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C. Camaschella · A. Roetto · P. Gasparini · A. Piperno
P. Fortina · S. Surrey · E. Rappaport

Allelic association of microsatellites of 6p in Italian hemochromatosis patients

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Abstract Hemochromatosis (HC) is an inherited disorder of iron metabolism and is frequently seen in Caucasians. The biochemical defect and the responsible gene are unknown, but the HC locus is closely linked to HLA-A on human chromosome 6 in the region 6p21.3. Although extensive studies have been performed in several populations, the precise location of the gene is still undefined. Linkage disequilibrium with HC has been detected for loci that are 3 cM apart: HLA class I and D6S105, which is located on the telomeric side of HLA-A. We have analyzed the inheritance of several multi-allele polymorphisms that map to 6p (D6S265, Y52, HLA-F, D6S306, D6S105, D6S464, D6S299) in 34 Italian HC families and in 17 unrelated patients. Significant association with HC was shown for alleles of multiple markers in the HLA-A region, for the distant marker D6S105, but not for the D6S299 marker at 4 cM from HLA-A on the telomeric side. HC status was unambiguously assigned to 70 affected and 63 unaffected chromosomes from family studies. Thirty five different haplotypes were found in 70 HC chromosomes when considering four markers most tighly associated with the disease. A predominant haplotype comprising alleles 1-3-1-8 (marker order D6S265, HLA-A, Y52, D6S105) accounted for 30% of the HC chromosomes and was absent in normals. A minority of other HC haplotypes could be related to the major haplotype by assuming single crossover events. Results of haplotype studies suggest a founder effect in the Italian population, as previously shown in Australian patients, and a possible common mutation shared with affected individuals of Celtic origin.

C. Camaschella (✉) · A. Roetto
Dipartimento di Scienze Biomediche e Oncologia Umana, Università di Torino, Ospedale San Luigi Gonzaga, I-10043 Orbassano Torino, Italy
Tel.: (11) 902 66 10, Fax: (11) 903 86 36

C. Camaschella
CNR CIOS Torino, Torino, Italy

P. Gasparini
IRCCS CSS, San Giovanni Rotondo, Italy

A. Piperno
Istituto di Scienze Biomediche, Ospedale S. Gerardo, Monza, Milan, Italy

P. Fortina · S. Surrey · E. Rappaport
Department of Pediatrics, The Children's Hospital of Philadelphia, University of Pennsylvania, School of Medicine, Philadelphia, PA, 19104, USA

Introduction

Hemochromatosis (HC) is a late onset genetic disease characterized by progressive iron overload. It is the most common autosomal recessive disorder in Caucasians with an estimated carrier frequency approximating 10% (Edwards and Kushner 1993). If untreated, HC can cause liver cirrhosis, diabetes, cardiopathy, hypogonadism, arthropathy and, in some cases, hepatocellular carcinoma, in midlife. Early diagnosis allows the prevention of complications by phlebothomies, which remove excess iron, thereby providing patients with a normal life expectancy. The biochemical defect and the gene causing the disease remain unidentified; however, genetic studies show that the HC gene is tightly linked to HLA-A, providing a starting point on 6p for positional cloning (Simon et al. 1976).

The finding of linkage disequilibrium with a specific marker is a useful tool for locating genes in disorders in which cytogenetic abnormalities are not available. If a mutation is predominant in a population, increased disequilibrium is observed for specific alleles of polymorphic markers close to the mutation site (Jorde 1995). Although the method is sensitive to population history and works better when applied to populations that have expanded from small founder communities (Jorde 1995), examples exist in the literature of the application of the same approach also to mixed populations (Kaplan et al. 1995). It is well known that HC is in linkage disequilibrium with HLA-A3 (Simon et al. 1976), and with several restriction fragment length polymorphisms in a restricted region surrounding HLA-A (Yaouanq et al. 1994). In addition, in

