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ORIGINAL ARTICLE

Single-nucleotide variations in the genes encoding the mitochondrial Hsp60/Hsp10 chaperone system and their disease-causing potential

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Abstract Molecular chaperones assist protein folding, and variations in their encoding genes may be disease-causing in themselves or influence the phenotypic expression of disease-associated or susceptibility-conferring variations in many different genes. We have screened three candidate patient groups for variations in the *HSPD1* and *HSPE1* genes encoding the mitochondrial Hsp60/Hsp10 chaperone complex: two patients with multiple mitochondrial enzyme deficiency, 61 sudden infant death syndrome cases (MIM: #272120), and 60 patients presenting with ethylmalonic aciduria carrying non-synonymous susceptibility variations in the *ACADS* gene (MIM: *606885 and #201470). Besides previously reported variations we detected six novel variations: two in the bidirectional promoter region, and one synonymous and three non-synonymous variations in the *HSPD1* coding region. One of the non-synonymous variations was polymorphic in patient and control samples, and the rare variations were each only found in single patients and absent in 100 control chromosomes. Functional investigation of the effects of the variations in the promoter region and the non-synonymous variations in the coding region indicated that none of them had a significant impact. Taken together, our data argue against the notion that the chaperonin genes play a major role in the investigated diseases. However, the described variations may represent genetic modifiers with subtle effects.

Keywords Hsp60 · Hsp10 · Mitochondria · Modifier gene · Molecular chaperone · Protein quality control · Short-chain acyl-CoA dehydrogenase · Sudden infant death syndrome

We dedicate this article to the memory of our colleague Etienne Agsteribbe.

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Introduction

In many monogenic diseases, the phenotype is not solely determined by gene variations in the primary target gene, but is subject to modification by so-called modifier genes and environmental influences (Dipple and McCabe 2000; Nadeau 2001). In complex multifactorial diseases, no single gene defect with a large impact is present, but rather a set of gene variants (also termed modifiers), each exerting a small effect in itself, can act together with environmental factors to produce the disease phenotype (Botstein and Risch 2003).

Modifying effects may be brought about by influencing the level of expression of a target gene product, its intracellular trafficking and folding, posttranslational modifications, activity and/or structural maintenance, and turnover. Molecular chaperones assist and supervise intracellular protein folding and are, together with specific proteases, involved in clearance of misfolded and damaged proteins. They typically interact with a large number of different proteins, and it has been proposed that genetic variations in chaperone genes may function as modifiers of amino acid substituting variations involved in many traits/diseases (Slavotinek and Biesecker 2001). Genetic screens of neurodegenerative disease models have indeed shown that molecular chaperones, intracellular proteases and other components involved in protein quality control represent a prominent group of such modifier genes (Fernandez-Funez et al. 2000; Bonini and Fortini 2003; Nollen et al. 2004). It has furthermore been demonstrated that even a simple decrease in the levels of the Hsp90 chaperone may enable a number of otherwise suppressed phenotypic traits to be expressed in natural fruitfly and Arabidopsis populations (Rutherford and Lindquist 1998; Queitsch et al. 2002). A similar buffering function against deleterious variations has also been accomplished by experimentally increasing the levels of the bacterial GroEL/ES chaperone complex (Fares et al. 2002).

The mammalian mitochondrial homolog of the bacterial GroEL/GroES chaperone system consists of Hsp60 and Hsp10. It is involved in folding of a subset of mitochondrial proteins (Dubaquie et al. 1998). Homozygosity for deleterious mutations in the *Drosophila melanogaster* gene encoding the fly Hsp60 homolog causes death early in embryogenesis establishing that Hsp60 is an essential protein (Perezgasga et al. 1999). We have shown that knock-down of the *HSPD1* gene by RNAi in human cells compromises folding of the mitochondrial matrix enzymes short-chain acyl-CoA dehydrogenase (SCAD) and medium-chain acyl-CoA dehydrogenase (MCAD), encoded by the *ACADS* and *ACADM* genes respectively, and mitochondrially targeted green fluorescent protein (Corydon et al. 2005). In addition, disease-causing mutant variants in the medium- and short-chain acyl-CoA dehydrogenases have been shown to remain in complex with the Hsp60 chaperonin for prolonged periods of time (Saijo et al. 1994; Pedersen et al. 2003), and elevated chaperonin levels could partially rescue the correct folding of some of them (Andresen et al. 2001; Bross et al. 1993, 1995).

