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Mutations in *NTRK3* suggest a novel signaling pathway in human congenital heart disease

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Abstract

Congenital heart defects (CHDs) are the most common major birth defects and the leading cause of death from congenital malformations. The etiology remains largely unknown, though genetic variants clearly contribute. In a previous study, we identified a large copy number variant (CNV) that deleted 46 genes in a patient with a malalignment type ventricular septal defect (VSD). The CNV included the gene *NTRK3* encoding neurotrophic tyrosine kinase receptor C (TrkC), which is essential for normal cardiogenesis in animal models. To evaluate the role of *NTRK3* in human CHDs, we studied 467 patients with related heart defects for *NTRK3* mutations. We identified four missense mutations in four patients with VSDs that were not found in ethnically matched controls and were predicted to be functionally deleterious. Functional analysis using neuroblastoma cell lines expressing mutant TrkC demonstrated that one of the mutations (c.278C>T, p.T93M) significantly reduced autophosphorylation of TrkC in response to ligand binding, subsequently decreasing phosphorylation of downstream target proteins. In addition compared to WT, three of the four cell lines expressing mutant TrkC showed altered cell growth in low-serum conditions without supplemental NT-3. These findings suggest a novel pathophysiological mechanism involving *NTRK3* in the development of VSDs.

Keywords

congenital heart disease; ventricular septal defect; copy number variants; CNV; *NTRK3*

Introduction

The etiology of congenital heart defects (CHDs) is poorly understood, even though cardiac malformations are the most common birth defects with a high perinatal morbidity and early

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mortality [Hoffman and Kaplan, 2002]. Despite advances in the identification of environmental as well as genetic factors [Jenkins et al., 2007; Pierpont et al., 2007; Richards and Garg, 2010], these observations explain only a small fraction of CHD cases so far. CHDs are frequently a component of genetic syndromes that are caused by chromosomal abnormalities. Detailed genetic analyses of deleted regions in syndromes such as DiGeorge and Alagille syndrome have identified a few genes responsible for CHDs including *TBX1* (T-BOX 1; MIM# 602054) and *JAG1* (JAGGED 1; MIM# 601920), respectively, [Pierpont, et al., 2007; Richards and Garg, 2010]. With recent advances in technology, additional copy number variants (CNVs) have been identified on genome wide scans, and rare CNVs have since been associated with disease risk in a variety of complex disorders, including CHDs [Cooper et al., 2011; Pinto et al., 2010; Soemedi et al., 2012; Xu et al., 2008]. Genes contained within these CNVs become prime candidates for the associated disease.

In a genome wide study undertaken to identify new candidate genes for human CHDs, we previously screened 58 patients with CHDs and other congenital anomalies with no identified genetic syndrome for CNVs. In a previous publication we described 12 patients with 12 novel CNVs that were absent in over 2,000 ethnically matched controls [Goldmuntz et al., 2011]. As previously described, one patient diagnosed with a posterior malalignment type of ventricular septal defect (pmVSD) had a 4.3-Mb deletion on chromosome 15q25-26 that encompassed 46 genes, including *NTRK3* (neurotrophic tyrosine kinase, receptor, type 3; MIM# 191316), which encodes the neurotrophic tyrosine kinase receptor C (TrkC) [Goldmuntz, et al., 2011]. Originally studied in the development of the nervous system, TrkC and its only ligand, neurotrophin 3 (NT-3) were found to regulate survival and differentiation in developing neural crest cells [reviewed in Barbacid, 1994; Bibel and Barde, 2000]. Upon binding NT-3, TrkC autophosphorylates and activates the PI3K/AKT and MAPK pathways [Caporali and Emanueli, 2009; Kaplan and Miller, 2000]. Although *NTRK3* has not yet been associated with human CHDs, animal models support its role in heart development [Donovan et al., 1996; Tessarollo et al., 1997]. Indeed, TrkC null (−/−) mice mostly die within the first weeks after birth due to severe cardiac defects. The heart defects seen in these mice resemble those commonly seen in humans, including atrial septal defects (ASD), VSDs, and tetralogy of Fallot (TOF), suggesting that *NTRK3* could also play a role in human cardiovascular malformation. Consequently, we chose to study *NTRK3* in our patient cohorts for disease-related sequence variations.

