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Deep Sequencing of *RYR3* gene identifies rare and common variants associated with increased carotid intima-media thickness (cIMT) in HIV-infected individuals:

RYR3 deep sequencing and subclinical atherosclerosis

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Abstract

Carotid intima-media thickness (cIMT) is a subclinical measure of atherosclerosis with mounting evidence that higher cIMT confers an increased risk of cardiovascular disease (CVD). The ryanodine receptor 3 gene (*RYR3*) has previously been linked to increased cIMT; however, the causal variants have not yet been localized. Therefore, we sequenced 339,480 bp encompassing 104 exons and 2 kb flanking region of the *RYR3* gene in 96 HIV-positive white men from the extremes of the distribution of common cIMT from the Fat Redistribution and Metabolic Changes in HIV infection Study (FRAM). We identified 2710 confirmed variants (2414 SNPs and 296 indels), with a mean count of 736 SNPs (ranging from 528–1032) and 170 indels (ranging from 128–214) distributed in each individual. There were 39 variants in the exons and 15 of these were non-synonymous of which with only 4 were common variants and the remaining 11 rare variants one was a novel SNP. We confirmed the common variant rs2229116 was significantly associated with cIMT in this design ($P<7.9X10^{-9}$), and observed 7 other significantly associated SNPs ($P<10^{-8}$). These variants including the private non-synonymous SNPs need to be followed up in larger sample size and also tested with clinical atherosclerotic outcomes.

Keywords

cIMT; extreme phenotype; *RYR3*; sequencing; subclinical atherosclerosis

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INTRODUCTION

Cardiovascular disease (CVD), specifically atherosclerotic vascular disease is emerging as a major comorbidity in HIV-infected (HIV+) individuals, who are living longer with effective treatments.[1] By the year 2015, it is estimated that over half of HIV+ individuals in the US will be aged 50 or greater. The risk of cardiovascular disease events is higher in HIV+ individuals than in HIV-negative individuals of the same age group. Previous studies have found similar levels of carotid intima-media thickness (cIMT), a subclinical measure of atherosclerosis, in HIV+ individuals and patients with coronary artery disease (CAD). HIV infection appears to increase the risk of atherosclerosis primarily through chronic immune hyperactivation and chronic inflammation.[2–5] Additionally, cIMT progression in the common carotid artery (CCA) is known to be accelerated among HIV+ individuals compared to HIV-negative controls.[6, 7] Several studies have shown that CCA cIMT is a reliable and valid subclinical measure of early atherosclerosis, and is a strong predictor of incident cardiovascular events in HIV-negative individuals.[8, 9] A systematic review and meta-analysis confirmed this strong relationship between CCA cIMT and future vascular events including CAD.[10,11] Thus, factors contributing to cIMT would provide insights to elucidate biological mechanisms underlying atherosclerosis.

Heritable factors account for 21–38% of the variance in CCA cIMT.[12, 13] Two SNPs (rs2229116 and rs7177922) in the *RYR3* gene, encoding a calcium ion (Ca^{2+}) channel mainly expressed in the endoplasmic reticulum (ER) of cardiac and endothelial cells, were significantly associated with CCA cIMT in a genome-wide association study (GWAS) in subjects from the Fat Redistribution and Metabolic Change in HIV Infection (FRAM) Study.[14] The association of one of these non-synonymous SNPs (rs2229116) was replicated in white men in the Multicenter AIDS Cohort Study (MACS).[15] Recently other variants in *RYR3* were associated with CCA cIMT in HIV-infected women in Women's Interagency HIV Study (WIHS).[16] Additionally, variants in *RYR3* increased risk of atherosclerotic cardiovascular outcomes, specifically heart failure, among hypertensive patients randomized to a Ca^{2+} channel blocker versus other antihypertensive drugs in the Genetics of Hypertension Associated Treatment (GenHAT) study.[17] All of these studies indicate an important role of RYR3, specifically hypothesized through its differential function in Ca^{2+} mobility in the endoplasmic reticulum that can lead to apoptosis of macrophages in atherosclerotic plaques in HIV patients.[5]

