Universidade de Aveiro Departamento de Biologia
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HLA AND HEMOCHROMATOSIS DISEASE ASSOCIATION IN SÃO MIGUEL ISLAND

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HLA AND HEMOCHROMATOSIS DISEASE ASSOCIATION IN SÃO MIGUEL ISLAND

dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Luísa Maria Quental Mota Vieira, Investigadora principal da Unidade de Genética e Patologia Moleculares do Hospital do Divino Espírito Santo de Ponta Delgada, EPE.

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o júri

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palavras-chave HLA, *HFE*, Mutação C282Y, Hemocromatose hereditária, Haplótipo, Açores

resumo

A hemocromatose hereditária uma doença autossómica recessiva do metabolismo do ferro, geralmente associada à mutação C282Y no gene *HFE*. Presume-se que a origem desta mutação tenha ocorrido por acaso no haplótipo HLA-A*03-B*07 de um indivíduo do noroeste da Europa. O presente trabalho visou caracterizar a associação entre os alelos e haplótipos dos *loci* HLA-A e -B com a mutação C282Y na população da ilha de São Miguel (Açores). Este estudo englobou 130 indivíduos, negativos para as mutações *HFE* H63D e S65C, que foram classificados em dois grupos: grupo C282Y (48 homozigóticos ou portadores da C282Y) e grupo controlo (82 dadores de sangue sem as três mutações no gene *HFE*). Para todos os indivíduos, foi efectuado a genotipagem HLA-A e -B por PCR-SSP e a detecção das mutações *HFE* por PCR-RFLP. A análise estatística revelou que quatro alelos – A*03 (p=0.003, OR=3.33), A*26 (p=0.003, OR=8.38), A*29 (p<0.001, OR=19.18) e B*45 (p=0.003, OR=8.37) – encontram-se significativamente aumentados no grupo C282Y. Os resultados demonstram, igualmente, uma associação significativa com a mutação C282Y para o haplótipo ancestral HLA-A*03-B*07 (p=0.006, OR=8.96) e dois haplótipos não ancestrais: A*02-B*58 (p<0.001, OR=19.78) e A*29-B*45 (p<0.001, OR=27.57). Além disso, outro haplótipo A*24-B*15 foi detectado por inferência directa num doente homozigótico para o HLA-A-B e para a mutação C282Y. Provavelmente, o mecanismo genético de recombinação gerou esta diversidade de haplótipos; no entanto, não se pode excluir a hipótese de uma mutação C282Y *de novo* no gene *HFE* associada ao haplótipo HLA-A*24-B*15. Em conclusão, além do haplótipo ancestral A*03-B*07, três novos haplótipos – A*02-B*58, A*24-B*15 e A*29-B*45 – sugerem estar associados à mutação C282Y na população da ilha de São Miguel. A elevada diversidade genética observada na população açoriana pode explicar a associação entre a mutação C282Y e os haplótipos HLA.

keywords HLA, *HFE*, C282Y mutation, Hereditary hemochromatosis, Haplotype, Azores