Materials and Methods

Patient cohort

Our study cohort consisted of 467 CHD patients with a conotruncal or related defect including cases with tetralogy of Fallot (TOF), truncus arteriosus (TA), interrupted aortic arch type B (IAA), and ventricular septal defect (VSD). The VSD cases were limited to those with conoventricular, posterior malalignment or conoseptal hypoplasia type VSDs (Table 1). Cases did not carry a 22q11.2 deletion and were not diagnosed with any recognizable genetic syndrome at the time of enrollment. Informed consent was obtained from all patients and parents following protocols approved by the Institutional Review Board for Human Research at The Children's Hospital of Philadelphia prior to collection of

samples. A three-generation pedigree was taken by a research genetic counselor and medical records were reviewed to identify additional congenital anomalies and/or familial disease. Case and parental DNA was extracted from whole blood, buccal swabs, or lymphoblastoid cell lines using standard methods (Gentra Puregene Blood kit by Qiagen, Valencia, CA). DNA from ethnically matched control subjects (see Table 2) was obtained from Coriell Institute for Medical Research (Camden, NJ).

Amplification and Sequencing

We evaluated all 20 coding exons of the 4 reference sequences for *NTRK3* (NM_002530.3, NM_001012338.2, NM_001007156.2, NM_001243101.1) in probands. PCR products with a size range of 213-399 bp spanning exonic sequences as well as exon/intron boundaries (see Supp. Table S1) were analyzed for sequence variations by high-resolution amplicon melting on a LightScanner (Idaho Technology Inc, Salt Lake City, UT) (except for exon 2, see below). Primer sequences were designed using OLIGO® 6.8 primer analysis software (Molecular Biology Insights, Inc., Cascade, CO) and are listed together with annealing temperatures, and product size in Supp. Table S1. Samples were amplified in a volume of 10 μl containing 1X LightScanner® Master Mix (Idaho Technology Inc.), 20 ng of DNA and 0.25 mM of each primer following the suggested PCR protocol, but with or without the inclusion of 3 touchdown cycling steps at the beginning starting 3°C above the annealing temperature, decreasing 1°C per step. PCR products were then transferred to the LightScanner for melting analysis. Melting curves were analyzed for sequence variations using the LightScanner Software. Samples that showed variation in the melting curves were chosen for sequencing. PCR products were re-amplified in a volume of 20 μl using AmpliTaq Gold Polymerase (Applied Biosystems, Life Technologies, Carlsbad, CA), with 20 ng of DNA and a final concentration of 0.2 mM dNTP, 2 mM $MgCl_2$, and 0.25 μM of each primer. PCR products were purified using the Agencourt AMPure XP system (Beckman Coulter Genomics, Danvers, MA) before sequencing using BigDye™ Terminator version 3.1 on an Applied Biosystems (ABI 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA) sequencer. Due to its high GC content, exon 2 was amplified either by using the GC Rich PCR system (Roche Applied Science, Indianapolis, IN) or using AmpliTaq Gold with 1M Betaine (Sigma-Aldrich, St. Louis, MO), 2mM $MgCl_2$, 0.375mM dNTP, 0.3 μM primers and 20 ng of DNA using a touchdown protocol. PCR products were submitted directly for sequencing after purification with the Agencourt AMPure XP system.

Sequence Analysis

Sequences were analyzed using Sequencher™ (Gene Codes, Ann Arbor, MI). Proband, parental and control DNA sequences were compared to the reference sequence for *NTRK3* (NM_001012338.2) to identify sequence variations. Identified variations were verified by Sanger sequencing. The novel variations have been submitted to dbSNP, http://www.ncbi.nlm.nih.gov/snp/ (ss# 825679011-825679013). The location of the altered nucleotide is based on the cDNA reference sequence NM_001012338.2.

Site-directed mutagenesis

A retroviral vector pLNCX (GenBank Acc#: M28247, Clontech Laboratories, Inc, Mountain View, CA) containing the *NTRK3* cDNA coding for the 825 AA TrkC protein (Reference sequence 2: NM_002530.2) was transformed into One Shot TOP10 competent cells (Invitrogen Corporation, Carlsbad CA). The vector was isolated using the Qiagen Plasmid Midi Kit (Qiagen,) and verified by sequencing. We introduced the four human variants identified into the wild type (WT) sequence of *NTRK3* by site directed mutagenesis using a QuickChange II Site- directed Mutagenesis Kit (Stratagene, La Jolla, CA) (see Table 2). The mutagenic oligonucleotide primers used for each of the variants were designed using the QuikChange Primer Design Program from Stratagene and are listed in Supp. Table S2. We also created a negative control through inactivation of the ATP bindings site by changing Lysine (AAG) at position 572 to Asparagine (AAC) [Eggert et al., 2000]. Mutated clones (see Table 3) were transformed into One Shot TOP 10 competent cells, isolated using a QIAprep Spin Miniprep Kit (Qiagen), and Sanger sequenced for verification.

