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Original Article

Tripartite motif-containing 55 identified as functional candidate for spontaneous cardiac hypertrophy in the rat locus cardiac mass 22

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Background: Left ventricular (LV) hypertrophy is a risk factor for cardiovascular death, but the genetic factors determining LV size and predisposition to hypertrophy are not well understood. We have previously linked the quantitative trait locus cardiac mass 22 (Cm22) on chromosome 2 with cardiac hypertrophy independent of blood pressure in the spontaneously hypertensive rat. From an original cross of spontaneously hypertensive rat with F344 rats, we derived a normotensive polygenic model of spontaneous cardiac hypertrophy, the hypertrophic heart rat (HHR) and its control strain, the normal heart rat (NHR).

Methods and results: To identify the genes and molecular mechanisms underlying spontaneous LV hypertrophy we sequenced the HHR genome with special focus on quantitative trait locus Cm22. For correlative analyses of function, we measured global RNA transcripts in LV of neonatal HHR and NHR and 198 neonatal rats of an HHR $\times$ NHR F2 crossbred population. Only one gene within locus Cm22 was differentially expressed in the parental generation: tripartite motif-containing 55 (*Trim55*), with mRNA downregulation in HHR ($P < 0.05$) and reduced protein expression. *Trim55* mRNA levels were negatively correlated with LV mass in the F2 cross ($r = -0.16$, $P = 0.025$). In exon nine of *Trim55* in HHR, we found one missense mutation that functionally alters protein structure. This mutation was strongly associated with *Trim*55 mRNA expression in F2 rats ($F = 10.35$, $P < 0.0001$). Similarly, in humans, we found reduced *Trim55* expression in hearts of subjects with idiopathic dilated cardiomyopathy.

Conclusion: Our study suggests that the *Trim55* gene, located in Cm22, is a novel candidate gene for polygenic LV hypertrophy independent of blood pressure.

Keywords: animal models, cardiomyopathy, functional genomics, microarray, transcriptome

Abbreviations: ABC transporters, adenosine triphosphate-binding cassette transporters; BWA, Burrows–Wheeler Aligner; CNV, copy number variation; Cm22, cardiac mass 22; CWI, cardiac weight index; GEO, gene expression omnibus; HHR, hypertrophic heart rat; InDel, insertion and deletion; LV, left ventricular; NHR, normal heart rat; qPCR, real-time quantitative PCR; QTL, quantitative trait locus; SHR, spontaneously hypertensive rat; SNP, single nucleotide polymorphism; Trim55, tripartite motif-containing 55

INTRODUCTION

Evidence for genetic determination of cardiac size comes from numerous twin and family studies, in which the heritability for cardiac mass has been estimated to be 30–84% [1–3]. Cardiac hypertrophy, in particular left ventricular (LV) hypertrophy, is the main predisposition factor to cardiovascular disease outcomes after age [4]. The molecular processes leading to an increase in cardiac size independent of blood pressure are still unknown.

The hypertrophic heart rat (HHR), a normotensive polygenic model of cardiac hypertrophy, is a unique model because it permits the study of cardiac mass without the confounding effects of high blood pressure. This model and its control, the normal heart rat (NHR), were derived from selectively crossing the spontaneously hypertensive rat (SHR) and Fisher 344 rats for over 13 generations [5]. At 2 days old, the HHR have smaller hearts with fewer and smaller cells when compared with the NHR defining hypertrophy later in life. By 12 weeks of age, however, the HHR presents established LV hypertrophy, which leads to

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premature death because of heart failure as early as 48 weeks of age [5,6].

Studies that identify genetic factors contributing to LV hypertrophy independent of pressure overload are crucial to understand the molecular mechanisms involved. The whole-genome sequencing of rat strains displaying diverse phenotypes [7,8] and the creation of the Rat Genome Database [9] permitted the discovery of a greater number of allelic variants among rat strains. These data, associated with gene expression studies, could identify disease-associated candidate genes [10]. Here, we aimed to take advantage of the HHR as a unique model of polygenic cardiac hypertrophy to investigate mutations in genes contributing to this condition.

In this study, we compared the whole-genome sequence of the HHR with the NHR and the reference laboratory rat, *Rattus norvegicus*. We focused particularly on a region of rat chromosome 2 that we identified in previous crosses of the SHR and Wistar Kyoto rats as linked with LV mass independent of blood pressure [11,12]. We originally designated this quantitative trait locus (QTL) as Lvm1, but it has been subsequently labelled as cardiac mass 22 (Cm22). To identify functional candidates in Cm22, we correlated sequence and expression data in HHR and NHR and tested their linkage with the LV mass phenotype in an F2 cross of the HHR and NHR.