We have recently reported the genomic structure of the human chaperonin genes and variations in these genes in a small set of independent control chromosomes (Hansen et al. 2003). Our genetic investigation of a French family with an autosomal dominant form of spastic paraplegia (SPG13; MIM 605280) identified a missense mutation in the *HSPD1* gene (c.292G>A/p.Val98Ile) that segregated with the disease phenotype and was shown to compromise chaperonin function (Hansen et al. 2002).

To further extend the search for disease-associated variations in the *HSPD1/HSPE1* genes we have in the present study screened three patient groups, in which an involvement of the Hsp60/Hsp10 chaperonin system has been indicated or suggested.

Primary disease gene group Two patients reported with multiple mitochondrial enzyme deficiency (Agsteribbe et al. 1993) who displayed decreased activities of a number of mitochondrial metabolic pathways; Hsp60 protein levels in cultured fibroblast cells from one of these patients were shown to be decreased (Briones et al. 1997) suggesting that a defect in the Hsp60 chaperonin might cause deficient folding of various enzymes involved in these pathways.

Modifier gene group Sixty patients presenting with ethylmalonic aciduria who carried either of two polymorphic SCAD missense variations conferring susceptibility to SCAD deficiency (Corydon et al. 2001); although both variant SCAD proteins are functional, they have been shown to cause prolonged association of the variant SCAD proteins with the Hsp60 chaperonin (Pedersen et al. 2003).

Genetic predisposition group Sixty-one cases of sudden infant death syndrome (SIDS); RFLP analysis has indicated overrepresentation of a certain *HSPD1* genotype in a group of SIDS cases (Rahim et al. 1996), and heat stress has been reported as risk factor for SIDS (Byard and Krous 2003).

In these patients, we sequenced all exons, exon-intron junctions and the bidirectional promoter region. The frequencies of the detected variations were analysed in control individuals, and functional analysis of potential effects of variations in the promoter region on transcription and of non-synonymous variations in the coding region on in vivo chaperonin function was performed.

Materials and methods

Patients

SIDS cases

The 61 patients included in the present study form part of an extensive forensic material comprising cases of sudden, unexpected death in infants and young children (0–10 years old) that have been autopsied at the Institute of Forensic Medicine, University of Aarhus, in the years 1989–1998. A part of the samples has previously been used in an investigation of the possible relationship between fatty acid oxidation disorders and sudden death in infancy (Lundemose et al. 1997). DNA was purified from cultured primary fibroblast cells isolated from Achilles tendon biopsies taken at the autopsy. The ethical committee (Den Videnskabsetiske Komité for Århus Amt) has approved the use of the SIDS material in the present study.

Patients with elevated urinary excretion of ethylmalonic acid and presence of SCAD susceptibility variations (hereafter called EMA/SCAD patients)

Patients referred to metabolic centres in Europe and the USA that displayed elevated levels of ethylmalonic acid in urine (>18 mmol/mol creatinine), and/or other biochemical parameters indicating SCAD deficiency, are routinely submitted to our laboratory for genetic analysis. A large fraction of these patients carry the polymorphic c.511C/T and/or c.625G/A variations in the *ACADS* gene in both alleles or in combination with rare, likely disease-causing mutations in the other allele. The polymorphic variations are present in homozygous or compound homozygous form in 14% of the population but strongly over-represented in patients indicating that other factors contribute. Based on the finding that interaction of the polymorphic SCAD variant proteins with the Hsp60/Hsp10 chaperone system had been shown to be altered (Pedersen et al. 2003), we investigated the *HSPD1* and *HSPE1* genes in 60 patients with the following *ACADS* genotypes (28 c.625A/c.625A, 2 c.625A/c.511T; 2 c.511T/c.511T, 24 c.625A/rare; 4 c.511T/rare).

Patients with multiple mitochondrial enzyme deficiency (MMED)

These were two patients described previously (Briones et al. 1997; Agsteribbe et al. 1993) in whom the activities of a series of mitochondrial enzymes were found to be decreased. Both patients died of their disorder at age 2 days and 4 1/2 years respectively. DNA purified from cultured skin fibroblasts was analysed.

