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# Novel deleterious nonsynonymous SNPs within *HLA-H* (*HFE*) gene can be used as diagnostic marker to predict hereditary Hemochromatosis: Using bioinformatics analysis

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## ABSTRACT

The hemochromatosis gene (HFE) is located on chromosome 6 in close proximity to *HLA-A* gene. HFE is a cell surface membrane protein; its function involved in iron homeostasis and directly regulates iron absorption. Defects of *HLA-H* (HFE) gene cause the majority cases of inherited haemochromatosis (HFE haemochromatosis -HC-). Identifying SNPs responsible for specific phenotypes appears to be problem that is very difficult to solve, and to overcome this problem of testing overwhelming numbers of SNPs (single nucleotide polymorphisms), would be priorities SNPs according to their functional significance. Bioinformatics help to discriminate between neutral and deleterious SNPs, which constitute the majority of genetic variation and SNPs of likely functional importance. The objective of the present study was to identify deleterious SNPs within (SNP database) located in coding-nonsynonymous, 3 untranslated regions and promoter regions of *HLA-H* gene. In this study many international databases such as (NCBI, Uniprot, PolymiRTS) were used to identify functional SNPs within (SNPs database -dbSNP-) located in coding-nonsynonymous, 3-untranslated region and promoter regions of *HLA-H* gene, in addition nsSNPs were analyzed using bioinformatics tools (SIFT, PolyPhen, I-mutant, CPH, Chimera). Analysis results were as follow, two new functional (pathological) nonsynonymous SNPs (M35T) and (T17I) had been detected, which may disrupt the protein function conformation, also in 3'UTR, 18 functionally SNPs disrupts a conserved of 52 miRNA site, and creates a new binding site of 41 miRNA. Furthermore nil SNP were found within predicted promoter regions. In conclude, these new SNPs can thus be used for diagnostic test as add to hemochromatosis (HH) SNPs profile, which might play an important role in investigation of disorder that results from excess of total body iron.

**Keywords:** Single-nucleotide polymorphisms (SNPs), hereditary hemochromatosis, HLA-H or HFE, insilico analysis, nonsynonymous SNPs (nsSNPs).

## 1. INTRODUCTION

HLA-H gene and its transcribed proteins have a lot of symbol name as (HFE; HH; HFE1; MVCD7; TFQTL2) [1]. Its chromosome located between HLA-A and HLA-G genes of 1029 base pairs length [2, 3]. It has an eight-exons structure similar to that of the MHC class I genes, but it is considered to be a pseudogene, which has a single-base-pair deletion in exon number four that result a premature stop codon at the end of this exon

[4]. The HLA-H protein predicted from the cDNA sequence is comprised of 343 amino acids, it is most analogous to major histocompatibility complex class I molecules that contain an extracellular peptide-binding region (a1 and a2 domains), an immunoglobulin like domain (a3), a transmembrane region, and a short cytoplasmic tail. HLA-H is presumed to contain intramolecular disulfide bridges that stabilize its tertiary structure [5]. Minor H antigens are immunogenic peptides derived from polymorphic self proteins presented in the context of HLA [6]. There are many Minor H antigen genotypes (HA-1, HA-2, HA-3, HA-8, HB-1, ACC-1, ACC-2, SP110, PANE1, UGT2B17, C19Orf48, LB-ECGF-1, CTSH, LRH-1, LB-ADIR and HY) [6]. HFE protein is normally present in most human cells [3], like basolateral surfaces of some epithelial cells in stomach, colon, biliary tract,and on sinusoidal lining cells of liver, the spread of this protein give possibility that it may have a different function at these sites. Perhaps its normal function in these sites is to serve as a barrier to iron transport, and loss of this function is a contributing factor in hereditary Haemochromatosis (HH). HLA-H is presumed to contain intramolecular disulfide bridges that stabilize its tertiary structure [5]. HFE is a cell surface membrane protein, its function involved in iron homeostasis its directly regulate iron absorption. Functional HFE believed to be required for the normal regulation of hepcidin synthesis, which is the main iron metabolism regulator, and mutations of this gene result in iron overload [7]. There are three single points mutations had been identified within HFE protein as direct causes of HFE HC (HFE Haemochromatosis) with different distributions as follow, about 80% of adult-onset have a substitution of a tyrosine to cysteine at position 282 (C282Y) [8]. A second mutation had been found at position 63, where histidine is replaced by as¬partate (H63D). This mutation may be homozygous mutation (H63D/H63D) or heterozygous (C282Y/H63D). These account for approximately 5% and 15% of the cases of he¬reditary hemochromatosis respectively. A third mutation, which results in the substitution of cysteine for serine at amino acid position 65 (S65C), has an estimated heterozygote frequency of 4%. This may be implicated in iron storage disease, usually in the compound heterozygous state with C282Y (C282Y/S65C) [9].

