Large copy-number variations in patients with statin-associated myopathy affecting statin myopathy-related loci.

Short title: CNVs and statin myopathy

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Summary

Background & aims: Some patients are susceptible to statin-associated myopathy (SAM) either because of genetic variations affecting statin uptake and metabolism, or because they predispose their carriers to muscular diseases. Among the frequent variants examined using the genome-wide association study approach, *SLCO1B1* c.521T>C represents the only validated predictor of SAM in patients treated with high-dose simvastatin. Our aim was to ascertain the overall contribution of large copy-number variations (CNVs) to SAM diagnosed in 86 patients.

Methods: CNVs were detected by whole genome genotyping using Illumina HumanOmni2.5 Exome BeadChips. Exome sequence data were used for validation of CNVs in SAM-related loci. In addition, we performed a specific search for CNVs in the *SLCO1B* region detected recently in Rotor syndrome subjects.

Results: Rare deletions possibly contributing to genetic predisposition to SAM were found in two patients: one removed *EYS* associated previously with SAM, the other was present in *LARGE* associated with congenital muscular dystrophy. Another two patients carried deletions in *CYP2C19*, which may predispose to clopidogrel-statin interactions. We found no common large CNVs potentially associated with SAM and no CNVs in the *SLCO1B* locus.

Conclusion: Our findings suggest that large CNVs do not play a substantial role in the aetiology of SAM.

Key words: statin myopathy, copy number variations, *SLCO1B*

Introduction

Statins acting as HMG-CoA inhibitors decrease total serum and LDL-cholesterol and reduce the risk of ischaemic heart disease. Although statins are generally well tolerated, a significant number of patients suffer from various side effects, including muscle pain and weakness. Myalgia without elevated serum creatine kinase (CK) activity and myopathy with elevated serum CK represent the most frequent causes of statin intolerance (Argov 2015). Although very rare, rhabdomyolysis is the most severe form of myopathy and results in muscle breakdown, myoglobinuria, kidney damage and death (Van Staa *et al.* 2014). The frequency of muscle problems increases with the daily dose of statins (Mosshammer *et al.* 2014). The mechanisms contributing to statin-associated myopathy (SAM) include decreased cholesterol content in muscle cell membranes, decreased production of ubiquinone, decreased prenylation of proteins, increased uptake of cholesterol and plant sterols, impaired metabolism of calcium and decreased production of selenoproteins (Mammen and Amato 2010, Thompson *et al.* 2003, Ucar *et al.* 2000). Moreover, administration of statins may unmask pre-existing myopathy due to heterozygous carrier status for muscle disease causing mutation (Argov 2015).

Family studies suggest the involvement of a strong genetic component in statin-induced muscle toxicity and susceptibility (Hedenmalm *et al.* 2015). Genetic factors predisposing carriers to SAM (Ghatak *et al.* 2010, Mosshammer *et al.* 2014) are divided into several groups – factors affecting (1) blood concentration of statins (variations in genes encoding transporters OATP1B1, OATP1B3, OATP2B1, ABCB1, ABCG2 and enzymes CYP3A4, CYP3A5, CYP2C8, CYP2D6, CYP2D9, CYP1A2, CYP2E1, CYP2A6 and CYP2B6), (2) muscle vascularisation (genes

encoding angiotensin receptor 1 (AGTR1) and NOS3 and (3) concentration of statins in muscle cells (genes encoding transporters OATP2B1, MRP1, MRP4 and MRP5). Additional numerous factors play a role in the aetiology of primary muscle diseases, classified as (4) rare variants underlying disorders of muscle cell energy metabolism in genes encoding myophosphorylase (*PYGM*), alpha-glucosidase (Stroes *et al.* 2015), carnitine palmitoyltransferase 2 (*CPT2*) and myoadenylate deaminase (*AMPD1*), (5) variants responsible for mitochondrial myopathies, (6) variants affecting production of coenzyme Q10 (genes *COQ10A* and *COQ10B*), (7) variants associated with muscle dystrophy (genes encoding dystrophin (*DMD*), myotilin (*MYOT*), lamin A/C (*LMNA*) and caveolin-3 (*CAV3*)) and (8) factors affecting calcium homeostasis (genes encoding the ryanodine receptor 1 (*RYR1*), Na^+/K^+-ATPase (*ATP1A1, ATP1A2, ATP1B1, ATP1G1*) and Ca^{2+}-ATPase (*ATP2A1*)).

