SHORT REPORT

The mitochondrial superoxide dismutase A16V polymorphism in the cardiomyopathy associated with hereditary haemochromatosis

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The A16V mitochondrial targeting sequence polymorphism influences the antioxidant activity of MnSOD, an enzyme involved in neutralising iron induced oxidative stress. Patients with hereditary haemochromatosis develop parenchymal iron overload, which may lead to cirrhosis, diabetes, hypogonadism, and heart disease. The objective of this study was to determine in patients with haemochromatosis whether the presence of the Val *MnSOD* allele, associated with reduced enzymatic activity, affects tissue damage, and in particular heart disease, as *MnSOD* knockout mice develop lethal cardiomyopathy. We studied 217 consecutive unrelated probands with haemochromatosis, and 212 healthy controls. *MnSOD* polymorphism was evaluated by restriction analysis. The frequency distribution of the polymorphism did not differ between patients and controls. Patients carrying the Val allele had higher prevalence of cardiomyopathy (A/A 4%, A/V 11%, V/V 30%, p = 0.0006) but not of cirrhosis, diabetes, or hypogonadism, independently of age, sex, alcohol misuse, diabetes, and iron overload (odds ratio 10.1 for V/V, p = 0.006). The frequency of the Val allele was higher in patients with cardiomyopathy (0.67 *v* 0.45, p = 0.003). The association was significant in both C282Y+/+ (p = 0.02), and in non-C282Y+/+ patients (p = 0.003), and for both dilated (p = 0.01) and non-dilated stage (p = 0.04) cardiomyopathy, but not for ischaemic heart disease. In patients with hereditary haemochromatosis, the *MnSOD* genotype affects the risk of cardiomyopathy related to iron overload and possibly to other known and unknown risk factors and could represent an iron toxicity modifier gene.

Hereditary haemochromatosis (HH), the most common autosomal recessive disease in white populations, is characterised by progressive iron overload with development of cirrhosis, diabetes, arthritis, hypogonadism, and cardiomyopathy.[1][2] In the majority of cases, HH is related to homozygosity for the C282Y mutation of the *HFE* gene,[3] but the penetrance is not complete, and population studies indicate that full blown clinical expression occurs in only 1–25% of C282Y homozygotes.[4–7] Inherited factors have been suggested to influence the phenotype, but genes involved in iron metabolism do not seem to play a major role.[8–10]

Iron toxicity is mediated by oxidative damage, which results in mitochondrial injury.[11][12] Manganese mitochondrial superoxide dismutase (MnSOD or SOD2) catalyses the removal of superoxide at its site of production, the matrix side of the inner mitochondrial membrane. Increased iron concentration induces MnSOD expression,

and overexpression of MnSOD prevents iron related oxidative damage.[13][14] Moreover, mice in which the *MnSOD* gene has been knocked out develop neonatally lethal cardiomyopathy and fatty liver.[15] The enzyme is encoded by nuclear DNA, and synthesised with a mitochondrial targeting sequence. A number of polymorphisms in this sequence have been described, but only Ala16Val has been demonstrated to have a functional significance.[16–18] The Val allele, by disrupting the α-helix structure of the protein, which is important for the translocation of the enzyme to the mitochondrial matrix where it exerts its function, causes the protein to be retained at the level of the mitochondrial inner membrane and has been associated with 30–40% lower activity compared with the Ala allele, and to increased susceptibility to oxidative stress.[18]

Hypothesising that MnSOD influences iron toxicity, we analysed the prevalence of the A16V polymorphism and its correlation with iron induced organ damage, especially cardiomyopathy, in a series of Italian patients with HH.

METHODS
Patients
We enrolled 217 consecutive unrelated probands with HH, 199 (92%) of whom were from northern, and 18 (8%) from southern Italy. The diagnosis[1][19] was based on the presence of a phenotype compatible with HH (iron removed to reach iron depletion >5 g in males, >3 g females; hepatic iron index[20] >1.9; typical parenchymal siderosis), in the absence of secondary causes of iron overload. Patients with juvenile HH were excluded from this study. All patients were referred because of iron overload and/or liver disease. Available clinical data at diagnosis included: age, sex, presence of alcohol misuse (>60 or 40 g/day in men and women, respectively, for >5 years), hepatitis B virus (HBV) surface antigen, anti-hepatitis C virus (HCV) antibodies, HCV RNA status, serum alanine aminotransferase, ferritin and transferrin saturation at diagnosis, amount of iron removed to reach depletion, *HFE* gene mutations, the presence of cirrhosis (histologically proven), diabetes (WHO criteria), hypogonadism (clinical and biochemical diagnosis), arthritis (clinical and radiological diagnosis),[21] and cardiomyopathy.[22–24] Mutations in the coding sequences of the transferrin receptor-2 and ferroportin-1 genes[25][26] were excluded in all of the non-C282Y+/+ patients. Cardiomyopathy stage was further classified as dilated[22] (increased ventricular size (left ventricular end diastolic diameter >56 mm) and decreased systolic function (ejection fraction <50%)), non-dilated (restrictive cardiomyopathy,[23] increased interventricular