several populations, a strong disequilibrium has been reported with a specific allele of D6S105 (Jazwinska et al. 1993; Worwood et al. 1994, 1995), a microsatellite marker mapping 3 cM from HLA-A (Volz et al. 1994). D6S105 is estimated to map approximately 2 Mb from HLA class I genes by fluorescence in situ hybridization (Stone et al. 1994) and at least 3.1 Mb from HLA class I by mapping radiation hybrids (Volz et al. 1994). The HC candidate region is therefore thought to span at least 3 Mb, including HLA-A and the D6S105 marker (Camaschella and Gasparini 1994). The centromeric boundary is close to HLA-A (Yaouanq et al. 1994), telomeric to HLA-C (Radisky et al. 1994), but the telomeric limit is still undefined. Efforts to isolate the HC gene by screening duodenal mucosa (El Kahloun et al. 1993; Goei et al. 1994) or liver transcripts (Wei et al. 1993) have focussed on the region surrounding HLA class I genes, but they have been unsuccessful so far. This failure is not surprising, since only a portion of the candidate region, available cloned in yeast artificial chromosomes (YACs; Abderrahim et al. 1994) has been analyzed. Present efforts are aimed at reducing the size of the candidate region in order to search more effectively for the gene.

We have evaluated seven highly polymorphic markers, mapping in the region of HLA-A and within 4 cM from HLA-A on the telomeric side, in a large series of HC and normal chromosomes obtained from Italian patients and their families. The aim of the study was both to evaluate allelic association with HC in our population and to identify haplotypes associated with the disorder with the final goal of refining the gene location. A fluorescence-based gel electrophoresis sizing technique and a semi-automated analysis of microsatellites were employed; these proved to be fast, susceptible to multiplexing, and reliable in assessing genotypes of more than 200 subjects.

Materials and methods

Patients and families

Criteria for the diagnosis of HC included transferrin saturation of more than 62% in men and more than 50% in females, and increased serum ferritin in the absence of other known causes of iron overload. Only patients with a hepatic iron index greater than 2 at diagnosis or patients who had completed a course of venesections with removal of more than 5 g iron were enrolled in the study (Powell et al. 1994). Some cases were included in a previous report (Gasparini et al. 1993; Totaro et al. 1995). The study group consisted of 34 HC families of two to three generations including a total of 171 subjects and 47 patients. A heterozygous-homozygous mating occurred in two families. A total of 64 unaffected and 70 affected unrelated chromosomes were obtained from this series. Seventeen unrelated HC patients were also studied, providing an additional 34 affected chromosomes. Thus, the total number of affected chromosomes studied was 104. For a disease with an heterozygote incidence of 0.10, the most reliable way of calculating unaffected chromosomes is to obtain them from HC heterozygote relatives of patients by subtracting the HC chromosome (Simon et al. 1987; David et al. 1989).

All cases were serologically typed for HLA class I by standard methods. In the calculation of the HLA-A3 allele frequency, only the chromosomes derived from family analysis were used. Chromosomes from unrelated patients and from blood donors were not considered, since, in the absence of parental chromosomes, it is impossible correctly to assign HLA-A3 alleles.

Molecular studies

High molecular weight DNA was extracted from peripheral blood leukocytes using a previously described protocol (Poncz et al. 1982). Deoxyoligoprimers were synthesized on a 380B DNA Synthesizer (Applied Biosystems, Foster City, Calif.) by the phosphoramidite method on a 0.2-mmol scale with (2-0-cyano-ethyl)-phosphoramidites, and were then deprotected and used without further purification (Caruthers et al. 1987). Fluorochrome-labeled primers were prepared by chemically attaching a fluorescent dye to the 5′-end of each forward oligonucleotide primer by employing the fluorescent amidite reagent, 6-FAM amidite (Applied Biosystems). Alternatively, fluorescent-dye-tagged oligonucleotide synthesis was accomplished via post-synthesis coupling using a reactive aminohexyl linker group (Aminolink; Draper and Gold 1980). Following cleavage from the solid support and deprotection, the modified primer was then reacted with the N-hydroxyl succinimide ester derivative of the dye (Applied Biosystem 1991). The dye-labeled primer was then removed from excess reactants via high pressure liquid chromatographic purification. Names and primer sequences for detecting the D6S105 (Weber et al. 1991), HLA-F (EMBL databank accession no. X17093), D6S265, D6S306, D6S299, and D6S464 (Gyapay et al. 1994) alleles were as previously described (Weber et al. 1991; Gyapay et al. 1994).