abstract

Hereditary hemochromatosis is an autosomal recessive disease of the iron metabolism, where *HFE* C282Y is commonly implicated. This mutation seems to have originated by chance on the HLA-A*03-B*07 haplotype in a northwestern European individual, and spread by migration. Given that recombination generates new haplotypes, the present investigation aimed to characterize the chromosomal background of C282Y in the São Miguel Island population (Azores). This study comprises 130 individuals, all negative for H63D and S65C, which were classified into two groups: 48 homozygous or carriers for C282Y, and 82 healthy individuals without these mutations. The subjects were HLA-A and -B genotyped by PCR-SSP, and *HFE* mutation detection was performed by PCR-RFLP. Statistical analysis revealed that four alleles – A*03 (p=0.003, OR=3.33), A*26 (p=0.003, OR=8.38), A*29 (p<0.001, OR=19.18) and B*45 (p=0.003 OR=8.37) – and the A*03-B*07 haplotype (p=0.006, OR=8.96) were significantly increased in the C282Y group. Two non-ancestral haplotypes were also significantly associated with C282Y: A*02-B*58 (p<0.001, OR=19.78) and A*29-B*45 (p<0.001, OR=27.57). This last haplotype showed the strongest association to the mutation in study, suggesting that it may be the principal hemochromatosis-haplotype in São Miguel Island population. Another haplotype – A*24-B*15 – was detected by direct inference in a C282Y and HLA-A-B homozygous patient. Recombination most probably generated these haplotypes, before or after the island settlement. However, we can not exclude the hypothesis of a recent *de novo* *HFE* C282Y mutation on the A*24-B*15 haplotype in an individual living in the São Miguel Island. Overall, in this population, besides the ancestral A*03-B*07, three new non-ancestral haplotypes – A*02-B*58, A*24-B*15 and A*29-B*45 – appear to be associated with C282Y. The association between this recessive mutation and these haplotypes undoubtedly reflects the high genetic diversity observed in the Azoreans.

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Abbreviations

A	Adenine
Asp	Aspartic acid
bp	Base pairs
C	Cytosine
ºC	Degrees Celsius
C282Y	Cys 282 Tyr
CI	Confidence interval
Cys	Cysteine
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphospate
G	Guanine
H63D	His 63 Asp
HFE	Hemochromatosis gene
HH	Hereditary hemochromatosis
His	Histidine
HLA	Human leukocyte antigen
$MgCl_2$	Magnesium chloride
min	Minutes
mL	Millilitres
mM	Millimolar
ng	Nanograms
OMIM	Online mendelian inheritance in man
OR	Odds ratio
PCR	Polymerase chain reaction
sec	Seconds
Ser	Serine
SSP	Specific-sequence primer
S65C	Ser 65 Cys
Tyr	Tyrosine
U	Units
µL	Microlitres
µM	Micromolar
%	Percentage

1. INTRODUCTION

Hereditary hemochromatosis (HH, OMIM 235200, *HFE* gene) is an autosomal recessive disease of the iron metabolism, common in northern European populations with a prevalence of 1 in 200-300 individuals (Adams *et al*., 2000; Camaschella *et al*., 2000). This disease is characterized by increased iron absorption and deposition in liver, pancreas, heart, joints and pituitary glands. If undiagnosed or untreated, death may occur due to cirrhosis, primary liver cancer, diabetes or cardiomyopathy. Early diagnosis and treatment can prevent the development of these clinical complications, allowing HH patients to have a normal life expectancy (Pietrangelo, 2006; Wheeler *et al*., 2006; Beutler, 2007). The *HFE* gene encodes an HLA-A class 1-like protein and is located on 6p21.3, 4 megabases (Mb) telomeric to the human leukocyte antigen region (HLA). Two *HFE* mutations – C282Y (845 G→A) and H63D (187 C→G) – are significantly correlated with HH. The majority (60% to 90%) of clinically diagnosed probands are homozygous for C282Y, and 5% are compound heterozygous for C282Y and H63D. In terms of molecular pathology, the C282Y is the most severe mutation, as the mutated protein is unable to bind to the β_2-microglobulin and, subsequently, its structure and function are affected (Feder *et al*., 1996).

The C282Y mutation seems to have originated by chance, before 4000 BC, on a HLA-A*03-B*07 haplotype in an individual from northwestern Europe, and early spread by Celts or Vikings (Simon *et al*., 1980; Milman & Pederson, 2003; Distante *et al*., 2004). By recombination and admixture, this mutation has been also found in non-ancestral HLA haplotypes, which were reported with significantly increased frequencies in Europe and European descent hemochromatosis patients (Simon *et al*., 1987; Porto & de Sousa, 2000; Yaouanq, 2000; Barton & Acton, 2002).