Common genetic variations do not seem to be important determinants of statin-induced muscle toxicity (Hopewell *et al.* 2014), with one exception: the SLC*O1B1* rs4149056 genotype (Link *et al.* 2008). In this study we focused on the potential contribution of large rare copy number variants (CNVs) affecting the above-mentioned candidate genes and/or genes involved in functionally related metabolic pathways. Moreover, since *SLCO1B1* c.521T>C (p.V174A) rs4149056 decreases liver uptake of hydrophilic statins and given that allele c.521C is associated with high-dose simvastatin (as found in the SEARCH Collaborative Study (Link *et al.* 2008)), we chose to examine our patients for the presence of known small deletions (Van De Steeg *et al.* 2012) and insertions (Kagawa *et al.* 2015) in the *SLCO1B* locus on chromosome 12p, found previously in patients with Rotor syndrome.

Methods

Selection of study subjects

Eighty-six patients treated with simva-, atorva- or rosuvastatin for familial or polygenic hypercholesterolaemia and suffering from SAM fulfilling the consensual definition of the American College of Cardiology (ACC)/American Heart Association (AHA)/National Heart, Lung and Blood Institute (NHLBI) (Pasternak *et al.* 2002) were enrolled. None of the patients was administered the known inhibitors of *SLCO1B1*/*OATP1B1* – cyclosporine, rifampicin, antibiotics, anticancer drugs, oestrogens, oral contraceptives – for at least one month prior to the onset of myopathy. Secondary causes of myopathy (e.g. hypothyroidism and other endocrine diseases, alcohol abuse, drug-induced) were excluded. Pregnancy was ruled out in fertile women. Consecutive patients who developed SAM over the course of the four-year study were included.

Written informed consent was obtained from all the study participants prior to any study-related procedure. The local ethics committee approved the conduct of the study, respecting the rules of the Declaration of Helsinki of 1975.

DNA genotyping and CNV identification

Genomic DNA of all available individuals was extracted from whole blood samples using the Qiagen DNA micro kit (QIAgen, Hilden, Germany). The quantity and quality of the isolated DNA were verified spectrophotometrically using the NanoDrop 2000 (Thermo Fisher Scientific, Prague, Czech Republic). Genotyping was performed using Illumina HumanOmni2.5 Exome BeadChips (San Diego, CA) at The Microarray Facility of The Centre of Applied Genomics of The Hospital for Sick Children in Toronto according to manufacturer protocol. Raw data were uploaded

into Illumina GenomeStudio version 2011.1 for genotype calling. All samples with a genotype call rate > 99% were subjected to further analysis. Relatedness of investigated subjects was assessed from obtained genotypes in PLINK (Purcell *et al.* 2007).

Extended homozygosity regions > 3 Mb were detected using the 3.2.0 Illumina cnvPartition CNV Analysis Plug-in within GenomeStudio software.

CNVs were identified using PennCNV (Wang *et al.* 2007) and the above-mentioned cnvPartition CNV Analysis Plug-in. Only gains and losses containing a minimum of 10 probes were reported. Gene content of CNVs of interest was functionally annotated in GeneDistiller (Seelow *et al.* 2008) and population frequencies of the identified changes were assessed in the curated catalogue of human genomic structural variations known as DGV (http://dgv.tcag.ca/dgv/app/home).

Validation of identified CNVs

The existence of selected CNVs was independently assessed in exome sequence data, which were made available for each of the samples. DNA for exome sequencing was enriched using SeqCap V3 (NimbleGen) and sequenced on the Illumina HiSeq 1500 system at the University Hospital in Motol (Prague, Czech Republic) as previously described (Kmoch *et al.* 2015).

The resulting FASTQ files were aligned to the human genome reference (hg19) using NovoAlign (Novocraft Technologies, Selangor, Malaysia). Following genome alignment, conversion of SAM format to BAM and duplicate removal were performed using Picard Tools v.1.129 (http://broadinstitute.github.io/picard/). The Genome Analysis Toolkit (GATK) (3.3) was used for local realignment around indels,

base recalibration, variant recalibration and genotyping. CNVs were identified from exome read counts using CONTRA 2.0.6 (Li *et al.* 2012) and CNVkit 0.74 (Talevich *et al.* 2014).

Detection of deletions in the SLCO1B locus found previously in subjects with Rotor syndrome

The 405 kb deletion NCBI37/hg19 g.(21,007,644)_(21,412,242)del(CA)ins, removing exons 3 – 15 of *SLCO1B3* and the whole gene *SLCO1B1*, and the 7.2 kb deletion NCBI37/hg19 chr12:g.(21,035,810)_(21,043,025)del(N205)ins removing *SLCO1B3* exon 12 were genotyped as described in (Van De Steeg *et al.* 2012). The heterozygous state for the 7.2 kb deletion was confirmed by simultaneous amplification of the deleted allele and the *SLCO1B3* exon 12 present in the non-deleted allele. We also screened patients for the presence of recently reported long interspersed element-1 (LINE-1) in intron 5 of *SLCO1B3* (Kagawa *et al.* 2015). For this purpose, we used the PCR-based technique reported in (Kagawa *et al.* 2015). Blood DNA samples taken from Rotor subjects homozygous for each of the tested mutations and from their heterozygous parents served as positive controls.

