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Exome Sequencing of families with Histiocytoid Cardiomyopathy reveals a Complex I Mitochondrial Etiology with *NDUFB11* Mutation

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Abstract

Histiocytoid cardiomyopathy (Histiocytoid CM) is a rare form of cardiomyopathy observed predominantly in newborn females that is fatal unless treated early in life. We have performed whole exome sequencing on five parent-proband trios and identified nuclear-encoded mitochondrial protein mutations in three cases. Two probands had *de novo* non-sense mutations in the second exon of the X-linked nuclear gene *NDUFB11*, which has not previously been implicated in any disease, despite evidence that deficiency for other mitochondrial electron transport complex I members leads to cardiomyopathy. A third proband was doubly heterozygous for inherited rare variants in additional components of complex I, *NDUFAF2* and *NDUFB9*, confirming that Histiocytoid CM is genetically heterogeneous. In a fourth case, the proband with Histiocytoid CM inherited a mitochondrial mutation from her heteroplasmic mother, as did her brother who presented with cardiac arrhythmia. Strong candidate recessive or compound heterozygous variants were not found for this individual or for the fifth case. Although *NDUFB11* has not been implicated before in cardiac pathology, morpholino-mediated knockdown of *Ndufb11* in zebrafish embryos generated defective cardiac tissue with looping defects, which confirms the causative role of *NDUFB11* in cardiac pathology. Therefore, the *NDUFB11* mutation represents a genetic basis of this heterogeneous disease.

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Internet Resources

Online Medelian Inheritance in Man (OMIM): http://www.omim.org/

RefSeq: http://www.ncbi.nlm.nih.gov/refseq/

Keywords

histiocytoid cardiomyopathy; NDUFB11; zebrafish; morpholinos; whole exome sequencing; *de novo* mutation

INTRODUCTION

Infantile histiocytoid cardiomyopathy (Histiocytoid CM, MIM 500000) is a rare, but distinctive arrhythmogenic disorder characterized by incessant ventricular tachycardia, cardiomegaly, and sudden death within the first two years of life if left untreated. Approximately 100 Histiocytoid CM cases have been reported in the literature [Bove et al., 1973; Ferrans et al., 1976; Shehata et al., 1998; Shehata et al., 2011; Ferrans et al., 1976; Gelb et al., 1993; Zimmermann et al., 1982; Malhotra et al., 1994; MacMahon et al., 1971; Andreu et al., 2000; Vallance et al., 2000; Ruszkiewicz et al., 1995; Prahlow et al., 1993; Heifetz et al., 1995], but the prevalence is likely to be higher since many cases of Histiocytoid CM may have been misdiagnosed as Sudden Infant Death Syndrome [Grech et al., 2000] [MIM 272120]. The disease was first confused with rhabdomyoma, benign tumors of the myocardium, and was not recognized as a separate pathologic entity until 1962 by Voth [Voth., 1962]. After observing familial tendency, the first author created an Histiocytoid CM registry in order to collect cases and perform analyses with the objective of identifying the causative gene(s). The registry can be found at spponline.org.

Although several disease mechanisms have been proposed, the etiology of Histiocytoid CM remains unknown. An early report [Andreu et al., 2000] of a missense mutation in Cytochrome *b* (*MTCYB* [MIM 516020]), a mitochondrion-encoded protein from complex III of the electron transport chain (ETC), suggested a mitochondrial pathology. However, analysis of additional 27 cases from the Histiocytoid CM registry through collaborative work with two institutions failed to verify this gene as a causal gene for Histiocytoid CM. [Andreu et al., 2000] A second mitochondrial mutation was found at A8344G within the *MT-TK* gene that encodes $tRNA^{LYS}$ [MIM 590060] in the mtDNA, and has previously been implicated in myoclonic epilepsy associated with ragged-red fibers syndrome [Vallance et al., 2004] (MERRF [MIM 545000]).

Whole transcriptome DASL profiling of heart tissue from 12 cases and 12 age-matched controls [Shehata et al., 2011] failed to provide evidence that differential gene expression of these putative causal genes for Histiocytoid CM from case studies may have a broader role in pathology. That study did identify seven differentially expressed genes in Histiocytoid CM that relate to interleukin signaling, but a causative role in cardiovascular pathology has not been demonstrated.

