



US 20080187931A1

(19) **United States**
(12) **Patent Application Publication**
Rothenberg et al.

(10) **Pub. No.: US 2008/0187931 A1**
(43) **Pub. Date: Aug. 7, 2008**

(54) **MUTATIONS ASSOCIATED WITH IRON DISORDERS**

of application No. 09/981,606, filed on Oct. 16, 2001, now Pat. No. 6,955,875, which is a continuation of application No. 09/277,457, filed on Mar. 26, 1999, now Pat. No. 6,355,425.

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Publication Classification

(51) **Int. Cl.**
C12Q 1/68 (2006.01)
(52) **U.S. Cl.** **435/6**

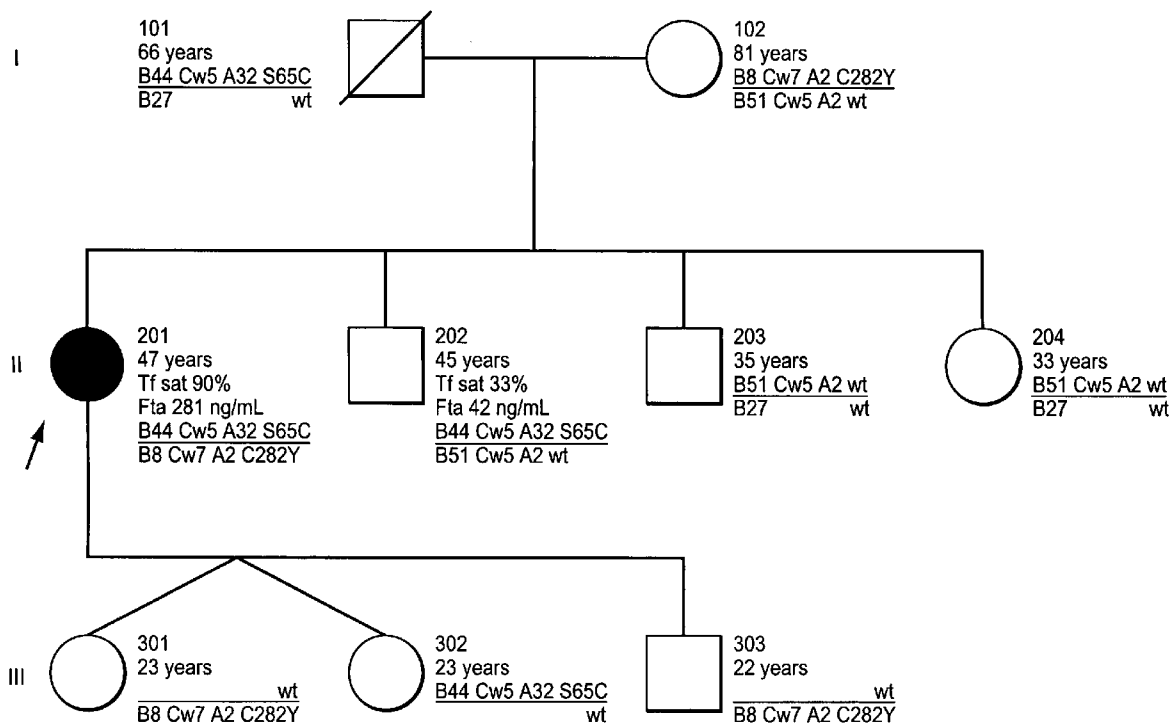
(21) Appl. No.: **12/005,791**
(22) Filed: **Dec. 28, 2007**

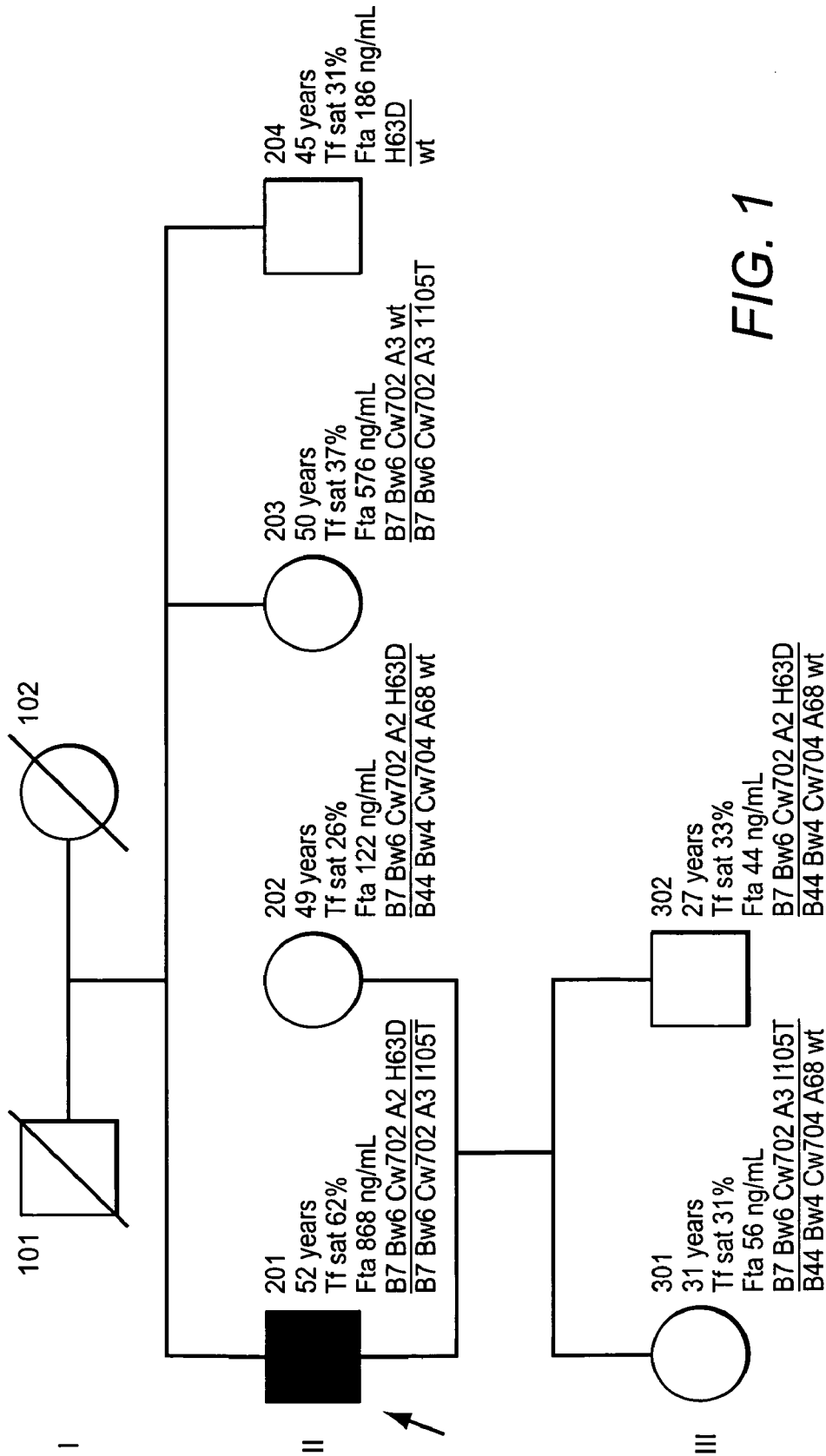
(57) **ABSTRACT**

The invention features a method of diagnosing an iron disorder, e.g., hemochromatosis, or a genetic susceptibility to developing such a disorder in a mammal by determining the presence of a mutation in exon 2 or in an intron of an HFE nucleic acid.

Related U.S. Application Data

(63) Continuation of application No. 11/252,452, filed on Oct. 18, 2005, now abandoned, which is a continuation