Transfection of SY5Y cells

The *NTRK3* vector constructs (see Table 3), and an empty vector pLNCX2 (Clontech Laboratories, Inc.) were each transfected separately into the packaging cell line BING (ATCC® #: CRL-11554) by electroporation. Virus containing supernatant (10 ml) from the Bing cells was used to infect SH-SY5Y, a subclone from the SK-N-SH neuroblastoma cell line [Biedler et al., 1973], with the addition of Lipofectamine (Invitrogen Corporation). For selection of transfected cells the culture media (RPMI 1640 containing 10% fetal bovine serum) was supplemented with 0.3 mg/ml Geneticin (Invitrogen Corporation). *NTRK3*-mutants, *NTRK3*-inact and *NTRK3*-WT clones were isolated from the corresponding cell lines and confirmed by sequencing. DNA was isolated from cells using Gentra Puregene Blood kit (Qiagen), PCR amplified and submitted for sequencing. Cells were then diluted to create stable clonal lines from single cells. Cells were maintained in RPMI-1640, with 10% fetal bovine serum, antibiotics and 0.3 mg/ml Geneticin (Invitrogen Corporation).

Transcription of WT or mutant *NTRK3* was verified by RT-PCR. mRNA from each cell line was isolated using the mRNA midi kit (Qiagen), treated with DNAse I (Invitrogen Corporation) and reverse transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen Corporation). To verify expression, amplification of part of the *NTRK3* clones as well as an endogenous control (Actin, Applied Biosystems/Ambion) was carried out on DNAse treated RNA and cDNA samples in a volume of 20 μl with a final concentration of 2 mM $MgCl_2$, 0.375 mM dNTPs, and 0.3 μM of each primer using AmpliTaq Gold using a touch down protocol. Primer sequences and annealing temperatures are given in Supp. Table S3. Quantitative PCR was carried out using a TaqMan Gene Expression Assay (Assay ID: Hs00176797_m1, Applied Biosystems/Ambion, Austin TX) and TaqMan Gene Expression Master Mix following the supplied protocol.

Sulforhodamine B assay (SRB) to assess cell growth

Cell growth was assessed using the SRB assay as described previously [Norris et al., 2011]. Cell lines were seeded in 96-well plates at a density of $5x10^3$ per well in 200 μl of RPMI media containing 1% serum. NT-3 was added to half of the samples 2 hrs after plating to a

final concentration of 100ng/ml. Media (with or without NT-3) was changed on day three and day five. Each condition was performed in quintuplicate in at least three separate experiments. At day one, three, five, and seven, ice cold 50μl of 50% trichloroaceic acid (TCA) was added to the wells after which the plates were incubated at 4°C for 30 min, then washed three times in distilled water and air dried. To measure cell growth the cells were stained with 0.4% SRB stain containing 1% acetic acid and incubated at room temperature for 30 min. After washing with 1% acetic acid the plates were then air-dried. Once dry, 100μl of 10mM Tris Base was added before measuring optical density at 520 nm using a Benchmark Plus Plate reader (Bio Rad, Hercules, CA). The average ratio of cell growth with or without NT-3 over at least three experiments was calculated and used to assess statistical differences using the two-sample *t*-test.

Western Assay

Stably transfected cell lines were grown in cell culture dishes (57 cm^2, 100 mm dia x20 mm H) to an approximate density of 75%. Cells were then starved for 2 hr in serum-free media followed for some by an incubation with recombinant human NT-3 (Peprotech, Rocky Hill, NJ) in a concentration of 100 ng/ml for 15 min at 37° C. Cells were washed in cold PBS and then collected in 30 μl of cell lysis buffer (Cell Signaling Technology, Danvers, MA). Protein concentration was measured in the supernatant collected after centrifugation at 10,000 rpm for 10 min. The samples (50 μg of protein) were separated by SDS-PAGE (NUPAGE Novex 4-12% BT precast gels; Invitrogen Corporation) electroblotted on nitrocellulose and analyzed by Western blot using Phospho-TrkA (Tyr490) antibody (Cell Signaling Technology), TrkC (C-14): sc-11 antibody, Phospho-Akt antibody (Thr308) (Cell Signaling Technology), AKT antibody, Phospho-p44/42 MAPK (ERK1/2)(Thr202/Thr204) antibody, p44/42 MAPK (Erk1/2) antibody, and Actin (C11) as a control (all Santa Cruz Biotechnology, Inc, Santa Cruz, CA). Phospho-TrkA antibody was used to detect TrkC when phosphorylated at Tyr516.

Films were scanned transparently using a flat bed scanner (Epson Perfection V500 Photo) and the density of bands was compared using the software program ImageJ (http://rsb.info.nih.gov/ij/index.html) [Abramoff et al., 2004]. Ratios were calculated using density values of phosphorylated protein and total protein for each clone. Three individual experiments were performed to calculate the average density ratio for each clone. For statistical analyses, the individual density values from the western blot assays were analyzed by the two-sample *t*-test.

