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VARIANTS OF THE MOLECULAR CHAPERONE HSPA8 AND HSPA1A GENES IN TRIMETHYLAMINURIA: A PILOT STUDY

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ABSTRACT

There is discrepancy in the phenotypic manifestations of trimethylaminuria (TMAU) between patients suggesting a certain diversity of etiological-pathogenic factors. Primary TMAU is linked to mutations in the FMO3 gene but a proportion of patients do not carry mutations in it or carry single nucleotide polymorphisms (SNPs) that do not have an impact on the gene's product, the enzyme FMO3. It remains to be established what other factors are pathogenic in TMAU underpinning the various phenotypes. We hypothesized that defective chaperones could contribute to the pathogenesis by, for example, failing to assist FMO3 in its folding and refolding cycles. In the initial screening reported here we investigated two chaperone genes, HSPA8 and HSPA1A in twelve TMAU patients and found that variants in the former were highly represented in comparison with controls. Further studies, including more patients are underway to firmly establish the prevalence of the variants and to begin elucidating molecular mechanisms.

1. Introduction

Trimethylaminuria (TMAU, OMIM#602079) is a metabolic disorder characterized by excretion of trimethylamine (TMA) in body fluids, such as sweat, urine, and via breath. Primary TMAU is a hereditary condition linked to germline mutations in the *FMO3* gene (*FMO3*, HGNC: 3771, 1q24.3) (1). *FMO3* encodes the flavin-containing dimethylaniline monooxygenase3 enzyme that catalyses *N*-oxidation of TMA, converting it to trimethylamine-*N*-oxide (TMAO). TMAO is physiologically excreted by urine. Trimethylamine is a malodorous compound and oxidation is a crucial reaction to eliminate its smell. Loss-of-function mutations in the *FMO3* gene result in failure of TMA malodour deactivation, and when it is excreted it smells of rotten fish. For this reason, TMAU is also known as "fish odour syndrome" (2). The disease is not lethal; however, because of the unpleasant odour emanating from the body, patients suffer devastating social discomfort that often leads to depression, psychiatric disorders, and even suicidal behaviour (3).

A proportion of patients with the TMAU phenotype do not carry mutations in the *FMO3* gene or carry single nucleotide polymorphisms (SNPs) that do not alter FMO3 enzymatic activity (4). It was hypothesized that these patients suffer a secondary form of TMAU.

These include TMAU phenotypes associated with hormonal deficiencies, chronic liver inflammation, certain medications, or alterations of the gut microbiota (5-7).

In the work reported here, we investigated another aspect of TMAU aetiology-pathogenesis that has not yet been discussed in the literature and pertains to the possible involvement of molecular chaperones. These are components of the chaperoning system (CS), which also includes co-chaperones, chaperone co-factors, and chaperone interactors and receptors (8). The canonical functions of chaperones are directed to the maintenance of protein homeostasis and include assistance of nascent polypeptides in their folding pathway to reach a native functional conformation, assembly of tertiary and quaternary structures, refolding of partially denatured proteins, translocation of proteins to their place of work, and ushering damaged or useless proteins to degradation machineries such as the ubiquitin proteasome system.