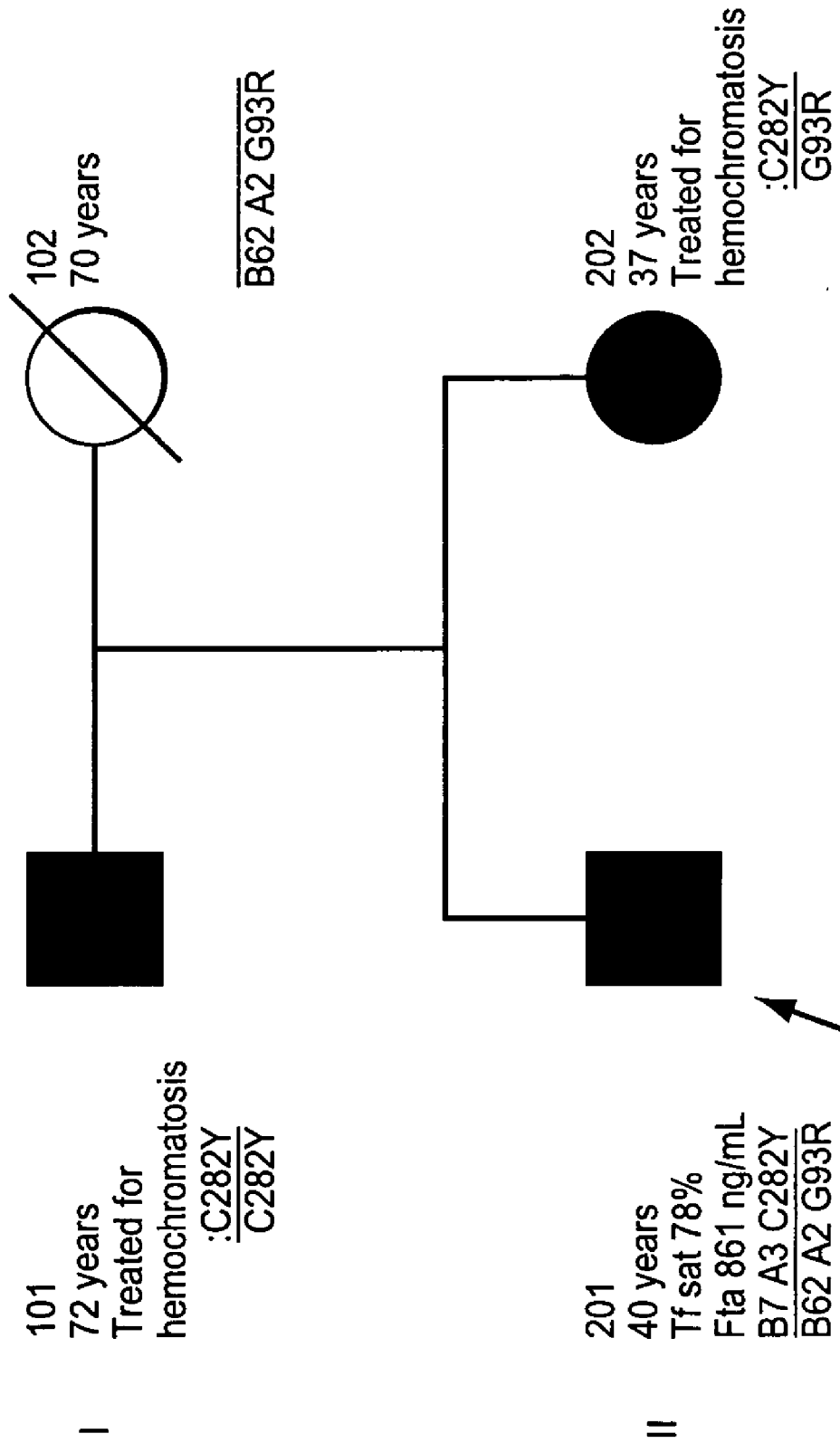
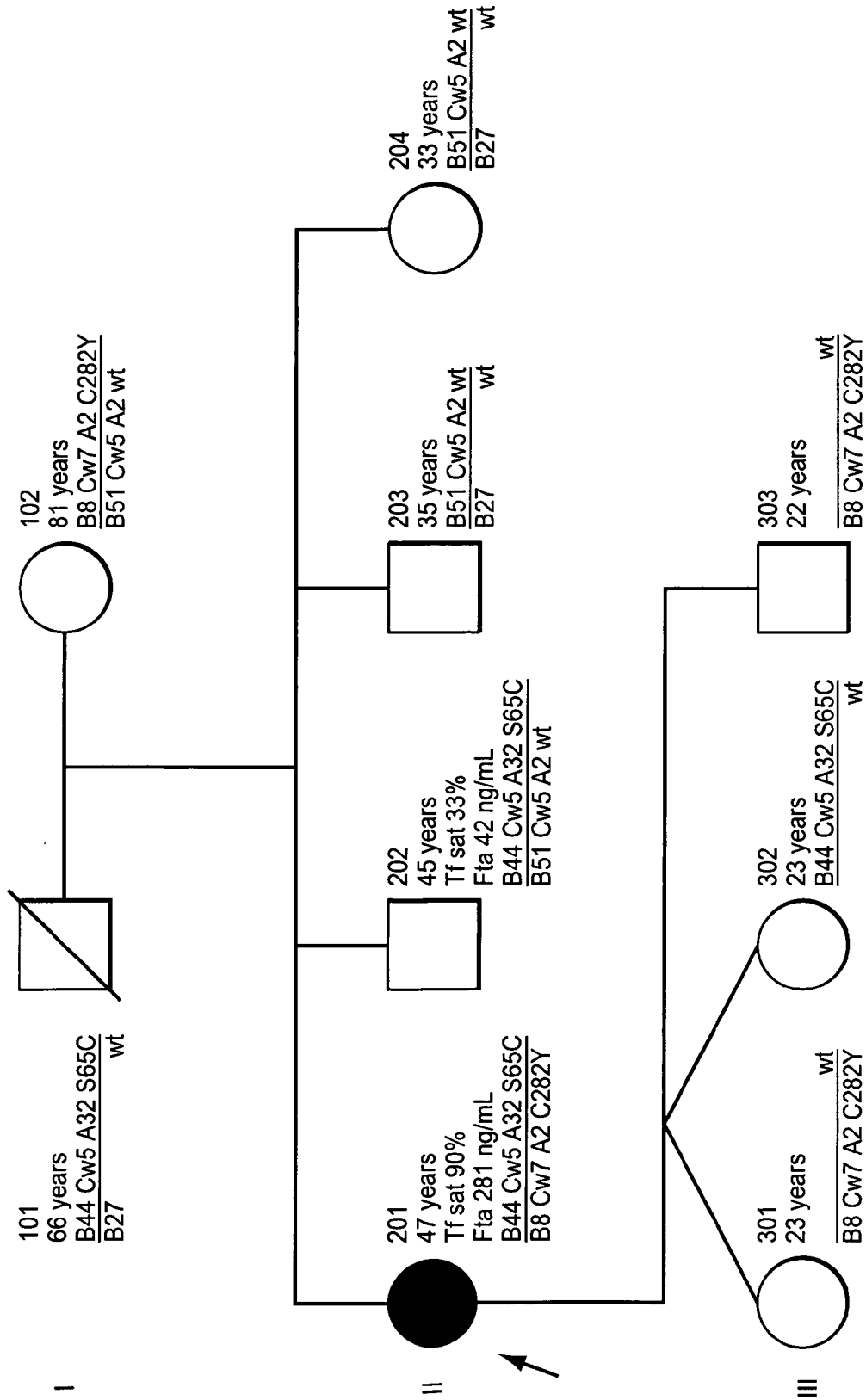


FIG. 2

FIG. 3



MUTATIONS ASSOCIATED WITH IRON DISORDERS

BACKGROUND OF THE INVENTION

[0001] Hemochromatosis is the most common progressive (and sometimes fatal) genetic disease in people of European descent. Hemochromatosis is a disease state characterized by an inappropriate increase in intestinal iron absorption. The increase can result in deposition of iron in organs such as the liver, pancreas, heart, and pituitary. Such iron deposition can lead to tissue damage and functional impairment of the organs.

[0002] In some populations, 60-100% of cases are attributable to homozygosity for a missense mutation at C282Y in the Histocompatibility iron (Fe) loading (HFE) gene, a major histocompatibility (MHC) non-classical class I gene located on chromosome 6p. Some patients are compound heterozygotes for C282Y and another mutation at H63D.

SUMMARY OF THE INVENTION

[0003] The invention is based on the discovery of novel mutations which are associated with aberrant iron metabolisms, absorption, or storage, or in advanced cases, clinical hemochromatosis. Accordingly, the invention features a

method of diagnosing an iron disorder, e.g., hemochromatosis or a genetic susceptibility to developing such a disorder, in a mammal by determining the presence of a mutation in exon 2 of an HFE nucleic acid. The mutation is not a C→G missense mutation at position 187 of SEQ ID NO:1 which leads to a H63D substitution. The nucleic acid is an RNA or DNA molecule in a biological sample taken from the mammal, e.g. a human patient, to be tested. The presence of the mutation is indicative of the disorder or a genetic susceptibility to developing it. An iron disorder is characterized by an aberrant serum iron level, ferritin level, or percent saturation of transferrin compared to the level associated with a normal control individual. An iron overload disorder is characterized by abnormally high iron absorption compared to a normal control individual. Clinical hemochromatosis is defined by an elevated fasting transferrin saturation level of greater than 45% saturation.

[0004] For example, the mutation is a missense mutation at nucleotide 314 of SEQ ID NO:1 such as 314C which leads to the expression of mutant HFE gene product with amino acid substitution I105T. The I105T mutation is located in the α 1 helix of the HFE protein and participates in a hydrophobic pocket (the "F" pocket). The alpha helix structure of the α 1 domain spans residues S80 to N108, inclusive. The I105T mutation is associated with an iron overload disorder.

TABLE 1

Human HFE cDNA sequence					
atgggcccg	cgagccaggc				
cgggccttct	ectoctgatg	cttttgca	ccgcggtcct	gcagggggcgc	ttgctgcggt
cacactctct	gcactaccctc	ttcatgggtg	cctcagagca	ggacctgggt	ctttccttgt
ttgaagcttt	gggctacgtg	gatgaccagc	tgttcgtggt	ctatgatcat	gagagtcgcc
				H63D	S65C
gtgtggagcc	ccgaactcca	tggtttcca	gtagaatttc	aagccagatg	tggtgcagc
tgagtcagag	tctgaagggg	tgggatcaca	tgttcactgt	tgactctctg	actattatgg
	G93R				I105T
aaaatcaca	ccacagcaag	gagtcccaca	cctgcagggt	catcctgggc	tgtgaaatgc
aagaagaca	cagtaccgag	ggctactgga	agtacgggta	tgatgggag	gaccacctcg
aattctgccc	tgacacactg	gattggagag	cagcagaacc	cagggcctgg	cccaccaagc
tgagtgagg	aaggcacaag	attcgggcca	ggcagaacag	ggcctacctg	gagagggact
gccctgcaca	gctgcagcag	ttgctggagc	tggggagagg	tgttttggac	caacaagtgc
ctcctttggt	gaagtgaca	catcatgtga	cctcttcagt	gaccaetcta	cggtgtcggg
ccttgaacta	ctacccccag	aacatcaca	tgaagtggct	gaaggataag	cagccaatgg
atgccaaagga	gttcgaacct	aaagacgtat	tgcccaatgg	ggatggggacc	taccagggct
ggataaacctt	ggctgtacc	cctggggaag	agcagagata	tacgtgccag	gtggagcacc
caggcctgga	tcagcccctc	attgtgatct	gggagccctc	accgtctggc	accctagtca
ttggagtcac	cagtggaatt	gctgtttttg	tcgtcatctt	gttcatttga	attttgttca
taataatgaag	gaagaggcag	ggttcaagag	gagccatggg	gacctactga	ttagctgaac
gtgagtgaca	cgcagcctgc	agactcactg	tgggaaggag	acaaaactag	agactcaaag
agggagtgca	tttatgatgt	cttcattgtt	caggagagag	ttgaacctaa	acatagaat
tgccctgacga	actccttgat	tttagccctc	tctgttcatt	tcctcaaaa	gatttccca
tttaggtttc	tgagttcctg	catgccgggt	atccctagct	gtgacctctc	ccctggaaat
gtctctcatg	aacctcaagc	tgcatctaga	ggcttccttc	atctctccg	tcacctcaga
gacatacacc	tatgtcattt	catttcctat	tttgggaaga	ccaaagcctt	aatttggggg
acttacatga	ttcattttaa	catctgagaa	aagccttgaa	ccctgggagc	tggtagtca
taaccttacc	agatttttac	acatgatct	atgcattttc	tggaacctgt	caacttttcc
tttgaatcct	ctctctgtgt	taccagtaaa	ctcatctgtc	acaaagcctt	ggggattctt
ccatctgatt	gtgatgtgag	ttgcacagct	atgaaggctg	tgcaactgac	gaatgggaaga
ggcactctgc	ccagaaaaag	catcatggct	atctgtgggt	agtatgatgg	gtgtttttag
caggtagggag	gcaaatatct	tgaagggggt	tgtaagagg	tggtttttct	aattggcatg
aagggtgcat	acagatttgc	aaagttaa	gggtccctca	tttgggatgc	tactctagta
ttccagacct	gaagaatcac	aataattttc	tacctggtct	ctccttgttc	tgataatgaa
aattatgata	aggatgataa	aagcacttac	ttcgtgtccg	acctctctga	gacctactct
acatgcatta	ctgcatgcac	ttcttacaat	aattctatga	gataggtaet	attatcccca
tttctttttt	aaatgaagaa	agtgaagcag	gccggggcagc	gtggctcgcg	cctgtggtcc
caggggtgctg	agattgcaag	tgtgagccac	cctgcccagc	cgtcaaaaga	ctcttaaat
atataccag	atggcatgtg	tttactttat	gttactacat	gcacttggct	gcataaatgt
ggtacaacca	ttctgtcttg	aagggcagggt	gcttcaggat	accatataca	gctcagaagt
ttctcttta	ggcattaaat	tttagcaaaag	atatctcatc	tctttttta	aaccttttcc
tttttttgtg	gttagaaaag	ttatgtagaa	aaaagtaaat	gtgatttacg	ctcatgtgag

TABLE 1-continued

Human HFE cDNA sequence
aaaagctata aatgaatac aattaaagct gttatttaaat tagccagtga aaaactatta acaactgtgc tattacctgt tagtattatt gttgcattaa aatgcatat actttaataa atgtacattg tattgtaaaa aaaaaaa (SEQ ID NO:1; GENBANK [®] Accession No. U60319)

TABLE 2

Human HFE gene product
MGPRRARPALLLLMLLQTAVLQG RLLRSHSHLHYLFMCASEQDLGLSLFEALGYVDDQLFVFDHESRRVRPR TPWVSSRISSQMWLQLSQSLKGGWDMFTVDFTTIMENHNHSHKESHTLQVI LGCEMQEDNSTEGYWKYGYDGDHLEFCPDTLDRWRAAEPRAWPTKLEWER HKIRARQNRAYLERDCPAQLQQLLELGRGVLDDQQVPLVKVTHHVTSSVT TLRCRALNYPQNIIMKWLKDKWPMDAKEFEKPKDVLPNGDGTYQGWIITLAV PPGEQRYTQCQWVEHPGLDQPLIIVTIWEPSPSGTLVIGVISGIAVFFVIL FIGILFIIILRKRQSGRGAMGHYVLAERE (SEQ ID NO: 2; GENBANK [®] Accession No. U60319)

Residues 1-22 = leader sequence; α1 domain underlined; residues 63, 65, 93, and 105 indicated in bold type)

Other mutations include nucleotide 277 of SEQ ID NO: 1, e.g., 277C which leads to expression of mutant HFE gene product G93R and one at nucleotide 193 of SEQ ID NO: 1, e.g., 193T, which leads to expression of mutant HFE gene product S65C.