The aim of the present study was to determine the frequencies of HLA-A and -B alleles and haplotypes associated with the C282Y mutation, in order to characterize the chromosomal background of this recessive mutation in the São Miguel Island population (Azores). This population is composed, to a great extent,

of mainland Portuguese descent with significant contributions of Jews, Africans and Europeans (Pacheco *et al*., 2005; Spínola *et al*., 2005; Branco *et al*., 2006; Branco *et al*., 2008; Branco *et al*., 2008).

2. MATERIAL AND METHODS

2.1. Population sample

The population sample, composed of 130 individuals from the São Miguel Island (Azores, Portugal), were divided into two groups, as follows: the C282Y mutated group with 36 heterozygous and 12 homozygous identified on medical care basis, and the control group of 82 apparently healthy blood donors (Mota-Vieira *et al*., 2005). All individuals were negative for the H63D and S65C mutations.

2.2. DNA extraction and *HFE* mutation analysis

Peripheral blood samples (7.5 mL) were collected by venipuncture into EDTA tubes and genomic DNA was extracted using the PUREGENE® kit (Gentra systems Inc.) from iron overload patients with presumptive clinical diagnosis of hemochromatosis.

The C282Y, H63D and S65C mutation detection was carried out by polymerase chain reaction (PCR), as previously described (Feder *et al*., 1996), and followed by specific restriction enzyme. The primer sequences used were: 5´-TGG CAA GGG TAA ACA GAT CC-3´ (forward) and 5´-CTC AGG CAC TCC TCT CAA CC-3´ (reverse) for the C282Y (exon 4), and 5´-ACA TGG TTA AGG CCT GTT GC-3´ (forward) and 5´-GCC ACA TCT GGC TTG AAA TT-3´ (reverse) for the H63D and S65C (exon 2). The PCR amplifications were performed in a 20 µL reaction mixture including 1 x PCR buffer, 1 x Q-solution, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 1.0 µM of forward and reverse amplification primers, 0.60 U of HotStarTaq DNA polymerase (QIAGEN) and 100 ng of genomic DNA, according to these conditions: 15 min at 95 ºC for initial denaturation and DNA polymerase activation followed by 35 cycles of 30 sec at 94 ºC for denaturation, 30 sec at 56.5 ºC for annealing, and 1 min at 72 ºC for extension; the last extension was carried out during 10 min at 72 ºC. The PCR products from exons 2 (208 base pairs, bp, for H63D and S65C) and 4 (390 bp for C282Y) were digested for 2 hours at 37 ºC with *Rsa*I for C282Y, *Mbo*I for H63D and *Hinf*I for S65C (New England Biolabs).

The digestion products were size resolved by electrophoresis on 4% agarose gel and visualized by SYBR® Green I nucleic acid gel stain (Molecular Probes). For the C282Y, the *Rsa*I produced two fragments of 250 and 140 bp in the wild type DNA and three fragments of 250, 111 and 29 bp in the mutated DNA. In the case of wild type DNA for H63D, the *Mbo*I generated two fragments of 138 and 70 bp, whereas for *Hinf*I the two fragments have 147 and 61 bp; both H63D and S65C mutated DNA were not cut.

2.3. HLA genotyping

HLA-A and -B genotyping was performed in the 130 individuals by PCR-SSP Olerup SSP™ (GenoVision Inc.), according to the manufacturers' instructions. After electrophoresis on a 4% agarose gel stained with SYBR® Green, the PCR products were visualized, followed by HLA allele identification using the Helmberg-SCORE™ "Sequence Compilation and Rearrangment Evaluation for Research only" software version 3.320T (*Olerup* SSP AB, Saltsjöbaden, Sweden).