Control individuals

Twenty unrelated control individuals were selected from the Danish Cell bank (Eiberg et al. 1983) including the ten individuals reported earlier (Hansen et al. 2003). To obtain a more precise estimate of the frequencies for rare variations detected in the promoter region (g.3172A>G, g.3191dupC, g.3198C>G) and the amino acid replacing variations in the coding region (c.1136A>G/p.Asp379Gly, c.1676G>A/p.Gly559p.Asp, c.1688G>C/p.Gly563Ala), 30 additional, unrelated control individuals from the Danish Cell bank were typed by sequencing the respective regions of the genome. The frequencies for the c.292G>A/p.Val98Ile variations associated with hereditary spastic paraplegia and the c.551A>G (p.Asn184Ser) variation in 400 unrelated control individuals have been determined previously (Hansen et al. 2002). For the polymorphic variation c.1688G>C/p.Gly563Ala an additional 114 unrelated control individuals were typed using a PCR/RFLP assay.

Sequencing

Screening for variations was performed by direct sequencing in both directions (MMED patients) or one direction (SIDS and SCAD/EMA patient groups) of PCR products spanning exons or the promoter region of the chaperonin genes as described previously (Hansen et al. 2003). For ambiguous stretches in the obtained sequences, amplification and sequencing was repeated. Due to limited availability of material from the SCAD/EMA patient group, repeating sequencing of ambiguous regions was not possible in all cases, but high quality sequences covered >95% of the sequenced regions in this patient group. Sequences were aligned using SEQUENCHER software (Version 3.1.1; Gene Codes Corporation, Mich., USA) and the entire chromatograms were reviewed manually. The presence of rare heterozygous variations was confirmed by repeating PCR amplification and direct sequencing of the respective region in the relevant samples.

Functional analysis of promoter variations

The detected promoter variations were introduced by site-directed mutagenesis (QuikChange; Stratagene, La Jolla, Calif., USA) into the two previously described (Hansen et al. 2003) luciferase promoter reporter plasmids containing a 656-bp long fragment of the intergenic

region between the *HSPD1* and *HSPE1* genes in either orientation. The promoter inserts were sequenced to verify the presence of the respective mutations and thereafter subcloned into another batch of the pGL3 (Promega) parent vector to rule out nucleotide variations introduced into the vector region during the mutagenesis procedure. Promoter activity was analysed after co-transfection of the human embryonic kidney cell line HEK-293 (ATCC) with the luciferase reporter constructs and the CMV-ß-galactosidase vector construct. HEK-293 cells were cultured at 37°C with 5% (v/v) CO_2 in DMEM (Gibco) supplemented with 5% heat-inactivated FCS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Leo, Denmark). Cells were seeded in 12-well plates 1 day prior to transfection. Each well received 75 ng pGL3 vector and 25 ng ß-galactosidase and carrier DNA (empty pcDNA3.1 vector; Invitrogen) to a total amount of 1.5 µg DNA before treatment with FuGENE6 transfection reagent (Roche Diagnostics). Cells were harvested 48 h post transfection in 200 µl lysis buffer (Tropix). Luciferase and ß-galactosidase activity was measured using a MicroLumatPlus LB96V luminometer (Berthold) in combination with the Luc-screen (Applied Biosystems) and Galacto-light plus kits (Tropix), respectively. Correction for transfection efficiency was done by normalising the luciferase activity to the ß-galactosidase activity.

Analysis of missense variations

The non-synonymous variations in the coding region (c.1136A>G/Asp379Gly, c1676G>A/Gly559Asp, and c.1688G>C/Gly563Ala) were introduced, by site-directed mutagenesis (QuikChange or megaprimer PCR-method), into the prokaryotic expression vector that contains cDNA encoding Hsp10 and the processed form of Hsp60 supplemented with a methionine initiation codon as an operon under control of an IPTG-inducible promoter (Hansen et al. 2002). Presence of the respective variations and absence of mutagenesis-induced PCR errors was established by sequencing and subcloning. The resulting vectors were used in the genetic complementation assay described previously (Hansen et al. 2002; Richardson et al. 2001).