MATERIALS AND METHODS

Samples and tissue collection

Two-day-old HHR ($n=8$ for arrays and $n=8$ for follow-up studies) and NHR ($n=8$ for arrays and $n=9$ for follow-up studies) were euthanized by decapitation; and 13 ($n=10$ HHR and $n=11$ NHR) and 33-week old ($n=7$ HHR and $n=9$ NHR) were euthanized with an overdose of pentobarbitone (Lethabarb; Vet N-Pet Direct, Brisbane, Australia). We crossed four male HHRs with four female NHRs to produce an F1 population ($n=9$). By crossing male and female F1 rats we derived an HHR × NHR F2 population ($n=198$). F2 animals were euthanized by decapitation at 2 days of age. Hearts were immediately removed, and LV dissected from the atria. Cardiac weight index (CWI, mg/g) was calculated from the total heart weight (mg) relative to total body weight (g) of the animal (Table S1, http://links.lww.com/HJH/A588; Figure S1, http://links.lww.com/HJH/A588). Tissues were first preserved in liquid nitrogen and later transferred to a −80°C freezer. The study was approved by the Animal Ethics Committees of the University of Melbourne and Deakin University, and ratified at Federation University Australia.

DNA and RNA extraction

DNA from LV tissue was extracted using the PureLink Genomic Extraction kit (Life Technologies, Carlsbad, California, USA). RNA was extracted using the miRNeasy kit (Qiagen, Hilden, Germany). Both RNA and DNA were quantified by spectrophotometry using a NanoDrop ND-100 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). RNA used in microarrays was also assessed for quality based on a RNA integrity number higher than 8 by electrophoresis in a 2100 Bioanalyzer (Agilent, Waldbronn, Germany). Figure S2, http://links.lww.com/HJH/A588, outlines the data analysis we used to identify a new candidate gene for polygenic LV hypertrophy.

Whole-genome sequencing and analysis

Three male 13-week-old NHR and three age-matched HHR (with established LV hypertrophy) were selected for whole-genome sequencing. DNA concentration and quantity were determined by Qubit Fluorometer (Life Technologies) and integrity was determined by agarose gel electrophoresis. The Illumina Cluster Station and Illumina HiSeq 2000 (San Diego, California, USA) were used for preparation of paired-end libraries with insert size of 500 bp. The whole-genome sequencing was performed on the Illumina HiSeq 2000 platform, where the sequences of each individual were generated as 90 bp paired-end reads. Quality filtered FASTQ paired-end sequences were mapped to the Brown Norway reference genome Rnor_5.0 from March 2012 (Rat Genome Sequencing Consortium, RGSC v5.0) using the Burrows–Wheeler Aligner (BWA-0.7.5a) software [13]. Preprocessing for sorting and removal of duplicates was performed using Picard tools [14]. We used default parameters for the BWA-maximal exact match algorithm to map reads and the Genome Analysis Tool Kit (GATK 3.1_1) [15] to analyze mapped sequences [8]. The sequences were realigned around insertions and deletions (InDels) and base quality scores were recalibrated for accurate base quality. We used Genome Analysis Tool Kit best practices [16] for variant calling analysis, to identify single nucleotide polymorphisms (SNPs) and SnpEff software [17] for functional annotation. Copy number variations (CNVs) were identified using Varscan [18] by simultaneously comparing reads counts, base quality, and allele frequency, and structural variants were identified using Seeksv to detect large insertions, deletions, and inversions.

We developed an in-house database for the storage and analysis of the variants identified in the genome sequencing. This database was used to identify the unique variants in HHR and NHR. Change rate, defined as the frequency of a substitution occurring in the genome, was calculated as the ratio between the number of changes and the genome region.

Whole-genome transcriptome and analysis

Affymetrix GeneChip Rat Gene 1.0 ST Arrays (Tokyo, Japan) were used for transcriptome-wide gene expression analysis. We analyzed eight neonatal HHR (four male, four female) and eight NHR (four male, four female). Each animal was considered an individual sample and no pooling was performed. Briefly, mRNA was converted to single-stranded DNA, labelled and hybridized to GeneChip Rat Gene 1.0 ST Arrays, which analyze 27 342 gene transcripts using 722 254 probe sets (on average 26 probes/gene), according to the manufacturer's instructions, and with the assistance of the Ramaciotti Centre for Gene Function Analysis (University of New South Wales, Sydney, Australia). The data set obtained has been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database according to Minimum Information About a Microarray Experiment guidelines with series accession number GSE38607

(http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=bjsxbiiaowmwozy&acc=GSE38607).

Results from arrays were normalized using robust-multi-array analysis. Differentially expressed genes were identified using a two-sample *t*-test in the Partek Genomics Suite (version 6.6) and selected based on their false discovery rate *q*-value less than 0.05.