2.4. Statistical analysis

The HLA-A and -B allele and haplotype frequencies were calculated using the Arlequin version 3.1 software (Excoffier *et al*., 2005). The haplotype frequencies, from multilocus genotype data, were computed using the Expectation-Maximization algorithm because, except for one individual, the gametic phase was unknown. The presence of association between the C282Y mutation and HLA alleles and haplotypes was estimated by Chi-square or Fisher´s tests, as appropriate, using GraphPad Prism software, version 5.01 for Windows, San Diego California USA (www.graphpad.com). A $p<0.05$ was considered as statistically significant. Odds ratio (OR) were also determined using the Odds Ratio Generator software, version 1.0 for windows (Devilly, 2005).

3. RESULTS

3.1. HLA-A and -B allele frequencies

A total of 130 individuals were HLA-A and -B genotyped in order to characterize the chromosomal background of the C282Y mutation in the São Miguel Island (Azores). The -A genotyping revealed 12 different alleles in the C282Y group (Appendix 1) and 16 in the control population. Only the A*28 was absent in the control population. The C282Y group has 11 homozygous individuals for the following alleles: A*02 (n=4), A*03 (n=6) and A*24 (n=1). The four most frequent observed in this group were A*02 (0.2917), A*03 (0.2083), A*01 (0.1250) and A*29 (0.1042). When we compare both groups, three alleles showed significant association with the C282Y mutation, namely: A*03 (p=0.003, OR=3.33, 95 % CI: 1.55–7.18), A*26 (p=0.003, OR=8.38, 95% CI: 1.77–39.64) and A*29 (p<0.001, OR=19.18, 95% CI: 2.41–152.32). This last allele has the strongest association with C282Y mutation.

Twenty HLA-B alleles were identified in the C282Y group in comparison with the control population (Appendix 2). Three C282Y individuals were homozygous for B*07, B*15 and B*44. The five most frequent alleles observed in the C282Y group were B*14 (0.0938), B*18 (0.0938), B*45 (0.0938), B*07 (0.0729) and B*08 (0.0729). From all alleles, only the B*45 was found to be statistically significant (p=0.003) and presented a strong association with the C282Y mutation (OR=8.37, 95% CI: 1.77–39.64).

3.2. HLA-A and -B haplotypes

The HLA-A and -B haplotype determination was performed to investigate which of them appeared to be associated with *HFE* C282Y mutation. A total of 106 different haplotypes were found in the population sample, being 29 (27.36%, Appendix 3) and 59 (55.66%, data not shown) only observed in the mutated and control groups, respectively.

The five most frequent haplotypes in the C282Y group were A*29-B*45 (0.0729), A*01-B*08 (0.0521), A*02-B*44 (0.0521), A*02-B*58 (0.0521) and A*03-B*07 (0.0521). Homozygosity was detected in two C282Y carriers for A*02-B*44 and A*03-B*07, and in one patient for A*24-B*15, being this last one a new non-ancestral haplotype. We found two other non-ancestral haplotypes – A*02-B*58 (p<0.001, OR=19.78, 95% CI: 1.08–362.00) and A*29-B*45 (p<0.001, OR=27.57, 95% CI: 1.56–488.70) –, both absent in the control population. The results also demonstrate that the ancestral A*03-B*07 haplotype was significantly increased in the C282Y group, when compared to the controls (p=0.006, OR=8.96, 95% CI: 1.03–77.84). Altogether, four HLA haplotypes associated with C282Y mutation were identified in the São Miguel Island population. However, due to the relatively low number of population sample, the OR estimated are significant, but some upper confidence intervals indicate that a strong conclusion can not be drawn.

4. DISCUSSION AND CONCLUSION

Extensive studies – population and family based – have been performed in several geographical areas to characterize the origin of *HFE* C282Y mutation through HLA haplotyping (Yaouanq, 2000; Barton & Acton; 2002; Olsson *et al*., 2008). These studies suggest that this mutation occurred once on a particular chromosome carrying the A*03-B*07 haplotype in northwestern Europe. By recombination and admixture, this ancestral haplotype has subsequently been modified, generating other HLA haplotypes: A*03 and non-A*03 bearing (Simon *et al*., 1987). In the present study, we describe the HLA haplotypes associated with C282Y mutation in the population living on the Azorean Island of São Miguel, located in the North Atlantic Ocean. The *HFE* mutation detection – C282Y, H63D and S65C – was determinant to increase the power of the population sample studied, assuring that the mutated group was only composed of C282Y subjects and the control group were negative for the three mutations. Due to the diminutive number of homozygous, the C282Y mutated group comprised homozygous and heterozygous individuals, even though homozygous are the ideal to study HLA haplotype associations (Pacho *et al*., 2004).