Results

Variations in the coding and promoter regions of the HSPD1 and HSPE1 genes

Sequencing of all *HSPD1* and *HSPE1* exons, exon/intron junctions and the common bidirectional promoter was performed on DNA from the 61 SIDS cases, 60 SCAD/EMA patients and two patients with multiple mitochondrial enzyme deficiency.

We found that the multiple mitochondrial enzyme deficiency patient described by Briones et al. (1997) was heterozygous for the rare g.3507T allele (formerly named IR 453C>T; nomenclature of sequence variations follows the recommendations of the Human Gene Variation Society (http://www.hgvs.org/mutnomen/) and numbering is based on accession number AJ250915) in the promoter region. This variation has been detected previously in a control individual, and functional analysis revealed no effect on promoter activity (Hansen et al. 2003). This patient was homozygous for the frequent variant (g.3175C) of the g.3175C/G polymorphism (formerly described as IR 121C/G) whereas the MMED patient described by Agsteribbe et al. (1993) was homozygous for the less frequent allele (g.3175G) of the g.3175C/G polymorphism (formerly described as IR 121C/G). Both patients were homozygous for the more frequent alleles of the silent variations in exons 2 and 3 (c.69T and c.273A; Fig. 1). Taken together, this defies the notion that mutations in the *HSPD1* and *HSPE1* promoter, coding regions and exon/intron junctions cause the phenotype.

Analysis of the SIDS and SCAD/EMA patients revealed six novel variations (Fig. 1; Tables 1, 2), namely two in the promoter region (g.3172A>G, g.3191dupC) and four in the coding region (one silent: c.27C>G and three amino acid replacing variations: c.1136A>G/p.Asp379Gly, c.1676G>A/p.Gly559Asp, c.1688G>C/p.Gly563Ala; amino acid numbering in this article starts with the initiator methionine; as for Hsp60, the first amino acid of the mature polypeptide corresponding to alanine-27 has commonly been used). No variations were detected in the exon/intron junctions.

With regard to the polymorphic variations, the g.3175C/G variation in the promoter, the c.-15C/T variation in the 5′UTR of the *HSPD1* transcript, and the silent c.273G>A variation in the *HSPD1* coding region displayed similar frequencies in the patient and control groups (Tables 1, 2). The silent c.69T variant in the coding region was found to be considerably less frequent (8%) in the 20 fully sequenced control individuals as compared to the patient groups (16.4% in the SIDS and 19% in the SCAD/EMA group, respectively). This variation is registered in the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/; see Table 1) and frequencies of 8 and 22% in a multinational and Japanese population, respectively, have been measured indicating that ethnic differences may be the reason for the discrepancy. The SCAD/EMA

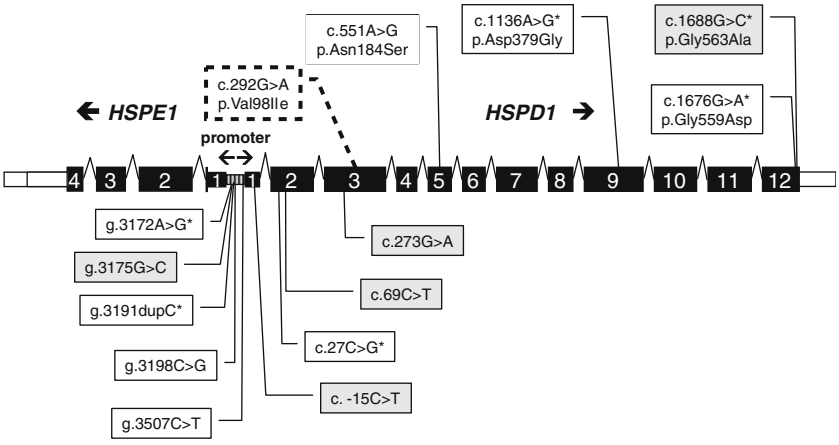

Fig. 1 Variations in the coding region of the *HSPD1* and *HSPE1* chaperonin genes and the bidirectional promoter. The exon and promoter regions are drawn to scale. The position of introns and the direction of transcription of the genes are indicated. Non-synonymous variations in the coding region are indicated *above* and variations in the promoter and silent variations in the coding region *below the drawing*. The mutation associated with hereditary spastic paraplegia in a French kindred is boxed with a *stippled line*. Polymorphic variations are shaded in *grey* and novel variations detected in the present study are marked with an *asterisk*. Numbering refers to the genomic and cDNA sequence coordinates from accession number AJ250915