Some physiological characteristics of the disease also support mitochondrial dysfunction in the etiology. Most cases of Histiocytoid CM show accumulation of aberrantly-shaped and excessive numbers of mitochondria in the cardiomyocytes. Lipids in small vacuoles are commonly observed to fill the intracellular space, generating a foamy cytoplasm that has been considered to result from failure of energy generation. Unlike other mitochondrial diseases, however, Histiocytoid CM does not affect all cardiomyocytes equally. The

observation that lesions tend to be clustered in the vicinity of the Purkinje fibers has also prompted the argument that the disease may be related to defective activity of this sub-type of cardiomyocyte that coordinate the cardiac action potential [Brunton et al., 1977].

MATERIALS AND METHODS

The material, collected over the last decade, includes over 100 cases, the majority of which are from autopsies, as well as over 20 cases collected from children who were diagnosed early and treated either with surgical excision or ablation of the arrhythmogenic foci. In cases where there is extensive involvement of Histiocytoid CM a cardiac transplant was performed. We estimate the sibling recurrence rate to be approximately 5%, but note that this is likely to be downward biased due to the incidence of recurrent miscarriages in some families.

In order to overcome the ambiguity of candidate gene studies, we have initiated whole exome sequencing [Ng et al., 2009] of Histiocytoid CM cases and here report results from five trios consisting of an affected proband and both biological parents (with one exception, Figure 1, HC4) who provided informed consent for the study. Each individual exome was sequenced to an average depth of 172× using Illumina TruSeq technology (Table SI). In addition to cataloguing all instances of recessive and compound heterozygous inheritance in each proband, custom perl scripts were written to identify all heterozygous mutations where both parents were homozygous for the HG19 reference allele. Synonymous substitutions were annotated using the SeattleSeq database [http://snp.gs.washington.edu/SeattleSeqAnnotation138/]. The results are summarized in Table I, which lists *de novo* and inherited rare variants (MAF<1%), and compound heterozygotes where at least one allele is predicted deleterious by the MutationTaster algorithm [Schwarz et al., 2010].

Sample Acquisition and DNA Isolation

Whole blood samples were obtained from patients and first degree relatives (parents and living siblings) after informed written consent was received under the research protocol and approved by the Emory University Institutional Review Board (IRB). This research is in compliance with the Helsinki Declaration for conduct of research utilizing human subjects. All the cases in this study have been confirmed by Dr Bahig Shehata as Histiocytoid CM. For isolation of DNA, whole blood was stored at 4°C until DNA extraction using the Mag-Bind SQ Blood DNA Isolation Kit (Omega Biotek, Atlanta, GA) according to the manufacturer's instructions. The concentration of DNA was confirmed on a Nanodrop spectrophotometer, and the RNA quantity values were greater than 1.9.

Exome Sequencing and Variant Prediction

To screen for causal mutations for Histiocytoid CM, the isolated DNA was enriched for 51MB of exonic DNA using Agilent SureSelect Solution probes (Agilent Technologies, Santa Clara, CA). Using the resulting exonic DNA, exome data was generated at Vanderbilt University (Nashville, TN) using high-throughput sequencing on an Illumina HiSeq2000, according to standardized operating procedures. The BWA short read alignment tool [Li et al., 2009] was used to map each mate-pair of reads (length 2×102 nt or 2×77 nt) to the

human genome (GRCh37). Samtools [Li et al., 2009] was then used to sort and index the bam files, and subsequently for generation of a pileup which was ported to VarScan [Koboldt et al., 2009] for variant calling. Calls with less than 10% of support from one strand were subsequently removed from further consideration. A total of 273,184 SNPs and 38,321 short indels were identified. Mitochondrial exons were included in the analysis, as they are pulled down passively with the Agilent SureSelect protocol, and the variants reported here had an average read depth of 40×.

Gene Annotation

Variants were analyzed against the RefSeq hg18 gene definitions including 18,933 genes. Potential causal mutations were then annotated with the software package SeattleSeq [SeattleSeq]. A list of rare homozygous deleterious (RHD) mutations was obtained after excluding SNP/Indel calls not within the CDS regions or with less than 20× depth. SNPs were reported as RHD if they had a minor allele frequency (MAF) of less than 0.01 or were not present in dbSNP137. The calls were then manually inspected using the Integrative Genome Viewer (IGV) [Thorvaldsdottir et al., 2013].