Gene functional annotation analysis

Gene functional annotation analysis was performed using the Database for Annotation, Visualization and Integrated Discovery [19,20] to ask which Kyoto Encyclopedia of Genes and Genomes pathways were enriched within the 1231 genes that contained nonsynonymous unique SNPs in the hypertrophic strain.

Quantitative trait loci characterisation

We previously described the QTL Cm22, also known as Lvm1, to be associated with LV hypertrophy independent of blood pressure [11,12]. QTL Cm22 is located on rat chromosome 2, nucleotide 98 037 122–177 517 146 in RGSC v5.0 [21].

Real-time quantitative PCR for gene expression analysis

First-strand complementary synthesis reaction was performed using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies). Primers were specifically designed around the most differentially expressed probe in the transcript cluster of each gene using Primer3 [22] and NCBI online tool Primer Blast. Primers were designed to flank an exon–exon junction. Primers and conditions used are shown in Table S2, http://links.lww.com/HJH/A588. Amplification reactions used the SensiFast SYBR Low-ROX Kit qPCR reagent system (Bioline, Foster City, California, USA) in a Viia7 qPCR instrument (Life Technologies). Samples were run in duplicates. The specificity of the qPCR was ensured by melting curve analysis and electrophoresis in agarose gels (data not shown). The glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as reference transcript. Significance was assessed by $2^{-\Delta\Delta CT}$ [23].

Protein measurements of tripartite motif-containing 55 by western blot

Whole soluble protein was extracted from left ventricle of 13-week-old HHR and NHR using radioimmunoprecipitation assay buffer (Sigma-Aldrich, Saint Louis, Missouri, USA) and 1% Halt Protease and Phosphatase Inhibitor Cocktail (Life Technologies, Rockford, Illinois, USA). One hundred micrograms of extracted rat protein were resolved by 4–15% Mini-PROTEAN TGX Precast Gel (BioRad, Hercules, California, USA) then electroblotted on to a Nitrocellulose Membrane (Life Technologies). Membranes were blocked for one hour at room temperature in 5% skim milk in Tris-buffered saline (TBS)-Tween 20, then incubated overnight at 4°C with antitripartite motif-containing 55 (anti-TRIM55) (N-terminal) Rabbit Polyclonal antibody (1 : 2500 dilution, ABIN405554, Antibodies-online Inc., Atlanta, Georgia, USA) in 0.5% skim milk/TBS-Tween 20. Membranes were stripped using Restore Western Blot Stripping Buffer (Life Technologies) and probed with β-actin (1 : 5000 dilution, 3700, Cell Signalling, Danvers, Massachusetts, USA) in 0.5% skim milk/TBS-Tween 20 solution for 1 h at room temperature, followed by four washing steps for 15 min in TBS-Tween 20 before detection using enhanced chemiluminescence SuperSignal West Pico Substrate (Life Technologies). Images were captured with a UVITEC Alliance digital imaging system (Thermo Scientific).

Three-dimensional protein prediction

The three-dimensional protein prediction of *Trim55* of HHR and NHR were generated after uploading the amino acid sequences separately into online software Phyre [24], using the normal modelling mode.

Statistical analyses

GraphPad Prism (version 6) package was used for graphing and statistical analyses. Data sets were tested for normal distribution using the D'Agostino and Pearson normality test, and equal variances were analyzed using the *F* test. An independent sample *t* test, Welch's test, or Mann–Whitney test were used to compare the data between the groups. Significance was set at P less than 0.05. A one-way analysis of variance (ANOVA) test was used to compare *Trim55* abundance and exonic variant genotypes. A linear regression test was performed to compare CWI and *Trim55* abundance (Figure S3, http://links.lww.com/HJH/A588).

Gene expression in human hearts

We used data in the repository GEO series GSE1145 to investigate the levels of *TRIM55* in human idiopathic dilated hearts ($n=11$ control hearts and $n=15$ idiopathic dilated hearts). We then performed a whole-genome analysis using the GEO tools to determine whether *TRIM55* was overexpressed in human idiopathic dilated hearts.

RESULTS

Whole-genome sequencing

We sequenced the whole-genome of the HHR and NHR and separately compared each to the Brown Norway reference genome (Rat Genome Sequencing Consortium, version 5.0). Postquality filtering was performed and duplicated sequences produced by PCR amplification were removed. Approximately 1.05 billion reads in the HHR and 977 million reads in the NHR were mapped to the Brown Norway genome, resulting in over 77% genome coverage and mean depth of whole-genome exceeding 25% for each strain.