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Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HH, hereditary haemochromatosis; MnSOD, manganese mitochondrial superoxide dismutase

septum thickness (>11 mm), and/or altered ultrasonographic structure, presence of conduction defects), or associated with ischaemic heart disease (previous myocardial infarction and/or ultrasonographic/electrocardiographic/angiographic evidence of regional ischaemia).

Mean (SD) age was 48.7 (12) years; 47 patients (22%) were females; 144 patients (66%) were C282Y homozygotes,[27] and 14 (6%) were C282Y/H63D compound heterozygotes; 28 (13%) had coexistent chronic viral hepatitis; and 37 (18%) were alcohol misusers. Mean (SD) serum ferritin at diagnosis was 1731 (1702) ng/ml (reference values: men <320, women <240 ng/ml), transferrin saturation 78.8 (21)% (men <45%, women <40%), iron removed to reach depletion 8.65 (7) g (<3 g), alanine aminotransferase 58.2 (54) U/l (<40 U/l). There were 92 patients (42%) with cirrhosis, and 43 (19%) had diabetes, 48 (22%) arthritis, 42 (19%) hypogonadism, and 27 (12%) cardiomyopathy.

Controls

The control group comprised 212 healthy blood donor subjects: 194 (92%) from northern, and 18 (8%) from central and southern Italy, without evidence of iron overload, liver disease, diabetes, or heart disease; the latter was excluded on the basis of clinical, electrocardiographic, and (when indicated) echocardiographic findings. There were 41 women (19%), and mean (SD) age was 44.7 (11) years. Serum ferritin was normal in all but three cases (male subjects; ferritin <450, 415, and 380 ng/ml). *HFE* genotypes were C282Y/wt in 5 cases (2%), H63D/H63D in 7 (3%), H63D/wt in 49 (23%), and wt/wt in 151 (72%). Informed written consent was obtained for each subject included in this study. *MnSOD* genotype was evaluated by restriction analysis[17] following validation of the method by sequencing.

Results were expressed as mean (SD). A two tailed value of p<0.05 was considered significant. Data were compared by *t* test, and frequencies by Fisher's exact test; χ^2 for trend was applied when comparing frequencies between A/A, A/V, and V/V *MnSOD* genotypes. Logistic regression analyses were performed both to adjust *MnSOD* polymorphism frequency distribution for age, sex, and geographical origin in patients versus controls, and to assess the relationship between *MnSOD* genotypes and HH related complications (independent variables considered are shown where appropriate).

RESULTS

The frequency distribution of the *MnSOD* polymorphisms was not significantly different between patients and controls (table 1).

Table 1 Frequency distribution of the Ala16Val *MnSOD* polymorphism in 217 patients with hereditary haemochromatosis (HH) and 212 healthy subjects

	MnSOD genotype, n (%)		
	A/A	A/V	V/V
HH (n=217)	48 (23)	132 (60)	37 (17)
Controls (n=212)	43 (20)	118 (56)	51 (24)
OR for HH	1 (RV)	1.1	0.7
95% CI		0.7 to 1.8	0.4 to 1.2

Odds ratio (OR) for haemochromatosis; logistic regression analysis adjusted for age, sex, and geographical origin (northern Italy versus others). RV, reference value; CI, confidence interval; HH, hereditary haemochromatosis; A/A, homozygosity for the Ala allele; A/V, heterozygosity for Ala and Val alleles; V/V, homozygosity for the Val allele.

