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Association between the MHC class I gene HFE polymorphisms and longevity: a study in Sicilian population

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Classes I and II human leukocyte antigens (HLA) genes encode highly polymorphic heterodimeric glycoproteins involved in the control of immune responses. The HLA class I gene HFE seemingly no longer participates in immunity because it has lost its ability to bind peptides and it has acquired the ability to form complex with the receptor for iron-binding transferrin by regulating iron uptake by intestinal cells. Thus, it indirectly regulates immune responses too, because iron availability plays a role in specific and non-specific immune responses. The distribution of HFE polymorphisms in Sicilian centenarians and nonagenarians was studied to evaluate if HFE alleles might be represented differently in people selected for longevity. DNA samples were obtained from 106 young controls (age range from 22 to 55 years; 40 men and 66 women) and 35 elderly subjects (age range from 91 to 105 years; seven men and 28 women). Samples were typed for C282Y, H63D and S65C alleles using polymerase chain reaction and sequence specific primers. Among the young individuals, none was heterozygous for the C282Y or for S65C mutation. Twenty-six were heterozygous for H63D mutation. Among the elderly subjects, 11 were heterozygous for the C282Y mutation or for H63D mutation. None was heterozygous for the S65C mutation. No compound heterozygous individuals (C282Y/H63D) were found. A highly significant difference was observed in frequencies of C282Y alleles between the young and the elderly subjects on the whole. By analysing polymorphisms according to gender, heterozygous subjects for C282Y were found both in old men and in old women, but by comparing the allele frequencies to those of young people significance was attained only in women. Concerning H63D polymorphisms, no significant differences were observed, between old and young people, both in men and in women. Possession of C282Y allele, known to be associated with an increase of iron uptake, significantly increases women possibility to reach longevity. Thus, present data adds another piece of evidence to the complex puzzle of genetic and environmental factors involved in control of lifespan expectancy in humans.
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Introduction

The human leukocyte antigens (HLA) complex contains over 200 genes divided into three classes. Most HLA genes involved in the immune response fall into classes I and II, which encode highly polymorphic heterodimeric glycoproteins. These molecules take up antigenic peptides intracellularly and emerge on the cell surface where processed peptides are presented to T cells, regulating T cell responses against specific antigens.[1,2] HFE, the most telomeric HLA class I gene, codes for a class I α chain, which seemingly no longer participates in immunity, because it has lost its ability to bind peptides owing to a definitive closure of the antigen binding cleft that prevents peptide binding and presentation. The HFE protein, expressed in crypt enterocytes of the duodenum, regulates the iron uptake by intestinal cells because it has acquired the ability to form complex with the receptor for iron-binding transferrin.[3–6] Thus, it indirectly regulates immune responses, because iron availability plays a role in specific and non-specific immune responses. In fact, iron deficiency may be associated with reversible abnormalities of immune function, although it is difficult to demonstrate the severity and relevance of these in observational studies.[6–8]

The C282Y mutation (a cysteine to tyrosine mutation at amino acid 282) in this gene has been identified as the

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main genetic basis of hereditary haemochromatosis (HH). It destroys its ability to make up a heterodimer with β2-microglobulin. The defective protein fails to associate with the transferrin receptor and the complex cannot be transported to the surface of the duodenal crypt cells. As a consequence, in homozygous people two to three times the normal amount of iron is absorbed from food by the intestine.[3,4,6,9] The disease is characterised by a progressive storage of iron in organs such as the liver, pancreas, pituitary gland and heart that results in end-organ damage and reduced life expectancy.[10] Two other mutations, H63D (a histidine to aspartate at amino acid 63) and S65C (a serine to cysteine at amino acid 65), appear to be associated with milder forms of HH. In fact, a small number of patients with HH were identified as homozygous for H63D or as compound heterozygous H63D/C282Y and enrichment of S65C among HH chromosomes has been reported.[6,9,11,12]

Several studies have shown a high prevalence of the C282Y mutation in northern European populations, whereas in those of the Mediterranean basin the prevalence seems low and almost absent in Far-East countries.[13–24] Besides, it usually occurs on the ancestral haplotype 7.1.[4,12,20,25] Thus, it has been suggested that an estimated 60 to 70 generations ago, this mutation occurred in the HFE gene of a Celtic individual who is the ancestor of more than 5% of white subjects now carrying the allele.[4,26] In contrast, the H63D substitution is not restricted to European populations, being found at allele frequencies of more than 5% in countries bordering the Mediterranean, in the Middle East, and in the Indian subcontinent, suggesting that the H63D substitution must have occurred earlier than the C282Y substitution.[12,13,19,27] Accordingly, it does not occur on such a large ancestral haplotype as C282Y.[12,27] A few studies have been performed on the distribution of the S65C mutation.[11,13,28]

It has been claimed that the great expansion of Celtic people can be in part explained by the widespread presence of this HFE gene mutation. This gave to heterozygous carriers selective advantages on the basis of improved survival during infancy, childhood, and pregnancy, by leading to increased iron absorption and accumulation of larger body iron stores because ancient diet consisted mainly of iron-poor grains and cereals, whereas meat was highly uncommon.[20] On the basis of this suggestion on the evolutionary significance of these mutations, we have evaluated their frequencies in a sample of nonagenarian and centenarian Sicilians. Our hypothesis was to find an increase of elderly people heterozygous for the mutations involved in iron sparing.

