S65C and Other Mutations in the Haemochromatosis Gene in the Czech Population

(haemochromatosis / *HFE* gene / S65C mutation / C282Y mutation / H63D mutation / allele frequency)

M. ČIMBUROVÁ[1], I. PŮTOVÁ[1], H. PROVAZNÍKOVÁ[2], D. PINTÉROVÁ[3], J. HORÁK[4]

[1]Center of Biomedical Sciences, Division of Cell and Molecular Biology
[2]Center of Preventive Medicine, Division of Health of Children and Youth
[3]Center of Biomedical Sciences
[4]Department of Medicine I, 3rd Faculty of Medicine, Charles University, Prague, Czech Republic

Abstract. HFE-linked hereditary haemochromatosis is a common autosomal recessive disease among Caucasians. The primary pathogenetic mechanism is excessive absorption of iron, which is deposited in various organs with their subsequent damage. In 1996 the gene responsible for haemochromatosis was detected – the *HFE* gene and its major mutation C282Y. The discovery of further mutations followed. Two sites of point mutations in the *HFE* gene, C282Y and H63D, are associated with more than 80% of haemochromatosis cases. Another mutation – S65C – was detected on 8% of chromosomes of haemochromatosis patients, which were negative for mutations C282Y or H63D. The objective of this study was to identify the allele frequency of S65C and other *HFE* mutations in the Czech population. DNA extracted from 481 randomly selected newborn screening cards (Guthrie cards) from all over the country was analysed by PCR-RFLP. No (0%) sample was identified as homozygous for S65C or C282Y mutation and 8 (1.67%) were homozygous for H63D mutation. Twelve (2.49%) samples were S65C heterozygous, 33 (6.86%) samples were C282Y heterozygous, and 128 (26.61%) were H63D heterozygous. Of these, 11 (2.29%) carried one copy of each mutation, i.e. were compound heterozygous. Two samples were S65C/H63D compound heterozygous and nine were C282Y/H63D compound heterozygous. Allele frequencies for S65C, C282Y, and H63D were 1.25% (95% CI, ± 0.70), 3.43% (95% CI, ± 1.15), and 14.97% (95% CI, ± 2.25), respectively. The observed genotype frequency for S65C, C282Y, and H63D mutations in the Czech Republic agrees with those reported for other Central European populations.

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Corresponding author: Markéta Čimburová, Division of Cell and Molecular Biology, 3rd Faculty of Medicine, Charles University, Ruská 87, CZ – 100 00 Prague 10, Czech Republic. Tel.: (+420) 267 102 657; e-mail: mcimbur@email.cz

HFE-linked hereditary haemochromatosis is an autosomal recessive disease affecting iron metabolism. The disease is frequent among Caucasians. The highest frequency of haemochromatosis, with 1 in 300–400 individuals affected, is found in populations of Northern European origin (Merryweather-Clarke et al., 2000). Haemochromatosis is characterized by excessive iron absorption, its deposition in organs (mainly parenchymal) and subsequent damage of the organism. Most frequently the liver is affected up to the degree of cirrhosis, a feared complication is the development of hepatocellular carcinoma. Other complications are diabetes mellitus, arthropathy, and hypogonadism. Early diagnosis and subsequent treatment by repeated phlebotomy can avert these serious complications. In 1996 the gene for haemochromatosis was detected on the short arm of chromosome 6 (6p21.3) – the *HFE* gene and its major mutation C282Y (Feder et al., 1996). The discovery of further mutations followed.