The comparative analysis revealed over 8 million variants in the HHR and 7 million in the NHR (Fig. 1a and b) relative to the Brown Norway reference genome. The majority of these variants were SNPs – over 5.6 million and 4.7 million in the HHR and NHR, respectively. To enrich our comparison between the two genomes under investigation, a comparison of the variants of the HHR to the NHR was performed to identify those unique to either strain. Unique variants represent 2.8 million and 1.7 million of the HHR and NHR genome, respectively (Fig. 1c and d). In contrast to the overall variant comparison, CNVs were the most prevalent type of variants between HHR and NHR strains.

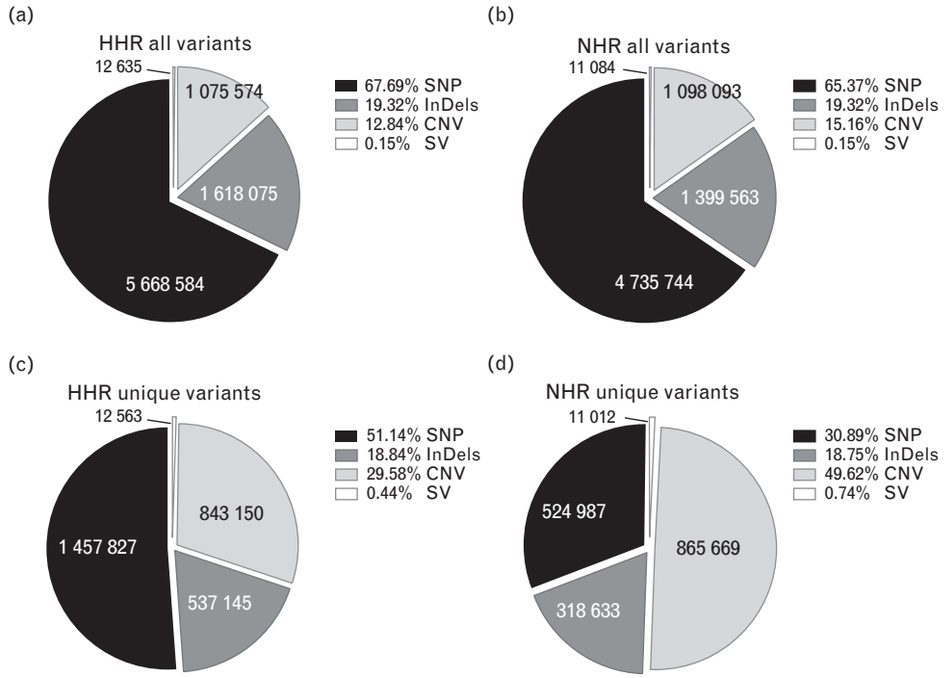

FIGURE 1 Whole-genome sequencing analysis of variants. (a) All variants in the HHR and (b) the NHR. (c) Unique variants in the HHR and (d) NHR. CNV, copy number variation; HHR, hypertrophic heart rat; InDel, insertions and deletions; NHR, normal heart rat; SNP, single nucleotide polymorphism.

We also investigated change rates in the HHR and NHR genomes according to analyses of SNPs and InDels. Overall, one variant is present in every 398 and 473 nucleotides in the HHR and NHR, respectively. According to our analysis, chromosome 20 and 12 have the highest change rate in the HHR and NHR, respectively. Meanwhile, the X chromosome has the lowest change rate in both genomes (Table S3, http://links.lww.com/HJH/A588).

We classified SNPs, insertions and deletions according to zygosity (Table S4, http://links.lww.com/HJH/A588). As expected, because of the inbred nature of both strains, most SNPs and InDels are homozygous. In regards to location, the majority of SNPs identified were intergenic, and the SNPs located in genomic regions were mostly present in introns in both genomes. Remarkably, no SNPs caused a frameshift in a coding region or loss of stop codon at a splice site (Table 1).

The SNPs located in coding regions were classified as synonymous or nonsynonymous. In our study, these SNPs were mostly synonymous not resulting in a change in the amino acid sequence. Furthermore, we classified the nonsynonymous SNPs into missense and nonsense. As expected, most SNPs were missense in both strains (Table S5, http://links.lww.com/HJH/A588).

Pathways associated with left ventricular hypertrophy

Among the genes with unique SNPs in the HHR, there was a significant overrepresentation of genes involved in adenosine triphosphate-binding cassette (ABC) transporters (rno02010), extracellular matrix-receptor interaction (rno04512), and tryptophan metabolism (rno00380), among others (Table S6, http://links.lww.com/HJH/A588).