Demographic features, the proportion of C282Y homozygous subjects, the degree of iron overload, the prevalence of alcohol misuse, coexistent chronic viral hepatitis, cirrhosis, diabetes, arthropathy, and hypogonadism were not significantly different when patients were subdivided according to *MnSOD* genotypes (table 2). This was true for the overall series of patients, for subjects with different *HFE* genotypes (C282Y+/+ and non-C282Y+/+), and after excluding from the analysis those with acquired risk factors (HBV/HCV infection, alcohol misuse). *MnSOD* genotypes containing the Val allele were associated with cardiomyopathy (A/A 2/48, 4%; A/V 14/132, 11%; V/V 11/37, 30%, p = 0.0006), the prevalence of the Val allele being 0.67 in patients with cardiomyopathy and 0.45 in those without (p = 0.003). Moreover, the frequency of the Val allele was significantly higher in patients with cardiomyopathy compared with control subjects (0.67 *v* 0.52; p = 0.04), and significantly lower in patients without cardiomyopathy than in controls (0.45 *v* 0.52; p = 0.048). No significant difference in the prevalence of the Val allele was observed in patients subdivided according to the presence of cirrhosis, diabetes, arthropathy, and hypogonadism. The prevalence and type of cardiomyopathy in patients subdivided according to *HFE* and *MnSOD* genotypes are shown in table 3. A/V and V/V genotypes were associated with either dilated (ejection fraction 41 (7)%), or non-dilated stage cardiomyopathy (p = 0.01 and p = 0.04 respectively at χ^2 for trend), but not with ischaemic related cardiomyopathy. Even after exclusion of subjects with coexistent hepatitis virus infection, *MnSOD* genotypes containing the Val allele were associated with cardiomyopathy in both C282Y (A/A 2/27, 7%; A/V 10/82, 12%; V/V 8/26, 31%; p = 0.017) and non-C282Y homozygotes (A/A 0/11; A/V 2/35, 6%; V/V 3/8, 38%; p = 0.009).

The prevalence of hypertension was 7/27 (26%) in patients with cardiomyopathy, not significantly different between the different types of heart disease or from that of patients without heart disease. Demographic, genetic, and clinical features of the 27 patients with HH and cardiopathy included in this study are shown in table 4.

Table 2 Demographic and clinical features at diagnosis of 217 patients with hereditary haemochromatosis subdivided on the basis of their *MnSOD* genotype

	MnSOD genotype		
	A/A (n = 48)	A/V (n = 132)	V/V (n = 37)
Female sex	10 (21%)	28 (21%)	9 (24%)
Age (years)	49 (13)	48.1 (12)	50.6 (13)
HFE			
C282Y/C282Y	29 (60%)	87 (66%)	28 (76%)
C282Y/H63D	3 (6%)	9 (7%)	2 (5%)
C282Y/wt	3 (6%)	4 (3%)	–
H63D/H63D	4 (8%)	3 (2%)	1 (3%)
TfR2 /ferroportin mutations	–	–	–
Alcohol misuse	8 (17%)	24 (18%)	5 (13%)
Ferritin (ng/ml)	1899 (2157)	1713 (1636)	1591 (1288)
Transferrin saturation (%)	81 (21)	77 (21)	82.6 (18)
Iron removed to depletion (g)	9.4 (7)	8.5 (6.9)	8.2 (7.9)
HCV/HBV chronic hepatitis	8 (17%)	17 (13%)	3 (8%)
ALT	56 (46	61 (59	50 (37
Liver cirrhosis	21 (44%)	53 (40%)	18 (49%)
Diabetes	7 (14%)	26 (20%)	10 (27%)
Arthropathy	11 (23%)	25 (21%)	12 (32%)
Hypogonadism	11 (23%)	23 (17%)	8 (22%)
Cardiomyopathy	2* (4%)	14* (11%)	11* (30%)

Results are n (%) as indicated, otherwise they are mean (SD). TfR2, transferrin receptor 2. *p = 0.0006.

Table 3 Prevalence and type of cardiomyopathy (dilated and non-dilated stage, and associated with ischaemic heart disease) in 217 patients with hereditary haemochromatosis, according to *HFE* status and *MnSOD* genotype

HFE status	MnSOD genotype	n	Cardiomyopathy, n				
			Overall	Dilated	Non-dilated	Ischaemic
C282Y +/+ (n = 144)	A/A	29	2* (7%)	0	1	1
	A/V	87	12* (14%)	4	5	3
	V/V	28	8* (29%)	3	4	1
Non-C282Y +/+ (n = 73)	A/A	19	0†	0‡	0	0
	A/V	45	2† (4%)	1‡	1	0
	V/V	9	3† (33%)	2‡	1	0

C282Y+/+: homozygotes for the C282Y *HFE* mutation. *p = 0.02, †p = 0.005, ‡p = 0.018; χ^2 test for trend.