The single-base substitution 845G→A (C282Y) is the main mutation responsible for haemochromatosis in all the population studies performed throughout the world. It is associated with more than 80% of haemochromatosis cases in the populations of Northern European descent and about 60% of cases from Mediterranean populations (Feder et al., 1996; Merryweather-Clarke et al., 2000). The C282Y mutation is speculated to abolish a conserved cysteine that impairs β2-microglobulin association and cell surface expression of the HFE protein. The C282Y mutation eliminates the interaction with TfR (transferrin receptor) that may be sufficient to cause iron storage overload (Feder et al., 1998). H63D mutation (187C→G substitution) is associated with a milder form of the disease representing 40% to 70% of non-C282Y haemochromatosis chromosomes (Merryweather-Clarke et al., 2000). This mutation leads to a loss of ability to reduce the TfR affinity for transferrin compared with wild-type *HFE* (Feder et al., 1998). Another mutation – S65C – was detected on 8% of chromosomes of haemochromatosis patients, which were negative for mutations C282Y or H63D. This substitu-

tion 193A→T, which leads to a cysteine to serine substitution (S65C), is localized in the vicinity of H63D. When combined with the C282Y mutation, the S65C mutation is associated with a mild form of haemochromatosis (Mura et al., 1999; Asberg et al., 2002). A large-scale study of European populations evidenced that the allele frequency was 6.6% for the C282Y mutation and 14.9% for H63D (Merryweather-Clarke et al., 2000). Regarding the recently described S65C mutation, various epidemiological studies have shown the frequency of S65C mutation about 0.5–3% among Caucasians (Mura et al., 1999; Beutler et al., 2000; Pietrapertosa et al., 2003).

The identification of mutations creates new opportunities for diagnosis – a genetic test which would enable early detection of the affected subjects. Therefore, population screening has been proposed in various countries. However, the precise level of risk for particular genotypes (C282Y homozygotes or compound heterozygotes) is not known yet because of incomplete penetrance of HFE mutations.

In our previous study we established the allele frequency for C282Y and H63D mutations in the Czech Republic (Cimburova et al., 2002). The objective of this study was to identify the allele frequency of S65C mutation together with C282Y and H63D mutations in the Czech population. Such data are essential when considering the question of screening for haemochromatosis in our country.

Material and Methods

Material

DNA analysis was performed on dry blood spots (Guthrie cards) from the Czech National Newborn Screening Program. The cards came from all regions of the country. The cards were randomly selected and anonymously tested. We analysed 481 cards for S65C, C282Y and H63D mutations.

DNA analysis

DNA was extracted from the samples by a NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany). The PCR-RFLP technique was performed for detection of mutations in *HFE* S65C, C282Y, and H63D. The region containing the S65C and H63D mutations was amplified by primers designed by Feder (Feder et al., 1996). Because the S65C mutation is located only six nucleotides away from the H63D change, it is amplified in the same PCR reaction. Digestion was performed with the *Hinf*I and *Bcl*I for S65C and H63D mutations, respectively, as previously described (Feder et al., 1996; Mura et al., 1999). At first Feder's primers for amplification of the region containing the C282Y mutation were used; later, new primers were designed: forward primer 20-mer 5'–AACCTTGGCTGTACCCCCTG–3' and reverse primer 20-mer 5'–GCCCACCCCCTAACAAAGAG–3'. The PCR product had a constant *Rsa*I site producing two fragments of 191 and 12 bp in the normal C allele, and another *Rsa*I site in the mutant Y allele generated two fragments as 162 and 29 bp cleavage of the 191 bp fragment. The resulting fragments were visualized with ethidium bromide staining following gel electrophoresis.

Statistical methods

The χ^2 test and Fisher's exact test were used to compare the frequency of genotypes of European populations published previously. A 95% confidence interval (CI) was calculated for the frequency of the alleles. The frequency of C282Y and S65C homozygotes was estimated using the Hardy-Weinberg equilibrium formula.

Results and Discussion

Complete results were obtained from 481 cards. No (0%) sample was identified as homozygous for S65C or C282Y mutation and eight (1.67%) were homozygous for H63D mutation. Twenty (2.49%) samples were S65C heterozygous, 33 (6.86%) samples were C282Y heterozygous, and 128 (26.61%) were H63D heterozygous. Of these, 11 (2.29%) carried one copy of each mutation, i.e. were compound heterozygous. Two samples (0.42%) were S65C/H63D compound heterozygotes and nine (1.87%) were C282Y/H63D compound heterozygotes. The results are shown in Table 1.