Polymerase chain reaction (PCR) amplification was performed on an automated Thermal cycler (Perkin Elmer, Norvalk, Conn.) using conditions previously described for each microsatellite marker. Following PCR amplification, 5 µl of each sample were precipitated by the addition of 400 µl 100% ethanol. After centrifugation, dried pellets were resuspended in 5 ml deionized water. When multiplexing was performed at the gel-loading level, 1 µl of each sample was mixed prior to ethanol precipitation. Loading buffer, consisting of 2 ml ultrapure grade deionized formamide (Bethesda Research Laboratories, Gaithersburg, Md.), 0.5 ml 2 × agarose loading buffer [50 mg/ml Ficoll 400-DL, 1.7 mg/ml dextran sulfate, 8.3 mg/ml blue dextran, 2 × TBE (178 m*M* TRIS-borate, 4 mM EDTA) (Applied Biosystems)], and 0.5 µl Genescan 2500 Rox internal lane standards (Applied Biosystems), was then added and samples were denatured by heating at 100° C for 2 min followed by chilling on ice. The sequencing reactions that were used to size the various dye-tagged alleles were prepared by sequencing pGem3Z (Promega) with the -21 M13 Dye primer cycle sequencing kit (Applied Biosystems). Each sequencing reaction was precipitated, resuspended in 6 µl deionized formamide/50 m*M* EDTA (5:1), and heat-denatured; 3 µl were then loaded in gel lanes 1 and 24.

Electrophoresis

Acrylamide gel electrophoresis was performed in 8 *M* urea containing 1 × TBE with the length and acrylamide percentage changing depending on the resolution required. For all gels, the acrylamide/bisacrylamide (N,N′-methylene-bis-acrylamide) ratio was 19:1 (w/w) for a cross-linking concentration of 5% C. Reactions were analyzed either on 4.75% or 6% (w/v) gels with 24 cm well-to-read distances.

Y52 repeat analysis

Y52 is a complex repeat derived from YAC clone 225B1 (Totaro et al. 1994), which was shown to be strongly associated with HC in a preliminary sample of Italian HC chromosomes (Totaro et al. 1995). For this reason, it was re-evaluated in this larger series. Alleles of this repeat were analyzed as previously reported (Totaro et al. 1994).