Many of SNPs are believed to cause phenotypic difference between individuals. However, identifying SNPs responsible for specific phenotypes appears to be problem that is very difficult to solve, to overcome this problem of testing overwhelming numbers of SNPs, would be priorities SNPs according to their functional significance. Bioinformatics help to discriminate between neutral and deleterious SNPs, which constitute the majority of genetic variation and SNPs of likely functional importance [10].
microRNAs (miRNAs) are small non-coding RNAs of 20–22 nucleotides found in human, plants, animals, and some viruses, which functions in RNA silencing and post-transcriptional regulation of gene expression [11]. Derive from a stem–loop precursor to regulate gene expression by binding primarily to the 3′-UTR of specific 'target' mRNAs, resulting in the disruption of mRNA stability and/or translation [12]. They are involved in crucial biological processes, including development, differentiation, apoptosis and proliferation [13]. miRNA involved in the normal functioning of eukaryotic cells, so has dysregulation of miRNA been associated with disease [14].

Defects of the HFE gene cause the majority cases of inherited haemochromatosis, which is therefore often referred to HFE haemochromatosis (HFE HC). Hereditary hemochromatosis (HH) is an autosomal recessive disorder associated with increased intestinal absorption of iron and deposition of excessive amounts of iron into the liver, pancreas, and other organs [15]. It encompasses a heterogeneous group of inherited iron overload disorders with distinct underlying molecular defects and varying clinical symptoms [16]. Its refers to the clinical disorder that results from excess of total body iron and organ failure due to iron toxicity, over time can cause serious organ dysfunctions leading to liver cirrhosis, diabetes mellitus, hypogonadism in addition of other endocrinopathies, cardiomyopathy, arthropathy, skin pigmentation, and in cirrhotic patients, increased susceptibility to liver cancer [17]. The main objective of this study, to identify functional SNPs within (dbSNP) located in coding-nonsynonymous, 3-untranslated region and promoter regions of HLA-H gene. Also to identify the crucial SNPs that would be expected to disrupt the native protein in the level of function and structure, using bioinformatics methods.

## 2. MATERIALS AND METHODS

### 2.1 Dataset

Total *HLA-H* gene SNPs and their protein sequences in the FASTA format were retrieved from the dbSNP database (http://www.ncbi.nlm.nih.gov/) and protein database (http://www.uniprot.org) for computational analysis. Total SNPs contain SNPs of 3′UTR/5′UTR near to gene, noncoding 3′UTR/5′UTR, intron, coding synonymous and coding nonsynonymous regions. For this study SNPs within exons, 3′UTR and 5′UTR had been selected for our investigation.

### 2.2 SIFT (Sorting Intolerant From Tolerant)

A software use to predict whether an amino acid substitution affect protein function based on sequence alignment and physical properties of amino acids [18]. SIFT is a sequence homology-based tool that sorting intolerant from tolerant amino acid substitutions, and predict whether an amino acid substitution in a protein will have a phenotypic effect. SIFT Score Ranges from (0 to 1). The amino acid substitution is predicted to be damaging if score is <= 0.05, and tolerated if the score is > 0.05. http://sift.bii.a-star.edu.sg/index.html