Results

Among the young individuals, none was heterozygous for the C282Y or for S65C mutation. Twenty-six were heterozygous for H63D mutation. Among the elderly people, 11 were heterozygous for the C282Y mutation or for H63D mutation. None was heterozygous for the S65C mutation. No compound heterozygous individuals (C282Y/H63D) were found. A highly significant difference ($P = 7.2 \times 10^{-7}$ by Fisher exact test) was observed in frequencies of C282Y alleles between young and oldest old subjects on the whole (Table 1).

Table 2 reports on allele frequencies for C282Y polymorphisms analysed according to gender. Heterozygous subjects for C282Y were found both in elderly men ($n = 2$) and in elderly women ($n = 9$), but by comparing the allele frequencies with those of young people significance ($P = 7.2 \times 10^{-7}$ by Fisher exact test) was attained only in women. However, it has to be taken into account that the small number of elderly men in the sample might not allow the identification of significant differences.

Table 3 reports on allele frequencies for H63D polymorphisms analysed according to gender. No significant differences were observed, between the elderly and young people, both in men and in women.

Finally, in 20 young and in 20 elderly subjects we evaluated other less common HFE mutations (V53M, V59M, H63H, Q127H, E168Q, E168X, W169X and Q283P)[29] and the recently described haemochromatosis related transferrin receptor 2 (TFR2) Y250X mutation,[30] but none was found heterozygous (data not shown).

Table 1 Allele frequencies for HFE polymorphisms in 106 young (<55 years) and 35 elderly (>90 years) Sicilians

	*C282Y**		*H63D*		*S65C*	
	Wild type	*Allele*	*Wild type*	*Allele*	*Wild type*	*Allele*
<55 years	212 (100%)	0 (0%)	186 (88%)	26 (12%)	212 (100%)	0 (0%)
>90 years	59 (84%)	11 (16%)	59 (84%)	11 (16%)	70 (100%)	0 (0%)

*$P = 7.2 \times 10^{-7}$ by Fisher exact test.

Table 2 Allele frequencies for C282Y HFE polymorphisms in 106 young (<55 years) and 35 elderly (>90 years) Sicilians analysed according to gender

	Men		*Women**	
	Wild type	*Allele*	*Wild type*	*Allele*
<55 years	80 (100%)	0 (0%)	132 (100%)	0 (0%)
>90 years	12 (86%)	2 (14%)	47 (84%)	9 (16%)

*$P = 8.3 \times 10^{-5}$ by Fisher exact test.

Table 3 Allele frequencies for H63D HFE polymorphisms in 106 young (<55 years) and 35 elderly (>90 years) Sicilians analysed according to gender

	Men		*Women*	
	Wild type	*Allele*	*Wild type*	*Allele*
<55 years	64 (80%)	16 (20%)	122 (92%)	10 (8%)
>90 years	14 (100%)	0 (0%)	45 (80%)	11 (20%)

Discussion

Our work shows that women carriers of C282Y mutation are at a higher frequency among the elderly compared with control women. These data suggests that this polymorphism plays a role in determining lifespan, at least in the Sicilian generation under study (see below).

In Italy, many authors have reported the prevalence of the HFE mutation pattern of high-risk groups and of general population, showing that the HFE gene mutations are more frequent in north than in south Italy.[13,20,24,31,32] However, in our study, in young controls, the prevalence of C282Y (and of S65C) gene mutations was surprisingly low. On the other hand, the frequencies observed by ourselves are in agreement with those recently reported in a large sample of the population from southern Italy (0.15%, 18.6% and 0.15%, for C282Y, H63D and S65C respectively),[13] being the C282Y mutation the lowest yet reported for a population of European origin. Incidentally, in our population it seems to have little, if any diagnostic relevance (our unpublished observations). Similar results have been obtained in Greece.[19]

Our results show an involvement of C282Y mutation in successful ageing. It is intriguing that HFE mutations have been suggested to be involved in unsuccessful ageing too. In fact, in Alzheimer disease (AD) patients carrying the H63D allele had a mean age at onset of 72 years *vs* 77 years of those who were homozygous for the wild-type allele. Thus, it seems that H63D mutations may anticipate sporadic late-onset AD clinical presentation in susceptible individuals.[33] In another study, in patients with familial AD (FAD) C282Y and H63D mutations were over-represented in men and under-represented in women with FAD.[34] Thus, the possibility that HFE mutations are important new genetic risk factors for AD should be pursued further.