In this report, we explore the genetic diversity of the *RYR3* gene in 96 HIV-positive white males in FRAM and investigate the associations of all the alleles with extreme common cIMT measurements. Sequencing of the extremes confers a substantial efficiency gain in association studies, because an excess of rare genetic variants among individuals at one extreme compared with other extreme underscores strong evidence of association between total mutational load and cIMT.[18]

MATERIALS AND METHODS

Source Population and Sequencing Study Design

FRAM is a multi-center (n=16) cohort study of 1,183 HIV+ men and women and 297 healthy adult subjects examined from 2000–02.[19] Carotid IMT measurements in FRAM were obtained by centrally-trained and certified ultrasonograpers with B-mode ultrasound in the common and internal carotid arteries using a standardized protocol developed by Dr. Daniel O'Leary.[20] For sequencing of the *RYR3* gene, 48 white males (average age = 44.3±7.1 yrs) with the lowest common cIMT measurements (average common cIMT = 0.729 ±0.075 mm) and 48 (average age = 54.6±8.7 yrs) with the highest common cIMT measurements (average common cIMT = 1.043 ±0.161 mm) within FRAM participants for the sequencing study.

The parent FRAM study and this sub-study conformed to the procedures for informed consent approved by institutional review boards at all participating sites and the genetic study at UAB and to human-experimentation guidelines set forth by the United States Department of Health and Human Services.

Fragment Libraries

A bait library for the Agilent SureSelect in-solution capture reagent was developed covering all 104 exonic and 2kb flanking region sequence of *RYR3* gene (total of 339,480 bp), located at chr15:33,601,161–34,159,307 of hg19 version of the human genome. Following the manufacturer's protocol for the SureSelect Target Enrichment System and as previously described,[21] two micrograms of genomic DNA was fragmented by sonication using the Covaris S2 to achieve a uniform distribution of fragments with a mean size of 150 bp. The sonicated DNA was purified using Agencourt's AMPure XP Solid Phase Reversible Immobilization paramagnetic bead (SPRI) followed by polishing of the DNA ends by removing the 3' overhangs and filling in the 5' overhangs resulting from sonication using T4 DNA polymerase and Klenow fragment (New England Biolabs). Following end polishing, a single 'A'-base was added to the 3' end of the DNA fragments using Klenow fragment (3' to 5' exo minus). This prepared the DNA fragments for ligation to specialized adaptors that have a 'T'-base overhang at their 3'ends. The end-repaired DNA with a single 'A'-base overhang was ligated to the Illumina paired-end adaptors in a standard ligation reaction using T4 DNA ligase and 3 uM final adaptor concentration. Following ligation, the samples were purified using SPRI beads, quality controlled by assessment on the Agilent Bioanalyzer and then amplified by six cycles of PCR in two identical reactions for each sample to maintain complexity and avoid bias due to amplification.

Hybrid Selection

Five-hundred nanograms of purified DNA (DNA library) was prepared for hybridization by adding the DNA library to Agilent blocking reagents, denaturing at 95˚C and incubating at 65˚C. All subsequent steps are performed at 65˚C. Hybridization buffer was added to the prepared library and the entire mix was then added to an aliquot of the Agilent SureSelect Capture Library and mixed. The DNA library and biotin-labeled Capture Library were hybridized by incubation at 65C for 24 hours. Following hybridization, streptavidin coated

magnetic beads were used to purify the RNA:DNA hybrids formed during hybridization. The RNA capture material was digested via acid hydrolysis following elution from the purification beads. The neutralized captured DNA was amplified for 12 cycles of PCR using Herculase II Fusion DNA polymerase. The libraries were purified following amplification and the library assessed using the Agilent Bioanalyzer and quantitated via a real-time PCR reaction. As previously described,[22] 48 libraries, each barcoded with a different sequence during library preparation, were pooled in molar equivalents to generate a 10nM final stock. The final library stock was then used in paired-end (PE) cluster generation at a final concentration of 6–8 pM to achieve a cluster density of ~600,000/mm^2 (on the Illumina HiSeq2000 instrument). The flow-cells were sequenced at a paired-end 50nt condition to average coverage of ~250x per sample. The RYR3 sequencing dataset has been deposited in the NCBI SRA with accession ID SRP047147.