**2.3 PolyPhen-2 (Polymorphism Phenotyping. v2)**
A Tool use to predict the possible impact of an amino acid substitution on the structure and function of protein [19]. PolyPhen generates multiple sequence alignment of homologous protein structures, calculate the position-specific independent counts (PSIC) score for each two variants, and then calculates the difference PSIC score between both allelic variants. The higher PSIC score refers to higher functional impact [20]. PolyPhen server discriminate nonsynonymous SNPs (nsSNPs) into three main categories with score range from 0 to 1, benign, possibly damaging, or probably damaging, plus provide the corresponding specificity and sensitivity values. The probably damaging nsSNPs are those that are predicted with high confidence and are expected to affect protein structure or function [21]. http://genetics.bwh.harvard.edu/pph2/index.shtml

**2.4 Protein stability prediction analysis (I-Mutant 3.0)**
Protein stability change is one of the main partner problems both for protein set and protein function [22]. I-Mutant is a suite of Support Vector Machine based predictors integrated in a unique web server, used to automatically predict the protein stability changes at single-site mutations starting from the protein structural or sequence information [23]. I-Mutant 3.0 is available at http://gpcr.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi

**2.5 Modeling of amino acid substitution effects due to SNPs on protein structure and visualization**
CPH server (CPHmodels-3.2): an automatic server use to predict protein 3D-structure, use of single protein sequence [24]. Server is available at: http://www.cbs.dtu.dk/services/CPHmodels. Then Chimera software used to visualize the PDB files. UCSF Chimera is a highly extensible program use for interactive visualization and analysis of molecular structures and related data, including density maps, supramolecular assemblies, sequence alignments, docking results, trajectories, and conformational ensembles. Chimera is available as a software package [25].

**2.6 Project HOPE-CMBI**
Fully automated program used to analyze the structural and functional effects of single point mutations. HOPE collect information from a wide range of sources including calculations on the 3D coordinates of the protein by using WHAT IF Web services, sequence annotations from the UniProt database, and prediction by DAS services. Homology models are built with YASARA. Data is stored in a database and used in a decision scheme to identify the effects of a mutation on the protein's 3D structure and function. HOPE builds a report with text, figures, and animations that is easy to use and understandable [26]. http://www.cmbi.ru.nl/hope/home.

**2.7 PolymiRTS server**
PolymiRTS database was designed specifically for SNPs that affect miRNA/mRNA targets in human and mouse. We used this computational server in order to determine functional SNPs in 3'UTR of *HLA-H* gene that may alter miRNA binding on target sites resulting in diverse functional consequences. All SNPs located in that region were selected and submitted to PolymiRTS (v3.0), available at: http://compbio.uthsc.edu/miRSNP/

**2.8 Identification of cis regulatory element - promoter region (Three programs was used)**
HFE gene sequence was obtained from Gene bank within National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/gene/) -Gene ID: 3077-, and submitted to subsequent tools for identifying promoter regions, and then search for any those 5′UTR SNPs located within predicted promoter regions.

**2.8.1 Promoter scan** is designed to find putative eukaryotic Pol II promoter sequences in primary sequence data. This program is experimental in nature, and should be used as an experimental tool. PROMOTER SCAN is best used to find regions in primary DNA sequence that might be good candidate regions to further test for promoter functionality [27]. PROSCAN version 1.7 Web Promoter Scan Service (http://bimas.dcrt.nih.gov/molbio/proscan/)

**2.8.2 Promoter 2.0 prediction server**, used to predict transcription start sites of vertebrate Pol II promoters in DNA sequences. It has been developed as a frequently updated database of simulated transcription factors that interact with sequences in promoter regions. It builds on principles that are common to neural networks and genetic algorithms. Through the use of genetic algorithms, the weights in the neural networks are optimized to maximally discriminate between promoters and non promoters [28]. Prediction output depends on the score, gives ignored when score below 0.5, marginal prediction (score 0.5-0.8), medium likely prediction (score 0.8-1.0), and highly likely prediction when score above 1.0. Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/services/Promoter/)

**2.8.3 TSSG** tool, an online algorithm predicts potential transcription start positions by linear discriminant function combining characteristics describing functional motifs and oligonucleotide composition of these sites. TSSG is the most accurate cis element prediction tool of Softberry database (the lowest % of false predictions) [29]. (http://www.softberry.com/).