Assay to test for apoptosis

Cell lines were grown in low medium conditions (RPMI with 1% serum) with or without the addition of NT-3 at a concentration of 100ng/ml in cell density of 1.6×10^4 per cm^2 in 60mm cell culture dishes. Media was changed on day three and day five. The Dead Cell Apoptosis Kit with Annexin V alexa Fluor 488 & Propidium Iodide kit (Life Technologies, Eugene, OR) was used to stain the cells. On day three, five and seven supernatant and cells were harvested and washed in cold PBS. Cells were diluted to an approximate concentration of 1×10^6 cells/ml in supplied 1X annexin-binding buffer and stained for 15 min according to the protocol before analysis. Cells undergoing apoptosis were assessed by flow cytometry

on a BD LSRFortessa (BD Biosciences, San Jose, CA). Analysis was done with BD FACSDiva software for three independent experiments for each day.

Results

Variants in NTRK3

Our study population consisted of 467 non-syndromic patients with ventricular septal and conotruncal defects (Table 1). We included patients with one of three types of VSD (conoventricular, posterior malalignment and conoseptal hypoplasia) as well as those with a subset of conotruncal defects (TOF, IAA, TA) given previous studies suggesting these lesions share a common genetic etiology [e.g. Peyvandi et al., 2013]. The cohort was of mixed ethnicity, with the majority being of Caucasian descent. We evaluated these patients for sequence variants in the coding region of *NTRK3* by high-resolution melt curve analysis. Samples showing unique melting curve profiles were sequenced to identify specific variants. We identified 6 non- synonymous variants in 7 unrelated patients of different ethnicity. One of these variants (c.61G>T, p.V21F) was found in two patients as well as Asian controls, and has been reported in the Japanese population of the 1000 Genomes project [rs200822610:G>T; Abecasis et al., 2010] with an overall allele frequency of 0.0018 (minor allele frequency: 0.022 in the JPT population). One of our patients with this variant (p.V21F) was of Asian descent, whereas the other patient was reported to be Caucasian but was also listed as adopted. Thus, the p.V21F variant was considered to be a polymorphism.

The remaining five variants were found in four patients diagnosed with a VSD and one patient with an IAA/VSD, and were not present in ethnically matched controls. If available, parental samples were screened for the same sequence variant (see Table 2). Two of these variants (c.211A>G, p.I71V, and c.278C>T, p.T93M) have since been reported in different populations. Variant p.I71V (rs200923715:A>G) was found with an allele frequency of 0.0007 in the cohort of the ClinSeq Project [Biesecker et al., 2009], and variant p.T93M (rs147992979:C>T) was found in the Japanese cohort (minor allele frequency: 0.006) of the 1000 Genomes Project with an overall allele frequency of 0.0005. In our study, variants p.T93M and p.I817M were inherited, whereas variant p.I71V was de novo (Table 2). Parental samples were not available for the two remaining patients. None of the patients reported a family history of CHDs.

A possible impact on protein function due to the resulting amino acid changes was predicted for four of the five variants by the online tool Polyphen-2 [Adzhubei et al., 2010] and for one by SIFT [Kumar et al., 2009](Supp. Table S4, S5). Neither algorithm predicted a functional change for the variant at position 71 (p.I71V) (Supp. Table S4, S6) such that only the four variants predicted to be damaging by Polyphen-2 (v2.1.0r367) were chosen for further functional assessment. These four mutations change amino acids that are highly conserved across numerous species, and three of the four mutations fall within functional domains of TrkC (Table 2, Fig. 1). Of interest, the p.T93M variant was the only one predicted by both Polyphen-2 and Sift to affect function but is the only one that does not fall within a conserved functional domain of TrkC. Of further note, the functional significance of the p.N163I (c.1597A>T) variant was subsequently changed from “possibly damaging” to “benign” (Supp. Table S7) by an updated Polyphen-2 version (v2.2.2r398).

Functional analysis

To examine possible functional consequences of the TrkC variants, we made use of a human neuroblastoma cell line, SH-SY5Y, which shows no endogenous expression of TrkC [Yamashiro et al., 1997]. We introduced the mutations into a retroviral vector carrying the WT *NTRK3* sequence (pLNCX-TrkC) and stably transfected the SH-SY5Y cells with the WT and mutant constructs. As controls, we also transfected a kinase-inactive *NTRK3* mutant (see Table 3) as well as an empty vector. Transcription of WT, mutant and inactive *NTRK3* was confirmed by RT-PCR and quantitative-PCR, demonstrating that expression in the cell lines was high and at comparable levels. No transcription of *NTRK3* was detected in the cell line transfected with the empty vector.