Results

Clinical and laboratory findings

Eighty-six patients (30 males and 56 females) aged 29 to 84 years (median – 65 years, interquartile range – 7 years) were selected from patient databases comprising approximately 2500 patients treated for dyslipidaemia at the lipid clinics of the 3rd Department of Medicine, 1st Medical Faculty of Charles University and of the Institute for Clinical and Experimental Medicine in Prague. Of these, 51 patients developed myopathy on atorvastatin on a daily dose of 10 - 20 mg (only 2 patients were given 40 mg daily), 20 patients developed muscle symptoms on simvastatin (daily dose 20 – 40 mg) and 12 patients on rosuvastatin (daily dose 10 – 20 mg, 2 patients were given 40 mg daily). One patient presented with muscle symptoms repeatedly on both atorvastatin and rosuvastatin and 2 patients complained while using simva-, atorva- and rosuvastatin.

Fifty-four patients (22 males and 32 females) met the recently published criteria (Vrablik *et al.* 2014) for definitive diagnosis of SAM, whereas in the remaining 32 patients (8 males and 24 females) myopathy was classified as possible.

The median time between the onset of statin therapy and myopathy, calculated from the data obtained from 78 patients, was 1 month. Later onset of myopathy was recorded between month 4 and month 10 only in 10 patients.

Treatment was temporarily interrupted in all examined patients. The median interval between statin withdrawal and myopathy remission, calculated from the data provided by 71 patients, was 10 days with a maximum of 30 days. No remission was achieved in two patients.

All 58 patients in whom serum activity of CK was measured before statin administration, at the onset of muscle problems and after their cessation, reached

the highest CK activity at the onset of the myopathy. However, in 9 patients the peak activity of CK did not exceed the upper normal limit (Table 1). Serum CK activity was not measured in 28 patients (Table 1). Most patients with severe CK elevation > 20 μkat/l also had a concomitant elevation in serum activity of aspartate aminotransferase (AST). In contrast, none of the patients with CK activity < 20 μkat/l had serum AST activity higher than twice the normal level.

CNV analysis

DNA genotyping was performed in all 86 subjects. All analysed samples passed the set genotyping criteria, although one sample had to be excluded from further analysis due to the detection of an abnormally high number of CNVs. Genotype analysis confirmed that all study subjects were genetically unrelated.

In the remaining 85 subjects, we identified a total of 2552 CNVs: 684 losses and 1868 gains. In this set, we searched for CNVs that were longer than 20 kb and located within ± 50 kb of any established gene loci. This analysis retrieved 503 CNVs: 164 losses and 339 gains.

The 164 losses ranging from 20 - 892 kb were present in 76 subjects. From these losses, 27 were recurrent (i.e. found repeatedly in the patient cohort) and 58 were singletons. The losses affected 205 distinct gene loci, from which 156 loci were functionally annotated in GeneDistiller (http://www.genedistiller.org/). Among these annotated gene loci, we searched using selected key words for the candidate genes listed in the introduction and the following genes: an additional 10 genes for nemaline myopathy (North and Ryan 2015); 7 genes for centronuclear/core myopathies (*BIN1, CCDC78, CNTN1, DNM2, MTM1, MYF6, MYH7*); 12 genes for congenital muscular dystrophies (Sparks *et al.* 2012); 6 genes underlying the congenital myopathies with prominent contractures (*EMD, FHL1, SEPN1, SYNE1,*

SYNE2, TMEM43); 16 genes for limb-girdle muscular dystrophies (Pegoraro and Hoffman 2012); 12 genes for congenital myasthenic syndromes (Abicht *et al.* 2012) and 24 genes associated with metabolic myopathies (*ACADL*, *ACADM, ACADVL*, *ACAD9, AGL, C10orf2, CPT1B, GYS1, HADHA, HADHB, LPIN1, OPA1, OPA3, PFKM, PGAM2, PGM1, PHKA1, POLG, POLG2, RRM2B, SLC22A5/OCTN2, SUCLA2, TK2, TYMP*). Moreover, we also considered published associations for each of the 156 annotated genes with myopathy or statin metabolism and their potential involvement in pathways reviewed in Ghatak *et al.* 2010 and Mosshammer *et al.* 2014. Using this approach we defined four singleton candidate deletions, which were confirmed from the exome sequence data (Table 2).

Gains ranging from 20 - 522 kb were present in 83 subjects. From the 339 identified gains, 41 were recurrent and 115 were singletons. The gains affected 359 distinct gene loci, from which 271 loci were functionally annotated in GeneDistiller and evaluated as above. Among the annotated gains we found no single candidate amplification that was independently confirmed in the exome sequence data.