Table 1 Frequencies of variations in the HSPD1 gene in patient groups and controls: promoter and 5'UTRs

Variation	Promoter					5′ UTR
	g.3172A>G*	g.3175C>G	g.3191dupC*	g.3198C>G	g.3507C>T	c.-15C>T
SIDS	0/122 <0.8%	33/122 27%	0/122 <0.8%	0/122 <0.8%	0/122 <0.8%	20/122 16.4%
EMA/SCAD	1/120 0.8%	28/120 23%	1/120 0.8%	0/120 <0.8%	0/120 <0.8%	18/116 16%
Controls (fully sequenced)	0/40 <2.5%	8/40 20%	0/40 <2.5%	1/40 2.5%	1/40 2.5%	3/20 15%
Frequency in extended control sample	0/100 <1%	25/100 25%	0/100 <1%	1/100 1%	–	
Our dbSNP accession number accession numbers for the same variation in dbSNP	ss65658187	ss65658181 rs1116734	ss65658188	ss65658182	ss65658183	ss65658184 rs3190642 rs3749095 rs13165 rs16874342

Nucleotide positions refer to accession number AJ250915 (genomic sequence) or the deduced cDNA sequence referring to the 'A' in the ATG start codon as position 1. Variations newly detected in the present study are marked with an asterisk. The number of detected variants per alleles studied and the allele frequencies in percent are given. For some variations, values including the 20 fully sequenced controls and an additional number of control alleles, which were only typed or sequenced in the respective regions, are also given. All variations were submitted to dbSNP (http://www.ncbi.nlm.nih.gov/SNP/). Accession numbers from our submission (publicly available starting with BUILD 127) and of previous submissions of the same variant sites are given

patients represent a multinational group whereas the SIDS cohort like the control material is mostly of Danish descent.

With the exception of the polymorphic variation in the coding region (c.1688G>C/p.Gly563Ala), all novel variations detected in this study were only found in one allele of a single patient and absent in the analysed control individuals. The non-synonymous c.551A>G/p.Asn184Ser variation present in one allele of the SIDS cohort was earlier found in a patient with spastic paraplegia, but was absent in affected family members from the same kindred (Hansen et al. 2002). It was also detected in 1 out of 800 alleles from 400 unrelated Danish control individuals, and functional analysis using a bacterial complementation system showed that it had no significant effect.

The overall number of variations detected in our studies in the *HSPD1-HSPE1* gene region is 14 (Fig. 1). Three (g.3175C>G, g.3507C>T, c.-15C>T) of the 14 nucleotide variations are localised in hypermutable CpG dinucleotides. Of the 14 variations that we have detected in the present and previous work in

Table 2 Frequencies of variations in the HSPD1 gene in patient groups and controls: coding region

Variation	Coding region							
	c.27C>G* silent	c.69C>T silent	c.273G>A silent	c.292G>A p.Val98Ile	c.551A>G p.Asn184Ser	c.1136A>G* p.Asp379Gly	c.1676G>A* p.Gly559Asp	c.1688G>C* p.Gly563Ala
SIDS	0/122	20/122	33/122	0/122	1/122	0/122	0/122	3/122
	<0.8%	16%	27%	<0.8%	0.8%	<0.8%	<0.8%	2.5%
EMA/SCAD (120)	1/120	23/120	28/120	0/120	0/120	1/120	1/120	3/120
	0.8%	19%	23%	<0.8%	<0.8%	0.8%	0.9%	2.5%
Controls (40)	0/40	3/40	8/40	0/40	0/40	0/40	0/40	0/40
	<2.5%	8%	20%	<2.5%	<2.5%	<2.5%	<2.5%	<2.5%
Frequency in extended control sample	–	–	–	0/800	1/800	0/100	0/100	3/228
	–	–	–	<0.125%	0.125%	<1%	<1%	1.3%
Our dbSNP accession number	ss65658189	ss65658185	ss65658186	ss65658194	ss65658193	ss65658190	ss65658191	ss65658192
accession numbers for the same variation in dbSNP	rs11551349 rs17857718 rs17844973	Rs1050347 rs17849711	rs3175511 rs17849710 rs8539					