Quantitative trait locus cardiac mass 22

We focused our gene expression and specific variants analysis present within the QTL Cm22. The overall number of variants and the unique variants were identified for each strain in this QTL (Table 2) and those variants were

TABLE 1. Classification of all single nucleotide polymorphisms according to type and region in the genome

Genome region	HHR	NHR
3′ UTR	14 842	12 251
5′ UTR	2295	2031
Downstream	241 403	203 590
Frameshift coding	–	–
Intergenic	4 253 768	3 554 880
Intronic	1 352 486	1 128 853
Processed pseudogene	6066	5133
Pseudogene	39 185	33 264
Splice site, 3′ UTR	21	16
Splice site, 5′ UTR	34	25
Splice site, intronic	2731	2303
Splice site, nonsynonymous coding	410	369
Splice site, synonymous coding	484	411
Start codon gained	356	297
Start codon lost	16	14
Stop gained	319	258
Stop gained at splice site	11	5
Stop lost	19	19
Stop lost at splice site	–	–
Upstream	247 516	209 474
Within mature microRNA	18 857	16 332
Synonymous coding	22 826	18 781
Nonsynonymous coding	19 033	16 002

HHR, hypertrophic heart rat; NHR normal heart rat; UTR, untranslated region.

TABLE 2. Variants identified in the quantitative trait locus cardiac mass 22

	All		Unique	
Type of variant	HHR	NHR	HHR	NHR
SNPs	182 404	153 527	45 731	16 854
InDels	46 787	10 038	15 954	10 038

HHR, hypertrophic heart rat; InDel, insertions and deletions; NHR normal heart rat; SNP, single nucleotide polymorphisms.

classified according to their type and region in the genome (Table 3).

The microarray data analysis showed that 109 genes were significantly differentially expressed (fold change >1.5) in the LV of neonatal HHR compared with neonatal NHR. The only gene differentially expressed in Cm22 was the gene for tripartite motif 55 (*Trim55*), being significantly downregulated in our data set (−1.52, adjusted $P = 0.0045$). Array results were validated using qPCR in LV from 2-day, 13 and 33-week-old animals confirming that *Trim55* was downregulated in HHR from neonatal to late adulthood (Fig. 2). Protein levels of *Trim55*, measured by western blot, were also lower in HHR than NHR (Fig. 3) in LV of 13-week-old animals.

To identify possible variant targets that could contribute to the hypertrophic phenotype, we explored unique variants found in the *Trim55* gene region (Chr2: 124 140 061–124 182 032) in addition to its upstream and downstream regions (Table 4). Our most significant finding was the exonic SNP present in the HHR. This exonic SNP is found in nucleotide position 124 181 039 of chromosome 2 (RGSC v5.0). This nonsynonymous variant leads to a missense mutation (C > T), resulting in a codon change (Ccc > Tcc) Tcc) and, therefore, a change from a proline to serine in the amino acid position 513 (P513S). In-silico protein modelling suggested this variant would lead to a change in the predicted three-dimensional protein structure (Fig. 4).

Trim55 mRNA levels were negatively correlated with LV mass in the F2 cross ($r = -0.16$, $P = 0.025$) at neonatal age. Although not statistically significant, *Trim55* mRNA abundance was also negatively correlated with CWI at 2 days ($r = -0.13$, $P = 0.05$), 13 weeks ($r = -1.04$, $P = 0.09$) and 33 weeks old ($r = -0.74$, $P = 0.07$) (Figure S3, http://links.lww.com/HJH/A588). We also found that the DNA sequence variant (P513S) was strongly associated with *Trim55* abundance ($F = 10.35$, $P < 0.0001$) (Fig. 5). The allele from the HHR was associated with a lower expression of *Trim55* in the F2 rats, consistent with the association observed in the parental strains.

Finally, we investigated the expression of *TRIM55* mRNA in human idiopathic dilated hearts. In these hearts, we found that the expression of TRIM55 was significantly downregulated compared with control hearts (−1.5, $P = 0.02$; Fig. 2d).

DISCUSSION

Our combined sequencing and expression analyses of the QTL Cm22, which we previously discovered on

TABLE 3. Single nucleotide polymorphisms in the quantitative trait locus cardiac mass 22 classified by type and region of the genome

	All		Unique	
Genome region	HHR	NHR	HHR	NHR
3′ UTR	305	254	60	9
5′ UTR	46	40	11	5
Downstream	3815	3296	853	334
Frameshift coding	–	–	–	–
Intergenic	148 512	124 158	38 712	14 358
Intronic	32 686	28 288	5896	2416
Processed pseudogene	62	47	21	6
Pseudogene	948	816	304	172
Splice site, 3′ UTR	–	–	–	–
Splice site, 5′ UTR	1	1	–	–
Splice site, intronic	43	38	6	1
Splice site, nonsynonymous coding	8	6	2	–
Splice site, synonymous coding	6	5	1	–
Start codon gained	8	7	2	1
Start codon lost	–	–	–	–
Stop gained	6	4	2	–
Stop gained at splice site	–	–	–	–
Stop lost	–	–	–	–
Stop lost at splice site	–	–	–	–
Upstream	3979	3533	871	425
Within mature microRNA	372	256	146	30
Synonymous coding	384	328	79	23
Nonsynonymous coding	404	372	73	41

HHR, hypertrophic heart rat; NHR normal heart rat; UTR, untranslated region.