[0005] Any biological sample containing an HFE nucleic acid or gene product is suitable for the diagnostic methods described herein. For example, the biological sample to be analyzed is whole blood, cord blood, serum, saliva, buccal tissue, plasma, effusions, ascites, urine, stool, semen, liver tissue, kidney tissue, cervical tissue, cells in amniotic fluid, cerebrospinal fluid, hair or tears. Prenatal testing can be done using methods used in the art, e.g., amniocentesis or chori-

onic villa sampling. Preferably, the biological sample is one that can be non-invasively obtained, e.g., cells in saliva or from hair follicles.

[0006] The assay is also used to screen individuals prior to donating blood to blood banks and to test organ tissue, e.g., a donor liver, prior to transplantation into a recipient patient. Both donors and recipients are screened.

[0007] In some cases, a nucleic acid is amplified prior to detecting a mutation. The nucleic acid is amplified using a first oligonucleotide primer which is 5' to exon 2 and a second oligonucleotide primer which is 3' to exon 2. To detect mutation at nucleotide 314 of SEQ ID NO: 1, a first oligonucleotide primer which is 5' to nucleotide 314 and a second oligonucleotide primer which is 3' to nucleotide 314 is used in a standard amplification procedure such as polymerase chain reaction (PCR). To amplify a nucleic acid containing nucleotide 277 of SEQ ID NO: 1, a first oligonucleotide primer which is 5' to nucleotide 277 and a second oligonucleotide primer which is 3' to nucleotide 277 is used. Similarly, a nucleic acid containing nucleotide 193 of SEQ ID NO: 1 is amplified using primers which flank that nucleotide. For example, for nucleotide 277, the first primer has a nucleotide sequence of SEQ ID NO: 3 and said second oligonucleotide primer has a nucleotide sequence of SEQ ID NO: 4, or the first primer has a nucleotide sequence of SEQ ID NO: 15 and said second oligonucleotide primer has a nucleotide sequence of SEQ ID NO: 16. Table 3, below, shows examples of primer pairs for amplification of nucleic acids in exons and introns of the HFE gene.

TABLE 3

Target DNA	Forward Primer	Reverse Primer
I. PRIMERS USED FOR AMPLIFICATION		
Exon 2	CCTCCTACTACACATGGTTAAGG (SEQ ID NO: 3)	GCTCTGACAACCTCAGGAAGG (SEQ ID NO: 4)
Exon 3	GGTGGAAATAGGGACCTATTCC (SEQ ID NO: 5)	CACTCTGCCACTAGCACTATAGG (SEQ ID NO: 6)
Exon 4	GTTCCAGTCTTCTGGCAAGG (SEQ ID NO: 7)	AAATGCTTCCATGGATGCCAG (SEQ ID NO: 8)
RT-PCR	AAAGGATCCACCATGGGCCGAGCCAGG (SEQ ID NO: 9)	GTGAGTCTGCAGGCTGCGTG (SEQ ID NO: 10)
Intron 4	GTTCCAGTCTTCTGGCAAGG (SEQ ID NO: 11)	AAATGCTTCCATGGATGCCAG (SEQ ID NO: 12)
Intron 5	GTTCCAGTCTTCTGGCAAGG (SEQ ID NO: 13)	AAATGCTTCCATGGATGCCAG (SEQ ID NO: 14)
II. PRIMERS USED FOR AMPLIFICATION		
Exon 2	GTGTGGAGCCTCAACATCCTG (SEQ ID NO: 15)	ACAAGACCTCAGACTTCCAGC (SEQ ID NO: 16)

TABLE 3-continued

Target DNA	Forward Primer	Reverse Primer
Exon 3	GGTGGAAATAGGGACCTATTCC (SEQ ID NO: 17)	CACTCTGCCACTAGAGTATAGG (SEQ ID NO: 18)
Exon 4	GTTCCAGTCTTCTGGCAAGG (SEQ ID NO: 19)	TTACCTCCTCAGGCACTCCTC (SEQ ID NO: 20)
RT-PCR	AAAGGATCCACCATGGGCCGCGAGCCAGG (SEQ ID NO: 21)	GTGAGTCTGCAGGCTGCCTG (SEQ ID NO: 22)
Intron 4	TGCCTGAGGAGGTAATTATGG (SEQ ID NO: 23)	AAATGCTTCCCATGGATGCCAG (SEQ ID NO: 24)
Intron 5	TGCCTGAGGAGGTAATTATGG (SEQ ID NO: 25)	AAATGCTTCCCATGGATGCCAG (SEQ ID NO: 26)

[0008] Mutations in introns of the HFE gene have now been associated with iron disorders and/or hemochromatosis. By “exon” is meant a segment of a gene the sequence of which is represented in a mature RNA product, and by “intron” is meant a segment of a gene the sequence of which is not represented in a mature RNA product. An intron is a part of a primary nuclear transcript which is subsequently spliced out to produce a mature RNA product, i.e., a mRNA, which is then transported to the cytoplasm. A method of diagnosing an iron disorder or a genetic susceptibility to developing the disorder is carried out by determining the presence or absence of a mutation in an intron of HFE genomic DNA in a biological sample. The presence of the mutation is indicative of the disorder or a genetic susceptibility to developing the disorder. The presence of a mutation in an intron is a marker for an exon mutation, e.g., a mutation in intron 4, e.g., at nucleotide 6884 of SEQ ID NO:27 is associated with the S65C mutation in exon 2. A mutation in intron 5, e.g., at nucleotide 7055 of SEQ ID NO:27 is associated with hemochromatosis. In some cases, intron mutations may adversely affect proper splicing of exons or may alter regulatory signals. Preferably, the intron 4 mutation is 6884C and the intron 5 mutation is 7055G. To amplify nucleic acid molecule containing nucleotide 6884 or 7055, primers which flank that nucleotide, e.g., those described in Table 3, are used according to standard methods. Nucleic acid-based diagnostic methods may or may not include a step of amplification to increase the number of copies of the nucleic acid to be analyzed. To detect a mutation in intron 4, a patient-derived nucleic acid may be amplified using a first oligonucleotide primer which is 5' to intron 4 and a second oligonucleotide primer which is 3' to intron 4, and to detect a mutation in intron 5, the nucleic acid may be amplified using a first oligonucleotide primer which is 5' to intron 5 and a second oligonucleotide primer which is 3' to intron 5 (see, e.g., Table 3).

[0009] In addition to nucleic acid-based diagnostic methods, the invention includes a method of diagnosing an iron overload disorder or a genetic susceptibility thereto by determining the presence of a mutation in a HFE gene product in a biological sample. For example, the mutation results in a decrease in intramolecular salt bridge formation in the mutant HFE gene product compared to salt bridge formation in a wild type HFE gene product. The mutation which affects salt bridge formation is at or proximal to residue 63 of SEQ ID NO:2, but is not amino acid substitution H63D. Preferably,

the mutation is between residues 23-113, inclusive of SEQ ID NO:2 (Table 2), more preferably, it is between residues 90-100, inclusive, of SEQ ID NO:2, more preferably, it is between residues 58-68, inclusive, of SEQ ID NO:2, and most preferably, the mutation is amino acid substitution S65C. Alternatively, the mutation which affects salt bridge formation is a mutation, e.g., an amino acid substitution at residue 95 or proximal to residue 95 of SEQ ID NO:2. Preferably, the mutation is G93R. Such an HFE mutation is detected by immunoassay or any other ligand binding assay such as binding of the HFE gene product to a transferrin receptor. Mutations are also detected by amino acid sequencing, analysis of the structural conformation of the protein, or by altered binding to a carbohydrate or peptide mimotope.

[0010] A mutation indicative of an iron disorder or a genetic susceptibility to developing such a disorder is located in the α 1 helix (e.g., which spans residues 80-108, inclusive, of SEQ ID NO:2) of an HFE gene product. The mutation may be an addition, deletion, or substitution of an amino acid in the wild type sequence. For example, the mutant HFE gene product contains the amino acid substitution I105T or G93R or in the loop of the β sheet of the HFE molecule, e.g., mutation S65C

[0011] Isolated nucleic acids encoding a mutated HFE gene products (and nucleic acids with nucleotide sequences complementary to such coding sequences) are also within the invention. Also included are nucleic acids which are at least 12 but less than 100 nucleotides in length. An isolated nucleic acid molecule is a nucleic acid molecule that is separated from the 5' and 3' sequences with which it is immediately contiguous in the naturally occurring genome of an organism. “Isolated” nucleic acid molecules include is nucleic acid molecules which are not naturally occurring. For example, an isolated nucleic acid is one that has been amplified in vitro, e.g., by PCR; recombinantly produced; purified, e.g., by enzyme cleavage and gel separation; or chemically synthesized. For example, the restriction enzyme, Bst4C I (Sib Enzyme Limited, Novosibirsk, Russia), can be used to detect the G93R mutation (point mutation 277C); this enzyme cuts the mutated HFE nucleic acid but not the wild type HFE nucleic acid. Such nucleic acids are used as markers or probes for disease states. For example, a marker is a nucleic acid molecule containing a nucleotide polymorphism, e.g., a point mutation, associated with an iron disorder disease state flanked by wild type HFE sequences. The invention also

encompasses nucleic acid molecules that hybridize, preferably under stringent conditions, to a nucleic acid molecule encoding a mutated HFE gene product (or a complementary strand of such a molecule). Preferably the hybridizing nucleic acid molecule is 400 nucleotides, more preferably 200 nucleotides, more preferably 100, more preferably 50, more preferably 25 nucleotides, more preferably 20 nucleotides, and most preferably 10-15 nucleotides, in length. For example, the nucleotide probe to detect a mutation is 13-15 nucleotides long. The nucleic acids are also used to produce recombinant peptides for generating antibodies specific for mutated HFE gene products. In preferred embodiments, an isolated nucleic acid molecule encodes an HFE polypeptide containing amino acid substitution I105T, G93R, or S65C, as well as nucleic acids the sequence of which are complementary to such nucleic acid which encode a mutant or wild type HFE gene product.