See footnote to Table 1 for explanations

altogether 326 chromosomes from patients and control individuals, 5 had previously been reported to the dbSNP database (see Tables 1, 2). Six non-synonymous variations in the *HSPD1* and *HSPE1* coding region reported in dbSNP have not been observed in any of the 326 chromosomes that we have sequenced. A large number of intron-less *HSPD1* and *HSPE1* pseudogenes are present in the human genome (see http://www.pseudogene.org and Hansen et al. 2003; Pochon and Mach 1996) and some of the reported variations likely arise from them. It is thus advisable to proceed with caution when using this information, and validate variations in the chaperonin genes by using PCR primers situated in the intron regions.

Based on our sequencing data, the nucleotide diversity in the patient groups is somewhat higher than in the control group (Table 3). In general, the nucleotide diversity in the *HSPD1* and *HSPE1* coding region in the patient groups and controls is markedly lower than the average gene diversity (1/346) obtained by Cargill and coworkers by screening of 106 potential disease related genes in 114 independent alleles (Cargill et al. 1999). It is likely that the lower diversity in the chaperonin genes reflects their high conservation throughout evolution.

Investigation of the effect of the variations situated in the promoter

The two newly detected variations (g.3172A>G, g.3191dupC) and the previously reported polymorphic g.3175C>G variation in the bidirectional promoter region were analysed using a luciferase reporter assay as described earlier (Hansen et al. 2003). The g.3172A>G variant showed a tendency to slightly increased basal promoter activity in the *HSPD1* direction (Fig. 2), whereas the other two variants displayed very similar basal promoter activity in both directions.

Investigation of the effect of the non-synonymous variations in the coding region

The three newly-detected non-synonymous variations in the *HSPD1* coding region (p.Asp379Gly, p.Gly559Asp, and p.Gly563Ala) were analysed using the *Escherichia coli* complementation system used previously (Hansen et al. 2002). In brief, plasmids carrying cDNA encoding the mature part of the respective Hsp60 variant proteins together with the Hsp10 co-chaperonin as an operon were transformed

Table 3 Variation in the *HSPD1* and *HSPE1* genes and the bidirectional promoter in patient groups and controls

Groups (number of alleles tested)	Coding region				Promoter + 5′ UTR	
	Total		Non-synonymous			
	Variant sites	Frequency (variations/bp)	Variant sites	Frequency (variations/bp)	Variant sites	Frequency (variations/bp)
SIDS (122)	57	1/4,347	4	1/61,945	53	1/1,280
SCAD/EMA (120)	57	1/4,276	5	1/48,744	48	1/1,390
Controls (40)	11	1/7,385	0	<1/81,240	12	1/1,853