To test for NT-3-mediated activation of TrkC, the transfected cell lines were grown for 2 hrs in low-serum conditions and then induced for 15 min with NT-3. Western blot analysis confirmed that TrkC was not phosphorylated when cells were grown in low-serum conditions without NT-3 (Fig. 2A). With the addition of NT-3, WT TrkC was strongly phosphorylated at Tyr516 where as the inactivated TrkC was not phosphorylated. Three of the four mutant TrkC proteins demonstrated similar levels of phosphorylation to that of the WT, whereas the most c-terminal mutation (p.T93M) conferred markedly decreased levels of TrkC phosphorylation as demonstrated by a significantly fainter signal ($p<0.05$) in three independent experiments (Fig, 2B).

To assess the effect on downstream targets of TrkC, we evaluated two signaling proteins from the phosphoinositide-3-kinase/AKT and the MAPK pathways. Both pathways are activated by TrkC signaling and essential for neuronal cell survival and differentiation [Brodeur et al., 2009; Kaplan and Miller, 2000]. Western blot analysis of phosphorylated AKT and MAPK supported the previous findings; the reduction in phosphorylated TrkC p.T93M also impaired the activation of the key signaling proteins of both pathways (Fig. 2B). No significant changes were observed in the phosphorylation of these two signaling proteins by the other three mutant TrkC proteins.

Next we evaluated whether cell growth was altered by expression of the mutant proteins in different conditions over a seven-day period. In serum-supplemented media (10% FBS), the cell lines grew equally well. Likewise in a serum reduced environment (1% FBS) with the addition of NT-3, there was no significant difference in cell growth between WT, null or mutant cell lines (Fig. 3A). However, substantial differences in growth between the mutant and WT cell lines were observed under low-serum conditions (1% FBS) in the absence of NT-3 (Fig. 3B). Cell lines that were transfected with an empty vector or the inactive TrkC vector grew equally well in low serum conditions with or without supplemental NT-3, where as WT cell lines demonstrated markedly reduced cell growth in the absence of NT-3 ($p<0.05$). While the cell line expressing the p.N163I mutant behaved like the WT , the growth of the cell line expressing TrkC p.I533F varied slightly from WT (Fig. 3C), and growth of cell lines expressing TrkC p.I817M, and p.T93M was markedly different as compared to WT (Fig. 3B and C). The ratio of cell growth with versus without NT-3 of the p.T93M and p1817M cell lines was statistically different from that of WT ($p<0.05$).

Previous reports have suggested that TrkC is a dependent receptor that induces cell death in the absence of NT-3 [e.g. Tauszig-Delamasure et al., 2007]. To see if cell death was responsible for the slow growth of the TrkC transfected cell lines in low serum conditions without supplemental NT-3, we used an annexin V/PI staining kit to detect early apoptosis and cell death by flow cytometry in the cell lines grown under the same conditions. Indeed, the cell line expressing WT TrkC showed early apoptosis and cell death in the absence of NT-3 (Fig. 4) whereas the cell line expressing an inactive form of TrkC did not (not shown). The cell lines expressing the mutant TrkC proteins also displayed apoptosis but three of the cell lines expressing mutant TrkC (p.I533F, p.I817M, p.T93M) showed a lower fraction of early apoptotic and dead cells than WT TrkC (e.g. p.T93M in Fig. 4). As would be expected from the pattern of cell growth, the TrkC p.N163I cell line was similar to WT TrkC (Fig. 4).

Discussion

A review of the 46 genes deleted in a VSD patient with a unique CNV [Goldmuntz, et al., 2011] identified *NTRK3* as a strong candidate gene for human CHDs due to findings in animal studies. This gene codes for the tyrosine kinase receptor C (TrkC), a member of the neurotrophic kinase receptor family, which plays a critical role in the development and maintenance of the nervous system. Upon binding to its sole ligand NT-3, TrkC autophosphorylates and activates different signaling pathways resulting in neuronal survival and cell differentiation. Gene targeted mice lacking TrkC or NT-3 expression, originally created to study neuronal cell survival, die unexpectedly in the first week after birth. The early death was explained by severe cardiovascular anomalies (including ASD, VSD, pulmonic stenosis, truncus arteriosus and valvular defects) in both mouse models, thus revealing a critical function of TrkC, and its ligand NT-3, in heart development [Donovan, et al., 1996; Tessarollo, et al., 1997]. Consequently, we chose to investigate the role of *NTRK3* in human CHDs by screening patients with a subset of VSDs and conotruncal defects previously shown to share a common genetic basis [e.g. Peyvandi et al., 2013].