Using logistic regression analysis that considered age (years), sex, iron removed to reach depletion (g), the presence of diabetes and of alcohol misuse, and *MnSOD* genotype (A/A and A/V versus V/V) as independent variables, cardiomyopathy was significantly associated with iron removed (p = 0.005; odds ratio 1.09; 95% confidence interval 1.03 to 1.15), age (p = 0.006; 1.07; 1.02 to 1.1), and V/V genotype (p = 0.006; 10.1, 2 to 54). The p value for the χ^2 model was <0.001.

DISCUSSION

In this study, we assessed whether a genetic variant of MnSOD, affecting detoxification of reactive oxygen species, influences iron induced tissue damage in patients with HH. Our results indicate that, independently of the degree of iron overload and the genetic background, the Val allele, previously linked to impaired enzymatic activity, is strongly associated with the presence of cardiomyopathy.

We considered a large and well characterised population of unrelated Italian patients with HH, comprising both C282Y+/+ subjects and patients with non *HFE*-related haemochromatosis,[27] fully representative of subjects who come to clinical attention in the Milan area, in whom the presence of organ involvement has systematically been assessed by reference methods.

Even after correction for age, sex, and geographical origin, the frequency distribution of the *MnSOD* polymorphism did not differ between patients and healthy controls, in whom it was in Hardy-Weinberg equilibrium. The degree of iron overload was not different among patients grouped according to *MnSOD* genotypes, consistent with a lack of effect of MnSOD on iron absorption. In contrast, Livesey *et al* recently suggested that a mitochondrial DNA mutation affecting mitochondrial redox status influences iron overload in C282Y+/+ subjects.[28] However, it should be noted that these authors analysed the effect of the mitochondrial DNA genotype on the expression of iron overload in C282Y+/+

Table 4 Demographic, genetic and clinical features of the 27 patients with cardiopathy and HH included in this study

No.	Age	Sex	HFE	MnSOD	Cardiological diagnosis	Iron removed (g)	Associated factors
1	64	M	282 +/+	V/V	NDCM	7	–
2	43	M	282 +/+	V/V	NDCM	16	Diabetes
3	46	M	282 +/+	V/V	DCM	44	Diabetes
4	68	F	282 +/+	V/V	DCM	7	–
5	57	F	282 +/+	V/V	IHD	10	Hypertension
6	33	M	282 +/+	V/V	DCM	5	Alcohol misuse
7	59	M	282 +/+	V/V	NDCM	5	–
8	82	F	282 +/+	V/V	NDCM	8	–
9	49	M	wt/wt	V/V	DCM	12	Diabetes, hypertension
10	50	M	wt/63	V/V	DCM	3	–
11	66	M	wt/wt	V/V	NDCM	10	Alcohol misuse
12	36	M	282 +/+	A/V	DCM	22	Dyslipidaemia, hypertension
13	41	M	282 +/+	A/V	NDCM	12	–
14	53	F	282 +/+	A/V	DCM	21	–
15	51	M	282 +/+	A/V	NDCM	39	Diabetes
16	72	F	282 +/+	A/V	NDCM	4	–
17	52	M	282 +/+	A/V	IHD	8	Hypertension
18	56	M	282 +/+	A/V	DCM	10	Diabetes
19	69	M	282 +/+	A/V	IHD	8	Diabetes, hypertension
20	67	M	282 +/+	A/V	DCM	4	–
21	55	M	282 +/+	A/V	NDCM	6	Diabetes
22	69	F	282 +/+	A/V	NDCM	4	Diabetes
23	59	F	282 +/+	A/V	IHD	5	Hypertension
24	68	M	wt/wt	A/V	DCM	7	Alcohol misuse
25	57	F	wt/wt	A/V	NDCM	25	–
26	38	M	282 +/+	A/A	NDCM	23	Diabetes
27	55	M	282 +/+	A/A	IHD	5	Alcohol misuse, hypertension

DCM, dilated stage cardiomyopathy; NDCM, non-dilated stage cardiomyopathy; IHD, ischaemic heart disease.

subjects, while our aim was to determine whether, independently from the genetic defect underlying HH, *MnSOD* genotype affects the organ damage related to iron overload.