MitoMap [Brandon et al., 2005] is a comprehensive database of human mitochondrial DNA from adult human tissues and serves as a universal reference of human mitochondrial DNA variation including tRNA/rRNA mutations, as well as coding and control region mutations. MitoMap was used to identify causal mitochondrial mutations in the disease and first-degree relative samples. We specifically focused on SNPs where the affected patient was heterozygous and the patients' parents were homozygous for the reference allele.

Zebrafish Husbandry and Morpholino Injection

Zebrafish (*Danio rerio*) used in this study were housed at the CSIR-Institute of Genomics and Integrative Biology following standard husbandry practices [Westerfield et al., 2000]. All experiments were performed in strict accordance with the recommendations and guidelines laid down by the CSIR Institute of Genomics and Integrative Biology, India, and the protocol was approved by the Institutional Animal Ethics Committee (IAEC) of the Institute. All efforts were made to minimize animal suffering.

Wildtype and transgenic zebrafish embryos were obtained by pair wise mating of adult. Tg*(cmlc2:mRFP)* that expresses red fluorescent protein in the heart. Alternatively, the Tg*(fli1:EGFP; gata1a:dsRed)* zebrafish line that expresses green fluorescent protein (GFP) in endothelial cells and red fluorescent protein in blood cells was used [Lalwani et al., 2012].

Morpholino (MO) oligonucleotides (Gene Tools, USA) were dissolved in nuclease free water (Ambion, USA) at a concentration of 1mM according to the protocols recommended by Gene Tools. 1mM stocks of MO oligos were stored at −80°C. Working aliquots of MO oligos were prepared and stored at 4°C. The *ndufb11* MO oligo sequences are GTTTCGAGACAGCTACCGCTTCGAG and AGACGTGAGAGCATTCTCCCGACTT. Microinjection glass capillary (World Precision) micropipettes were pulled using Sutter

Instrument (USA) and clipped appropriately to deliver 1–3 nl solution into 1–2 cell zebrafish embryos.

RESULTS

In two of the Histiocytoid CM cases, GHCT and GHCG, distinct non-sense *de novo* mutations were detected in exon 2 of the *NDUFB11* gene [MIM 300403, Refseq accession number NC_000023.10]. Given an average of one non-sense mutation per individual, the probability that two of the five cases would have disruptions in the same gene is less than 1 in 4,500. Including a prior expectation of disrupted mitochondrial function, the odds of this observation are considerably smaller. Furthermore, the Exome Variant Server lists just 4 mis-sense rare variants in the gene, only two of which are possibly deleterious, fewer than any other *NDUF* gene in the database and implying a high level of purifying selection. *NDUFB11* encodes the NADH-ubiquinone oxidoreductase ESSS subunit component of complex 1, and the two truncating mutations are likely to have a deleterious effect on the stability of the complex, compromising energy production. One of the two *de novo* mutations is an A→C transversion that changes a tyrosine at codon 108 to a premature stop, while the other is a C→T transition that changes a tryptophan at codon 85 to a premature stop. Both were confirmed by Sanger sequencing (Supplemental Figure S1). Since there is only a single intron in the gene, it is unlikely that alternative splicing would rescue protein function bypassing the premature stop codons, but no attempt was made to exclude this possibility since RNA was not available for sequencing.

A complete list of *de novo*, rare homozygous recessive, and deleterious compound heterozygous variants in each of the four trio probands and one additional case with missing paternal data is provided in Table SIII, while all discovered polymorphisms are listed in Table SIV. For one case we have been unable to highlight a likely causal genotype. The proband from trio HC7 was doubly heterozygous for mutations in two different genes, in *NDUFAF2* and *NDUFB9*, both inherited from singly heterozygous parents, and both predicted by MutationTaster [Schwarz et al., 2010] to be deleterious. Although activity at each locus is presumably provided by the alternate allele, we postulate (in light of the *NDUFB11* results) that compound loss of function of these two components of the same electron transport chain is sufficient to promote Histiocytoid CM. Furthermore, *NDUFAF2* and *NDUFB9* have both been implicated previously in mitochondrial complex I deficiency [Schlehe et al, 2013; Haack et al, 2012].