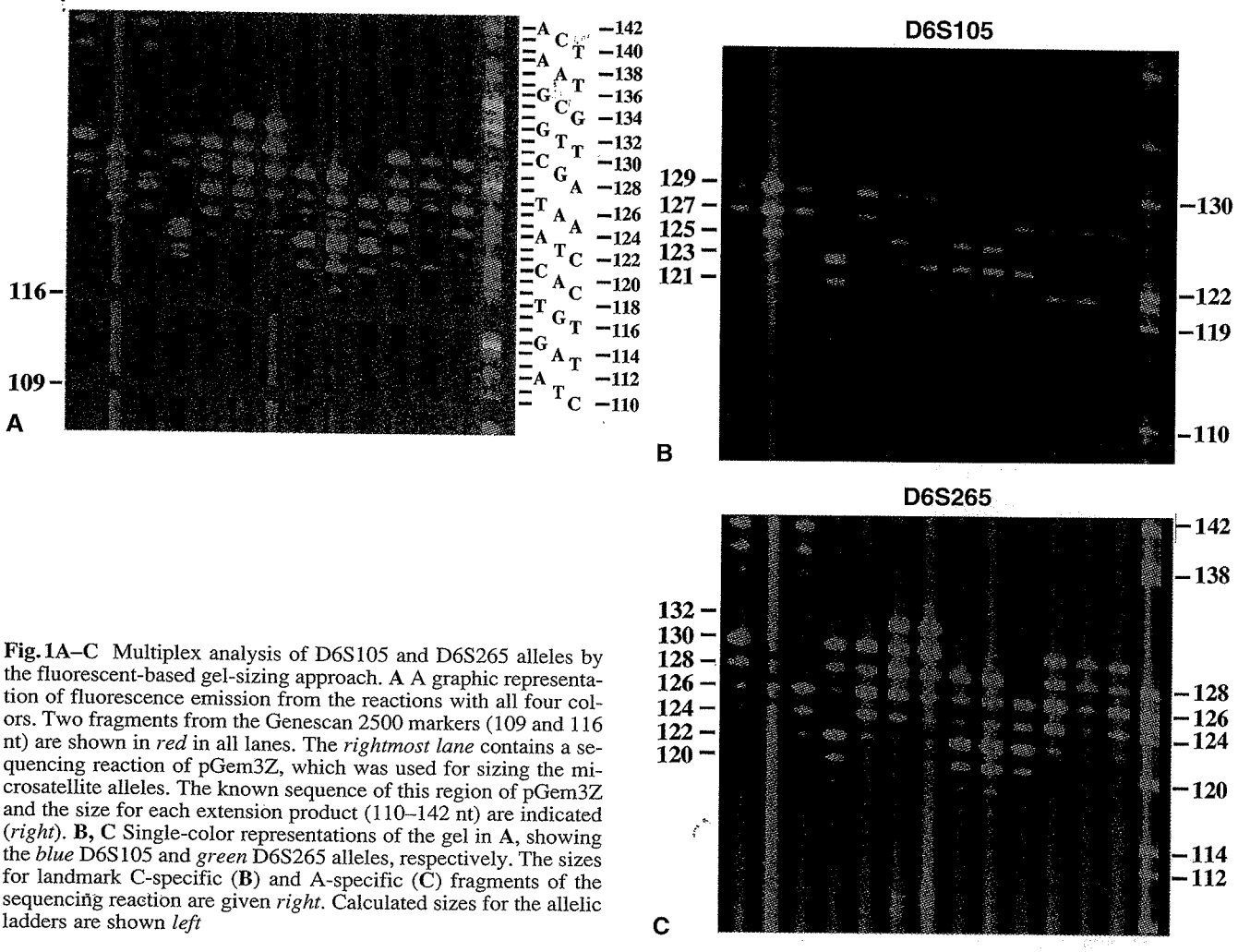

Fig. 1A–C Multiplex analysis of D6S105 and D6S265 alleles by the fluorescent-based gel-sizing approach. **A** A graphic representation of fluorescence emission from the reactions with all four colors. Two fragments from the Genescan 2500 markers (109 and 116 nt) are shown in *red* in all lanes. The *rightmost lane* contains a sequencing reaction of pGem3Z, which was used for sizing the microsatellite alleles. The known sequence of this region of pGem3Z and the size for each extension product (110–142 nt) are indicated (*right*). **B, C** Single-color representations of the gel in **A**, showing the *blue* D6S105 and *green* D6S265 alleles, respectively. The sizes for landmark C-specific (**B**) and A-specific (**C**) fragments of the sequencing reaction are given *right*. Calculated sizes for the allelic ladders are shown *left*

Data collection and analysis

Data was collected using the GeneScan Data Collection program version 1.1 and analyzed using GeneScan Data Analysis software version 1.1, 1.2 or 1.2.1b1 (Applied Biosystems). Whenever allele sizes calculated from the Genescan-2500 markers by the software were at variance with published results, fluorescent sequencing reactions from pGem3Z (Promega) were used to determine size.

HC haplotype definition

The study of inheritance of multiple microsatellite alleles in several affected and unaffected family members in large pedigrees allowed us unambigously to define haplotypes associated with normal and affected alleles. Haplotypes were defined by using a combination of the four markers (D6S265, HLA-A, Y52, and D6S105) most tightly linked to HC (see below).