Our studies identified four missense variants in VSD patients in highly conserved amino acids of *NTRK3* that were not found in our control cohort (Table 2) and were initially predicted to confer functional changes. Three of the four variants were also located in conserved functional domains, two in the catalytic tyrosine kinase domain and one in the LRRCT domain (Fig. 1). The p.N163I variant also altered a glycosylation site (Conserved Domain Database, http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).

Although NTRK3 participates in neuronal development, only one of the four patients with a mutation was reported to have developmental delay. However, reported clinical features were limited to those recorded in the medical record such that neurologic or developmental anomalies might not be detected without further screening. Two presumably normal parents were observed to carry a mutation implicated as disease-related. Previous studies demonstrate enormous variability in expressivity and penetrance of mutations related to CHD. For example, less than 50% of patients with trisomy 21 have CHD, and the type of CHD is variable. This observation and other studies suggest that CHDs are complex traits with multiple genetic and environmental factors contributing to disease risk and manifestations in any one person. Moreover, the parents of our cases and public control

populations have not been screened by echocardiography to identify subclinical congenital cardiac alterations. As such, the fact that two parents were found to be carriers does not obviate the potential contribution that each mutation makes to disease risk. Instead in the context of complex traits, we propose that at least three of the four mutations contribute to the risk of CHD, but may not be sufficient to cause CHD.

An evaluation of phosphorylation of TrkC at Tyr516, one of its four phosphorylation sites,revealed that the ability of TrkC to autophosphorylate at this site was significantly impaired by one of the variants (p.T93M). Consequently, activation of essential downstream signaling pathways in the cells carrying this particular mutant TrkC was almost completely inhibited, indicating a loss of function of TrkC in the patient carrying this mutation. Loss of function of TrkC in mice resulted in similar cardiac phenotypes as seen in this patient.

Although the remaining three mutations did not significantly alter the phosphorylation of TrkC and activation of downstream targets, functional differences for three of the four mutants were supported by cell proliferation studies under low serum conditions. We observed altered cell growth in the absence of NT-3 in three of the four mutant TrkC expressing cell lines as compared to WT. Our results suggest that cells expressing WT TrkC undergo apoptosis in the absence of NT-3 under low serum conditions, where as three of the mutants showed lower levels of apoptosis than the WT. Indeed, previous studies showed that TrkC can trigger cell death in the absence of NT-3 in neuronal as well as neuroblastoma cells [Bouzas-Rodriguez et al., 2010; Nikoletopoulou et al., 2010; Svensson et al., 1997; Tauszig-Delamasure, et al., 2007; Yamashiro, et al., 1997]. Cell growth, proliferation, migration, and apoptosis are essential processes for cardiac morphogenesis [Pexieder, 1975; Soufan et al., 2006].

The role of TrkC in heart development is not yet fully understood. The cardiac defects described in the gene-targeted mice are generally attributed to an alteration in cardiac neural crest cell (CNCC) function, which contributes to the developing outflow tract (OFT) and the proximal great vessels. It has therefore been suggested that NT-3 and TrkC are essential for regulating the development of the CNCCs [Donovan, et al., 1996; Sieber-Blum, 2004; Srivastava and Olson, 1996; Tessarollo, et al., 1997]. Indeed, expression of TrkC in a subset of migrating mouse neural crest cells was confirmed [Youn et al., 2003]. Furthermore, neural crest stem cells lacking TrkC expression differentiated prematurely into fate-restricted cells. This could ultimately affect the migration of undifferentiated cells into the conotruncal ridges, resulting in stationary cell growth and consequent thickening of the OFT wall, as seen in TrkC null mice [Youn, et al., 2003]. In addition, NT-3 and TrkC are present in the OFT of the chicken heart before arrival of CNCC suggesting that myocardial cells from the secondary heart field also express TrkC [Bernd et al., 2004]. Lin et al., [2000] found that TrkC was expressed by cardiac myocytes and may be responsible for ventricular trabeculation in the first week of chicken development. They conclude that impairment of TrkC function is likely to be responsible for the defects in ventricular septation and ventricular wall thinning seen in TrkC-deficient mice due to reduced myocyte proliferation.