The fourth family, HC4, included a female infant who died of Histiocytoid CM and her brother who, like his mother, has symptoms of arrhythmia and tachycardia. The mother is heteroplasmic for A15924G in the mtDNA, affecting *MT-TT* ($tRNA^{THR}$), and both children seem to be homoplasmic for the same mutation, which was previously reported to cause two cases of lethal infantile mitochondrial myopathy (LIMM [MIM 551000]) [Yoon et al., 1991; Brown et al., 1992]. However, according to dbSNP [Sherry et al., 2001] the variant is at a prevalence of 2.3% in humans so would appear to have variable penetrance and expressivity and cannot reliably be considered the cause of Histiocytoid CM in this proband. Table SIII includes evaluation of the potential deleteriousness of each site using the AACDS classification scheme [Preeprem et al., 2013]. A handful of variants were found that are

simultaneously highly likely to be deleterious and in genes associated with diseases or traits (AACDS categories 2B and 3B). Proband GHC-G is also homozygous for a non-synonymous substitution in a gene (*RAI14*) previously linked to linked left ventricular mass, but which is predicted to be benign, while HC4 is homozygous for a recessive variant thought to contribute to colorectal cancer (*KMT2C* Leucine to Phenylalanine substitution) and for a predicted benign mutation in *COL17A1*, a hemidesmosomal component gene that has also been repeatedly linked to epidermolysis bullosa [Vanotti et al., 2013].

In order to examine the role of *NDUFB11* in cardiac development and function, we have employed the zebrafish vertebrate model, which is well established for the study of cardiac dysfunction [Liechke et al., 2007]. *Danio rerio ndufb11* is known to be expressed ubiquitously during embryogenesis [Thisse et al., 2001]. Morpholino (MO) mediated transient translational suppression of *ndufb11* in zebrafish embryos was performed with two different morpholinos injected into one-cell zebrafish embryos at a final dose of 0.4mM (Figure 2A and Supplemental Figure S2). Knockdown of *ndufb11* in zebrafish embryos carrying the heart marker Tg{*cmlc2*:mRFP} displayed edema and abnormal heart structure in approximately 80% of injected animals. Detailed analysis revealed heart structure defects such as loss of the S-shaped heart at 3 days post fertilization (dpf), resulting in a linear heart tube consistent with defective cardiac looping (Figure 2 E,G; Supplemental Movies 1,2). This linear heart tube defect was confirmed by whole mount *in situ* hybridization of the *gata5* transcript, which is expressed in heart tissue along with the gut and pharynx (Figure 2H,I). Suppression of *ndufb11* expression in Tg {*fli1*: EGFP: *gata1*: dsRED} zebrafish embryos also revealed defects in angiogenic vessels (Figure 3). We did not observe any sign of apoptosis associated with *ndufb11* morpholino injections compared to non-injected zebrafish embryos as determined by whole mount TUNEL assay (Supplemental Figure S3). In summary, knockdown of *ndufb11* in developing zebrafish embryos results in abnormal heart structure suggesting its putative role in cardiac development in zebrafish embryos.

DISCUSSION

A causal role for truncation of *NDUFB11* in the etiology of Histiocytoid CM helps to explain the female-bias of the disease, which presents in girls in 75% of cases. Whereas most complex I deficiencies are thought to be inherited in a Mendelian recessive manner, these two *de novo* mutations establish a dominant haploinsufficient phenotype. The gene is located on the short arm of the X-chromosome at interval Xp11.23. It is tempting to speculate that similar mutations occurring in males are embryonic lethal and cause miscarriage, since there would be no residual protein activity. By contrast, male bias for mitochondrial encephalomyopathy was associated with recessive X-linked *NDUFA1* mutations [Fernandez-Moreira et al., 2007].

Our data also confirm that Histiocytoid CM is genetically heterogeneous since *NDUFB11* did not carry mutations in the other three probands. Thus, some cases are likely due to autosomal mutations that may segregate in a recessive manner, some of which may also be modified in male and female backgrounds.