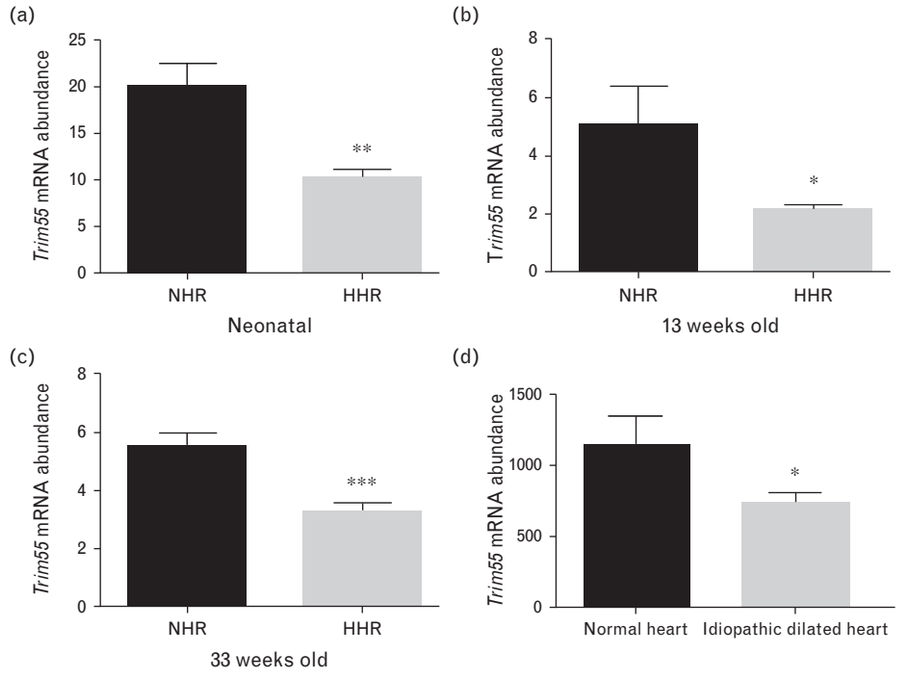

FIGURE 2 *Trim55* mRNA expression in the hypertrophic heart rat (HHR), normal heart rat (NHR), and human hearts. *Trim55* is downregulated in (a) neonatal, (b) 13-week-old, (c) 33-week-old animals, and (d) human idiopathic dilated hearts. Graphs represent mean and error bars represent standard error. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. *Trim55*, tripartite motif-containing 55.

chromosome 2, identified the gene encoding *Trim55* (alias *MuRF2*) as a functional candidate for cardiac hypertrophy. We discovered an exonic variant in *Trim55* that is strongly associated with *Trim55* gene expression in the HHR and the HHR × NHR F2 population. This variant was also associated with reduced Trim55 protein levels in the heart and the in-silico analysis predicted that the resultant amino acid change would alter protein folding. Furthermore, we have found that *Trim55* mRNA levels were reduced in the hearts from patients with human idiopathic dilated cardiomyopathy.

Our primary goal in this study was to delineate the genetic factors that program the characteristics of the heart in neonatal HHR that predisposes to adult hypertrophy. The clue to the origins of the hypertrophy was our previous discovery of reduced numbers of cardiomyocytes in the adult HHR [6]. Importantly, we found that soon after birth (neonates) HHR hearts tend to be smaller than NHR and possess fewer cardiomyocytes [6]. Around 6–10 weeks of age (at the age of reproductive maturity) cardiac hypertrophy begins to become apparent in the HHR hearts to become clearly established in adulthood. The implications of starting life with fewer cardiomyocytes are far reaching, as the loss of ability to replicate after the neonatal period (switch from proliferative to hypertrophic cellular growth) means that the deficit in cell numbers cannot be restored [25]. HHR hearts entering adolescence with fewer cardiomyocytes are less able to cope with the considerable physiological stresses of body growth (volume and pressure loads). Individual cells must hypertrophy rapidly not only to make up for missing cells but also for the relative reduction in contractile efficiency that often accompanies such hypertrophy. This exaggerated hypertrophy results in a bigger heart than normal in relation to body size after sexual maturity. In our study, we find *Trim55* as a primary candidate for the neonatal programming that predetermines adult cardiac hypertrophy and eventual failure in HHR.