[0012] Also within the invention are substantially pure mutant HFE gene products, e.g., an HFE polypeptide containing amino acid substitution I105T, G93R, or S65C. Substantially pure or isolated HFE polypeptides include those that correspond to various functional domains of HFE or fragments thereof, e.g., a fragment of HFE that contains the $\alpha 1$ domain.

[0013] Wild type HFE binds to the transferrin receptor and regulates the affinity of transferrin receptor binding to transferrin. For example, a C282Y mutation in the HFE gene product reduces binding to the transferrin receptor, thus allowing the transferrin receptor to bind to transferrin (which leads to increased iron absorption).

[0014] The polypeptides of the invention encompass amino acid sequences that are substantially identical to the amino acid sequence shown in Table 2 (SEQ ID NO:2). Polypeptides of the invention are recombinantly produced, chemically synthesized, or purified from tissues in which they are naturally expressed according to standard biochemical methods of purification. Biologically active or functional polypeptides are those which possess one or more of the biological functions or activities of wild type HFE, e.g., binding to the transferrin receptor or regulation of binding of transferrin to the transferrin receptor. A functional polypeptide is also considered within the scope of the invention if it serves as an antigen for production of antibodies that specifically bind to an HFE epitope. In many cases, functional polypeptides retain one or more domains present in the naturally-occurring form of HFE.

[0015] The functional polypeptides may contain a primary amino acid sequence that has been altered from those disclosed herein. Preferably, the cysteine residues in exons 3 and 4 remain unchanged. Preferably the modifications consist of conservative amino acid substitutions. The terms "gene product", "protein", and "polypeptide" are used herein to describe any chain of amino acids, regardless of length or post-translational modification (for example, glycosylation or phosphorylation). Thus, the term "HFE polypeptide or gene product" includes full-length, naturally occurring HFE protein, as well a recombinantly or synthetically produced polypeptide that correspond to a full-length naturally occurring HFE or to a particular domain or portion of it.

[0016] The term "purified" as used herein refers to a nucleic acid or peptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Polypeptides are

said to be "substantially pure" when they are within preparations that are at least 60% by weight (dry weight) the compound of interest. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight the compound of interest. Purity can be measured by any appropriate standard method, for example, by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

[0017] Diagnostic kits for identifying individuals suffering from or at risk of developing an iron disorder are also within the invention. A kit for detecting a nucleotide polymorphism associated with an iron disorder or a genetic susceptibility thereto contains an isolated nucleic acid which encodes at least a portion of the wild type or mutated HFE gene product, e.g., a portion which spans a mutation diagnostic for an iron disorder or hemochromatosis (or a nucleic acid the sequence of which is complementary to such a coding sequence). A kit for the detection of the presence of a mutation in exon 2 of an HFE nucleic acid contains a first oligonucleotide primer which is 5' to exon 2 and a second oligonucleotide primer is 3' to exon 2, and a kit for an antibody-based diagnostic assay includes an antibody which preferentially binds to an epitope of a mutant HFE gene product, e.g., an HFE polypeptide containing amino acid substitution I105T, G93R, or S65C, compared to its binding to the wild type HFE polypeptide. An increase in binding of the mutant HFE-specific antibody to a patient-derived sample (compared to the level of binding detected in a wild type sample or sample derived from a known normal control individual) indicates the presence of a mutation which is diagnostic of an iron disorder, i.e., that the patient from which the sample was taken has an iron disorder or is at risk of developing one. The kit may also contain an antibody which binds to an epitope of wild type HFE which contains residue 105, 93, or 65. In the latter case, reduced binding of the antibody to a patient-derived HFE gene product (compared to the binding to a wild type HFE gene product or a gene product derived from a normal control individual) indicates the presence of a mutation which is diagnostic of an iron disorder, i.e., that the patient from which the sample was taken has an iron disorder or is at risk of developing one.

[0018] Individual mutations and combinations of mutations in the HFE gene are associated with varying severity of iron disorders. For example, the C282Y mutation in exon 4 is typically associated with clinical hemochromatosis, whereas other HFE mutations or combinations of mutations in HFE nucleic acids are associated with disorders of varying prognosis. In some cases, hemochromatosis patients have been identified which do not have a C282Y mutation. The I105T and G93R mutations are each alone associated with an increased risk of iron overload (compared to, e.g., the H63D mutation alone), and the presence of both the I105T and H63D mutation is associated with hemochromatosis. Accordingly, the invention includes a method of determining the prognosis for hemochromatosis in a mammal suffering from or at risk of developing said hemochromatosis by (a) detecting the presence or absence of a first mutation in exon 4 in each allele of an HFE nucleic acid, e.g., patient-derived chromosomal DNA, and (b) detecting the presence of a second mutation in exon 2 in each allele of the nucleic acid. The presence of the first mutation in both chromosomes, i.e. an exon 4 homozygote such as a C282Y homozygote, indicates a more negative prognosis compared to the presence of the second mutation in one or both chromosomes, i.e., an exon 2 heterozygote or homozygote. An exon 4 mutation homozy-

gote is also associated with a more negative prognosis compared to the presence of a first mutation (exon 4) in one allele and the presence of the second mutation (exon 2) in one allele, i.e., a compound heterozygote.

[0019] Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] FIG. 1 is a diagram of the family of proband 1 (HFE genotype H63D/I105T). □=male, ●=female, ⊙=deceased, ■=hemochromatosis phenotype. Proband 1 is indicated by an arrow. Phenotype and genotype data: age in year saturation; % Ftn=serum ferritin concentration. I105 separate chromosomes. The sister of the proband (II, 203) has hyperferritinemia.

[0021] FIG. 2 is a diagram of the family of proband 2 (HFE genotype C282Y/G93R). Symbols and abbreviations are the same as those described for FIG. 1. Proband 2 is indicated with an arrow. G93R, C282Y, and wt alleles are known to exist only on separate chromosomes. The father and sister of the proband are being treated for hemochromatosis.

[0022] FIG. 3 is a diagram of the family of proband 3 (HFE genotype C282Y/S65C) Symbols and abbreviations are the same as those described for FIG. 1. Proband 3 is indicated with an arrow. S65C, C282Y, and wt alleles are known to exist only on separate chromosomes. Proband 3 also has porphyria cutanea tarda, and her brother (II, 203) has ankylosing spondylitis.

DETAILED DESCRIPTION

[0023] A proband is the first individual in a family identified to be affected by hemochromatosis. Forward and reverse sequencing of HFE exons 2, 3, 4, and 5, and of portions of HFE introns 2, 4, and 5 was carried out on biological samples taken from twenty hemochromatosis probands who lacked C282Y homozygosity, C282Y/H63D compound heterozygosity, or H63D homozygosity. Four probands had novel HFE coding region mutations. Probands 1 and 2 were heterozygous for previously undescribed mutations: exon 2, nt 314T→C (314C; I105T), and exon 2, nt 277G→C (277C; G93R) respectively; these probands were also heterozygous for H63D and C282Y, respectively. Probands 3 and 4 were heterozygous for an HFE mutation in exon 2, nt 193A→T (193T; S65C) Twelve other probands did not have an exon 2 HFE exon mutation; four were heterozygous for H63D. In probands 1, 2, 3, and 4, the amino acid substitutions I105T, G93R, and S65C (respectively) occurred on separate chromosomes from those with the C282Y or H63D mutations. In 176 normal control subjects, two were heterozygous for S65C; I105T and G93R were not detected in controls. Nine probands were heterozygous and two probands were homozygous for a base-pair change at intron 2, nt 4919T/C (SEQ ID NO:27). Heterozygosity for a base-pair change in intron 4 (nt 6884T→C) was detected only in probands 3 and 4, both of whom also had S65C and HLA-A32. The intron 2 mutation is not diagnostic of an iron disorder and appears randomly in the population. One proband was heterozygous for a base-pair change at intron 5 (nt 7055A→G)

[0024] The data described herein indicate that, in addition to the C282Y and H63D HFE mutations, the HFE exon and intron 5 mutations described herein are diagnostic (and prognostic) of iron disorders.

Pathology of Iron Overload

[0025] Iron plays an essential role in normal growth and development, but in elevated concentrations, iron is a toxic inorganic molecule and is the leading cause of death in children by poisoning. It has been implicated in the pathophysiology of a number of common diseases, e.g., hepatitis, cancer, heart disease, reperfusion injury, rheumatoid arthritis, diabetes, AIDS, and psychological abnormalities (e.g. depression).

[0026] The incidence of cancer (especially liver cancer) rises dramatically in the course of hemochromatosis. Iron, acting alone or in synergy with other environmental agents, catalyzes free radical formation. These free radicals which mediate tissue damage also cause DNA double strand breaks and oncogene activation. Iron may also play a role in the pathogenesis of rheumatic diseases and in predisposition to heart disease. High levels of iron can also cause diabetes with 2% of diabetics being hemochromatosis patients. High levels of iron may also affect the disease progression of many viral diseases. Individuals infected with such viruses as hepatitis (e.g., hepatitis B or C) or HIV should be tested for HFE mutations because of the impact increased iron stores have on the treatment and prognosis of such diseases.

[0027] Excessive iron stores and iron deposition is also a major contributing factor in the pathology and treatment of non-valvular heart disease. These conditions include dilated cardiomyopathy caused by deposition of iron in myocardial fibers; myocardial injury the product of anthracycline cardiomyopathy and re-perfusion injury. Increased iron stores may also be a contributing factor in myocardial infarction due to atherosclerosis. Some evidence suggests a significant increase in the incidence of reported heart disease in probands (cardiac symptoms-32%, insulin-dependent diabetes-18%, cardiac arrhythmia-17%, clinically significant coronary artery atherosclerosis-9%, and congestive heart failure-7%. Cardiac complications have been detected in 30% of patients. These include EKG abnormalities, congestive heart failure and cardiac arrhythmias. An increased frequency of HFE mutations in individuals with porphyria cutanea tarda indicates that HFE mutations may predispose an individual to developing this syndrome.

[0028] The effect of iron overload is irreparable damage to vital organs and a multiplicity of associated pathologies described above. The multiplicity of clinical symptoms (and associated pathologies) often causes misdiagnosis of hemochromatosis or failure to diagnose hemochromatosis.

[0029] Untreated hemochromatosis is characterized by iron overload of parenchymal cells, which is toxic and the probable cause of various complications including cirrhosis, and liver cancer, arthropathy, hypogonadotropic hypogonadism, marrow aplasia, skin disorders, diabetes mellitus, and cardiomyopathy. There are 1.5 to 2 million active cases in the U.S. of which 40% have progressive liver disease because they have not been properly diagnosed or treated.

[0030] In untreated hemochromatosis, iron is universally deposited in the hepatocytes of the liver. The iron is found primarily in the cytoplasm of hepatocytes, and by electron microscopy in lysosomal vacuoles, and in more severe cases iron has also been reported deposited in mitochondria. Other liver toxins such as alcohol, and hepatitis exacerbate the

damage caused by the iron deposition. Patients with hemochromatosis are advised not to drink, because of increased liver damage, or to smoke, as iron deposition can also occur in the lungs.