Targeted genotyping for known Rotor syndrome deletions and insertions affecting *SLCO1B1* and *SLCO1B3* was performed in all 86 studied individuals. None of the investigated patients carried any of these three tested candidate variants.

Discussion

Biological mechanisms and genetic factors contributing to SAM are heterogeneous and remain enigmatic. Previous genetic studies have focused almost exclusively on the role of common genetic variants, mostly single nucleotide polymorphisms, and have identified only a small fraction of the expected heritability (Feng 2014, Vrablik *et al.* 2014).

Copy number variations constitute a substantial fraction of the total genetic variability known to cause, predispose to, and modulate, human diseases (Zarrei *et al.* 2015). The distribution of larger CNVs in the general population remains largely unexplored. Because rare pathogenic copy number variations are often large and contain multiple genes, identification of the underlying genetic drivers has proven difficult. In our analysis, we searched for recurrent or individually rare CNVs that may affect genes involved in drug metabolism and muscle function. We did not find any cases of recurring CNVs that would be present in higher frequencies when compared to the general population reported in the curated catalogue of human genomic structural variation (DGV) and in our internal database. Our findings thus suggest that in addition to common single nucleotide variants, large CNVs do not seem to play a substantial role in the aetiology of SAM.

In two studied cases, a rare CNV that could be considered an individually contributing genetic factor was detected. In one patient, we found a 28 kb deletion in intron 12 of *EYS* which may have impaired its splicing pattern. Common variants in *EYS* were found to be associated with SAM in a large study based on the GWAS approach (Isackson *et al.* 2011). However, this finding has not been validated in further studies.

A 6.4 kb deletion located in intron 1 of *LARGE* was detected in the other patient. Pathogenic mutations in *LARGE*, when present on both alleles, are known to cause autosomal recessive dystroglycanopathy, which is considered a subtype of congenital muscular dystrophy. Interestingly, a large intronic deletion in exon 10 of *LARGE* has recently been identified as causing the disease in a consanguineous family from Lebanon (Clarke *et al.* 2011).

In another two patients, we found 176 kb and 57 kb deletions affecting the *CYP2C18/CYP2C19* locus. While one patient completely lacked both *CYP2C18* and *CYP2C19*, the smaller deletion present in the other patient removed only the first five exons of *CYP2C19*. *CYP2C19* is known to metabolise a significant number of drugs except for statins (Hirota *et al.* 2013). However, haploinsufficiency of *CYP2C19* contributes to the altered metabolism of clopidogrel concomitantly used with atorvastatin (Tantry *et al.* 2014).

In conclusion, our findings suggest that the participation of common large CNVs in genetic predisposition to SAM is unlikely, whereas rare CNVs may play some role in SAM pathogenesis.

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Table 1: Clinical and laboratory findings in enrolled patients with statin-induced myopathy grouped according to their serum CK activity levels.

Serum CK activity	No. of pts	Sex (m/f)	Median age (IQR)	Susp/proven diagnosis	Atorva/ Simva/ Rosuva (%)	Fibrate users	SAM type
> 5x ULN	12	10/2	60 (53-66)	6 suspect hFH, all 12 polygenic	50/25/25	4 (33%)	11 (92%) defin 1 (8%) prob
1 - 5x ULN	37	13/24	67 (60-71)	4 suspect hFH, 2 hFH proven 35 polygenic	62/25/13	7 (19%)	25 (68%) defin 12 (32%) prob
< ULN	9	3/6	67 (58-70)	4 suspect hFH, 1 hFH proven 8 polygenic	78/11/11	2 (22%)	6 (67%) defin 3 (33%) prob
not available	28	4/24	63 (58-68)	3 suspect hFH, all 28 polygenic	50/25/25	6 (21%)	12 (43%) defin 16 (57%) prob

ULN – upper limit of normal, pts – patients, IQR – interquartile range, hFH – heterozygous familial hypercholesterolaemia, defin – definitive, prob – probable.

Table 2: Rare large deletions leading to heterozygous losses in genes potentially involved in SAM pathogenesis and in clopidogrel-statin interactions.

Patient no.	Chromosome no.	Begin (GRCh37/hg19)	End (GRCh37/hg19)	Size (bp)	Gene name	*DGV gold std var. frequency (%)	Potential functional implication
28	10	96443782	96620554	176772	*CYP2C18, CYP2C19*	0.9	clopidogrel-statin interaction
30	6	65786994	65815520	28526	*EYS*	0.3	SAM?
41	10	96499710	96557336	57626	*CYP2C19*	3.0	clopidogrel-statin interaction
47	22	34265402	34271782	6380	*LARGE*	0.1	SAM?

*DGV – catalogue of human genomic structural variation.