Statistical analysis

Chi-square analysis for 2↔2 tables with Yates' correction was applied to evaluate the significance of the distribution of microsatellite alleles among normal and affected chromosomes. The delta disequilibrium coefficient (Δ) was obtained by using the following calculation:

$$\Delta = \frac{(ad)-(bc)}{\sqrt{(a+b)(c+d)(a+c)(b+d)}}$$

where a was the percentage of HC chromosomes with a specific allele, b was the percentage of the normal chromosomes with the same allele, and c and d represented the percentage of chromosomes without the specific allele in HC and non-HC, respectively. The odds ratio (o.r.) was used to approximate relative risk, as an additional method to measure the strength of allelic association according to Ott (1991).

YAC clones

YAC clones 225B1 and 421F7 were kindly supplied by Drs. D. Cohen and D. Le Paslier (CEPH, Paris). YAC clones 753H12, 20BF3, and 11HH2, containing inserts of the region surrounding the microsatellite D6S105, were courtesy of Drs. C. Sala and D. Toniolo (DIBIT-HSR and IGBE-CNR, Milan, Italy). The characterization of YAC clones 225B1 and 421F7 has been reported elsewhere (Abderrahim et al. 1994; Totaro et al. 1995), whereas the characterization of YACs 753H12, 20BF3, and 11HH2 is still in progress. The localization of all the analyzed microsatellites to particular YAC clones was established or confirmed by PCR.

Table 1 Alleles in linkage disequilibrium with HC in Italian patients (Δ Delta disequilibrium coefficient, *o.r.* odds ratio)

Marker	Allele size (bp)	χ^2	P	Δ	o.r.
D6S265 - allele 1	122	12.28	< 0.005	0.32	6.5
HLA-A3[a]		21.16	< 0.0001	0.43	18.04
Y52 – allele 1		17.94	< 0.0005	0.41	15.06
HLA-F allele 2	262	10.88	< 0.01	0.30	4.04
D6S306 – allele 4	238	13.23	< 0.005	0.35	5.07
D6S105 – allele 8	123	12.45	< 0.005	0.32	7.98
D6S464 – allele 4	210	7.64	< 0.05	0.29	3.20

[a] Data refer to A3 gene not to A3 antigen

Results

Six polymorphic markers were typed in 202 individuals using a fluorescent-based gel-sizing approach. Results for D6S105 and D6S265 microsatellite alleles are shown in Fig. 1. A sequencing reaction of the pGEM3Z extension products (right of Fig. 1A) and the length of these products was used to calibrate the sizes of individual alleles (E. Rappaport et al. in preparation).

A minimum of eight alleles was found for each microsatellite, except for HLA-F, which shows four alleles (Camaschella et al. 1993) and the three-allelic repeat Y52 (Totaro et al. 1994). One finding deserves mention: D6S105 (allele 8) in HC patients was strongly associated with HLA-A3. Of 34 HLA-A3 alleles, 29 were associated with D6S105 (allele 8). Otherwise, D6S105 (allele 8) was found in one out of seven HLA-A1 alleles and in one out of 21 HLA-A2 alleles.

Marker alleles showing significant association with HC, including HLA-A, are shown in Table 1. The highest degree of allelic association was seen for HLA-A3 (χ^2 = 21.16; $P < 0.0001$, o.r. = 18.04) and Y52 ($\chi^2 = 17.94$; $P <$ 0.0005; o.r. = 15.60). The association was slightly lower for D6S265 ($\chi^2 = 12.28$; $P < 0.005$; o.r. = 6.50), HLA F ($\chi^2 = 10.88$; $P < 0.01$; o.r. = 4.04), D6S306 ($\chi^2 = 13.23$; $P <$ 0.005; o.r. = 5.07) and D6S105 ($\chi^2 = 12.45$; $P < 0.005$; o.r. = 7.98). The level of association decreased at D6S464 ($\chi^2 = 7.64$; $P < 0.05$; o.r. = 3.20), whereas D6S299 showed no significant association ($\chi^2 = 4.15$; P = N.S.; o.r. = 3.30).