[0031] Individuals which are homozygous (and to a lesser extent heterozygous) for an HFE mutation are at risk for developing increased levels of blood lead. Thus, it is important to identify heterozygous as well as homozygous patients.

[0032] Identification and detection of mutations in the HFE gene are critical to understanding the general mechanisms of iron disorders and diagnosing iron-related pathologies.

Nucleic Acid-based Assays for HFE Mutations

[0033] A biological sample containing RNA or DNA is obtained from an individual and the nucleic acid extracted. Optionally, the nucleic acid is amplified according to standard procedures such as PCR. A nucleic acid polymorphism, e.g., a single base pair polymorphism, is detected using methods well known in the art of molecular biology. For example, a mutation is detected using a standard sequencing assay, nucleic acid hybridization, e.g., using standard Southern, Northern, or dot blot hybridization assay systems and an HFE-specific oligonucleotide probe, restriction enzyme fragment polymorphism analysis, oligonucleotide ligation assay (OLA; Nikerson et al., 1990, *Nucl. Acids Res.* 18:8923-8927), primer extension analysis (Nikiforov et al., 1994, *Nucl. Acids Res.* 22:4167-4175), single strand conformation polymorphism (SSCP) analysis, allele-specific PCR (Rust et al., 1993, *Nucl. Acids Res.* 21:3623-3629), denaturing gradient gel electrophoresis (DGGE), fluorescent probe melting curve analysis (Bernard et al., 1998, *Am. J. Pathol.* 153:1055-61), RNA mismatch cleavage assay, capillary hybridization, or TaqMan™ assay (PE Applied Biosystems, Foster City, Calif.). Nucleic acid hybridization assays are also carried out using a bioelectronic microchip technology known in the art, e.g., that described in Sosnowski et al., 1997, *Proc. Natl. Acad. Sci. U.S.A.* 94:1119-1123; Cheng et al. 1998, *Nature Biotechnology* 16:541-546; or Edman et al., 1997, *Nucl. Acids Res.* 25:4907-4914.

Detection of Mutations Using Antibodies and Other HFE Ligands

[0034] Anti-HFE antibodies are known in the art, e.g., those described by Feder et al., 1997, *J. Biol. Chem.* 272:14025-14028, or are obtained using standard techniques. Such antibodies can be polyclonal or monoclonal. Polyclonal antibodies can be obtained, for example, by the methods described in Ghose et al., *Methods in Enzymology*, Vol. 93, 326-327, 1983. An HFE polypeptide, or an antigenic fragment thereof, is used as an immunogen to stimulate the production of HFE-reactive polyclonal antibodies in the antisera of animals such as rabbits, goats, sheep, rodents and the like. HFE antibodies specific for mutated HFE gene products are raised by immunizing animals with a polypeptide spanning the mutation, e.g., a polypeptide which contains the mutations described herein. For example, the entire $\alpha 1$ domain of a mutant HFE gene product is used as an immunogen. Monoclonal antibodies are obtained by the process described by Milstein and Kohler in *Nature*, 256:495-97, 1975, or as modified by Gerhard, *Monoclonal Antibodies*, Plenum Press, 1980, pages 370-371. Hybridomas are screened to identify those producing antibodies that are highly specific for an HFE polypeptide containing a mutation characteristic of an iron metabolism abnor-

malty or clinical hemochromatosis. Preferably, the antibody has an affinity of at least about 10^5 liters/mole, preferably at least 10^6 liters/mole, more preferably at least 10^3 liters/mole, and most preferably, an affinity of at least about 10^9 liters/mole.

[0035] Antibodies specific for the wild type HFE can also be used to diagnose hemochromatosis or iron metabolism abnormalities. Such antibodies are also useful research tools to identify novel mutations indicative of iron disorders or hemochromatosis. A reduction in binding to a wild type HFE-specific antibody indicates the presence of a mutation. Antibody binding is detected using known methods.

[0036] For example, an ELISA assay involves coating a substrate, e.g., a plastic dish, with an antigen, e.g., a patient-derived biological sample containing an HFE gene product. An antibody preparation is then added to the well. Antibodies specific for a mutant HFE gene product bind or fail to bind to a patient-derived sample in the well. Non-binding material is washed away and a marker enzyme e.g., horse radish peroxidase or alkaline phosphatase, coupled to a second antibody directed against the antigen-specific primary antibody is added in excess and the nonadherent material is washed away. An enzyme substrate is added to the well and the enzyme catalyzed conversion is monitored as indicative of presence of the mutation. Antibodies are also labelled with various sizes of colloidal gold particles or latex particles for detection of binding.

[0037] The invention employs not only intact monoclonal or polyclonal antibodies, but also an immunologically-active antibody fragment, for example, a Fab or (Fab)₂ fragment; an antibody heavy chain, an antibody light chain; a genetically engineered single-chain Fv molecule (Ladner et al., U.S. Pat. No. 4,946,778).

EXAMPLE 1

Selection and Characterization of Subjects

[0038] All individuals studied were Caucasians, 18 years of age or older, and from central Alabama. Twenty probands were identified that were either heterozygous for C282Y or H63D, or lacked these mutations. Hemochromatosis is typically diagnosed by detecting elevated saturation of transferrin, with elevated serum ferritin levels, combined with liver biopsy. Each proband patient described below was previously diagnosed to have hemochromatosis by the working diagnostic criterion for hemochromatosis of the American College of Pathologists (elevated fasting transferrin saturation of greater than 60% saturation for males and greater than 50% saturation for females) on at least two occasions in the absence of other known causes. Probands were interviewed regarding their general medical history, diet (including estimated iron content and ethanol consumption), medicinal iron use, receipt of blood transfusion, prior significant hemorrhage, blood donation for transfusion and/or therapeutic phlebotomy, and pregnancy and lactation. Each proband was also evaluated for viral hepatitis B and C and other hepatic disorders, excess ethanol intake, and hereditary, and acquired anemia. Iron overload was defined as evidence of systemic iron overload demonstrated by otherwise unexplained elevated serum ferritin concentration (≥ 300 ng/mL in men, ≥ 200 ng/mL in women), increased hepatic iron content determined using hepatic biopsy specimens, or iron >4 g mobilized by phlebotomy. Complications of iron overload were evaluated and treated, and therapeutic phlebotomy was performed using

standard methods. HFE mutation analysis for C282Y and H63D and human leukocyte antigen (HLA) immunophenotyping or molecular typing were performed using known methods. In some family members, HLA haplotyping had been performed previously for other disease associations, or their HLA type could be deduced from analysis of their kinship and HFE genotyping results. Measurement of serum iron and other clinical laboratory parameters and analysis of hepatic biopsy specimens were performed using routine methods. Control subjects (n=176) who were in apparently good health and were unrelated to the hemochromatosis probands were recruited from the general population. Iron parameters were measured and HLA typing was performed in two control subjects after HFE genotyping revealed that they had the S65C mutation.

EXAMPLE 2

HFE Gene Analysis

[0039] PCR amplification was used to detect mutations. Genomic DNA was prepared from peripheral blood buffy coat or saliva using the QIAmpBlood Kit (QIAGEN, Valencia, Calif.) or FTA Paper and FTA purification reagent (Fitzco Inc., Maple Plain, Minn.), respectively. Fragments were amplified from genomic DNA using eLONGase (Life Technologies, Gaithersburg, Md.) or HotStarTaq DNA polymerase (QIAGEN, Valencia, Calif.).

[0040] Primers used to amplify each exon are shown in Table 3.

TABLE 4

Human HFE genomic DNA						
1	ggatccttta	accgaggaga	ttattatagc	cgagactctg	aagcagcaat	ctcagttcct
61	gtgatagtg	gcaaagaact	acaaactaac	accaaaatgc	aagcttaaa	caaagtttat
121	tgaagcacia	taatacactc	tgagggacag	cggtcttatt	tctgcgaagt	gaactcagca
181	cttctttaca	gagctcaagg	tgcttttatg	gggtttgtgg	ggaggagtgt	aggtttgggc
241	tgtatctgag	tgacaggatg	atgttatttg	attgaagttt	atagctatac	aatctaaaat
301	taaacctgac	atggtcttac	ctataatctg	ttaagaaaag	cctcccaggg	atgggggggc
361	aaaaccgat	gtaaatctta	ttataatgat	ggcatgatga	acttgggggt	aaacttaaga
421	caggcttttg	tggttgtggg	catgtgcccac	cttaggggat	tccaccctgt	accctccttt
481	ctctttctcc	aggatatttt	ggccacagac	ttatcataaa	actccatccc	ttagggttgc
541	atagggttag	tcttgggctt	gaatttaggt	gggccagtgg	ctgtcttagt	gacagccttt
601	ccgctctctt	ctgtcatccc	ctcccactgc	ctaatgtcta	actacctaac	aattaccctt
661	taaatcagtg	tgtctggggt	taggagcagg	cctcaatag	tttaactcatt	ctccagataa
721	tccaataact	gtaaaagtgt	tgaaacactt	gtcagataat	tcaattatga	aggctgtgga
781	acgtgtttca	gtaggatcta	attgggtaat	gttatgactt	aattaatttg	aatcaaaaaa
841	caaaattgaaa	aagctttata	ttctcaagtc	aaataagaca	taagttggtc	taaggttgag
901	ataaaatttt	taaatgtatg	attgaatttt	gaaaatcata	aatattttaa	tatctaaagt
961	tcagatcaga	acattgcgaa	gctactttcc	ccaatcaaca	acacccttcc	aggattttaa
1021	aaccaagggg	gacactggat	cacctagtg	ttcacaagca	agctccttcc	gctctaggag
1081	agagagaact	aaagtctctg	aagacctgtt	gcttttcacc	aggaagtttt	actgggcatc
1141	tcttgagcct	aggcaatagc	tgtagggtga	cttctggagc	catccccggt	tccccgcccc
1201	ccaaaagaag	cgagagattt	acggggacgt	gcgcccatag	ctggggaaat	ggggcccgca
1261	gccaggcccg	cgctctctct	cctgatgctt	tgcagaccg	cggtcctgca	ggggcgctg
1321	ctgcgtgagt	ccgagggcgt	cgggcgaaact	aggggcccgg	cggggggtgga	aaaatcgaaa
1381	ctagcttttt	dtttgcgctt	gggagtttgc	taacttttga	ggacctgtcc	aacctaatcc
1441	gcaagcccct	ctccctactt	tctgcgtcca	gaccccgtag	gggagtgctt	accactgaac
1501	tgacagatag	ggctcctcgc	cccaggacct	gccccctccc	ccggtctgcc	cggtctgctg
1561	gagtgacttt	tggaaaccgc	cactcccctc	cccccaactg	aatgctttta	aataaatctc
1621	gtagttcctc	acttgagccg	agctaagcct	ggggctcctt	gaacctgaaa	ctcgggttta
1681	tttccaatgt	cagctgtgca	gttttttccc	cagtcacttc	caaacagaaa	gttcttccct
1741	gagtgcttgc	cgagaaggct	gagcaaaacc	acagcaggtg	ccctccagct	tttccacctc
1801	agaacgaatg	cggtggggcg	tggggggcgg	aaagagtggt	gttggggatc	tgaattcttc
1861	accattccac	ccacttttgg	tgagacctgg	ggtggaggtc	tctagggtgg	gaggctcctg
1921	agagatgctt	acctcgggcc	tttcccactt	ctggcaattg	ttcttttgc	ctggaaaatt
1981	aagtatatgt	tagttttgaa	cgtttgaact	gaaccaattc	cttttcggct	aggctctatt
2041	gatttgcgat	gtgctgtgta	attaagagcc	ctctctacaa	agttactgat	acgaacatgt
2101	aagcaatgca	ctcaccttcta	agttacattc	atatctgata	ttatttgatt	ttcactaggc
2161	atagggaggt	aggagctaat	aatacgttta	tttactaga	agttaactgg	aatctcagatt
2221	ataaactctt	tttcaggtta	caaagaacat	aaataactgg	ttttctgatg	ttatttcaa
2281	gtactacagc	tgcttcta	cttagttgac	agtgattttg	ccctgtagtg	tagcagctgt
2341	ttctgtgggt	cacacgcccg	cctcagcaca	gcactttgag	ttttggcact	acgtgtatcc
2401	acattttaca	catgacaaga	atgagggcatg	gcacggcctg	cttccctggca	aatattattca
2461	atggatcacg	gggtcttggg	ggcagagctc	atgtctccac	ttcatagcta	ttctcttaa
2521	acatcacact	gcattagagg	ttgaataata	aaatttcatg	ttgagcagaa	atattcat
2581	tttacaagtg	taaatgagtc	ccagccatgt	gttgcaactg	tcaagcccca	agggagagag
2641	cagggaaaca	agcttttacc	ctttgatatt	ttgcattcta	ttggggagaga	tgacaataag
2701	caaatgagca	gaaagatata	caacatcagg	aaatcatggg	tgttgtgaga	agcagagaag
2761	ccagggcaag	tcactctggg	gctgacactt	gagcagagac	atgaagggaa	taagaatgat
2821	atgactggg	agcagtattt	cccagggcaa	ctgagtgggc	ccctgcaagt	ggattaaaaa
2881	gogggttttc	tcagcaactac	tcattgtgtg	gtgtgtgggg	ggggggggcg	cgtgggggtg
2941	ggaaggtgga	ctaccatctg	catgtaggat	gtctagcagt	atcctgtcct	ccctactcac
3001	taggtgctag	gagcactccc	ccagctctga	caacccaaaa	ttctctaaa	ctttgccaca
3061	tgtcacctag	tagacaaaact	cctgggttaa	aagctcgggt	tgaaaaaaat	aaacaagtga
3121	tgtctggggag	tagagggcaa	gaagtaggta	atgggctcag	aagaggagcc	acaaacaagg
3181	ttgtgcaggc	gcctgtaggc	tgtgggtgta	attctagcca	aggagtaaca	gtgatctgtc
3241	acaggctttt	aaaagattgc	tctgctgctt	atgtggaag	cagaatgaag	ggagcaacag