Table 1. Distribution of HFE genotypes

HFE genotypes	N	(%)	Frequency
S65C homozygous	0	(0.00)	0.0002[a]
S65C heterozygous	12	(2.49)	0.0249
C282Y homozygous	0	(0.00)	0.0012[a]
C282Y heterozygous	33	(6.86)	0.0686
H63D homozygous	8	(1.67)	0.0166
H63D heterozygous	128	(26.61)	0.2661

Total number of tested samples N = 481 (962 chromosomes).
[a]The frequency of S65C and C282Y homozygotes was estimated using the Hardy-Weinberg equilibrium formula.

Allele frequencies for S65C, C282Y, and H63D were 1.25% (95% CI, ± 0.70), 3.43% (95% CI, ± 1.15), and 14.97% (95% CI, ± 2.25), respectively.

Our results showed the S65C frequency of 1.25%, which is not significantly different from the frequencies found in other European populations (0.5–3.0%), as demonstrated in Table 2. This is the first report of the S65C mutation in the Czech population.

The highest frequency of S65C has been reported in the Saami population of Northern Scandinavia (3.0%) (Beckman et al., 2001), a slightly lower frequency was found in Caucasians of Northern European origin (1.5–1.95%) (Mura et al., 1999; Beutler et al., 2000;

Table 2. Frequencies of the HFE gene mutations in different populations

		Allele frequencies %		
Population	**Reference**	**S65C**	**C282Y**	**H63D**
Norway	(Milman et al., 2005)	1.5	6.6	11.2
Denmark	(Milman et al., 2005)	1.5	5.7	12.8
Sweden	(Holmstrom et al., 2002)	1.6	6.2	11.4
Sweden, Saami	(Beckman et al., 2001)	3.0	2.0	7.9
Finland	(Milman et al., 2005)	1.6	3.6	9.8
Faroe Islands	(Milman et al., 2005)	1.0	8.0	17.5
Ireland	(Ryan et al., 1998)	–	14.0	17.9
UK	(Merryweather-Clarke et al., 2000)	–	8.1	15.2
France	(Mura et al., 1999)	1.95	7.7	14.0
Germany	(Merryweather-Clarke et al., 2000)	–	3.8	13.2
Poland	(Moczulski et al., 2001)	–	3.1	16.2
Czech Republic	Present study	1.25	3.4	14.9
Austria	(Merryweather-Clarke et al., 2000)	–	3.7	12.9
Croatia	(Ristic et al., 2003)	1.8	3.3	14.5
Slovenia	(Ristic et al., 2003)	0.5	4.0	14.5
Russia	(Mikhailova et al., 2003)	1.7	3.7	13.3
Bulgaria	(Merryweather-Clarke et al., 2000)	–	0.0	23.0
Nothern Italy	(Mariani et al., 2003)	1.3	3.2	13.4
Italy, Apulian	(Pietrapertosa et al., 2003)	0.5	1.5	14.0
Greece	(Merryweather-Clarke et al., 2000)	–	1.3	13.5
Nothern Spain	(Altes et al., 2004)	1.0	3.0	20.0
Spain, Basque	(Merryweather-Clarke et al., 2000)	–	3.6	30.4
Portugal	(Porto et al., 1998)	–	2.8	23.0
USA, Caucasian	(Beutler et al., 2000)	1.6	6.3	15.2
USA, Hispanic	(Beutler et al., 2000)	0.6	2.7	12.4
USA, Asian	(Beutler et al., 2000)	0	0.2	3.3
USA, Black	(Beutler et al., 2000)	0.7	1.1	5.1

Holmstrom et al., 2002; Milman et al., 2005;) and Central Europe and Mediterranean (0.5–1.8%) (Mariani et al., 2003; Mikhailova et al., 2003; Pietrapertosa et al., 2003; Ristic et al., 2003; Altes et al., 2004) The lowest frequency was seen in individuals of Hispanic, Asian, or African origin (0.0–0.7%) (Beutler et al., 2000). Mutation S65C has been described as a cause of haemochromatosis in combination with C282Y mutation. This genotype is supposed to be associated with a mild form of iron overload (Mura et al., 1999).