The physical location was known or obtained for all microsatellites studied: D6S265, HLA-F, and Y52 map close to HLA-A (Jazwinska et al. 1995, Worwood et al. 1995; Totaro et al. 1995). D6S265 is centromeric (Totaro et al. 1995; Jazwinska et al. 1995) and HLA-F telomeric to HLA-A (Campbell and Trowsdale 1993). We were unable to establish whether Y52 is telomeric or centromeric to HLA-A, since it was roughly mapped by PCR to the region of approximately 150 Kb overlapping YAC clones 225B1 and 421F7. D6S464 and D6S306 were localized, by PCR, close to and on opposite sites of D6S105 by using three YAC clones (753H12, 20BF3, 11HH2) forming a contig around D6S105 (data not shown). Sequences from D6S299 were not amplified using any of the YAC clones positive for D6S105.

Seventy affected and 63 unaffected haplotypes were unambigously assigned in suitable pedigrees. The distribution of haplotypes determined by using a combination of four markers among affected and unaffected chromosomes is shown in Table 2. Some 35 different haplotypes were identified for 70 HC chromosomes. However, a single haplotype (D6S265-1, HLA-A3, Y52-1, D6S105-8) was present in 30% of the HC chromosomes. The same

Table 2 Haplotypes for D6S265, HLA-A, Y52, and D6S105 among 70 HC (*HC*) and 63 normal (*N*) chromosomes. Allele numbers refer to published sizes (see Jazwinska et al. 1995 for D6S265 and D6S105; Totaro et al. 1994 for Y52.)

	No. of chromosomes			No. of chromosomes	
	HC	N		HC	N
1– 3– 1– 8	23	–	10–19– 2– 5	1	–
1– 3– 1– 7	1	–	6–19– 2– 4	1	–
1– 3– 1– 4	1	–	3–19– 2–11	1	2
1– 3– 1– 3	1	–	3–19– 2– 9	1	–
3– 3– 2– 8	1	–	3–19– 2– 5	1	–
3– 3– 2– 5	2	1	1– 3– 1– 5	–	1
6– 2– 2– 7	2	–	6– 2– 2–10	–	1
6– 2– 2– 6	2	4	6– 2– 2– 9	–	1
6– 2– 2– 5	2	1	5– 2– 2– 2	–	1
6– 2– 2– 4	1	–	3– 2– 2– 5	–	2
5– 2– 2– 9	2	1	1– 2– 1–11	–	1
5– 2– 2– 8	1	1	6– 1– 2–X[a]	–	1
5– 2– 2– 7	1	–	3– 1– 2– 7	–	2
5– 2– 2– 6	4	4	1– 1– 2– 8	–	1
5– 2– 2– 5	1	2	1– 1– 2– 4	–	1
5– 2– 2– 4	1	–	5– 9– 2– 6	–	1
5– 2– 2– 3	1	–	4– 9– 2– 5	–	1
4– 2– 2–12	1	–	3– 9– 2– 9	–	1
4– 2– 2– 7	1	–	3– 9– 2– 4	–	1
3– 2– 2– 7	1	2	4–11– 2– 6	–	2
3– 1– 2– 9	1	1	7–19– – 7	–	1
3– 1– 2– 8	1	1	6–19– 2– 5	–	1
3– 1– 2– 6	2	2	1–19– 2– 5	–	1
3– 1– 2– 5	2	1	5–10– 2– 7	–	1
3– 1– 2– 4	1	1	4–10– 2– 7	–	1
3– 9– 2– 6	5	3	4–10– 2– 6	–	3
3– 9– 2– 5	2	2	4–10– 2– 3	–	1
4–11– 2– 9	1	1	6–21– 3– 3	–	1
4–11– 2– 7	3	2	6–28– 3– 6	–	1
4–11– 2– 5	1	2			

[a] X = 140 bp

haplotype was not found in 63 normal chromosomes segregating in the HC families. No other haplotype was found exclusively associated with HC. Forty-three different haplotypes were discerned in normals, the majority not represented in disease chromosomes.