The expression of TrkC at different time points as well as in different cell types suggests that TrkC has a variety of functions during heart development. Thus different *NTRK3*

mutations may confer different developmental and morphologic consequences, and the assays undertaken in this study may not detect changes in all of its roles or settings. The p.T93M mutation likely confers a loss of protein function given the near absence of autophosphorylation in the presence of ligand and similar cell growth patterns to the null mutant cell line. Such a loss of function might result in premature differentiation and/or reduced myocyte proliferation resulting in abnormal ventricular septation. Two of the mutations (p.I533F, p.I817M) do not alter TrkC auto- phosphorylation in our assay, but demonstrate increased cell growth with less apoptosis than WT in the absence of ligand in restricted serum conditions. These results suggest that these mutations might permit increased cell growth under developmental conditions where morphologic changes require cell death. The last mutation, p.N163I, was ultimately predicted to be benign by an updated Polyphen program and demonstrates no particular functional change in the cell growth assays. Therefore this very rare variant may be benign. However, as noted, TrkC is present in a variety of cell types at different developmental stages suggesting different developmental roles. Therefore the particular assays completed in this study may not detect all changes of functional significance. Additional functional studies of our mutant TrkC proteins in CNCC, cardiac myocytes, or animal models might help to further define such potential functional deficiencies of our other mutant TrkC proteins and more importantly, the role of TrkC in heart development and defects.

Our findings, in conjunction with the known involvement of *NTRK3*/TrkC in heart development, suggest that mutations in this gene contribute to the etiology of the heart defects seen in our patients. Since heart defects are recognized to be complex traits, additional factors, such as incomplete penetrance, differential expressivity, epigenetic modification, other interacting genetic factors and environmental factors, could explain the fact that a parent, although carrying the same variant, remains seemingly unaffected. Alternatively, a detailed examination of the parent is rarely available, making it possible that the parent harbors a mild and undiagnosed heart defect. Furthermore, the low number of variations found in our patients is not unexpected considering the genetic heterogeneity and complexity of CHDs. Mutations in other genes in patients with CHDs show similar frequencies [McElhinney et al., 2003; Posch et al., 2008; Thienpont et al., 2010; Tomita-Mitchell et al., 2007].

In conclusion, our study supports a possible role for *NTRK3* in the development of CHDs. Sequence analysis of *NTRK3* in over 400 patients with heart defects identified four missense mutations altering amino acids in highly conserved regions, three of which seemed to alter protein function in cell based assays. Taken together with the observed heart defects in gene-targeted mice lacking expression of either TrkC or its ligand NT-3, our findings suggest that *NTRK3* mutations contribute to the risk for VSDs. These findings implicate a novel candidate signaling-pathway, the disruption of which may contribute to human congenital cardiac defects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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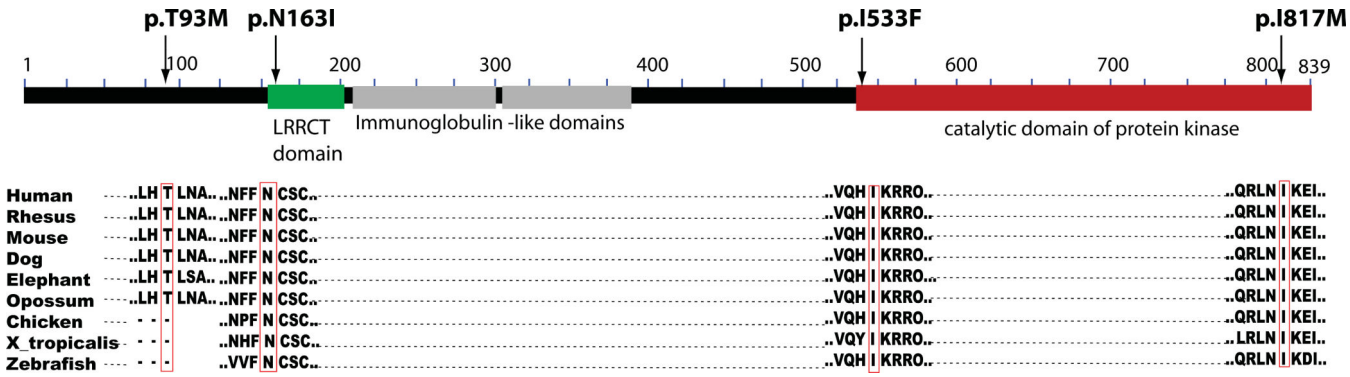

Figure 1.

Schematic showing TrkC with the conserved protein domains. Location and description of the mutations studied in our patients are noted above by arrows. Comparative alignment of mutated regions in homologous proteins in various species indicating the affected amino acids.