C282Y allele frequencies are distributed along a decreasing line from northwest to southeast of Europe (Table 2) consistent with putative Celtic or Viking origin of the mutation (Lucotte and Dieterlen, 2003; Milman and Pedersen, 2003). The highest allele frequency of C282Y was found in Ireland (14%) (Ryan et al., 1998). C282Y allele frequencies of 5.7–8.1% are observed in Norway, Sweden, the Faroe Islands, Denmark, Great Britain, and Brittany (Merryweather-Clarke et al., 2000; Milman et al., 2005). Intermediate allele frequencies (3.1–4.0%) are seen in the populations in Central Europe. Low allele frequencies (0–3.6%) are recognized in populations in Southern Europe and the Mediterranean (Merryweather-Clarke et al., 2000; Mariani et al., 2003; Pietrapertosa et al., 2003; Altes et al., 2004). The 3.43% frequency of C282Y allele in the Czech population is not different from this allele frequencies reported in other Central European populations. Significantly higher frequency ($P < 0.001$) than in the Czech population was found in the population of Ireland, Great Britain, Norway, France, and Denmark. C282Y allele frequency is significantly lower in Southern European populations (Greece, Italy, Bulgaria) than in the Czech population (Table 2).

The H63D mutation is more common than C282Y. Most European populations studied have H63D allele frequency between 10–20% (Table 2). The frequencies above 20% were observed in the Bulgarians, Spanish and Portuguese. The highest reported H63D allele frequency is 30.4% in the Basque population (Merryweather-Clarke et al., 2000). Our results show the H63D allele frequency of 14.97%, which is consistent with frequencies in other Central Europeans. Signifi-

cantly higher frequency than in the Czech population was found in the population of Spain ($P < 0.001$), Bulgaria ($P < 0.01$), and Portugal ($P < 0.05$) (Table 2).

The discrepancies between the frequency of homozygotes and compound heterozygotes and the number of cases of haemochromatosis imply that the penetrance of *HFE* gene mutations is incomplete. According to various studies the penetrance of C282Y homozygotes is estimated to be 1–85% (Olynyk et al., 1999; Bulaj et al., 2000; Beutler et al., 2002; McCune et al., 2002; Phatak et al., 2002). The penetrance of compound heterozygotes is even lower (Moirand et al., 1999; Phatak et al., 2002). However, these studies differ in their definition of iron overload, with using biochemical values of iron metabolism or clinical symptoms of haemochromatosis. Therefore, population screening for haemochromatosis is controversial because of the uncertainty regarding the real frequency of the disease in each country and discrepancies observed among population studies about the clinical penetrance of the disease. On the other hand, the family screening as an opportunity to identify relatives at risk of haemochromatosis is recommended. In the population of 10,000,000 persons in the Czech Republic approximately 11,800 individuals should be C282Y homozygous. The estimated frequency of compound heterozygotes C282Y/S65C and C282Y/H63D in the Czech population is 0.086% and 1.03%, respectively. Unfortunately, according to the unclear penetrance of *HFE* gene mutation we are not sure what is the real number of individuals at risk for haemochromatosis in our country.

In conclusion, we provide the prevalence of three main mutations in the *HFE* gene in the Czech Republic. These results answer the first question in screening – prevalence of casual mutations. These data have an important clinical implication, but further studies especially concerning the penetrance of the mutations are needed to translate these data to the screening strategies in our country.

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