## 3. RESULTS AND DISCUSSION
This study identified the total Homo sapiens SNPs located in coding-nonsynonymous, 3′ and 5′/UTR of *HLA-H* gene, within SNPs database (world wide SNPs), then for SNPs located in exon regions, insilico tools had been used to predict sorting tolerant or benign SNPs (Not have a pathological effect) from intolerant or

damaging SNPs (have a pathological effect). After that bioinformatics tools had been used to recognize the functional SNPs in miRNA binding sites and SNPs within promoter regions. *HLA-H* gene was contained a total of 692 SNPs, of which 34 were nonsynonymous SNPs (~4.9% of total), 75 SNPs were within 3'UTR which form (~10.8 %), and 10 SNPs within 5'UTR (~1.4 %).

Total nsSNPs were submitted respectively to SIFT and Polyphen servers, SNPs of double positive results (predicted by both servers) just which had been taken. Two SNPs (rs143662783, rs2242956) of total nsSNPs had predicted. Table 1. SNPs with double positive results were submitted in I-mutant server, and prediction results showed protein stability change due to new type of protein residue Table 2.

First SNP (rs2242956), Methionine residue changed to Threonine at position 35. Figure (1) shows the schematic structures and homology modeling of the original and mutant amino acid. Each amino acid has its own specific size, charge, and hydrophobicity value. The mutant residue is smaller than the wild-type, and this will cause a possible loss of external interactions. The wild-type residue is more hydrophobic than the mutant residue and this mutation might cause loss of hydrophobic interactions with other molecules on the surface of the protein and thus may cause function loss or change. By the same token second SNP (rs143662783) a Threonine shift to Isoleucine at position 17. Figure (2) shows the schematic structures of the original and the mutant amino acid. The mutant residue is bigger and more hydrophobic than the wild-type residue. The wild type residue forms a hydrogen bond with the Methionine on position 13. The size difference between wild-type and mutant residue makes that the new residue is not in the correct position to make the same hydrogen bond as the original wild-type residue did. This difference in hydrophobicity will affect hydrogen bond formation.

Through SNPs Within 3'UTR (seed region) Result shown, Among 75 total SNPs, 18 functional SNPs predicted effected miRNA binding sites, these 18 SNPs functionally disrupts a conserved of 52 miRNA site, and creates a new binding site of 41 miRNA (ancestral allele with support >=3) Table 3.