Bioinformatics analysis

Reads were aligned to the hg19 reference sequence using BWA.[23] SNPs and small indels (Insertion/deletion) were called using Genome Analysis Toolkit (GATK) software package.[24] wANNOVAR (accessed Jan 19 2014) was used to annotate the genetic variants, amino acid change, SIFT scores, PolyPhen2 scores and identify variants in dbSNP.[25, 26] Linkage disequilibrium between SNPs present in the HapMap CEU and 1000 genomes project samples was retrieved from the SNAP server (http://www.broadinstitute.org/mpg/snap/, v2.2).

Association analysis

While individuals with the lowest and highest cIMT were chosen for sequencing, Alf and Abraham (1975) showed that analyzing data as continuous variables from extremes instead of treating them qualitatively as a case-control analysis provides better statistical power.[27] Thus, linear regression model was used in PLINK (version 1.07) to analyze genetic associations of individual variants with quantitative cIMT. This is a valid analytical strategy since the normality assumption in linear models is for residuals rather than trait measurements. Statistical association analyses with cIMT were performed using the Genotypic (2 df) test, with adjustments for age, duration of d4t and genetic principle component 1. SNP-set (Sequence) Kernel Association Test (SKAT) [28] with default parameters and the same covariates was used to test the association of multiple variants with the continuous trait of cIMT in sliding windows along the gene, with a window size of 4kb and a skip step of 2kb.

RESULTS

Description of sequencing data (with function annotations)

Of the 3015 variants identified in the *RYR3* gene region, 2710 variants (2414 SNPs and 296 indels) passed the standard GATK filtering with an overall Ts/Tv ratio of 2.1, and 2707 of them were annotated by ANNOVAR (Table 1). A vast majority of these SNPs were intronic, with only 39 in the exons. Each individual had a mean count of 736 SNPs (ranging from 528–1032) and 170 indels (ranging from 128–214), of which there was an average of 7.6 private variants (including singletons and private doubletons) per person (ranging from 0–

57). Novel variants (516) were enriched for upstream (P=0.0001, chi-squared test), non-synonymous exonic (P=0.0009), and 3' UTR variants (P<0.0001).

Single-variant Association Analysis

Association analysis was conducted with cIMT for 1861 SNPs and indels with minimal minor allele frequency (MAF) of 1% (excluding singletons with a variant in only one chromosome of an individual). The regional plot for association is shown in Figure 1a. The top significantly associated variants (P<0.001, corresponding to top 30 non-redundant association signals) are shown in Table 2 (MAF. Not surprisingly, one of the top hits (rs2229116) was the GWAS hit, previously reported in the larger cohort, confirming both genotyping and true association in the extreme study design. Additionally, immediately around the GWAS hit, there are multiple variants within about 30kb downstream, with the strongest signals (p=5.74E-10) located about 7–8 kb. Outside of the region with strong LD to the GWAS hit, there are two regions that showed statistically significant associations with cIMT. The first region was upstream of this SNP, spanning chr15:33762337–33768118 bp in chromosome 15 with strongest p-value 2.04E-4. This region is surrounding the second exon, but no exonic variants were identified. The second region is downstream of this SNP spanning chr15:34123709–34126522 bp with strongest p-value 3.76E-4.

Coding and Functional Variant Analysis

Among the 39 exonic variants, 15 were missense mutations and are listed in Table 3. Among the 4 non-singletons, the previously identified rs2229116, causing V494I mutation, was significantly associated with cIMT at $p<7.9X10^{-9}$. None of the SNPs in the promoter were associated with cIMT after Bonferroni correction.

Multivariate analysis

SKAT [28] was used to test the association of multiple variants in sliding windows along the gene, with a window size of 4kb and a skip step of 2kb. As seen in Figure 1b, the pattern of the association was largely consistent with single-variant association, although the region around the previous GWAS hit rs2229116 (position 33905410) did not show any significant associations with cIMT after stringent Bonferroni correction. In addition, some peaks were found in the first intron, but no known genomic features were annotated.

In addition, we investigated the effect of rare variants by comparing the frequencies of private variants (singletons) of individuals with high versus low cIMT. Interestingly, although there is no overall different (P=0.14, T test) over the entire length of the sequenced segment, the middle part of the gene Chr15:33.75M-33.95M, had enriched for high cIMT specific private variants (confidence band of LOESS regression is above 0) (Figure 2).