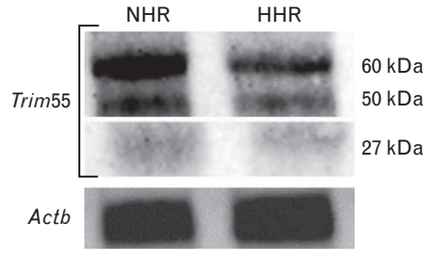

FIGURE 3 Representative western blot analysis showing protein levels of tripartite motif-containing 55 (*Trim55*) in the left ventricle of the hypertrophic heart rat (HHR) and the normal heart rat (NHR) at 13 weeks of age.

TABLE 4. Genomic region of variants in *Trim55* unique to each rat strain

Type of variant	Genomic region	HHR	NHR
	Upstream	0	0
	Exonic	1	0
SNP	Intronic	8	0
	Downstream	1	0
	Upstream	3	1
InDel	Exonic	0	0
	Intronic	7	12
	Downstream	0	2

HHR, hypertrophic heart rat; InDel, insertions and deletions; NHR normal heart rat; SNP, single nucleotide polymorphisms.

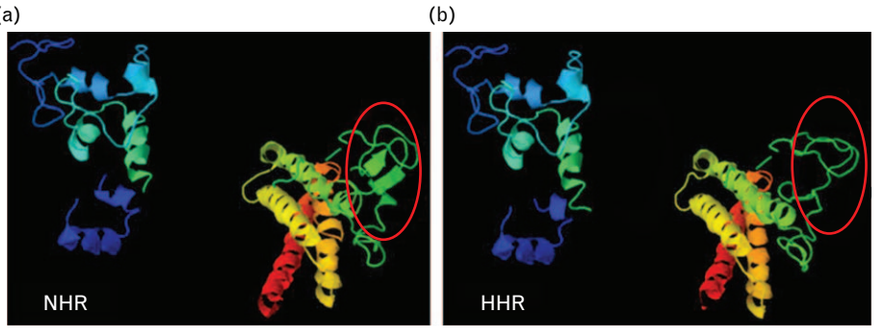

FIGURE 4 In-silico three-dimensional modelling of *Trim55* protein structure. (a) The predicted structure in the normal protein sequence and (b) including exonic SNP variant present in the HHR sequence using online software Phyre. A predicted change in the protein domain is circled in red. HHR, hypertrophic heart rat; NHR, normal heart rat; SNP, single nucleotide polymorphism; *Trim55*, tripartite motif-containing 55.

Trim55 and the cognate gene *Trim63* (alias *MuRF1*) encode striated muscle-specific proteins, which are localized at the sarcomere M-line [26] – the site of myosin crosslinking attachment at the titin C-terminal. This sarcomere region is understood to be of key importance in the transduction of sarcomeric mechanical stress. *Trim55* and *Trim63* are members of a subfamily of the really interesting new gene-finger E3 ubiquitin ligases involved in mediating replacement turnover of sarcomeric proteins via proteosomal degradation [27]. Thus, altered regulation of *Trim55* coding could be implicated in impaired sarcomeric protein turnover as a trigger for the hypertrophic growth response [28]. Trim55 knockout mice display severe diabetic cardiomyopathy characterized by early onset systolic dysfunction, higher LV mass, and increased heart weight when compared with wild-type littermates [29]. Moreover, neonatal *Trim55/Trim63* double knockout mice have severe LV hypertrophy and high mortality rate after birth [30]. *Trim63* was not differentially expressed in our data set and variants were not present (data not shown). However, the presence of a variant in the *Trim55* coding region leading to a change in the amino acid and protein sequence causing a change in the three-dimensional protein structure could influence the protein–protein interaction of *Trim55* and *Trim63* causing a disarray in the sarcomere assembly of cardiac muscle cells, as previously suggested [28]. Furthermore, the prevalence of variants in TRIM55 and TRIM63 is higher in patients with LV hypertrophy than in healthy controls and could contribute to increased risk of LV hypertrophy [26].

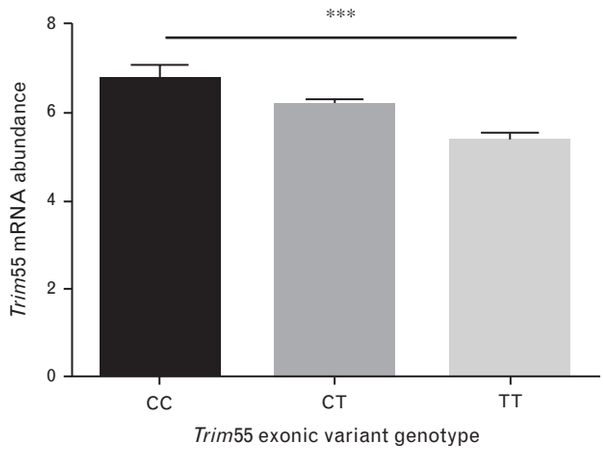

FIGURE 5 *Trim55* abundance in 2-day-old rats from the HHR × NHR F2 population according to exonic variant genotype. Graphs represent mean and error bars represent standard error. HHR, hypertrophic heart rat; NHR, normal heart rat; *Trim55*, tripartite motif-containing 55. $F = 10.35$, $P < 0.0001$.