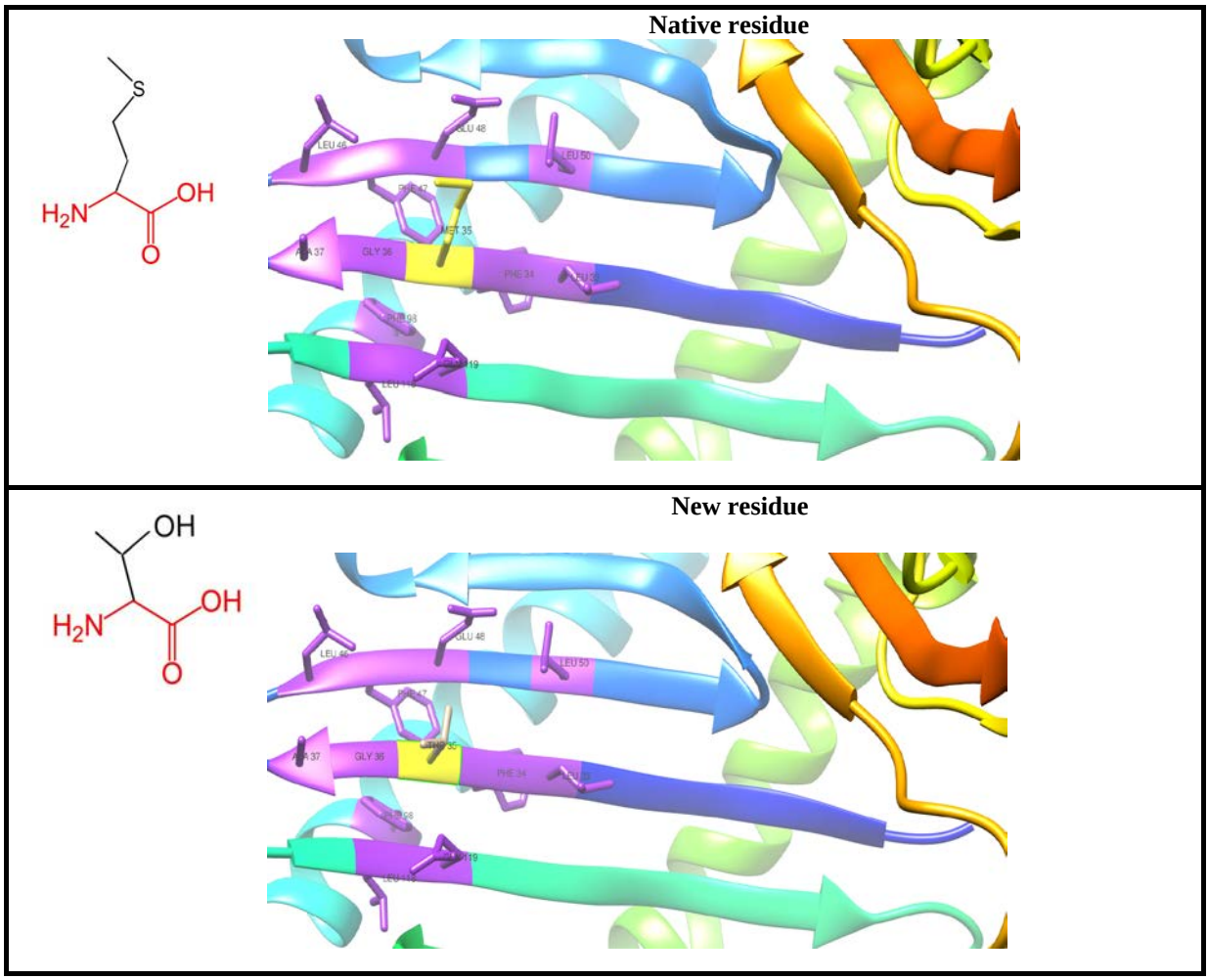


**Figure 1.** Shows position of native and new protein residues in yellow colour used Chimera program v1.8 and Hope server to get schematic structures in upper left, which shows the original (first) which is Methionine, and the mutant (second) which is

Threonine amino acid in position 35. The backbone, which is the same for each amino acid, is coloured red and the side chain, unique for each amino acid, is coloured black.

**Table 1.** Prediction result for nsSNPs with double positive used SIFT and PolyPhen

| Gene name | SNP ID | Amino acid substitution | SIFT prediction | Score | PolyPhen prediction | score |
|---|---|---|---|---|---|---|
| HLA-H | rs143662783 | T 17 I | Damaging | 0.01 | Possibly damaging | 0.845 |
| | rs2242956 | M 35 T | Damaging | 0.02 | Possibly damaging | 0.944 |

SIFT result: Score Ranges from (0 to 1). The amino acid substitution is predicted damaging is the score is <= 0.05, and tolerated if the score is > 0.05.PolyPhen-2 result: POROBABLY DAMAGING (more confident prediction) / POSSIBLY DAMAGING (less confident prediction).

**Table 2.** Prediction result of I-Mutant software

| Gene name | SNP ID | Amino acid position | WT | MT | SVM2 Prediction Effect | DDG Value Prediction: Kcal/mol | RI |
|---|---|---|---|---|---|---|---|
| HLA-H | rs143662783 | 17 | T | I | Decrease | -0.77 | 4 |
| | rs2242956 | 35 | M | T | Decrease | -1.59 | 6 |

For all the predictions, pH and temperature were selected as 7.0 and 25 °C, respectively. WT: Wild type amino acid, MT Mutant type amino acid, DDG: DG (New Protein)-DG(Wild Type) in Kcal/mol (DDG<0: Decrease stability, DDG>0: Increase stability), RI: Reliability index