DISCUSSION

The objective of this study was to comprehensively evaluate the sequence variations present in the exonic and flanking regions of the *RYR3* gene and to determine their associations with cIMT. The genetic architecture of the *RYR3* gene was examined in 96 HIV-positive white males with extremes of cIMT measurements. These results expand on the previously

consistently reported SNP, but the region is not fully explained for functional genomics. While the previously reported GWAS hit (rs2229116) was validated in a different study design ($p<7.93X10^{-9}$), multiple independent association signals were also identified throughout the 500kb *RYR3* gene region, including the most significant association with rs28501179, rs10519841, and rs7168848 ($p<5.74X10^{-10}$).

We report 2707 variants in the *RYR3* gene region, including 516 (19%) novel ones that have not been reported in known genetic variant database. A majority of the novel variants were rare (MAF <0.01). Because the sample size of our study is n=96, the rare mutation is really singletons (1/192). To date, while there are 670 variants (5 frame shift, 414 missense, 3 nonsense and 250 synonymous) within the coding regions in *RYR3* reported in dbSNP in NCBI, only 12 have MAF greater than 0.05, of which 4 are non-synonymous. In our study, only 15 non-synonymous variants were observed, including the 4 with MAF greater than 0.05 and only 1 rare SNP is novel (Table 2). Some of these variants reported, which still lack data in different populations including in dbSNP, might be specific to ethnic groups (e.g. white versus other races), or uniquely found within the extremes of common cIMT, or associated with an unknown phenotype in these HIV-infected white men. Interestingly, high cIMT individuals were enriched for private (singleton) variants in the middle part of the *RYR3* gene. This is intriguing as the overall load of private mutations was not significantly different between low and high cIMT individuals. We used LOESS regression which is a commonly-used non-parametric smoothing method but it is rarely used in the context of rare variant association. It captures the density of private mutations throughout the length of the *RYR3* gene, which spans over 500kb region. Our results capture the enrichment signal but it is can be tricky to claim any statistical significance. However, all of the rare variants in the coding regions, specifically the ones that are private to the extremes and their concentration in specific region need to be investigated in larger population, both HIV-positive and HIV-negative general population.

This study has several important limitations. The associations of rs2229116 (only exonic in the top 30 – Table 2) and other variants around it in the region are still not biologically explained. Evaluations in larger cohorts need to be conducted to examine if these are the true causal variants or are tagging multiple rare functional variants that we observed. Further, genetic associations of these variants need to assessed for functional studies. The SKAT analysis did not show significant associations beyond Bonferroni corrections, but this might be the result of low power of SKAT in identifying causal high frequency variants. The sample size was relatively small and we may have missed the identification of certain rare functional variants. However, a study design of extreme cIMT was optimized to provide a cost-effective method to discover causal genetic variations. Allelic heterogeneity is a possibility and thus individuals with extreme cIMT might have rare and/or low frequency funcational *RYR3* variants not previously reported in the literature. Lanktree et al. reported that “to obtain similar statistical power, the number needed to genotype is 4 times greater when using a population-based approach compared to an approach based on targeted genotyping of individuals at the extremes of unexplained atherosclerosis and unexplained protection.”[29] We did not examine other clinical outcomes in these individuals; however cIMT is a valid subclinical surrogate for atherosclerosis.

In summary, this is the first study to comprehensively examine all the genetic variants through target deep-sequencing of *RYR3* gene and evaluate their associations with cIMT in HIV-infected patients. The results from this study lend further strong support to the role of *RYR3* gene in atherosclerosis and provide detailed sequence information of the gene, which can be potentially insightful in understanding the function in the etiology of high cIMT in HIV patients. Even with the small sample size, several significant associations were observed; however, these SNPs need to be followed up in larger sample size and also tested with clinical atherosclerotic outcomes.