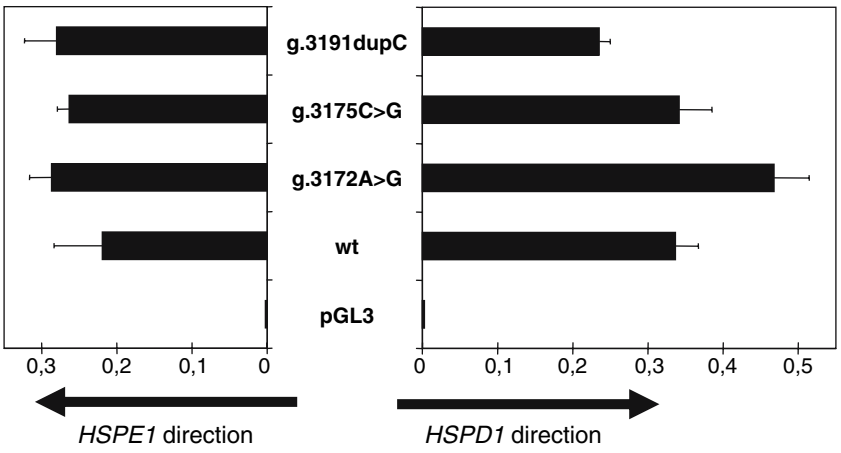

Fig. 2 Analysis of the effect of the detected variations on activity of the bidirectional promoter. Luciferase reporter plasmids carrying a 656 bp fragment comprising the bidirectional promoter with the indicated promoter variations in both the *HSPD1* and *HSPE1* orientation were, together with a plasmid carrying a cytomegalo virus promoter driven ß-galactosidase cDNA, used to transfect HEK-293 cells (see Materials and methods for details). Cells were grown at 37°C, harvested after 48 h and luciferase and ß-galactosidase activity were determined. Triplicate transfections were performed with two different DNA preparations of each construct. The quotient of the values obtained from the luciferase and ß-galactosidase measurements normalised to the wild type in the *HSPD1* or *HSPE1* direction, respectively, are given. As the two data sets had equal variances, the values from both series were combined and common standard deviations (*error bars*) were calculated. 'wt' refers to the sequence carrying the most frequent alleles in all variant positions; the variants carry the indicated variant nucleotide in relation to 'wt'

into *E. coli* B174 cells. The endogenous *groESL* chaperonin genes were subsequently deleted using phage transduction and homologous recombination. As the bacterial chaperonins are indispensable genes, the ability of the respective expressed Hsp60 variant protein together with wild type Hsp10 to support growth of *E. coli* cells lacking the endogenous chaperonins was scored as a measure for its functional capacity. All three variants were able to functionally complement the deletion of the *E. coli groESL* genes suggesting that none of the mutations exerts a major effect on chaperonin function. Furthermore, no apparent differences in growth characteristics at various temperatures could be observed between the transductants carrying plasmids encoding the different Hsp60 variants as compared to transductants with the plasmid encoding wild type Hsp60 (data not shown).

Discussion

Although molecular chaperones have been in focus for more than two decades, and variations in their corresponding genes have repeatedly been suggested as potential modifiers in various disease processes, surprisingly few studies have addressed variations in these genes in humans. Recently, variations in the Hsp90alpha and Hsp90beta genes in 73 Caucasians have been reported (Passarino et al. 2003) and subsequent investigations of their functional effects using a yeast-based assay system revealed that the non-synonymous variant p.Gln488His in the alpha-Hsp90 gene was severely defective (MacLean et al. 2005). Altogether 14 variations in the three highly inducible cytosolic Hsp70 genes *HSP70-1, HSP70-2* and *HSP70-HOM* have been described, and some of them have been shown to be associated with various diseases, such as rheumatoid arthritis, multiple sclerosis, insulin-dependent diabetes mellitus and ageing just to name a few (see Singh et al. 2004 and references therein).

In the present study, we selected a spectrum of diseases where it was indicated that variations in the mitochondrial chaperonin genes could represent disease-associated or disease-modifying factors. In the MMED patients, decreased activities of a series of mitochondrial enzymes were observed. Detailed analysis of one patient showed low levels of Hsp60 protein and a partial deficiency in Hsp60 was suggested as the likely cause (Huckriede et al. 1995; Huckriede and Agsteribbe 1994; Agsteribbe et al. 1993). Our sequencing results of this patient revealed only polymorphic variations in the chaperonin gene coding, intron/exon junction and promoter regions. The patient reported by Briones et al. (1997) displayed heterozygosity for the g.3507C>T variation in the promoter. This variation has been reported previously, and functional analysis of its impact for promoter activity revealed no significant effect (Hansen et al.

2003), arguing against the notion that mutations in the chaperonin genes play a role in these patients.

For the SCAD/EMA patient group, a modifying effect of variations in the chaperonin genes on SCAD enzyme folding was suggested by the observation that the two non-synonymous polymorphic *ACADS* gene variants c.511T and c.625A, which are strongly over-represented in the patient group compared to controls (Corydon et al. 1996; Corydon et al. 2001), cause prolonged interaction of their encoded SCAD variant proteins with the Hsp60 chaperone and delayed folding and assembly to the active tetramer (Pedersen et al. 2003). Variations in the chaperonin genes that compromise their function or affect their expression could possibly exacerbate this mild effect and trigger disease. In 60 patients presenting with ethylmalonic aciduria and carrying one of these two rare *ACADS* variations in at least one allele, we detected two variations in the *HSPD1/HSPE1* promoter region (g.3172A>G, g.3191dupC) and two non-synonymous variations in the *HSPD1* coding region (c.1136A>G/p.Asp379Gly, c.1676G>A/p.Gly559Asp). These variations were found in one allele of single patients each and could potentially explain the defect in these patients. However, analysis of their potential effects on expression or function revealed no significant impact. Because, in addition, no significant over- or under-representation of the polymorphic variations in the mitochondrial chaperonin genes could be observed, we conclude that variations in the mitochondrial chaperonin genes play no significant role in the SCAD/EMA patient group.