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Thus, chaperones are cytoprotective, but they can contribute to the mechanism of disease if they are abnormal in structure, function, quantity, or location. Disease in which defective chaperones play and etiological-pathogenic role are the chaperonopathies (9). These can be genetic or acquired with the former caused by a pathogenic variant in a chaperone gene, which affects or eliminates the functions of the chaperone protein. Typically, genetic chaperonopathies present a clear clinical picture of relatively easy diagnosis, but in many cases, they may go undiagnosed, mostly because the medical community is still largely unaware of the existence of these diseases. The entity chaperonopathy does not usually enter in the differential diagnosis algorithm and the condition is misdiagnosed. Furthermore, chaperonopathies might be hidden under the manifestation of a well-known disease, which however does show also signs and symptoms that do not fit within the standard picture for the condition (10). A chaperonopathy is an etiological-pathogenic factor added to the canonical aetiology of the disease. An example of the latter is provided by a fraction of cases of phenylketonuria (PKU). Typically, PKU is associated with mutations in the gene coding for the enzyme phenylalanine hydroxylase (PAH), but a small proportion of patients have tetrahydrobiopterin (BH4) deficiency and show neurotransmitter abnormalities. However, there are cases with PKU clinical picture that do not have PAH mutations or BH4 deficiency but bear pathogenic mutations in the gene encoding the co-chaperone DnaJC12 (11-14). This molecule is a member the chaperoning system and as such plays a critical role together with its partner chaperones in the maturation of the enzymes phenylalanine, tyrosine, and neuronal tryptophan hydroxylases. When DnaJC12 is genetically defective the activity of these three enzymes is diminished or abolished, causing HPA and neurotransmitter deficiency.

We hypothesize that a similar mechanism might be in operation in some patients with TMAU, considering the variations in the clinical manifestations observed in them. A chaperonopathy may be at the basis of defective FMO3, which may not fold correctly to achieve a full functional configuration, or may be degraded faster than normally. To initiate our search, we focused on two chaperones that belong to the Hsp70 family which has 17 members: the Heat shock cognate 71 kDa protein (Hsc70), which is constitutively expressed, and Heat shock 70 kDa protein 1A(Hsp72) which is stress-inducible (15); the current standard names of these human chaperones are HSPA8 and HSPA1A, respectively (16). We searched variants of the genes encoding these two chaperones in TMAU patients and controls and correlated the finding with phenotype.

2. Methods

Patients

Our cohort was made by 12 (n = 12) Sicilian patients with diagnosed TMAU who were previously screened for the *FMO3* gene. They were included in our search for variants of *HSPA8* and *HSPA1A.* The group was heterogeneous for sex and age and all members showed the TMAU phenotype.

Control group

The control group consisted of 150 healthy subjects (n = 150). They were all Sicilian and randomly selected. We selected Sicilian controls reflecting the geographical origin of the patients. Genetically, Sicilian population is very peculiar due to the several dominations over the centuries (17-19). The genes *HSPA8* and *HSPA1A* were examined by mutational analysis was performed, the same as for the patients.

DNA purification and analysis

DNA was purified from whole peripheral blood (buffy coat) by the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), following manufacturer's protocol. *FMO3*, *HSPA8* and *HSPA1A*coding regions and exon-intron boundaries were amplified by polymerase chain reaction (PCR). Primer sequences and PCR conditions are available upon request. Sanger sequencing was carried out by using the Big Dye Terminator© v3.1 Cycle Sequencing Kit chemistry and run on a 3130*xl* Genetic Analyzer (Applied Biosystems™, Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

Variants showing significant differences in frequencies between patients and controls were analyzed to calculate statistical significance. The Association Study was conducted by SPSS® Statistics (IBM Analytics, Armonk, New York, USA). Because of the low sample number, statistical significance was estimated by the Fisher test.

3. Results

FMO3 screening in the patients revealed variants in 10 of them, and the other two patients showed the wild-type *FMO3* nucleotide sequence (Table 1).

Most of the variants were SNPs and most of them are reported in the Human Genome Mutation Database (HGMD, http://www.hgmd.cf.ac.uk/ac/index.php).*FMO3*variants were mostly heterozygous, while TMAU phenotype usually arises as autosomal recessive condition.

For *HSPA8* and *HSPA1A*, 15 and 13 SNPs were found, respectively (Tables 2 and 3).

Fisher's test applied to calculate statistical significance revealed no significant association between HSPA1A SNPs and TMAU phenotype. In contrast, 7 SNPs in the HSPA8 gene were highly represented in the patients, when compared to the healthy controls (Table 4). Of these SNPs, the rs1136141c.-11C>T affects the 5'-UTR; and rs4935825, rs10892958, rs3057456, rs201521469, and rs770829808 fall within non-coding exons, spanning 100 nucleotides from the exon splice sites. The rs1064585 c.1455A>C; p.Ile485= is a synonymous variant.