In patients with HH, the Val allele, (which confers 30–40% decreased enzymatic activity and superoxide detoxification, because of the impaired transport of the resultant protein across the inner mitochondrial membrane[18]), was associated with the presence of cardiomyopathy, both dilated and non-dilated. The 16Val allele was significantly more prevalent in patients with heart disease compared both with those without disease and with the control subjects, while subjects without cardiomyopathy had a significantly lower frequency of the Val allele compared with controls. The presence of the Val allele was not associated with ischaemic heart disease, in the pathogenesis of which the role of iron is still controversial.[29 30] Moreover, the Val/Val genotype conferred a 10 fold increased risk of heart disease, independently of confounding factors (age, sex, alcohol misuse, diabetes, and iron overload; the latter, as previously reported,[22] strongly associated with the risk of heart disease).

As HH is a heterogeneous disease in Italy, and different genetic defects may influence iron overload and tissue iron distribution, we analysed the effect of *MnSOD* genotype in C282Y+/+ and non-C282Y+/+ subjects. The association between the Val allele and cardiomyopathy was significant in both the genetically homogeneous group of C282Y homozygotes, and in the non-C282Y homozygotes, even after exclusion of patients with chronic viral hepatitis (potentially affecting liver disease and iron overload), suggesting that the decreased MnSOD activity in patients with a phenotype compatible with HH increases the risk of heart injury independently of the genetic defect responsible for iron overload, and the associated acquired factors.

Experimental data indicate that increased MnSOD activity prevents iron induced oxidative damage,[16] and knockout *MnSOD* mice develop dilated cardiomyopathy,[15] which is neonatally lethal, probably due to the critically high mitochondrial concentration and oxygen tension in the heart. Interestingly, it has recently been reported that *HFE*−/− mice develop cardiac iron overload with increased reactive oxygen species content and decreased MnSOD activity, and are at increased susceptibility to oxidative damage.[31] However, as *MnSOD*−/+ mice do not develop heart disease, it is possible that a partial defect in MnSOD activity becomes clinically significant in the presence of iron overload or other factors able to induce oxidative stress. Similarly, Val/Val control subjects are very probably not at increased risk of cardiomyopathy in the absence of a triggering factor. However, Hiroi *et al*[32] have reported a twofold increase in the risk of idiopathic dilated cardiomyopathy in subjects carrying the *MnSOD* 16Val/Val genotype, associated with a decreased processing of the *MnSOD* leader peptide, raising the possibility that it plays a role, even if smaller, also in the general population.

In conclusion, the functional A16V *MnSOD* polymorphism affects the risk of cardiomyopathy related to iron overload and possibly to other known and unknown risk factors, and may represent an iron toxicity modifier. Whether the *MnSOD* genotype influences the development of heart disease in transfusion dependent patients with secondary iron overload and the iron related myocardial toxic effects of antracyclines,[33 34] and interacts with DNA mitochondrial variants,[26] warrants further investigation. It would be also interesting to assess whether *HFE* and *MnSOD* genotypes interact in determining the risk of cardiomyopathy in the general population.

ACKNOWLEDGEMENTS

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ECHO

Dynamic changes of gene expression profiles during postnatal development of the heart in mice

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Objective: To study postnatal cardiac differentiation in the mouse.

Hypothesis: There might be mechanisms or factors in cardiac differentiation that could be identified by systematic gene expression analysis during postnatal cardiac development.

Methods: Expression of 6144 genes was examined in mouse heart, from the newborn period (day 0), through day 7 and day 14, to adulthood, using the cDNA microarray approach. Northern blotting and immunohistochemical techniques were used to confirm the microarray results.

Results: Various cardiac development related genes involving the cell cycle (cyclin B1, proliferating cell nuclear antigen (PCNA), and Ki67), growth factors (IGF-II, pleiotrophin (PTN), and midkine (MK)), and transcriptional regulation, cytoskeleton, and detoxification enzymes were identified by microarray analysis. Some of these genes were also confirmed by Northern blotting and immunohistochemistry of their RNA and protein content. In vivo treatment with PTN (20 ng/g) increased bromodeoxyuridine incorporation (by 2.24-fold) and PCNA expression (by 1.71-fold) during day 7 to day 14, indicating that PTN induces cell proliferation in mouse heart.

Conclusions: Global gene expression analysis in the whole heart may be useful in understanding the orchestrated process of postnatal development or terminal differentiation in the cardiac environment. These data are likely to be helpful in studying developmental anomalies of the heart in neonates.

▲ *Heart* 2004;**90**:927–934.