Finally, in the SIDS cohort study, a possible link to the mitochondrial chaperonin genes was suggested by the finding that hyperthermia, which is known to induce *HSPD1* and *HSPE1* transcription, represents a risk factor (Gozal 1996). In the 61 SIDS cases investigated there was no significant over- or under-representation of polymorphic sequence variations. One patient was heterozygous for the non-synonymous c.551A>G/p.Asn184Ser variation. This variation has been reported earlier, as has the functional analysis of its p.Asn184Ser variant protein in a bacterial complementation assay, which showed no dramatic effect on chaperonin function (Hansen et al. 2002). These results suggest that *HSPD1* and *HSPE1* gene variations represent no significant factor for the development of SIDS. The MspI RFLP observed with a *HSPD1* cDNA probe, and reported to be overrepresented in a SIDS cohort (Rahim et al. 1996), most likely represents a polymorphism that resides in a *HSPD1* pseudogene, since the MspI sites in this chromosome region predicted by the complete human genome sequence are not compatible with the chromosomal DNA fragments observed by these authors (data not shown). Furthermore, the statistical significance of the finding has been challenged (Tanner et al. 1997).

The rare variations in the promoter region and the non-synonymous variations in the coding region detected in this report may still exert mild effects contributing to the phenotype in a few patients. Only for the g.3172A variant was a small increase of promoter activity indicated experimentally. The facts that the g.3172A>G variant site, like the g.3175G>C g.3191dupC sites, is situated in a short sequence stretch that is absent in the mouse and rat promoter sequences (Hansen et al. 2003), and that all five so far detected variations in the *HSPD1/HSPE1* promoter region are situated distantly from the heat-shock factor binding elements and the CHOP binding motif involved in the heat-shock response and a mitochondria-specific stress-response, respectively (Zhao et al. 2002), argue against a major impact of any of them.

Functional testing of the non-synonymous variations in the coding region using the bacterial complementation assay that is capable of revealing dramatic effects, such as the one exerted by the mutation (c.292G>A; p. Val98Ile) associated with hereditary spastic paraplegia (Hansen et al. 2002), showed no detectable effect, arguing against them having a major impact. Two of the newly detected variations that alter amino acids in Hsp60 are localised in a stretch encoding the Gly-Gly-Met iterations in the carboxy terminus of the protein. Similar sequences are present in the carboxy terminus of many, but not all, type I chaperonins (Brocchieri and Karlin 2000). A four-fold Gly-Gly-Met repeat region is present in *E. coli* GroEL C-terminus. Its structure is not resolved in the X-ray crystals suggesting a flexible conformation. Deletion of the entire repeat region in GroEL has indicated that it is dispensable, although deletion had some effect on ATPase activity and the ability to suppress temperature-sensitive mutations (Mclennan et al. 1993). Moreover, a recent study showed that replacement of the methionine residues by alanine decelerated folding of a mutant maltose binding protein (Tang et al. 2006).

The variant positions of the p.Asn184Ser and the p.Asp379Gly variations correspond to lysine-160 and glutamic acid-354 in *E. coli* GroEL, respectively. In the *E. coli* GroEL structure, both residues are situated at the outer surface of the ring structure. Inspection of alignments shows that the mutated asparagine-184 is not well conserved, but a negatively charged residue at the position corresponding to aspartic acid-379 in human Hsp60 is conserved in the large majority of chaperonins.

In summary, we have described a series of variations in the *HSPD1/HSPE1* promoter and coding regions that may exert potential effects on chaperonin expression and function. Although the detected variations do not play a major role in the disease groups investigated, they may still represent modifiers with subtle effects. Although variations in chaperone genes have been used in a large number of association studies, studies on functional tests of the impact of these variations have been reported very rarely. Clearly, functional testing of variations that exert small effects is difficult, and devising appropriate assay systems that can reveal small effects presents a challenge for future research in the field of multifactorial diseases and modifier genes.

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