Many previous studies have implicated 16 different *NDUF* genes in respiratory chain complex I deficiency, which presents with highly variable morbidity due to exercise intolerance and muscle wasting, and may also result in neonatal mortality [Von Kleist-Retzow JC et al., 1998; Calvo et al., 2012; Swalwell et al., 2011]. Typical features include lactic acidosis, neuropathy, and neurodegeneration, and in approximately one quarter of cases, cardiomyopathy. This raises two questions: why has *NDUFB11* escaped identification as a disease gene until now, and why did these two cases present with such a specific and severe cardiac defect?

A possible explanation for the severe defect is that NDUFB11 may be particularly critical in heart tissue. Intriguingly, the BioGPSserver [Wu et al., 2009] reports three-fold elevated expression of *NDUFB11* transcripts in the heart over most other tissues, a feature it shares with another cardiomyopathy-associated family member *NDUFS2* [MIM 602985]. Most other *NDUF* genes do not have elevated cardiac expression. However, the correlation is not complete as the Leigh Syndrome [MIM 25600] associated *NDUFS7* [MIM 601825] and *NDUFS8* [MIM 602141] also show relatively high expression in the heart. We consider it more likely that the Histiocytoid CM phenotype arises as a result of modification of the primary defect in mitochondrial electron transport by the genetic background, either additional rare variants such as those mentioned in the text, or unknown common variant contributions to mitochondrial function.