VARIANT		PATIENT (P) AND GENOTYPE[1]											
Variant ID (dbSNP)	**Coding sequence – amino acid changes**	*P1*	*P2*	*P3*	*P4*	*P5*	*P6*	*P7*	*P8*	*P9*	*P10*	*P11*	*P12*
rs1136141	*c.-11C>T*	C/T	C/C	C/C	C/T	C/C	C/C	C/C	C/C	C/C	C/T	C/C	C/C
rs145277103	*c.-6+83_6+84insA*	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/A	-/-	-/-	-/-
rs4935825	*c.-5-328T>G*	T/T	T/G	T/T	G/G	G/G	G/G	T/G	G/G	T/G	T/T	T/G	T/G
rs10892958	*c.-5-88G>A*	G/G	G/A	G/G	G/G	G/G	G/G	G/G	G/G	G/A	G/A	G/G	G/G
rs3057456	*c.206-24_206-23delGT*	GT/GT	GT/-	GT/GT	GT/-	GT/GT	GT/GT	GT/GT	GT/GT	GT/-	GT/-	GT/GT	GT/GT
rs74853789	*c.411+33C>T*	C/C	C/C	C/C	C/C	C/C	C/T	C/C	C/C	C/C	C/C	C/C	C/C
rs149047184	*c.412-100_412-99insG*	-/-	-/G	-/-	-/G	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
rs201521469	*c.1120+5A>C*	A/A	C/C	A/A	C/C	A/A	A/A	A/A	A/A	C/C	C/C	A/A	A/A
rs770829808	*c.1120+77_1120+79delTTC*	TTC/TTC	TTC/-	TTC/TTC	TTC/-	TTC/TTC	TTC/TTC	TTC/TTC	TTC/TTC	TTC/-	TTC/-	TTC/TTC	TTC/TTC
rs1461496	*c.1324-8dT>C*	T/C	C/C	T/T	C/C	C/C	C/C	T/C	C/C	C/C	T/C	C/C	T/C
rs1064585	*c.1455A>C;p.Ile485=*	A/A	A/C	A/A	A/C	A/A	A/A	A/A	A/A	A/C	A/C	A/A	A/A
rs77374206	*c.1523-9C>A*	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/A	C/C	C/C	C/C
rs4502	*c.1761T>C;p.Ala587=*	T/T	T/C	T/T	T/C	T/T	T/T	T/T	T/T	T/C	T/C	T/T	T/T
rs7948948	*c.*267T>C*	T/T	T/C	T/T	T/C	T/T	T/T	T/T	T/T	T/C	T/C	T/T	T/T
rs1181171965	*c.1815C>A;p.Pro605=*	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/C	C/A	C/C	C/C

Table 1. *FMO3* variants in TMAU patients (Variants are in boldface)