This study is the first to describe genomic variation in a model of spontaneous cardiac hypertrophy, the HHR, and revealed many potential variants associated with cardiac mass independent of blood pressure that included SNPs, CNVs, and InDels.

The HHR strain carried a larger number of variants relative to the NHR strain. Both genomes, however, carried more SNPs and InDels than either of their parental strains, the SHR and Fischer 344 (approximately 3.8 million SNPs and 1.2 million InDels, and 3.4 million SNPs and 1.1 million InDels, respectively) [7,8]. In this context, the identification of unique variants to the HHR strain could assist the discovery of genetic variants, and therefore, candidate genes leading to the hypertrophic phenotype. Moreover, variants unique to NHR could present a protection or compensatory mechanism maintaining the normal phenotype.

Surprisingly, the frequency of variants is not uniform throughout the genome, and appeared to show no relation to chromosome size (Table S3, http://links.lww.com/HJH/A588). Chromosome 1, the largest rat chromosome, had the third largest change rate in the HHR and the seventh in the NHR. In contrast, the seventh largest rat chromosome, the X chromosome, had the largest change rate in both strains. These findings indicate a possible hotspot for mutations in the HHR that could contribute to the development of cardiac disease.

SNPs comprise the largest source of genetic variability in both humans and rats. The location and type of a SNP can differently affect gene expression and lead to diseases and phenotypical changes [31]. Nonsynonymous SNPs in an exon affect the amino acid and, consequently, the protein sequence and are believed to lead to the highest phenotypical changes in any genome [31,32]. Meanwhile, intronic and synonymous SNPs do not affect the amino acid sequence of a gene but could interfere with the splicing site region making site recognition difficult [33,34] or affect gene and protein expression by changing the shape of the mRNA hindering its interaction with the ribosome [35]. The intronic SNPs present in the *Trim55* could be hindering

the recognition of a splice site, which may influence mRNA levels of this gene.

Apart from our *Trim55* findings, our gene functional annotation analysis identified the ABC transporters pathway with the highest fold enrichment in our gene set. ABC transporters are membrane proteins involved in energy-dependant transport of substrates across biologic membranes [27]. Mutations in its components have been previously described to cause dilated cardiomyopathy in humans [36]. Moreover, other enriched pathways in our dataset (e.g. extracellular matrix remodelling, tryptophan metabolism, and lysosomal compartments) have also been associated with cardiac disease and remodelling [37–39]. Nonsynonymous mutations in genes from these pathways may be influencing the LV hypertrophy observed in the HHR.

The study has some limitations. It would be preferable to sequence a larger number of samples of each strain. Other studies with similar design, however, have also analyzed only one sample per strain [40]. Moreover, microarrays were analyzed in neonatal heart, before LV hypertrophy is established. The analyses of microarrays in hearts from animals after the development of LV hypertrophy could identify other genes in the QTL Cm22 differentially expressed with relevant function in this condition.

In conclusion, we have characterized unique variants present in the genome and identified *Trim55* as a candidate gene contributing to LV hypertrophy. Furthermore, we identified *Trim55* in the QTL Cm22 as differentially expressed in LV hypertrophy in both model and humans rendering a novel candidate gene for polygenic LV hypertrophy independent of blood pressure.

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Conflicts of interest

There are no conflicts of interest.

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Reviewers' Summary Evaluations

Reviewer 1

Cardiac hypertrophy, in particular left ventricular (LV) hypertrophy, is a risk factor for cardiovascular death, but the genetic factors determining LV size and predisposition to hypertrophy are not well understood. In this study, the authors found that Trim55 gene, located in Cm22, is a novel candidate gene for polygenic LV hypertrophy independent of blood pressure. This finding will be helpful both in clinical and basic research. In the future, the authors should focus on the molecular mechanism of Trim55 in the development of LV hypertrophy.

Reviewer 2

Left ventricular hypertrophy is a risk factor for cardiovascular death. However, the genetic factors that contribute to cardiac hypertrophy are not well understood. The paper by Prestes *et al.* identifies Trim55 as a functional candidate for spontaneous cardiac hypertrophy in the rat locus Cm22. Generally, the strength of the manuscript comes from its strong genetic data support. One minor concern is that many of genetic data were collected from neonate rats. Furthermore, selective Trim55 gene manipulation in cardiomyocytes will be recommended to test if this gene is functionally related to cardiomyopathy in cardiovascular diseases in future studies.