Regarding the biological significance of HFE heterozygous status, this phenotype was recently defined.[35,36] The mean serum iron concentrations, ferritin levels and transferrin-saturation values were higher in heterozygous subjects than in normal ones, as were mean haemoglobin levels and mean corpuscular volume. The prevalence of iron deficiency anaemia was lower in women who carried HFE mutations. Concerning then the significance of iron in immune responses, a delicate balance exists inside the host cell. Too much iron down-regulates microbicidal effector mechanisms and favours the growth of the pathogen, whereas too little iron is inhibitory to the induction of antimicrobial processes.[37] However, it is undeniable the importance of iron in the regulation of immune responses.[7,8]

The role of genetics in determining lifespan is complex. On the basis of results obtained from the population-based Scandinavian Twin Registries,[38,39] a maximum of around one-third of the variance of 'longevity' is attributable to genetics. However, the real contribution of the genetics to human longevity has not yet been sufficiently addressed. Therefore, the role of genetics in longevity might be much higher than predicted.[2]

As pointed out by Shachter *et al*[40] two strategies can be exploited to identify genes that influence human lifespan, ie sibling pair analysis or case-control studies. In a case-control study, allele and genotype frequencies at polymorphic marker loci (for instance HFE gene) are compared between a long-lived group and a control group of randomly selected adults. Case-control studies might be subjected to a number of possible confounding factors, the total number of patients (elderly people in our study) and controls and the homogeneity of the population in terms of geographical origin among others. Although based on a relatively reduced number of controls, as reported above, our frequencies are in agreement with those obtained in southern Italy, suggesting the absence of population biases. In fact, both the elderly and young people were Sicilians, and the Sicilian population is a very homogeneous population because the last genetic admixtures there were several centuries ago, at the time of Arabian conquest.[41]

Several findings point out that gender is a major variable in the genetics of longevity and suggest as a working hypothesis that men and women follow different strategies to reach longevity.[42–46] Present data indicates that C282Y allele distribution is different in elderly women in respect to controls, but not in elderly men. Even if the small number of sample elderly males might not allow the identification of significant differences, these data seem to suggest that possession of C282Y allele, known to be associated with an increase of iron uptake,[3,4,6,9] significantly increases only in women the possibility to reach longevity. In this respect, it has to be remembered that for the generation of elderly people under study lifestyle, including diet, was quite different for men and women. For instance, in Sicily, like in other pre-industrial countries, a lot of pregnancies (and abortions) were yet the rule for the women born at the beginning of the last century and the their diet consisted mainly in iron-poor grains, vegetables and fruits (if any), whereas meat was highly uncommon and in any case put aside for men and children.[41,47] Thus, it is to be taken into account that gene variants representing a genetic advantage for one gender might not be automatically relevant for the other gender in terms of successful or unsuccessful ageing.

In conclusion, present data showing the relevance of C282Y for womens survival to late age, adds another piece of evidence to the complex puzzle of genetic and environmental factors involved in control of lifespan expectancy in humans.

Materials and methods

Subjects

One hundred forty-one unrelated healthy Sicilians randomly selected were studied: 106 young controls (age range from 22 to 55 years; 40 men and 66 women) and 35 elderly subjects (age range from 91 to 105 years; seven men and 28 women). Reported age was verified by researching archival records in the City Hall and/or Church registries, paying attention to the concordance between reported age and personal chronologies (age of marriage, age of military service for males, age of first and last pregnancy for women, age of children, among others). Written informed consent for enrolling in the study and for personal data management had been previously obtained from all the subjects according to Italian laws. Blood specimens were collected in tripotassium EDTA sterile tubes, immediately stored at –70°C and in a further time processed for HFE genotyping. Genomic DNA extraction was carried out according to Miller *et al*[48] and stored at –20°C for the HFE gene analysis.

Analysis of HFE gene mutations

Samples were typed for C282Y, H63D and S65C alleles using polymerase chain reaction and sequence specific primers listed in Table 4.