VARIANT		PATIENT (P)ANDGENOTYPE[1]											
Variant ID (dbSNP)	**Coding sequence – amino acid changes**	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**	**P10**	**P11**	**P12**
rs1008438	*c.-128A>C*	A/A	A/A	A/A	A/A	A/A	A/C	A/A	A/A	A/C	A/A	A/A	A/A
rs11557922	*c.-97T>C*	T/T	T/T	T/T	T/T	T/T	T/C	T/T	T/T	T/T	T/T	T/T	T/T
rs201753050	*c.-37A>G*	A/A	A/G	A/A	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/A
rs200771637	*c.-21G>C*	G/G	G/C	G/G	G/C	G/C	G/C	G/C	G/C	G/C	G/C	G/C	G/G
rs1043618	*c.-27G>C*	G/G	G/C	G/G	G/C	G/C	G/C	G/C	G/C	G/C	G/C	G/C	G/G
rs780554980	*c.-18_-17delAG*	G/G	AG/-	AG/-	AG/-	AG/-	AG/-	AG/-	AG/-	AG/-	AG/-	AG/-	AG/-
rs2242660	*c.-7A>C*	A/A	A/C	A/C	A/C	A/C	A/C	A/C	A/C	A/C	A/C	A/C	A/C
rs10434630	*c.222T>C p.Ile74=*	T/T	T/T	C/C	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	C/C
rs562047	*c.330 G>C;p.Glu110Asp*	G/G	G/G	G/C	G/G	G/G	G/C	G/C	G/C	G/C	G/G	G/G	G/C
rs112190359	*c.348C>T;p.Pro116=*	C/C	C/C	C/C	C/C	C/C	C/C	C/T	C/T	C/C	C/C	C/C	C/T
rs1061581	*c.1053G>A;p.Gln351=*	G/G	G/G	G/G	G/A	G/A	G/G	G/A	G/A	G/G	G/A	G/A	G/G
rs5506770	*c.1695G>A;p.Ala565=*	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A
rs5341340	*c.1710G>T p.Val570=*	G/T	G/T	G/T	G/T	G/T	G/T	G/T	G/T	G/T	G/T	G/T	G/T

Table 2. *HSPA8* variants in TMAU patients (Variants are in boldface)

Variant ID (dbSNP)	Coding sequence – amino acid changes	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
rs1008438	*c.-126A>C*	A/A	A/A	A/A	A/A	A/A	A/C	A/A	A/A	A/C	A/A	A/A	A/A
rs11557922	*c.-97T>C*	T/T	T/T	T/T	T/T	T/T	T/C	T/T	T/T	T/T	T/T	T/T	T/T
rs201753050	*c.-37A>G*	A/A	A/G	A/A	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/A
rs200771637	*c.-21G>C*	G/G	G/C	G/G	G/C	G/C	G/C	G/C	G/C	G/C	G/C	G/C	G/G
rs1043618	*c.-27G>C*	G/G	G/C	G/G	G/C	G/C	G/C	G/C	G/C	G/C	G/C	G/C	G/G
rs760554980	*c.-18_-17delAG*	G/G	AG/-	AG/-	AG/-	AG/-	AG/-	AG/-	AG/-	AG/-	AG/-	AG/-	AG/-
rs2242667	*c.-7A>C*	A/A	A/C	A/C	A/C	A/C	A/C	A/C	A/C	A/C	A/C	A/C	A/C
rs1043620	*c.2222T>C p.Ile74=*	T/T	T/T	C/C	T/T	T/T	T/T	T/T	T/T	T/T	T/T	T/T	C/C
rs562047	*c.330 G>Cp.Glu110Asp*	G/G	G/G	G/C	G/G	G/G	G/C	G/C	G/C	G/C	G/G	G/G	G/C
rs12190359	*c.348C>Tp.Pro116 =*	C/C	C/C	C/C	C/C	C/C	C/C	C/T	C/T	C/C	C/C	C/C	C/T
rs1061581	*c.1053G>Ap.Gln35 1=*	G/G	G/G	G/G	G/A	G/A	G/G	G/A	G/A	G/G	G/A	G/A	G/G
rs506770	*c.1695G>Ap.Ala56 5=*	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A
rs541340	*c.1710G>T p.Val570=*	G/T	G/T	G/T	G/T	G/T	G/T	G/T	G/T	G/T	G/T	G/T	G/T

Table 3. *HSPA1A* variants in TMAU patients (Variants are in boldface)