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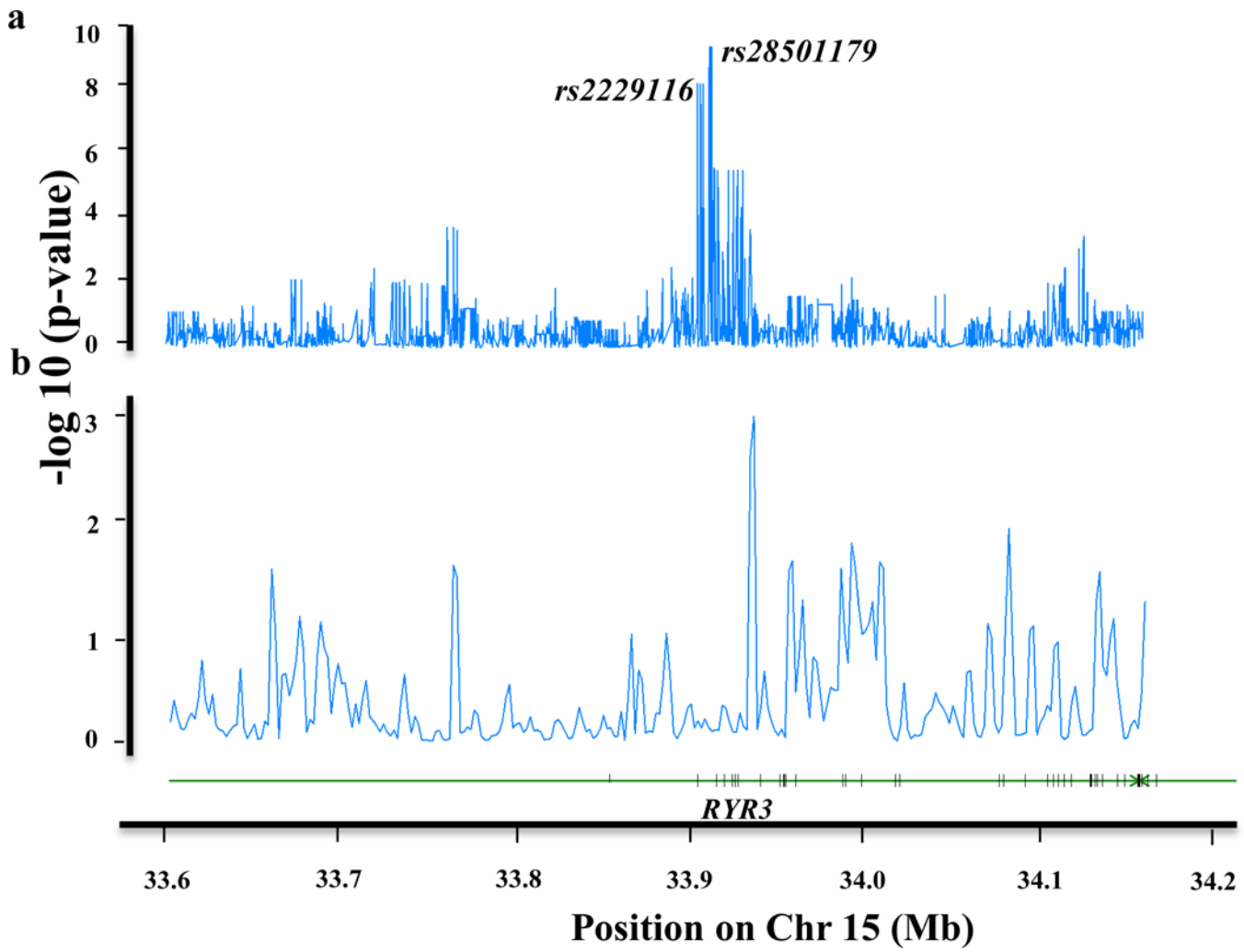

Figure 1:
RYR3 gene regional plot of genetic associations with cIMT extremes in FRAM (-log p-value) - a) single variant association p-values; b) p-values from SNP-set (Sequence) Kernel Association Test (SKAT) using 4kb sliding window.

