (187C→G), causing aspartate to substitute for histidine at amino acid position 63 in the *HFE* protein. The study population consisted of 350 unrelated subjects comprising of 150 iron deficiency anemic patients and 200 ethnically matched controls of central Indian population were included in this study. Significant level of change has been seen in distribution of HFE genotypes in Healthy Control (HC) group as compared to iron deficient patient group. **[21,24].** Healthy control (HC) group showed increase in common 'CC' genotype as compared to Patients of iron deficient (87.0% *vs.76.0*% respectively). Similarly, 'GG' genotype was present at lesser frequency in of iron deficient patients group 3.0% and also in control group 1.0%. The overall genotype was statistically nonsignificant (χ^2 =9.958, P=0.0069). Major allele 'C' was found at lower frequency in iron deficient group (86.5%) as compared to HC group (93.00%) whereas allele 'CG' was present in slightly higher frequency in the disease group (21.0% in patients and 12.0% in control) and the difference was statistically significant (χ^2 = 9.958,P=0.0069). An odds ratio of 2.109 of rare allele 'G' shows moderate effect of minor allele in iron deficient susceptibility. **[20-25].**

The present study shows that H63D was also associated with serum transferrin and is consistent with previous finding studies carried out by Blanco-Rojo et al., 2011 in Spanish population. In Australian population results

also found similar and showing H63D associated risk factors. All of these studies provide insight into haematopoietic pathways and reveal genetic variants that may predispose some individuals to iron deficiency and related disorders. **[26-30].**The present study is part of a wider project aimed at investigating the influence of dietary, physiological, and genetic risk factors on iron deficiency anaemia. Any finding of association between a genotype with a frequency that varies among populations and a phenotype that may vary for a number of reasons raises the question of whether there is a true cause-and-effect relationship between the allele and the phenotype or whether some form of population stratification is causing the apparent association. Further investigations in anaemic and non-anaemic subjects should be designed to increase the existing knowledge of the relationship between genetic variants and iron deficiency anaemia, and the possible modulating effect of diet and menstruation **[31-36].**

Our results may be also useful to increase knowledge on iron overload disorders. It is also important to study the possible gene-disease interaction effects on the recovery of iron status. Finally, other studies should be carried out to explore the mechanisms by which these variants, or combination of variants as in haplotypes, affect iron metabolism at the transcriptional and posttranscriptional levels and also at the functional level. The development of genome-wide association studies (GWAS) make it possible to associate genetic variations, such as single nucleotides polymorphisms (SNPs), with traits that could be related to a disease. To resolve the mystery of metabolic error a large sample size GWAS study with function genomic study is recommended **[35-37].**

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