One further interesting finding arose from the comparison of exome sequence data derived from cardiac tissue and whole blood for the proband GHCG with the *de novo* Trp85 non-sense mutation. A second non-sense mutation was detected in *Cytochrome b*, but only at a frequency of 20% and only in the cardiac tissue, possibly indicating clonal selection of a somatic mutation in the diseased heart. This individual also showed a Val→Met mis-sense substitution in *ACSS1* [MIM 614355], which encodes a mitochondrial acetyl-coA synthetase. Since the variant is common in all human populations is not causal for Histiocytoid CM, but may have modified the phenotype as well. Whole transcriptome expression profiling of 12 cases and 12 age-matched controls [Shehata et al., 2011] detected significant differential expression of 1356 probes enriched for cell death as well as cardiac development and function. Consequently, there are many possible avenues through which the expressivity of defects in complex I activity might be regulated, giving rise to diseases as diverse as Histiocytoid CM and Leigh syndrome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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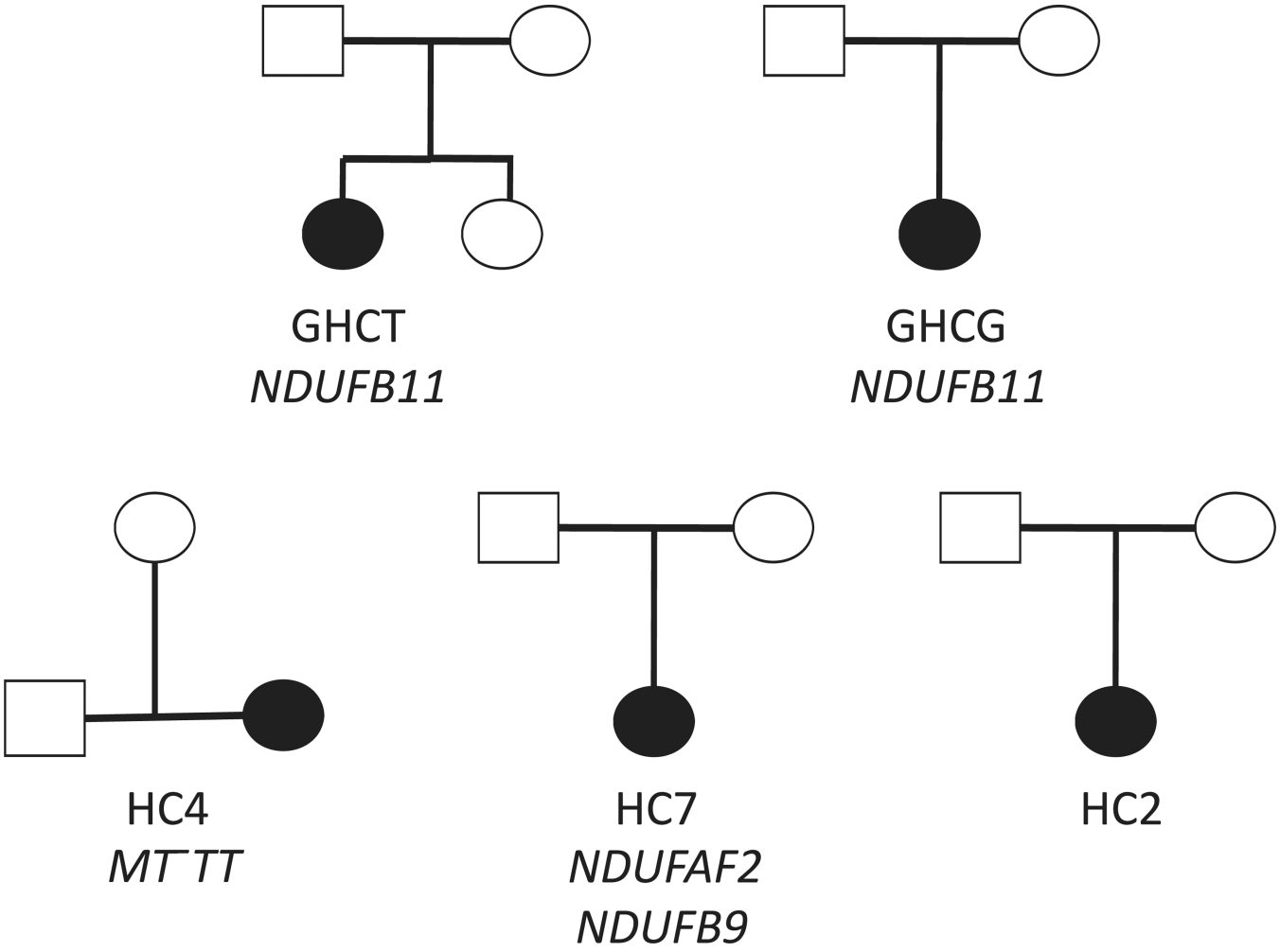