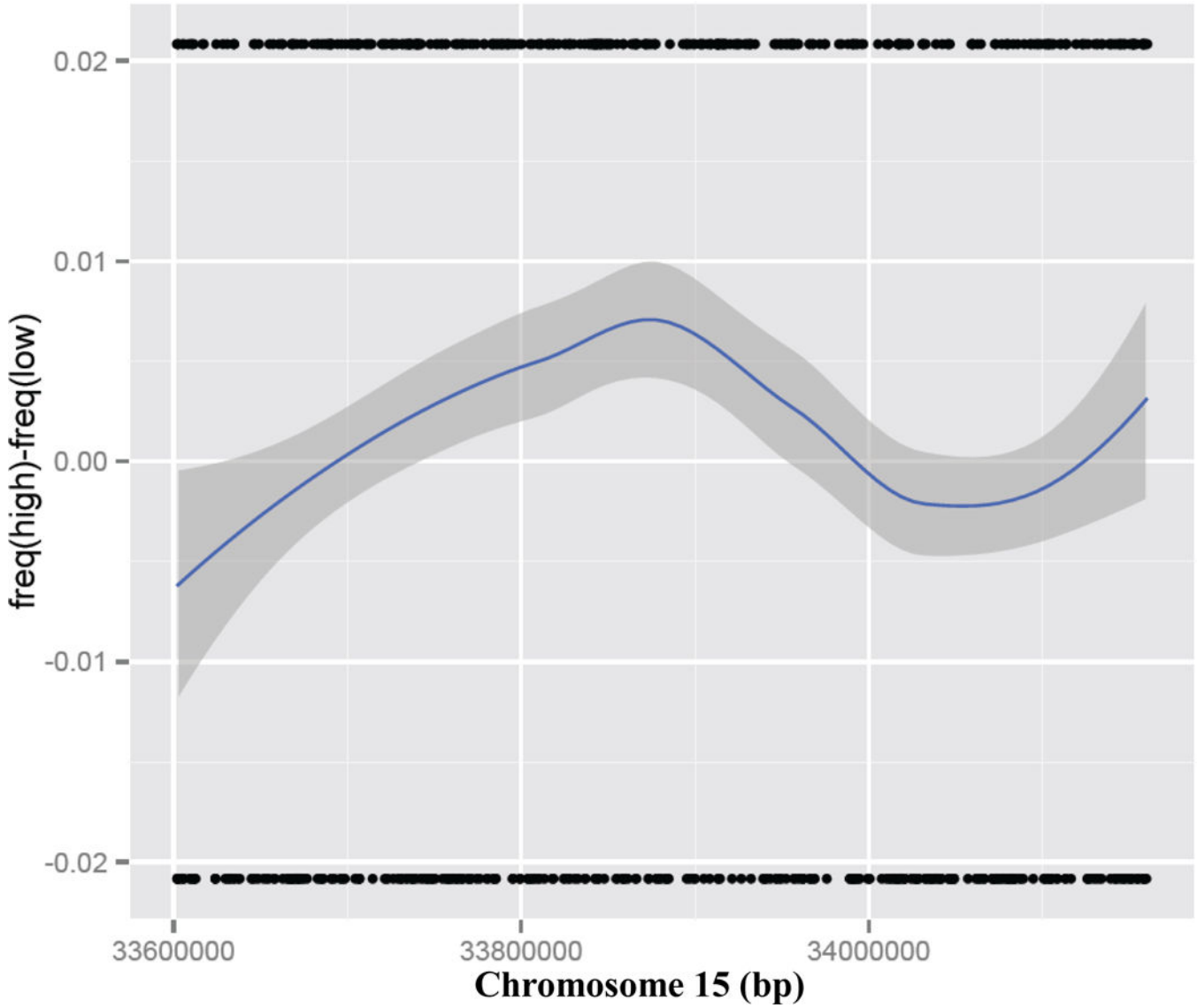

Figure 2:

FRAM high cIMT extremes are enriched for singleton mutations in chr15:33.75M-33.95M. Raw mutation frequencies are plotted as dots at ± 0.0208(=1/48). LOESS smoothed line are shown in blue with confidence band in grey.

Table 1

Overall, characterization of sequence variants in *RYR3* gene

Type of variant	Count	known (dbSNP135)	novel
upstream	18	8	10
5’ UTR[1]	0	0	0
exonic	39	34	5
synonymous	23	19	4
nonsynonymous	15	14	1
nonframeshift deletion	1	1	0
intronic	2622	2135	487
3’UTR[1]	24	11	13
downstream	4	3	1
Total	2707	2191	516