C282Y mutation was typed according to Takeuchi *et al*[49] using a multiple PCR protocol. Briefly 20 μl reaction mixtures containing 50 ng of template DNA, 0.5 U TaqGold-DNA polymerase (PE BioSystem, Milan, Italy), and a final concentration of 200 mM each deoxynucleotide and 1 × reaction buffer (PE Biosystem), 1 mM of F1, R1 and F2M primers and 0.2 mM of R2W primer. Cycling was performed at 96°C for 7 min and 35 cycles at 95°C for 30 s, 64°C for 30 s and 72°C for 30 s, followed by a final extension of 10 min at 72°C. PCR products were detected by electrophoresis on 2% agarose, obtaining a 521 bp product and a 257 and/or 136 bp bands from mutated or wild HFE Y282C sequences. H63D alleles were typed with the amplification refractory mutation system/polymerase chain reaction (ARMS/PCR) using two separated primer couples (F3/R3 and F4/R3)[50] in the following conditions. 50 ng of template DNA were mixed in a final volume of 20 μl with 0.5 U TaqGold-DNA polymerase (PE BioSystem), 200 mM each deoxynucleotide and 1 × reaction buffer and 0.5 mM of each specific primer. Cycling was performed at 96°C for 10 min and 35 cycles at 95°C for 30 s, 64°C for 30 s and 72°C for 30 s, followed by a final extension of 10 min at 72°C. PCR products were evaluated by electrophoresis on 2% agarose. Detection of a 178 bp PCR product in one or both electrophoresis lanes allows to identify homozygous or heterozygous subjects for H63D genotypes. S65C specificities were evaluated after H63D genotyping using the same ARMS/PCR protocol and appropriated primer couples. 63H homozygous subjects were typed using F5/R3 and F6/R3 primer mixes whereas 63D subjects were typed using F7/R3 and F8/R3. All the four primer mixes were used to type H63D heterozygous subjects.

To validate our results and evaluate the presence of other less common HFE mutation and of the recently described haemochromatosis related TFR2 Y250X mutation[30] a commercially purchased Reverse-Hybridisation assay was used (Haemochromatosis Kit, Nuclear Laser Medicine, Settala Milanese, Italy). This method, originally described by Oberkanins *et al*[29] enables us to type C282Y, H63D and S65C HFE mutations together with V53M, V59M, H63H, Q127H, E168Q, E168X, W169X and Q283P. Moreover the kit allows to evaluate Y250X mutation at TFR2 gene sequence. The test was performed according to the manufacturers instructions. Briefly, multiplex PCR products (cycling was performed at 94°C for 2 min and 30 cycles at 94°C for 15 s, 58°C for 30 s and 72°C for 30 s, followed by a final extension of 3 min at 72°C) obtained using biotinylated primers were hybridised to aminomodified oligonucleotides immobilized on a nylon membrane able to hybridise normal or mutated HFE and TFR2 sequences. Strips containing oligoprobes were hybridised with 10 μl amplified DNA denatured in an equal volume of NaOH 1N for 5 min and then incubated in an adequate volume of hybridisation solution (SSC 6× 0.1% SDS) in a shaking bath at 45°C for 30 min. After two washes in hybridisation solution at room temperature, stringent wash was performed at 45°C for 30 min in prewarmed SSC 6× 0.1% SDS. After two washes in SSC 1× 0.1% SDS, membranes were incubated with the appropriate alkaline phospatase conjugated streptavidin dilution for 15 min at room temperature. After two other washes, bound PCR fragments were detected using colour substrate (NBT/BCIP) development.

Table 4 Details of reaction mixtures used for HFE genotyping

Primer No.	*Sense/ antisense*	*Primer sequence*	*HFE nucleotide annealing position*
F1	S	AAGCAGCCAATGGATGCCAAG	948–968
F2M	S	GGGAAGAGCAGAGATATACGTA	1045–1066
F3	S	AGCTGTTCGTGTTCTATGATC	388–408
F4	S	AGCTGTTCGTGTTCTATGATG	388–408
F5	S	TTCGTGTTCTATGATCATGAGA	393–414
F6	S	TTCGTGTTCTATGATCATGAGT	393–414
F7	S	TTCGTGTTCTATGATGATGAGA	393–414
F8	S	TTCGTGTTCTATGATGATGAGT	393–414
R1	AS	CCACTGATGACTACTCCAATGACTA	1135–1156
R2W	AS	CCTGGGTGCTCCACCTGGC	1066–1085
R3	AS	CTGTGGTTGTGATTTTCCATAA	535–566

Specificity of the primers is determined by the underlined 3′ nucleotide. Primers F1, R1 and R3 are consensus non-allele specific primers. References in the text. Nucleotides are numbered according to Feder *et al*[3] (Genbank accession number U60319).

Statistical analysis

Allele frequencies were evaluated by gene count and 2 × 2 tables were constructed to determine statistical significance (Fisher exact test) of differences in allele frequency for the HFE polymorphisms between oldest old and controls. According to Bonferroni, obtained *P* values were corrected by multiplying for the number of alleles under study, ie 6.

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