In the São Miguel Island population, the C282Y mutation seems to be associated with four haplotypes. One of them – A*03-B*07 – is the ancestral and the most common haplotype in hemochromatosis patients (Barton & Acton, 2002; Yaouanq, 2000). With time, the recombination of this haplotype with other B-alleles generated new HLA-A*03 haplotypes that migrated with the mutation (Simon *et al*., 1987). Here, seven HLA-A*03 bearing haplotypes – A*03-B*18, A*03-B*27, A*03-B*50, A*03-B*51, A*03-B*53, A*03-B*55 and A*03-B*57 – were only present in the C282Y group. From these, four were previously described in patients from other regions, namely: A*03-B*18 and A*03-B*51 in mainland Portugal (Cruz *et al*., 2006) and A*03-B*27 and A*03-B*57 in Alabama (Barton & Acton, 2002). Knowing the strategic position of the Azores, the presence of these HLA-A*03 haplotypes in São Miguel Island may be explained by the admixture of their inhabitants,

descendants from individuals of different origins, including the first settlers of the 15th and 16th centuries.

The present study also revealed three new non-ancestral haplotypes –A*02-B*58, A*24-B*15 and A*29-B*45 –, being the last one the most frequent (7.29%) and presenting the strongest association with the mutation (p<0.001, OR=27.57, 95% CI: 1.56–488.70). These findings suggest that A*29-B*45 haplotype appeared predominantly linked to the C282Y in the São Miguel Island population. Considering that this population is mainly of Portuguese descent, the A*29-B*45 may have arisen by recombination of the A*29-B*37 reported in mainland (Cruz *et al*., 2006). Even though rarely associated with the C282Y, the A*29 was found in linkage disequilibrium with two other *HFE* mutations, the H63D in the mainland (Cardoso *et al*., 2002) and the S65C in the Azorean Island of Terceira (Couto *et al*., 2003). Other non-ancestral haplotypes – A*01-B*08 and A*11-B*35 – have also been identified in specific geographical areas, such as Sweden (Olsson *et al*., 2007; Olsson *et al*., 2008) and Brittany (Simon *et al*., 1987), respectively. Although the evolution mechanisms underlying these associations remain unclear, the non-A*03 haplotypes are thought to originate from recent recombinations (Cruz *et al*., 2006; Yaouanq, 2000).

HLA genotyping of the twelve C282Y homozygous patients revealed, by direct inference, the A*24-B*15. To our knowledge this is a new haplotype, generated probably by recombination, and reinforces the association between C282Y and A*24 (-B*18, -B*35 and -B*57) detected in mainland Portuguese homozygous patients (Cruz *et al*., 2006). However, we can not exclude the hypothesis of a *de novo* mutation on the A*24-B*15, because this haplotype was also present in our control population. Most likely, one of these events occurred recently in an individual living in the São Miguel, since it only appeared in one homozygous patient.

In the Spanish population, Pacho and colleagues (2004) considered *protector* haplotypes, those present with high frequency in the control group and absent in C282Y homozygous individuals. According to these criteria, in São Miguel population we could consider five *protector* haplotypes: A*02-B*35 (3.35%),

A*01-B*51 (3.05%), A*68-B*49 (3.05%), A*30-B*18 (2.44%) and A*68-B*15 (2.44%).