Discussion

Mapping studies have been greatly facilitated by semi-automated fluorescence-based microsatellite analysis (Reed et al. 1994). Among the advantages of this approach are the ability to analyze multiple markers in the same lane, using both size and color differences to increase data throughout a single gel. In addition, the availability of in-lane molecular weight standards provides the basis for semi-automated allele sizing; this is highly relevant to allelic association studies.

Allelic association mapping has been successfully applied to gene localization, especially in cases where recombination rates between disease genes and markers is too small to be of practical use. Integration of genetic and physical maps is important for the aim of localizing genes. The physical location is roughly known for all the markers that we have examined. In our study, HLA-A and Y52 show the highest degree of association with HC, confirming previous results in Italian patients (Gasparini et al. 1993; Totaro et al. 1995). This indicates that they are the closest markers to the HC mutation. Nevertheless, the persistent association with markers in the region of D6S105 (Table 1) and the results obtained in other populations complicate this simple interpretation. High degrees of allelic association extending from HLA-A to D6S105 (with a peak at this marker) have recently been found in Australia (Jazwinska et al. 1993, 1995). High degrees of disequilibrium with D6S105 are also known for patients from Wales (Worwood et al. 1994) and Brittany (Worwood et al. 1995). The HC gene is thought to have been introduced into northern Europeans by Celtic colonization. Brittany and Wales were Celtic settlements in ancient times and people included in the Australian study were of Irish/Scottish ancestry (Jazwinska et al. 1993). One of the problems encountered in the application of the disequilibrium analysis to the Italian population is its complex genetic history. The heterogeneous genetic background of the Italian population (Piazza et al. 1988) may be partially responsible for the results obtained, especially for the lower association with HC observed for D6S105, compared with other studies. Although Celtic colonization is historically established in some Italian regions, and a Celtic component is certainly included in our sample (see below), molecular differences between southern (Mediterraneans) and northern Europeans are well known for HLA haplotypes and other genetic disorders (Estivill et al. 1988; Dianzani et al. 1994). Complex patterns of linkage disequilibrium have been observed in other genetic disorders (MacDonald et al. 1991) and may preclude the precise localization of a gene.

Our analysis shows that a common haplotype (D6S265-1, HLA-A3, Y52-1, and D6S105-8) accounts for 30% of the affected chromosomes and is absent in normals. It shares the same HLA-A, D6S265, HLA F, and D6S105 alleles with the most frequent haplotype reported in Australian patients of Irish/Scottish origin (Jazwinska et al. 1995) and in approximately 50% of HC chromosomes in Wales (Worwood et al. 1995) and Brittany (Yaouanq et al. 1994). The finding of an identical chromosomal background that has been maintained for centuries and that is associated with HC in different populations strenghens the hypothesis that it is the ancestral "Celtic" haplotype carrying an ancient HC mutation (Jazwinska et al. 1995). The other haplotypes common among HC chromosomes are also common among normals. It is unlikely that they resulted from the spread of the same mutation by multiple crossover events, since they differ from the main haplotypes at all the analyzed sites. We favor the hypothesis that these different haplotypes contain different mutations.

The finding of an ancestral haplotype in several populations offers a tool for mapping the gene, as shown in other genetic disorders (MacDonald et al. 1992; Bowcock et al. 1994; Ramsay et al. 1993). This type of analysis, when preliminarily applied to Australian patients, localizes the gene in the large region between HLA-F and D6S105, since specific alleles of these two markers are present in almost 50% of the HC chromosomes. In our samples, we cannot draw the same conclusion, since D6S105 allele 8 and HLAF allele 2 are restricted to the ancestral haplotype. We have found that D6S299 does not show allelic association to HC; in addition, several different alleles of this marker are associated with the ancestral haplotype, confirming that it is outside the candidate region. The other HC haplotypes are extremely heterogeneous, suggesting that multiple mutations might be responsible for HC in Italy. The combination of different mutations in patients carrying different haplotypes is probably responsible for the heterogeneous clinical presentation and for the variable degrees of iron overload observed. Preliminary data (A. Piperno et al. in preparation) obtained in Italian HC patients suggest that the presence of the ancestral haplotype is associated with the most severe phenotype in terms of iron overload.

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