Figure 1.

Patient pedigrees and mutations. Probands are indicated as filled circles (all are females). For HC4, the father was unavailable and is denoted in gray. Implicated causal mutations are shown underneath each trio.

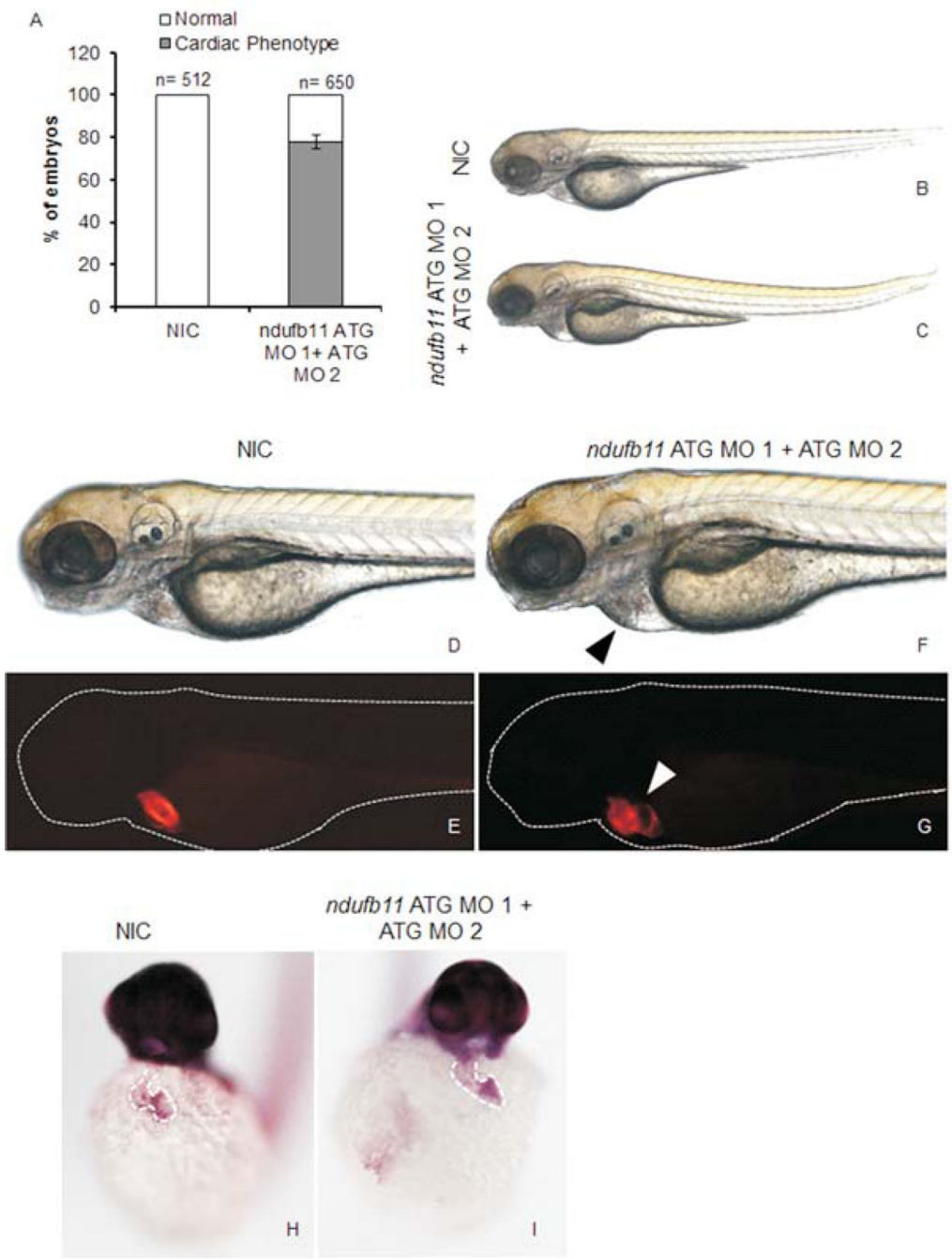

Figure 2.

Microinjection of ndufb11morpholino (MO) in zebrafish embryos leads to cardiac tissue defects. (A) Bar graph showing cardiac defect phenotype in *ndufb11*ATG MO 1+ATG MO 2 injected Tg*(cmlc2: mRFP)* zebrafish embryos at 3 days post fertilization (dpf). Data is represented as mean percentage ± SD (standard deviation) collected over 7 independent experiments and n is number of embryos analyzed. (B–G) Representative images of cardiac defects in zebrafish embryos at 3 dpf. (B,D,E) Non-injected control embryos (NIC) with normal cardiac development and (C,F,G) ndufb11 MO injected embryos displaying defect in

cardiac structure. Arrowheads indicate regions with cardiac tissue defect. (H, I) Expression of *gata5* transcript in 48 hpf zebrafish embryos. (B,C) 2.5× magnification, (D–G) 5× magnification.