In summary, this study suggest that in São Miguel population the C282Y mutation is not only associated with the ancestral haplotype – A*03–B*07 –, but also with three new non-ancestral A*29-B*45, A*02-B*58 and A*24-B*15 haplotypes. Recombination most probably generated these haplotypes, before or after settlement. However, we also hypothesize a recent *de novo HFE* C282Y mutation on the A*24-B*15 haplotype in an individual living in São Miguel Island. The existence of several haplotypes associated with this recessive mutation is corroborated by the Azorean high genetic diversity.

5. PERSPECTIVES

The present thesis is part of an ongoing research project on the genetic features of hereditary hemochromatosis in the São Miguel Island population (Azores). During the last five years, this population has been the object of several studies, concerning in particular its genetic structure and diversity.

Here, we demonstrate that the principal mutation implicated with the hereditary hemochromatosis – the C282Y on *HFE* gene – may be associated with several HLA-A-B haplotypes, namely A*03-B*07, A*02-B*58, A*24-B*15 and A*29-B*45. However, a further study with an increase sample size would contribute to narrow the confidence intervals observed in some odds ratio values. Nevertheless, the scientific information obtained can be used in planning investigations of other recessive mutations, as well as in the characterization of the molecular consanguinity in the São Miguel Island population.

Furthermore, this HLA-hemochromatosis association study has lead to the initiation of another research project, the HLA (class II) susceptibility with leptospirosis, an infectious disease which is a severe public health problem in the São Miguel and Terceira Islands.

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APPENDICES

Appendix 1. HLA-A allele frequencies in the *HFE* C282Y mutated and control groups

	Allele frequencies			
HLA	C282Y (n=96)	Controls (n=164)	*p*-value (Chi-square)	OR (95% CI)
A*01	0.1250	0.1585	NS	0.76
A*02	0.2917	0.2805	NS	1.05
A*03	**0.2083**	**0.0732**	**0.003**	**3.33 (1.55–7.18)**
A*11	0.0312	0.0549	NS	0.55
A*24	0.0938	0.1037	NS	0.89
A*26	**0.0208**	**0.0122**	**0.003**	**8.38 (1.77–39.64)**
A*28	0.0104	0.0000	NS	5.17
A*29	**0.1042**	**0.0061**	**<0.001**	**19.18 (2.41–152.32)**
A*31	0.0208	0.0183	NS	1.14
A*33	0.0417	0.0183	NS	2.33
A*66	0.0104	0.0061	NS	1.72
A*68	0.0417	0.0976	NS	0.40

n: total number of chromosomes in each group, OR: Odds ratio, CI: Confidence interval, *p*: Significance level, NS: Not significant.

Appendix 2. HLA-B allele frequencies in the *HFE* C282Y mutated and control groups

HLA	Allele frequencies		*p*-value (Chi-square)	OR (95% CI)
	C282Y (n=96)	Controls (n=164)		
B*07	0.0729	0.0610	NS	1.21
B*08	0.0729	0.1220	NS	0.57
B*13	0.0208	0.0122	NS	1.72
B*14	0.0938	0.0732	NS	1.31
B*15	0.0312	0.0549	NS	0.55
B*18	0.0938	0.0427	NS	2.32
B*27	0.0521	0.0305	NS	1.75
B*35	0.0625	0.0793	NS	0.77
B*37	0.0312	0.0061	NS	5.26
B*38	0.0104	0.0244	NS	0.42
B*40	0.0312	0.0488	NS	0.63
B*44	0.0521	0.1341	NS	0.35
B*45	**0.0938**	**0.0122**	**0.003**	**8.37 (1.77–39.64)**
B*49	0.0521	0.0549	NS	1.07
B*50	0.0104	0.0427	NS	0.24
B*51	0.0417	0.0976	NS	0.40
B*53	0.0208	0.0183	NS	1.14
B*55	0.0521	0.0122	NS	4.45
B*57	0.0521	0.0183	NS	2.95
B*58	0.0521	0.0183	NS	2.95

n: total number of chromosomes in each group, OR: Odds ratio, CI: Confidence interval, *p*: Significance level, NS: Not significant.