Gene	Variant id.[1]	Patient: variant carriers (total cohort: n = 12)		Controls: variant carriers (total cohort: n = 150)		p-Value (Fisher test)
		Number	Frequency	Number	Frequency	
HSPA8	rs1136141*	4	25	1	0.67	0.0012
	rs145277103	1	8.3	18	12	1
	rs4935825*	9	75 (44.4% homozygous)	1	0.67	< 0.00001
	rs10892958*	4	25	0	0	0.0003
	rs3057456*	3	33.3	7	4.7	0.0044
	rs74852789	1	8.3	15	10	1
	rs149047184	2	16.7	26	17.3	1
	rs201521469*	3	33.3	3	2	0.0006
	rs770829808*	3	33.3	2	1.3	0.0002
	rs1461496	11	91.7 (77.8% homozygous)	97	64.7 (46.4% homozygous)	0.0628
	rs1064585	3	33.3	6	4	0.0029
	rs77374206*	1	8.3	21	14	1
	rs4802	3	33.3	49	32.7	1
	rs7948948	3	33.3	47	31.3	1
	rs118171965	1	8.3	29	19.3	0.6979
HSPA1A	rs1008438	2	16.7	37	24.7	0.7323
	rs11557922	1	8.3	26	17.3	0.6924
	rs201753050	9	75	71	47.3	0.0776
	rs200771637	9	75	67	44.7	0.0683
	rs1043618	9	75	99	66	0.7522
	rs760554980	11	91.7	106	70.7	0.1817
	rs2242667	11	91.7	113	75.3	0.2974
	rs1043620	2	16.7 (homozygous)	34	22.7	1
	rs562047	6	50	93	62	0.5402
	rs12190359	3	25	54	36	0.5428
	rs1061581	6	50	46	30.7	0.2023
	rs506770	12	100	127	84.7	0.2188
	rs541340	12	100	124	82.7	0.2171

Table 4. Association of HSPA8 and HSPA1A variants with TMAU phenotype (*significant p-Value ≤0.05).

4. Discussion

Seven HSPA8 SNPs were identified as highly represented in TMAU patients, when compared to 150 healthy subjects.

The functional consequences of these variants are difficult to predict due to the large number of HSPA8 alternative transcripts and the scarce knowledge about them.

At least 24 alternative transcripts were indeed detected and seven of these are non-coding transcripts showing regulatory functions (https://www.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000109971;r=11:123057489-123063230).

Six of the seven variants we detected in our cohort of patients involve exons that are non-coding in the canonical isoform. Intronic variants were already reported as associated to several diseases being they able to alter splicing process (20)

The other variant, rs1064585, involves the coding region; however, it leads to no amino acid change (1455A>C; p.Ile485=).

HSPA8 has been shown to be involved in clathrin uncoating of endocytic vesicles, vesicle-mediated transport, and chaperone-mediated autophagy (CMA) (21,22). CMA is an autophagy-lysosome pathway that degrade cytosolic proteins in lysosomes without vesicle formation. Phenotypes associated to HSPA8 gene variants include non-insulin-dependent diabetes mellitus (23,24). Moreover, in the liver, HSPA8 is involved in lipid homeostasis (25). Therefore, our current hypothesis is that HSPA8 is necessary for amino compound metabolism through its active role in FMO3 folding, or degradation in lysosomes.

5. Conclusion and perspectives

The results reported were derived from a pilot study devised to obtain preliminary information that might help in determining if chaperonopathies contribute to pathogenesis in TMAU. The initial hypothesis postulates that in some TMAU patients a defective chaperone contributes to the malfunctioning of the FMO3 enzyme by failing to assist it in its folding pathway and/or in stabilizing its functional conformation, or by accelerating its degradation. *HSPA8* gene variants were highly represented in TMAU patients in comparison with controls. Although, to date, there is little evidence of interaction between HSPA8 and FMO3, two possible mechanisms for further investigation can be considered. The first regards the role of HSPA8 in FMO3 folding, assembly, and stabilization. The second one is related to HSPA8 as mediator of FMO3 degradation.

The results of this pilot study clearly encourage its continuation with more patients and experimental models to assess the role of HSPA8 abnormalities in TMAU. This research is likely to open new avenues for treatment of this psychologically devasting disease by focusing on the chaperone.

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