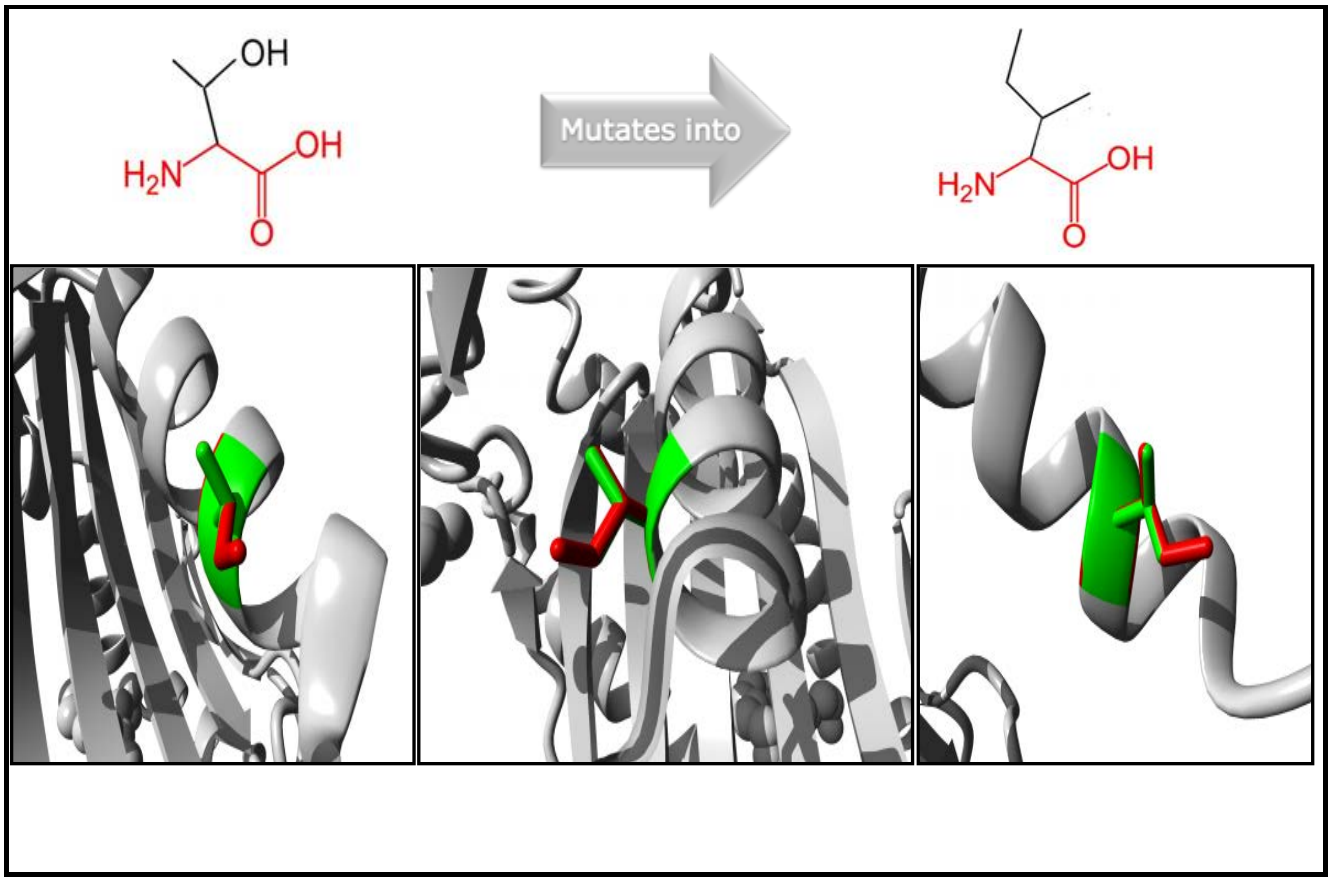


**Figure 2.** Shows the schematic structures of the original (upper left) which is Threonin, and the mutant (upper right) which is Isoleucine amino acid. The backbone, which is the same for each amino acid, is coloured red and the side chain, unique for each amino acid, is coloured black. In addition figure shows Close-up of the mutation (seen from different angles). The protein is coloured grey, wide type residue colored green and new one colored red in position 17.