Appendix 3. HLA-A-B haplotype frequencies in the *HFE* C282Y mutated and control groups

	Haplotype frequencies			
HLA	C282Y (n=96)	Controls (n=164)	*p*-value (Chi-square)	OR (95% CI)
A*01-B*08	0.0521	0.0976	NS	0.51
A*01-B*14	0.0104	0.0061	NS	1.72
A*01-B*35	0.0313	0.0000	NS	12.32
A*01-B*57	0.0313	0.0061	NS	5.26
A*02-B*07	0.0104	0.0000	NS	5.17
A*02-B*13	0.0104	0.0061	NS	1.72
A*02-B*14	0.0238	0.0000	NS	8.70
A*02-B*38	0.0104	0.0122	NS	0.85
A*02-B*40	0.0208	0.0244	NS	0.85
A*02-B*44	0.0521	0.0938	NS	0.55
A*02-B*45	0.0104	0.0000	NS	5.17
A*02-B*49	0.0283	0.0000	NS	12.32
A*02-B*51	0.0313	0.0272	NS	1.29
A*02-B*55	0.0417	0.0000	NS	16.01
A*02-B*58	**0.0521**	**0.0000**	**<0.001**	**19.78 (1.08–362.00)**
A*03-B*07	**0.0521**	**0.0061**	**0.006**	**8.96 (1.03–77.84)**
A*03-B*14	0.0283	0.0122	NS	2.61
A*03-B*18	0.0208	0.0000	NS	8.70
A*03-B*27	0.0208	0.0000	NS	8.70
A*03-B*35	0.0104	0.0183	NS	0.56
A*03-B*49	0.0134	0.0061	NS	1.71
A*03-B*50	0.0104	0.0000	NS	5.17
A*03-B*51	0.0104	0.0000	NS	5.17
A*03-B*53	0.0104	0.0000	NS	5.17
A*03-B*55	0.0104	0.0000	NS	5.17

Appendix 3. (cont.) HLA-A-B haplotype frequencies in the *HFE* C282Y mutated and control groups

	Haplotype frequencies			
HLA	C282Y (n=96)	Controls (n=164)	*p*-value (Chi-square)	OR (95% CI)
A*03-B*57	0.0208	0.0000	NS	8.7
A*11-B*18	0.0104	0.0000	NS	5.17
A*11-B*35	0.0104	0.0122	NS	0.85
A*11-B*37	0.0104	0.0000	NS	5.17
A*24-B*08	0.0104	0.0244	NS	0.42
A*24-B*15[a]	0.0313	0.0061	0.006	5.25 (0.54–51.28)
A*24-B*18	0.0313	0.0000	NS	12.32
A*24-B*27	0.0104	0.0108	NS	0.85
A*24-B*35	0.0104	0.0136	NS	0.85
A*26-B*49	0.0104	0.0000	NS	5.17
A*26-B*53	0.0104	0.0000	NS	5.17
A*28-B*37	0.0104	0.0000	NS	5.17
A*29-B*08	0.0104	0.0000	NS	5.17
A*29-B*27	0.0208	0.0000	NS	8.70
A*29-B*45	0.0729	0.0000	**<0.001**	**27.57 (1.56–488.70)**
A*31-B*07	0.0104	0.0000	NS	5.17
A*31-B*13	0.0104	0.0000	NS	5.17
A*33-B*14	0.0313	0.0122	NS	2.61
A*33-B*45	0.0104	0.0000	NS	5.17
A*66-B*40	0.0104	0.0000	NS	5.17
A*68-B*18	0.0313	0.0000	NS	12.32
A*68-B*37	0.0104	0.0000	NS	5.17

[a] This haplotype is also associated with C282Y, since patient was homozygous for HLA-A and -B alleles, n: total number of chromosomes in each group, OR: Odds ratio, CI: Confidence interval, *p*: Significance level, NS: Not significant.