[1]UTR: untranslated region

Table 2

List of top 30 variants in *RYR3* gene region associated with cIMT

Position	dbSNP ID	Reference/alternate allele	MAF	P-value[1]	Non-redundant association signals
33912652	rs28501179	A/G	0.208	5.74E-10	1
33913277	rs10519841	C/T	0.208	5.74E-10	1
33913487	rs7168848	A/T	0.208	5.74E-10	1
33911933	rs7161819	T/G	0.214	7.49E-09	2
33905410	rs2229116[2]	A/G	0.198	7.93E-09	3
33907233	rs2339313	G/A	0.198	7.93E-09	3
33908619	rs7177922	G/A	0.198	7.93E-09	3
33914901	rs67514754	C/CT	0.198	3.08E-06	4
33915088	rs17817440	T/C	0.188	3.50E-06	5
33917103	rs59753482	A/G	0.177	3.59E-06	6
33923237	rs28696769	C/A	0.182	3.59E-06	6
33925879	rs28582856	G/T	0.182	3.59E-06	6
33928500	rs2291734	G/A	0.177	3.59E-06	6
33931264	rs28608677	A/T	0.182	3.59E-06	6
33930493	rs28576933	G/C	0.266	4.80E-05	7
33908919	rs1874279	G/A	0.427	5.20E-05	8
33908279	rs13379896	G/C	0.427	5.20E-05	8
33908316	rs10519839	G/T	0.427	5.20E-05	8
33908535	rs56013212	G/A	0.427	5.20E-05	8
33908945	rs2101818	G/C	0.427	5.20E-05	8
33927764	rs1495280	C/T	0.391	1.61E-04	9
33913340	-[3]	A/AT	0.464	1.62E-04	9
33762337	rs62015588	G/A	0.234	2.04E-04	10
33765803	rs2278470	A/T	0.234	2.04E-04	10
33935674	rs118139432	G/T	0.010	2.33E-04	11
33768118	rs11637662	G/C	0.245	2.50E-04	12
34126522	rs71759695	CACAGTG/C	0.224	3.76E-04	13
33915632	-[3]	CTG/C	0.271	5.38E-04	14
34123709	rs6495268	G/A	0.245	9.45E-04	15
34123836	rs72716804	T/C	0.245	9.45E-04	15

[1] P-value: linear regression p-value of cIMT association.

[2] previous GWAS hit; only exonic SNP, all others are in the intron

[3] novel SNPs with no rs# assigned in NCBI

All information except p-values are derived from wANNOVAR.

Table 3

List of known and novel non-synonymous variants in RYR3 gene identified from deep sequencing and associated with cIMT

Position	dbSNP ID	Reference/alternate allele	MAF Study Population[1]	NHLBI-ESP[2]	Mutation	P-value[3]
33873751	rs2077268	G/A	0.15	0.14	V494I	0.997
33895346	rs368209451[4]	G/C	0.005	0.0002	G649R	
33905410	rs2229116	A/G	0.19	0.19	I731V	7.93×10^{-9}
33916136	rs199500216[4]	G/A	0.005	0.001	R829H	
33954652	rs4780144	C/T	0.03	0.13	R1641C	0.244
33992014	rs370232598[5]	A/G	0.005	0.0002	E2120G	
33999253	rs181264765[5]	A/C	0.005	0.0002	N2206T	
34016274	rs6495228	G/A	0.18	0.15	G2270E	0.523
34016315	rs201633381[5]	G/A	0.005	0.0007	A2284T	
34030756	-[4,6]	C/A	-	-	L2541I	
34072417	rs139568967[5]	G/A/T	0.005 (A) 0.005(T)	0.002 (A) 0.0005(T)	R3048H/L	
34072528	rs61996335[4]	C/G	0.005	0.003	P3085R	
34077949	rs200830195[5]	G/A	0.005	0.0017	E3119K	
34080628	rs182972491[5]	C/T	0.005	0.0001	P3267S	
34130020	rs201375567[4]	G/A	0.005	0.0007	A3942T	

[1] MAF - minor allele frequency in the study population (N = 96)

[2] MAF (if known) in National Heart, Lungs and Blood Institute– Exome Sequencing Project (NHLBI-ESP)

[3] P-value: linear regression p-value of cIMT association.

[4] private SNP in highest cIMT extreme

[5] private SNP in the lowest cIMT extreme

[6] novel SNP with no rs# assigned in NCBI

All information except p-values and MAF are derived from wANNOVAR