TABLE 4-continued

Human HFE genomic DNA						
3301	taaaagcagg	gagcccagcc	aggaagctgt	tacacagtcc	aggcaagagg	tagtggagtg
3361	ggctgggtgg	gaacagaaaa	gggagtgaca	aaccattgtc	tectgaatat	attctgaagg
3421	aagtgtctga	aggattctat	gttgtgtgag	agaaagagaa	gaattggctg	ggtgtagttag
3481	ctcatgccaa	ggaggagccc	aaggagagca	gattcctgag	ctcaggagtt	caagaccagc
3541	ctgggcaaca	cagcaaaacc	cctctctctac	aaaaaataca	aaaatttagct	gggtgtgggtg
3601	gcattgcacct	gtgatccctag	ctactcggga	ggctgagggtg	gagggtattg	cttgagccca
3661	ggaagttag	cctgcagtg	gccatgactg	tgccactgta	cttcagccta	ggtgacagag
3721	caagaccctg	tctcccctga	ccccctgaaa	aatagaagag	ttaaagttag	ctttgttctt
3781	tattttaatt	ttattggcct	gagcagtg	gtaattggca	atgccatttc	tgagatggtg
3841	aaggcagagg	aaagagcagt	ttggggtaaa	tcaaggatct	gcatttggac	atggttaagt
3901	tgagattcca	gtcaggcttc	caagtggtag	ggccacatag	gcagttcagt	gtagaattc
3961	aggaccaagg	cagggcactg	tggtcactt	ctgtaatccc	agcactttgg	tggtgaggc
4021	aggtagatca	tttgaggtca	ggagttag	acaagcttgg	ccaacatggt	gaaacccat
4081	gtctactaaa	aatacaaaaa	ttagcctggt	gtgggtggcg	acgcctatag	tcccaggtt
4141	tcaggaggct	taggtaggag	aatcccttga	accaggagg	tgagggttgc	agttagctga
4201	gattgtgcca	ctgcactcca	gcctgggtga	tagagtgaga	ctctgtctca	aaaaaaaaaa
4261	aaaaaaaaaa	aaaaaaaaaa	aactgaagga	attattcctc	aggatttggg	tctaatttgc
4321	cctgagcacc	aactcctgag	ttcaactacc	atggctagac	acaccttaac	attttctaga
4381	atcccaccag	tttagtgag	tctgtctaat	catgagtatt	ggaataggat	ctggggcag
4441	tgaggggggtg	gcagccacgt	gtggcagaga	aaagcacaca	agtaaagagc	accaggact
4501	gtcatatgga	agaaagacag	gactgcaact	caccttcac	aaaatgagga	ccagacacag
4561	ctgatggtat	gagttgagtc	aggtgtgtgg	agcctcaaca	tctgtctccc	ctcctactac
4621	acatggttaa	ggcctgttgc	tctgtctcca	ggttcacact	ctctgacta	cctctctcatg
4681	ggtgcctcag	agcaggacct	tggtctttcc	ttgtttgaag	ctttgggcta	cgtggatgac
4741	cagctgttcg	tgttctatga	tcatgagagt	cgccgtgtgg	agccccgaac	tccatgggtt
4801	tccagtagaa	tttcaagcca	gatgtggctg	cagctgagtc	agagtctgaa	agggtgggat
4861	cacatgttca	ctgttgactt	ctggactatt	atggaaaatc	acaaccacag	caagggtatg
4921	tgagaggggg	gcttcacctt	cctgaggttg	tcagagcttt	tcactctttc	atgcatcttg
4981	aaggaacag	ctggaagtct	gaggtcttgt	gtgagcaggg	aagaggggaag	gaatttgcct
5041	cctgagatca	tttggctcct	ggggatgggtg	gaaataggga	cctattcctt	tggttgcagt
5101	taacaagct	ggggattttt	ccagagtccc	acacctgca	ggtcatcctg	ggctgtgaaa
5161	tgcaagaaga	caacagtagc	gagggctact	ggaagtacgg	gtatgatggg	caggaccacc
5221	ttgaattctg	ccctgacaca	ctggattgga	gagcagcaga	accaggggcc	tgccccacca
5281	agctggagtg	ggaaagccac	aagatctggg	ccaggcagaa	cagggcctac	ctggagaggg
5341	actgccttgc	acagctgcag	cagttgctgg	agctggggag	aggcgttctg	gaccaacaat
5401	gtatggtgga	aacacacttc	tgcccctata	ctctagtggc	agagtggagg	agggtgcagg
5461	gcacggaaatc	ccttgggtgga	gtttcagagg	tggtgaggg	tggtgtcctc	tccaaattct
5521	gggaagggac	tttctcaatc	ctagagtctc	tacctataaa	ttgagatgta	tgagacagcc
5581	acaagtcatg	ggtttaattt	cttttctcca	tgcatatggc	tcaaaaggaa	gtgtctatgg
5641	cccttgcttt	ttatttaacc	aataatcttt	tgatatatta	tacctgttaa	aaattcagaa
5701	atgtcaaggc	cgggcacggg	ggctcaccct	tgtaatccca	gcacttttgg	aggccgaggg
5761	gggtgggtcac	aaggtcagga	gtttgagacc	agcctgacca	acatgggtgaa	accctgtctc
5821	aaaaaaaaatc	gaaattagc	tggtccacagt	catgcccacc	Egtagtccca	gctaattgga
5881	atgctgaggc	aggagcatcg	cttgaacctg	ggaagcggaa	gttgcactga	gccaagatcg
5941	cgccactgca	ctccagccta	ggcagcagag	tgagactcca	tcttaaaaaa	aaaaaaaaaa
6001	aaaaagagaa	ttcagagatc	tcagctatca	tatgaatacc	aggacaaaat	atcaagttag
6061	gocacttatc	agagtagaag	aatcctttag	gttaaaagt	tctttcatag	aacatagcaa
6121	taactactga	agctaccctat	cttacaagtc	cgcttcttat	aaacatggct	cctaggttga
6181	cccaggtgaa	cctgagcctc	tgatattcaat	cattttcaat	gcacataaag	ggcaattata
6241	tctatcagaa	caaagaacat	gggtaacaga	tatgtatatt	tacatgtgag	gagaacaagc
6301	tgatctgact	gctctccaaag	tgacactgtg	ttagagtcca	atcttaggac	acaaaatggt
6361	gtctctcctg	tagcttgttt	ttctctgaaa	agggtatttc	ctttcctccaa	cctatagaag
6421	gaagtgaag	ttccagtctt	cctggcaagg	gtaaacagat	cccctctcct	catccttctc
6481	cttctctgtca	agtgccctcct	tttgggtgaa	gtgacacatc	atgtgacctc	ttcagtgacc
6541	actctacggg	gtcgggcctt	gaactactac	cccagaaca	tcacatgaa	gtggctgaag
6601	gataagcagc	caatggatgc	caaggagtcc	gaacctaaag	acgtatggcc	caatggggat
6661	gggacctacc	agggctggat	aaccttggct	gtaccccctg	gggaagagca	gagatatacg
6721	tgccaggtgg	agcaccocagg	cctggatcag	cccctcattg	tgatctgggg	tatgtgactg
6781	atgagagcca	ggagctgaga	aaatctattg	gggggtgaga	ggagtgcctg	aggaggtaat
6841	tatggcagtg	agatgaggat	ctgctctttg	ttaggggatg	ggctgagggt	ggcaatcaaa
6901	ggctttaact	tgcttttctc	gttttagagc	cctcaccgtc	tggaacctca	gtcattggag
6961	tcatcagtg	aattgctgtt	tttgtctca	tcttgttcc	tggaattttg	ttcataaat
7021	taaggaaag	gcaggggtca	agtgagtagg	aaacaggggg	aaagtctcta	gtacctctgc
7081	cccagggcac	agtggggaaga	ggggcagagg	ggatctggca	tcocatgggaa	gcatttttct
7141	catttatatt	ctttggggac	accagcagct	ccctgggaga	cagaaaaata	tggttctccg
7201	cagaatgaaa	gtctctaatt	caacaaaact	cttcagagca	cctactattt	tgcaagagct
7261	gtttaaggtg	gtcaccgggc	tttgaggttg	agaagtcact	gtggctattc	tcagaaccca
7321	aatctggtag	ggaatgaaat	tgatagcaag	taaatgtagt	taagaagac	cccatgaggtt
7381	cctaaagcag	gcaggaagca	aatgcttagg	gtgtcaaagg	aaagaatgat	cacattcagc
7441	tggggatcaa	gatagccttc	tggtcttga	aggagaagct	ggatctccat	agggtgaggt
7501	gaagatgatg	ggaggtctac	acagacggag	caacctgccc	aagtaggaga	gtataaggca
7561	tactgggaga	ttagaataaa	ttactgtacc	ttaaccttga	gtttgcttag	ctatcactca
7621	ccaattatgc	atttctacc	ccttaacatc	tggtgtgtag	ggaaaagaga	atcagaagaa
7681	agccagctca	tacagagtcc	aagggtcttt	tggtatattg	ggttatgatc	actgggggtg