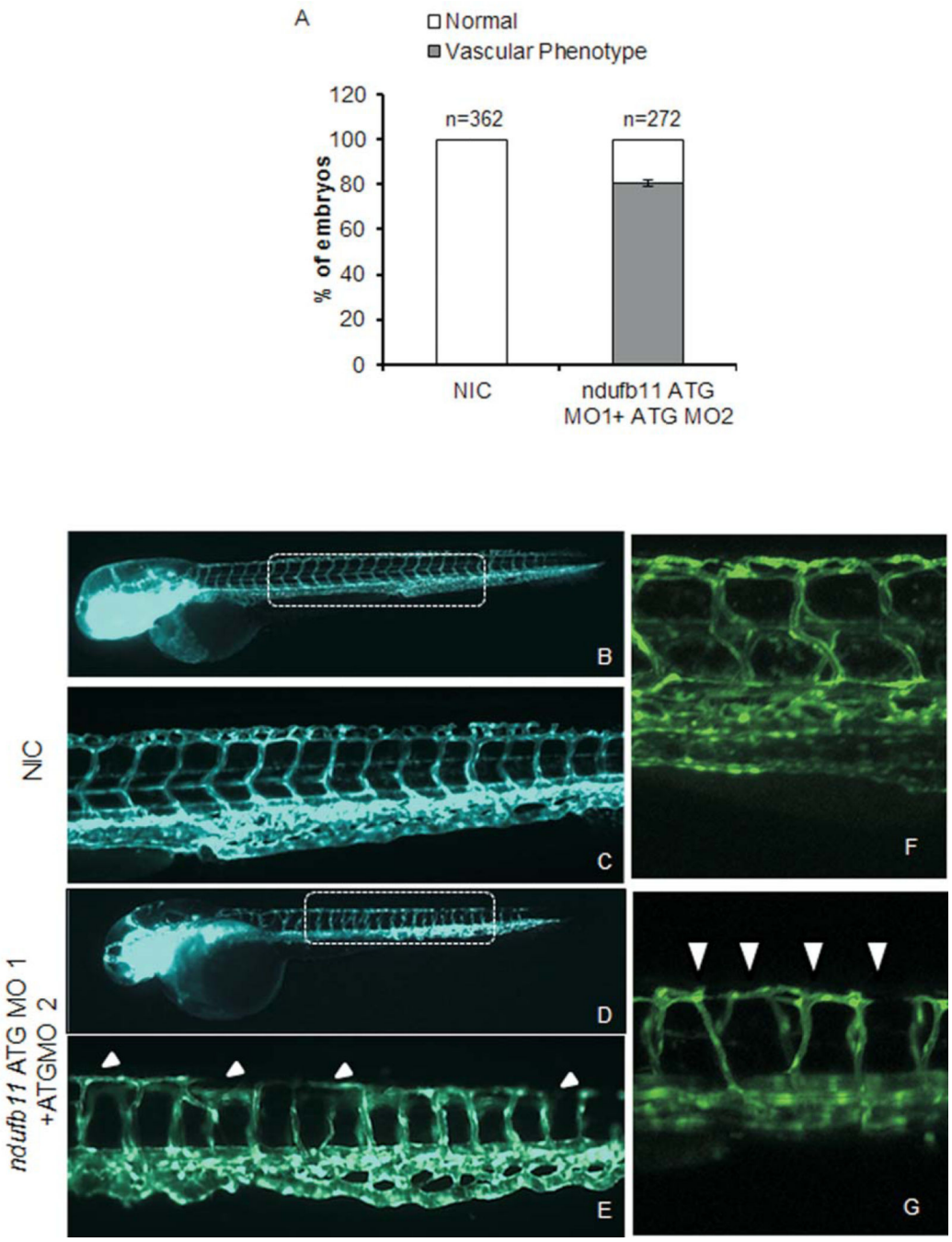

Figure 3.

Microinjection of ndufb11morpholino (MO) in zebrafish embryos leads to vasculature defects. (A) Bar graph showing embryos with normal and defective inter-segmental vessel in non-injected control (NIC) and 400 μM ndufb11ATG MO 1+ ATG MO 2 morpholino injected embryos at 72 hpf. (B–G) Representative images of 72 hpfTg*(fli1:EGFP, gata1a: dsRed)* zebrafish embryos. (B,C,F) Non-injected control embryos (NIC) with normal vasculature and (D,G,H) ndufb11 MO injected embryos displaying vasculature defect. (B–E) 2.5× magnification. (F,G) 20× magnification. Images are arranged in a lateral view and

inset displaying intersegmental vessels from the trunk region. Arrowheads indicate regions with vascular defects.

Table I

Summary of Exome Sequencing Results[#]

Individual	De novo non-sense	De novo mis-sense	RHD non-syn[1]	RHD syn[2]	DCH[3]	Candidate mutation
GHC-T	2	0	15	17	1	*NDUFB11*
GHC-G	1	0	15	10	2	*NDUFB11*
HC2	0	0	9	12	1	
HC4	n/a[4]	n/a	n/a	n/a	n/a	*MT-TT*
HC7	0	1	19	16	7	*NDUFAF2/B9*

[#]See Supplemental Table S3 for a description of the rare or de novo variants at each locus.

[1]Rare homozygous non-synonymous substitutions inherited from carrier parents

[2]Rare homozygous synonymous substitutions inherited from carrier parents

[3]Deleterious compound heterozygotes, at least one allele predicted deleterious by Mutation Taster

[4]Data not available since one parent missing

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