**Table 3.** Prediction result of PolymiRTS database.

| dbSNP ID | miR ID | Conservation | miRSite | Function Class |
|---|---|---|---|---|
| rs200222294 | hsa-miR-1184 | 8 | gcagcCTGCAGAc | D |
| | hsa-miR-1205 | 7 | gcagCCTGCAGAc | D |
| | hsa-miR-17-3p | 8 | gcagcCTGCAGAc | D |
| | hsa-miR-3158-5p | 8 | gcagcCTGCAGAc | D |
| | hsa-miR-6511b-5p | 7 | gcaGCCTGCAgac | D |
| | hsa-miR-6811-5p | 7 | gcaGCCTGCAgac | D |
| | hsa-miR-4734 | 7 | gcagCCCGCAGAc | C |
| | hsa-miR-4767 | 7 | gcaGCCCGCAgac | C |
| | hsa-miR-6829-5p | 6 | GCAGCCCgcagac | C |
| rs192228238 | hsa-miR-8055 | 6 | catttcCTCAAAA | D |
| rs140664305 | hsa-miR-766-5p | 3 | TTCCTCCgtcacc | D |
| | hsa-miR-3125 | 3 | TTCCTCTgtcacc | C |
| | hsa-miR-3916 | 3 | TTCCTCTgtcacc | C |
| | hsa-miR-6859-5p | 3 | TTCCTCTgtcacc | C |
| rs3177318 | hsa-miR-7855-5p | 7 | tgTCACCAAgcct | D |
| | hsa-miR-3606-5p | 7 | tgTCACTAAgcct | C |
| | hsa-miR-921 | 7 | tgTCACTAAgcct | C |
| rs62625352 | hsa-miR-6077 | 5 | atTCTTCCAtctg | D |
| | hsa-miR-7-5p | 5 | atTCTTCCAtctg | D |
| rs62625353 | hsa-miR-204-3p | 5 | cctgTCCCAGAaa | D |
| | hsa-miR-3192-5p | 5 | cctgTCCCAGAaa | D |
| | hsa-miR-4314 | 5 | cctgTCCCAGAAa | D |
| | hsa-miR-4646-5p | 5 | cctgTCCCAGAaa | D |
| | hsa-miR-619-5p | 5 | cctgTCCCAGAaa | D |
| | hsa-miR-6506-5p | 5 | cctgTCCCAGAaa | D |
| | hsa-miR-4251 | 5 | cctgTCTCAGAaa | C |
| | hsa-miR-4303 | 5 | cctgtCTCAGAAa | C |
| | hsa-miR-6761-5p | 5 | cctgTCTCAGAaa | C |
| rs149600437 | hsa-miR-1322 | 6 | aaaaaGCATCATg | D |
| rs62625355 | hsa-miR-124-3p | 3 | aatgGTGCCTTca | D |
| | hsa-miR-4493 | 4 | aatggtGCCTTCA | D |
| | hsa-miR-506-3p | 3 | aatgGTGCCTTca | D |
| | hsa-miR-593-5p | 3 | aaTGGTGCCttca | D |
| | hsa-miR-605-3p | 4 | aatggTGCCTTCA | D |
| | hsa-miR-6126 | 4 | aatggtGCCTTCA | D |
| | hsa-miR-767-5p | 3 | aATGGTGCcttca | D |
| | hsa-miR-1252-5p | 4 | aatggTTCCTTCA | C |
| | hsa-miR-3914 | 3 | aatGGTTCCTtca | C |
| rs62625356 | hsa-miR-4496 | 3 | taaTTTCCTAcct | C |
| rs192191139 | hsa-miR-3653 | 6 | CTTCTTAcaataa | D |
| | hsa-miR-5701 | 6 | cttcttACAATAA | D |
| | hsa-miR-137 | 5 | cttcttGCAATAA | C |
| | hsa-miR-25-3p | 5 | cttctTGCAATAa | C |
| | hsa-miR-32-5p | 5 | cttctTGCAATAa | C |
| | hsa-miR-363-3p | 5 | cttctTGCAATAa | C |
| | hsa-miR-367-3p | 6 | cttctTGCAATAa | C |
| | hsa-miR-4696 | 6 | ctTCTTGCAataa | C |
| | hsa-miR-92a-3p | 6 | cttctTGCAATAa | C |
| | hsa-miR-92b-3p | 6 | cttctTGCAATAa | C |
| rs184512625 | hsa-miR-5701 | 4 | ttcttACAATAAt | D |
| | hsa-miR-3653 | 6 | TTCTTAAaataat | C |
| | hsa-miR-3658 | 6 | TTCTTAAAataat | C |
| | hsa-miR-7856-5p | 6 | ttCTTAAAAtaat | C |