TABLE 4-continued

Human HFE genomic DNA						
7741	cattgaagga	tcctaagaaa	ggaggaccac	gatctccctt	atatggtgaa	tgtgttgta
7801	agaagttaga	tgagaggtga	ggagaccagt	tagaaagcca	ataagcattt	ccagatgaga
7861	gataatgggt	cttgaaatcc	aatagtgccc	aggtcctaat	tgagatgggt	gaatgaggaa
7921	aataaggaag	agagaagagg	caagatggtg	cctaggtttg	tgatgcctct	ttcctgggtc
7981	tcttgtctcc	acaggaggag	ccatggggca	ctacgtctta	gctgaacgtg	agtgcacgc
8041	agcctgcaga	ctcactgtgg	gaaggagaca	aaactagaga	ctcaaagagg	gagtgcatttt
8101	atgagctctt	catgtttcag	gagagagttg	aacctaaaca	tagaaattgc	ctgacgaaact
8161	ccttgatttt	agccttctct	gttcattttc	tcaaaaagat	ttccccattt	aggtttctga
8221	gttcctgcac	gccggtgatc	cctagctgtg	acctctcccc	tggaactgtc	tctcatgaac
8281	ctcaagctgc	acttagaggg	ttccttcatt	tcctccgtca	cctcagagac	atacacctat
8341	gtcattttcat	ttcctatttt	tggaagagga	ctccttaaat	ttgggggact	tacatgattc
8401	atTTTTtaacat	ctgagaaaag	ctttgaacc	tgggacgtgg	ctagtcataa	ccttaccaga
8461	TTTTTtacaca	tgtatctacg	cttttctgg	accogttcaa	cttttcttt	gaatcctctc
8521	tctgtgttac	ccagcaactc	atctgtcacc	aagccttggg	gattcttcca	tctgattgtg
8581	atgtgagttg	cacagctatg	aaggctgtac	actgcacgaa	tggaagaggc	acctgtccca
8641	gaaaaagcat	gagctctatc	tggtggtagt	atgatgggtg	TTTTtagcag	gtaggaggca
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9181	ccagctactc	ggaaggtgca	ggcaggagaa	tggcatgaac	ccaggaggca	gagcttcag
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9301	aaaataaaaa	taaaaataaaa	aaaatgaaaa	aaaaaagaaa	gtgaagtata	gagtatctca
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9421	cagaccacc	tacatccagt	agtttagatg	cctagaataa	atagagaagg	aaggagatgg
9481	cccttctcct	gtctcattgt	gtttcttctg	aatgagcttg	aatcacatga	aggggaacag
9541	cagaaaacaa	ccaactgacc	ctcagctgtc	atgtttcctt	taaaagtccc	tgaaggaagg
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9901	ggacattcag	gaattgctag	atctgggaa	atcagttcac	catgttcaaa	agagtctttt
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11761	gactatcatt	taaatatttc	tgactttcaa	attaaagatt	ttcacatgca	ggctgatatt
11821	tgtaatgtg	attctctctg	taggctttgg	gtataatgtg	ttcttttctc	tttttgcatc
11881	agcgattaac	ttctcactc	taacatgtag	aatgttacta	caaatataaa	gtattttgta
11941	tgacaatttt	atTTTgaaagc	ctaggatgctg	ttgacatcct	gcatgcattt	attacttgat

TABLE 4-continued

Human HFE genomic DNA	
12001	atgcatgcat tctggtatct caagcattct atttctgagt aattgtttaa ggtgtagaag
12061	agatagatat ggtggatttg gagttgatac ttatatattt tetatttctt ggatggatga
12121	atgtgtacat taaaagtttt ccatgg
	(SEQ ID NO:27; GENBANK [®] Accession No. Z92910)

[0041] Exon 1 spans nt 1028-1324, inclusive; exon 2 spans nt 4652-4915, inclusive; exon 3 spans nt 5125-5400, inclusive; exon 4 spans nt 6494-6769, inclusive; exon 5 spans nt 6928-7041, inclusive; exon 6 spans nt 7995-9050, inclusive, and exon 7 spans nt 10206-10637, inclusive. Intron 4 spans nt 6770-6927, inclusive, and intron 5 spans nt 7042-7994, inclusive.

[0042] Total RNA for the RT-PCR was prepared from 1.5 mL of whole blood using the RNeasy Blood Kit (QIAGEN, Valencia, Calif.). Total messenger RNA encoding the HFE gene was transcribed and amplified with the primers shown above using standard methods, e.g., the Superscript ONE-STEP RT-PCR System (Life Technologies, Gaithersburg, Md.). The amplified product was directly subcloned into the pCR2.1-TOPO vector and transfected into TOP 10 bacteria (Invitrogen, Carlsbad, Calif.). Plasmid DNAs isolated from the subcloning were prepared with the UltraClean Mini Prep Kit (Mo Bio, Solana Beach, Calif.) and sequenced.

[0043] DNA sequencing was performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, Calif.) and analyzed on an ABI Prism 377.

[0044] To detect mutations in exon 2 of the HFE gene, the genomic DNA of probands and normal control subjects were amplified and subjected to a dot blot hybridization assay. 1.0 µl of each resulting PCR product was then applied to a Magna Graph nylon membrane (MSI, Westboro, Mass.). The membranes were treated with 0.5 N NaOH/1.5 M NaCl to denature the DNA, neutralized with 0.5 M Tris-HCl (pH 8.0)/1.5 M NaCl, and rinsed with 2×SSC (1×SSC=0.15 M NaCl/0.015 M sodium citrate, pH 7.0). The DNAs were fixed on the membrane by UV irradiation using a Stratilinker 1800 (Stratagene, Inc., La Jolla, Calif.). The ECL 3'-oligolabelling and detection system (Amersham, Arlington Heights, Ill.) was used for synthesis of labeled oligonucleotide probes, hybridization, and signal detection. The oligonucleotide sequences used to detect each point mutation were (substituted bases are shown as upper case letters):

TABLE 5

Oligonucleotide Probes	
Point Mutation	Oligonucleotide
G93R mutation	gtctgaaaCggtgggat (SEQ ID NO:28)
I105T mutation	acttctggactaCtatgg (SEQ ID NO:29)
S65C mutation	atcatgagTgtcgccgt (SEQ ID NO:30)

For signal detection, each oligonucleotide was labeled with fluorescein-11-dUTP using terminal deoxynucleotidyl trans-

ferase according to the manufacturer's instructions (Amersham Ltd., Arlington Heights, Ill.). The membranes were prehybridized in 5×SSC, 0.1% Hybridization buffer component, 0.02% SDS, 5% LiquidBlock at 42° C. for approximately 2 hours. Labeled oligonucleotide probes were added to individual bags containing the membranes and prehybridization buffer and incubated at 42° C. overnight. The blots were washed twice with 5×SSC, 0.1% SDS for 5 minutes at room temperature. Stringency washes for hybridization with oligonucleotides having the sequence of SEQ ID NO: 30 or 28 were performed twice in 0.2×SSC/0.1% SDS for 15 minutes at 42° C. Membranes probed with an oligonucleotide having the sequence of SEQ ID NO:29 was washed twice under less stringent conditions (0.5×SSC/0.1% SDS, 15 minutes at 42° C.). Detection of a fluorescent signal was performed according to standard methods.

EXAMPLE 3

Characterization of Probands

[0045] The mean age of the twenty probands was 44±11 years (range 27-62 years); thirteen (65.0%) were men and seven (35.0%) were women. Eleven had iron overload. One had hepatic cirrhosis, two had diabetes mellitus, four had arthropathy, and two had hypogonadotrophic hypogonadism. One proband also had hereditary stomatocytosis, another had beta-thalassemia trait, a third had ethanol intake>60 g daily, and a fourth had porphyria cutanea tarda. No proband is had evidence of excess oral or parenteral iron intake, or of viral hepatitis B or C. At diagnosis of hemochromatosis, evaluation for common HFE mutations revealed that eleven probands were C282Y heterozygotes, five were H63D heterozygotes, and four did not inherit C282Y or H63D.

[0046] The mean age of the initial 176 control subjects was 52±15 years (range 18-86 years); 79 (44.9%) were men and 97 (55.1%) were women. There was no significant difference in the mean ages of men and women. Frequencies of HFE genotypes among the control subjects are shown in Table 6. These values are similar to those previously reported from normal persons from the same geographic area.

TABLE 6

Frequencies of HFE Genotypes in Alabama Subjects.		
HFE Genotype	Hemochromatosis Probands with "Atypical" HFE Genotypes, % (n)	Normal Control Subjects, % (n)
wt/wt	15.00 (3)	60.23 (106)
C282Y/wt	45.00 (9)	13.06 (23)
H63D/wt	20.00 (4)	15.34 (27)
S65C/wt	5.00 (1)	1.14 (2)
C282Y/S65C	5.00 (1)	0
C282Y/G93R	5.00 (1)	0

TABLE 6-continued

Frequencies of HFE Genotypes in Alabama Subjects.		
HFE Genotype	Hemochromatosis Probands with "Atypical" HFE Genotypes, % (n)	Normal Control Subjects, % (n)
H63D/I105T	5.00 (1)	0
H63D/C282Y	0	6.82 (12)
H63D/H63D	0	3.41 (6)

Results are expressed as percentage (n). The wild-type (wt) allele was defined as the HFE configuration in which the mutations C282Y, H63D, S65C, I105T, or G93R were not detected.

EXAMPLE 4

Identification of Novel HFE Mutations in Hemochromatosis Probands

[0047] The following novel mutations (missense mutations) were identified in probands 1 and 2: exon 2, nt 314T→C (I105T), and exon 2, nt 277G→C (G93R), respectively (Table 7; FIGS. 1 and 2). Probands 3 and 4 had a S65C mutation. The S65C mutation has been observed in hemochromatosis patients but has not been deemed to be indicative of a disease state. In contrast, the data presented herein indicate

that the S65C mutation is diagnostic of a disease state. This result is surprising in view of earlier observations. Other than C282Y or H63D, no HFE exon mutations were detected in the remaining sixteen of the twenty probands (Table 6). Nine probands were heterozygous for a base-pair change at intron 2, nt 4919T/C (SEQ ID NO:27); two probands were homozygous for this base-pair change. Heterozygosity for a base-pair change in intron 4 (nt 6884T→C) was detected only in probands 3 and 4, both of whom also inherited S65C. One proband was heterozygous for a base-pair change at intron 5, nt 7055A→G.

[0048] Using dot blot methodology, heterozygosity for the S65C mutation was detected in two of 176 normal control subjects (Table 6). The G93R or I105T mutations were not detected in normal control subjects (Tables 6 and 8).

EXAMPLE 5

Association of Novel HFE Coding Region Mutations to C282Y and H63D and HFE Intron Alleles

[0049] In proband 1, two mutations of exon 2 (H63D and I105T) were detected. After subcloning the genomic fragment, the subclones revealed that these mutations occurred on separate chromosomes; this observation was confirmed by family studies indicating segregation of I105T

TABLE 7

Phenotypes and Uncommon HFE Genotypes in Alabama Subjects*							
Subject†	Age (years), HFE		HLA Type	Transferrin Saturation, %	Serum Ferritin, ng/mL	Hepatocyte Iron Grade	Phlebotomy, Units
	Sex	Genotype					
Proband 1	52 M	H63D/I105T	A2, 3; B7, 7	62	868	2+	20
Proband 2‡	40 M	C282Y/G93R	A2, 3; B7, 62	78	861	4+	34
Proband 3§	47 F	C282Y/S65C	A2, 32; B8, 44; Bw4, 6; Cw5, 7	90	281	3+	37
Proband 4**	81 F	S65C/wt	A2, 32; B14, 62	100	5,135	N.D.	37
Normal Control 1	28 M	S65C/wt	A2, 31; B35, 60	28	141	N.D.	N.D.
Normal Control 2	69 M	S65C/wt	A24, 26; B8, B37; Bw4, 6; Cw6, 5 (or 7)	42	747	2+	N.D.

*Serum transferrin saturation, serum ferritin concentration, and percutaneous hepatic biopsy were performed before therapeutic phlebotomy was initiated. Reference ranges for these parameters are 15-45%; 20-300 ng/mL (men) and 20-200 ng/mL (women); and 0-1+, respectively. Iron depletion (serum ferritin \leq 20 ng/mL) was induced by removing the indicated numbers of units of blood. None of these persons had evidence of hepatic cirrhosis, diabetes mellitus, hemochromatosis-associated arthropathy, hypogonadotropic hypogonadism, other endocrinopathy, or cardiomyopathy. N.D. = not done. The mutations indicated are exon 4, nt 845G→A (C282Y); exon 2, nt 187C→G (H63D); exon 2, nt 314T→C (I105T); exon 2, nt 277G→C (G93R); and exon 2, nt 193A→T (S65C). The wild-type (wt) allele was defined as an HFE allele in which the mutations C282Y, H63D, S65C, I105T, or G93R were not detected.

†Countries of origin: Probands 1 and 2, England; Proband 3, Wales, England, and Americas (Cherokee); Proband 4, England and Ireland; Normal Control 1, England; Normal Control 2, The Netherlands.

‡The father and sister of Proband 2 are presently undergoing therapy for hemochromatosis and iron overload, but their clinical and genetic data were unavailable.

§Proband 3 had porphyria cutanea tarda alleviated with therapeutic phlebotomy.

**Proband 4 had hereditary stomatocytosis unaffected by phlebotomy treatments. 37 units of blood were removed by phlebotomy before treatment was discontinued due to stroke apparently unrelated to anemia or iron overload (post-treatment serum ferritin 1,561 ng/mL). Her 59 year-old daughter (who does not have hereditary stomatocytosis) had transferrin saturation 42%, serum ferritin 62 ng/mL, HLA type A1, 32; B14, 15; Bw4, 6; Cw3, 8, and HFE genotype S65C/H63D. These data permitted assignment of the S65C mutation in this family to a haplotype carrying HLA-A32; linkage of S65C and HLA-A32 was also observed in the family of Proband 3.

TABLE 8

Frequencies of HFE Alleles in Alabama Subjects.						
	wt*	C282Y	H63D	S65C†	I105T	G93R
Hemochromatosis Probands with "Atypical" HFE Genotypes (n = 20)	0.500	0.275	0.125	0.050	0.025	0.025
Normal Control Subjects (n = 176)	0.750	0.099	0.145	0.006	‡	‡

The wild-type (wt) allele was defined as an HFE allele in which the mutations C282Y, H63D, S65C, I105T, or G93R were not detected.

†S65C was detected in 2 of 22 (0.091) proband chromosomes and in 2 of 266 (0.0075) control chromosomes that did not bear the C282Y, H63D, S65C, I105T, or G93R mutation.

‡Based on this data set, the frequency of the I105T and G93R HFE alleles is estimated to be <0.0028, respectively.

and H63D (FIG. 1). In proband 2 (HFE genotype C282Y/G93R), RT-PCR analysis (with subsequent subcloning and sequencing) revealed that these HFE mutations occurred on separate chromosomes. Family studies of proband 3 (HFE genotype C282Y/S65C) indicated that the C282Y and S65C HFE alleles segregated independently, establishing their occurrence on separate chromosomes (Table 7, FIG. 3).

[0050] In proband 1 (HFE genotype H63D/I105T), the I105T mutation was co-inherited with HLA-A3, B7. In probands 3 and 4 and their respective families, S65C was inherited on the same chromosome as HLA-A32, indicating that HLA-A32 is a marker for chromosomes bearing the S65C mutation, and individuals with HLA-A32 have an increased risk for developing hemochromatosis. The G93R mutation is associated with HLA-A2, and individuals with that haplotype have an increased risk for developing hemochromatosis. The I105T mutation is associated with HLA-A3, e.g., HLA-A3, B7, and individuals with that haplotype have an increased risk for developing hemochromatosis. Among twenty probands tested, the nucleotide polymorphism in intron 4 (nt 6884T→C) was detected in probands 3 and 4, both of whom also had S65C. Subjects that tested positive for the S65C mutation all were found to have the

intron 4 (6884T→C) mutation, including two probands (3 and 4), their families, and two normal controls.

EXAMPLE 6

HFE Coding Region Mutations and Clinical Phenotype

[0051] The I105T and G93R mutations were associated with a hemochromatosis clinical phenotype in probands 1 and 2 who also inherited H63D and C282Y, respectively. Proband 3 had clinical evidence of hemochromatosis, iron overload, and porphyria cutanea tarda associated with compound heterozygosity for C282Y and S65C. Proband 4 had severe iron overload associated with heterozygosity for S65C and co-inheritance of hereditary stomatocytosis (Table 7). The sister of proband 1 (HFE genotype I105T/wt) was not completely evaluated for hyperferritinemia (FIG. 1). Otherwise, family members of probands who were heterozygous for novel HFE mutations described herein had little or no evidence of abnormal iron parameters, a hemochromatosis phenotype, or of iron overload (Table 7 and 9; FIGS. 1 and 3). Normal Control 1 who had HFE genotype S65C/wt had a

TABLE 9

Hemochromatosis (HC) Family study/patient							
Subject/Age/Sex	HLA Type	exon 2	exon 4	intron 4 5636bp	Tf sat** %	Ftn** ng/ml	Diagnosis/Hepatocyte Iron grade
Proband 1/57M (201)	A2, 3; B7, 7	H63D/H, I105T/1	Wt	T	62	868	HC/2+
brother/45M(204)		H63D/H	Wt	T*	31	186	
sister/50F(203)	A3, 3; B7, 7	I105T	Wt*	T*	37	576	
daughter/31F(301)	A32, 68; B7, 44	I105T/1	Wt*	T*	31	56	
son/27M(302)	A2, 68; B7, 44	H63D/H	Wt*	T*	33	44	
Proband 2/40M	A2, 3; B7, 62	G93R/G	C282Y/C	T	78	861	HC/4+
Father		Wt	C282Y/Y*	T*			HC
Sister		G93R/G	C282Y/C*	T*			HC
Proband 3/47(201)	A2, 32; B8, 44	S65C/S	C282Y/C	T/C	90	281	HC/3+
brother/45M(202)	A2, 32; B44, 51	S65C/S	Wt	T/C	33	42	
mother/81F(102)	A2, 2; B8, 51	Wt	C282Y/C	T*	NT	NT	
sister/33F(204)	A2, 7; B27, 51	Wt	Wt	T*	NT	NT	
brother/35M(203)	A2, 7; B27, 51	Wt	Wt*	T*	NT	NT	
sister		Wt	C282Y/C*	T*			
sister		S65C/S	Wt*	T/C*			
Proband 4/81F	A2, 32; B14, 62	S65C/S	Wt	T/C	100	S135	HC + stomatocytosis
daughter/59*	A1, 32; B14, 15	H63D/H, S65C/S	Wt*	T/C	42	62	
Control 1/28M	A2, 31; B35, 60	S65C/S	Wt	T/C	28	141	
Control 2/69M	A24, 26; B8, 37	S65C/S	Wt	T/C	42	747	2+

*RE cut

**normal (15-45%)

***20-300 ng/ml(men)

2C-200 ng/ml(women)

normal iron phenotype (Table 7) Normal Control 2, who also had the HFE genotype S65C/wt, had hyperferritinemia and mildly increased stainable hepatocellular iron deposition, but had no symptoms or other objective findings attributable to iron overload (Table 7). These data indicate that S65C heterozygosity is associated with abnormal iron parameters.

EXAMPLE 7

HLA Gene Linkage

[0052] In the family of proband 1, the I105T mutation was linked to HLA-A3, B7, markers which are often linked to the C282Y mutation and its ancestral haplotype. HLA-A3, B7 is also significantly more common among C282Y-negative hemochromatosis probands than in normal control subjects tested. S65C was linked to HLA-A32 in probands 3 and 4 (and their respective families). The base-pair change in intron is 4 (nt 6884T→C) was detected only in probands who inherited the S65C mutation. These data indicate that an intron 4 mutation (nt 6884→C) is a marker for chromosomes bearing

the S65C HFE allele. Three of four probands who inherited mutated HFE exon 2 mutations described herein also inherited the C282Y or H63D mutations on separate chromosomes. In a fourth proband, the co-inheritance of S65C heterozygosity and hereditary stomatocytosis was associated with severe iron overload.

[0053] Altered interactions of transferrin receptor, transferrin, and C282Y and H63D mutant HFE protein contribute to the pathology of hemochromatosis. The S65C, G93R, and I105T mutations are located within the $\alpha 1$ domain: in the $\alpha 1$ helix of the HFE class I-like heavy chain (I105T and G93R), and at the tip of the A chain loop of the β -pleated sheet (S65C). These mutations affect the overall structure of the HFE gene product, and specifically affect the salt bridge between residues H63 and D95. The I105T substitution also inhibits proper folding of the $\alpha 1$ domain of the HFE gene product, and specifically affects the hydrophobicity of the hydrophobic F pocket.

[0054] Other embodiments are within the following claims.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 30

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<211> LENGTH: 2506

<212> TYPE: DNA

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 1

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1-15. (canceled)

16. A kit for diagnosing an iron disorder or a genetic susceptibility to developing said disorder in a mammal, comprising an antibody which preferentially binds to an epitope of a mutant HFE gene product, wherein said gene product comprises amino acid substitution I105T, G93R, or 865C.

17. A kit for diagnosing an iron disorder or a genetic susceptibility to developing said disorder in a mammal, comprising an antibody which preferentially binds to an epitope of a wild type HFE gene product, wherein said gene product comprises amino acid substitution I105, G93, or 865.

* * * * *