miR ID: Link to miRBase, Conservation: Occurrence of the miRNA site in other vertebrate genomes in addition to the query genome, miRSite: Sequence context of the miRNA site, Bases complementary to the seed region are in capital letters and SNPs are highlighted in red, FuncClass: D: The derived allele disrupts a conserved miRNA site, C: The derived allele creates a new miRNA site (ancestral allele with support >= 3).

### 3.1 Analysis of cis regulatory elements (promoter region)

According to the next results used three tools, nil 5′UTR SNP was detected within any *HLA-H* gene promoter regions.

### 3.2 PROSCAN version 1.7

Promoter region predicted on forward strand in region between 418 bp to 668 bp

### 3.3 Softberry TSSG

Two promoter(s) were predicted (LDF= Linear discriminant function). Position: 826 LDF and Position: 8083 LDF. In addition, TATA box predicted at 8042 and transcription factor binding sites: for promoter at position 826.

**Table 4.** Promoter 2.0 Prediction Server

| Position | Score | Likelihood |
|---|---|---|
| 700 | 0.541 | Marginal prediction |
| 1300 | 0.609 | Marginal prediction |
| 1900 | 0.649 | Marginal prediction |
| 2600 | 0.639 | Marginal prediction |
| 3100 | 0.539 | Marginal prediction |
| 5500 | 0.703 | Marginal prediction |
| 6100 | 0.644 | Marginal prediction |
| 7400 | 0.588 | Marginal prediction |

**Position**: is a position in the sequence, **Score**: is the prediction score for a transcription start site occurring within 100 base pairs upstream from that position and, **Likelihood**: is a descriptive label associated with that score. The scores are always positive numbers; they are labeled as follows, ignored (below 0.5), Marginal prediction (0.5 - 0.8), Medium likely prediction (0.8 - 1.0) or highly likely prediction (above 1.0).

Previews studies showed the correlation between mutation HFE protein and hereditary hemochromatosis. This study is agree with many studies, such as first, study done by Feder [8]. Through Feder results, two missense mutations C282Y (rs1800562) and H63D (rs1799945) are relatively common. C282Y is most common in Northern European populations and H63D has a global distribution. Other study showed substitution of Serine to Cysteine at amino acid position 65 (S65C). These three mutations that already correlated with HH are confirmed using bioinformatics tools, and the result as follow, the C282Y substitution was found damaging (pathological), H63D found as benign and that's because this SNP has a global distribution, therefore multiple sequence alignment of bioinformatics tools gave benign result. In addition the third one (S65C) was found as damaging SNP. These findings show the agreement between this study and previous studies.

Many studies showed the role of autosomally encoded of minor H antigens in related renal transplantation [30, 31]. It plays a role in solid organ graft survival. HA-1 might be involved in chronic rejection [32], but can also lead to transplantation tolerance [33]. Apart from minor H antigens, polymorphisms in the adhesion molecule CD31 (PECAM-1) might influence the outcome of renal transplantation [34], mHAags are non MHC-encoded cell surface processed peptides which, in association with MHC, contribute to graft rejection compared with polymorphic MHC molecules, minor histocompatibility antigens can evoke only MHC restricted human, mH-specific T-cell responses have also been described after organ transplantation and blood transfusions [35].

## 4. CONCLUSION

In the current work the influence of functional SNPs in the *HLA-H* gene was investigated through various computational methods. Determined that two crucial mutations -Methionine mutated into a Threonine at position 35- (rs2242956) and Threonine mutated into an Isoleucine at position 17 (rs143662783) may disrupt the protein function. These results show that, new SNPs have a potential functional impact and can thus be used for diagnostic test as add to HH SNPs profile, which might play an important role in investigation of disorder that results from excess of total body iron. In addition this study draws attention to SNPs (variation) within non-coding 3UTR.

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