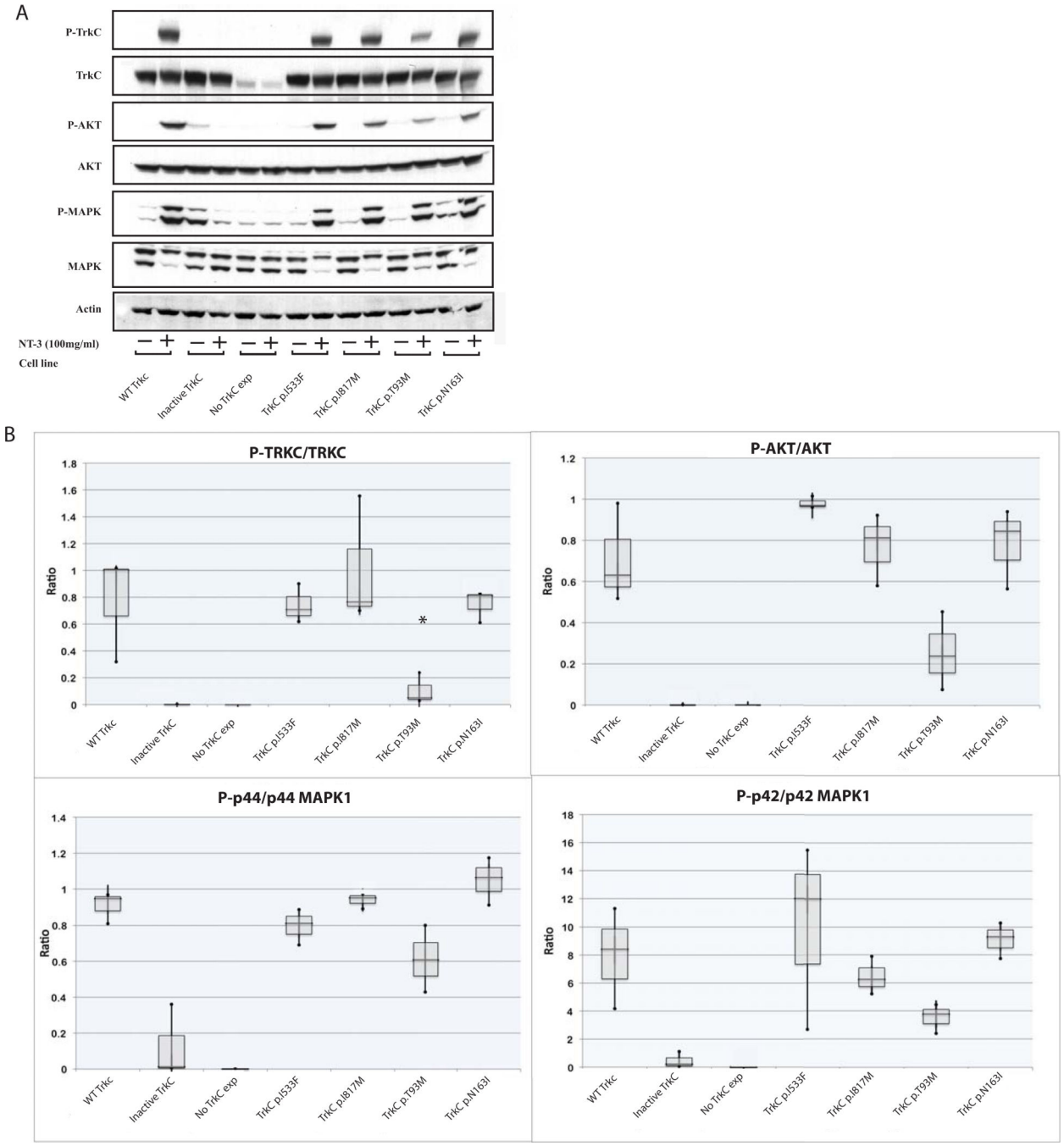

Figure 2.

Western blot analysis of SY5Y cells transfected with WT (WT TrkC) or mutant TrkC (TrkC p.I533F, TrkC p.I817M TrkC p.T93M., TrkC p.N163I) and controls (No TrkC (empty vector), Inact-TrkC). **A**: Phopshorylation of TrkC (P-TrkC), AKT (P-AKT) and MAPK (PMAPK) with (+) or without (-) NT-3 treatment. Blots were stripped and rehybridized with antibodies against TrkC, AKT, MAPK, and Actin as a control. **B:** Densitometry: Box plots showing the distribution of the ratios of densitometry values from Western blot analysis in stably transfected SY5Y cell lines. P-TrkC/TrkC: Ratio of phosphorylated to

unphosphorylated TrkC (* significant difference of TrkC p.T93M to WT TrkC, $p<0.05$), P-AKT/AKT: Ratio of phosphorylated to unphosphorylated AKT, P-p44/p44 MAPK: Ratio of phosphorylated to unphosphorylated p44 MAPK1, and P-p42/p42 MAPK1: Ratio of phosphorylated to unphosphorylated p42 MAPK1.

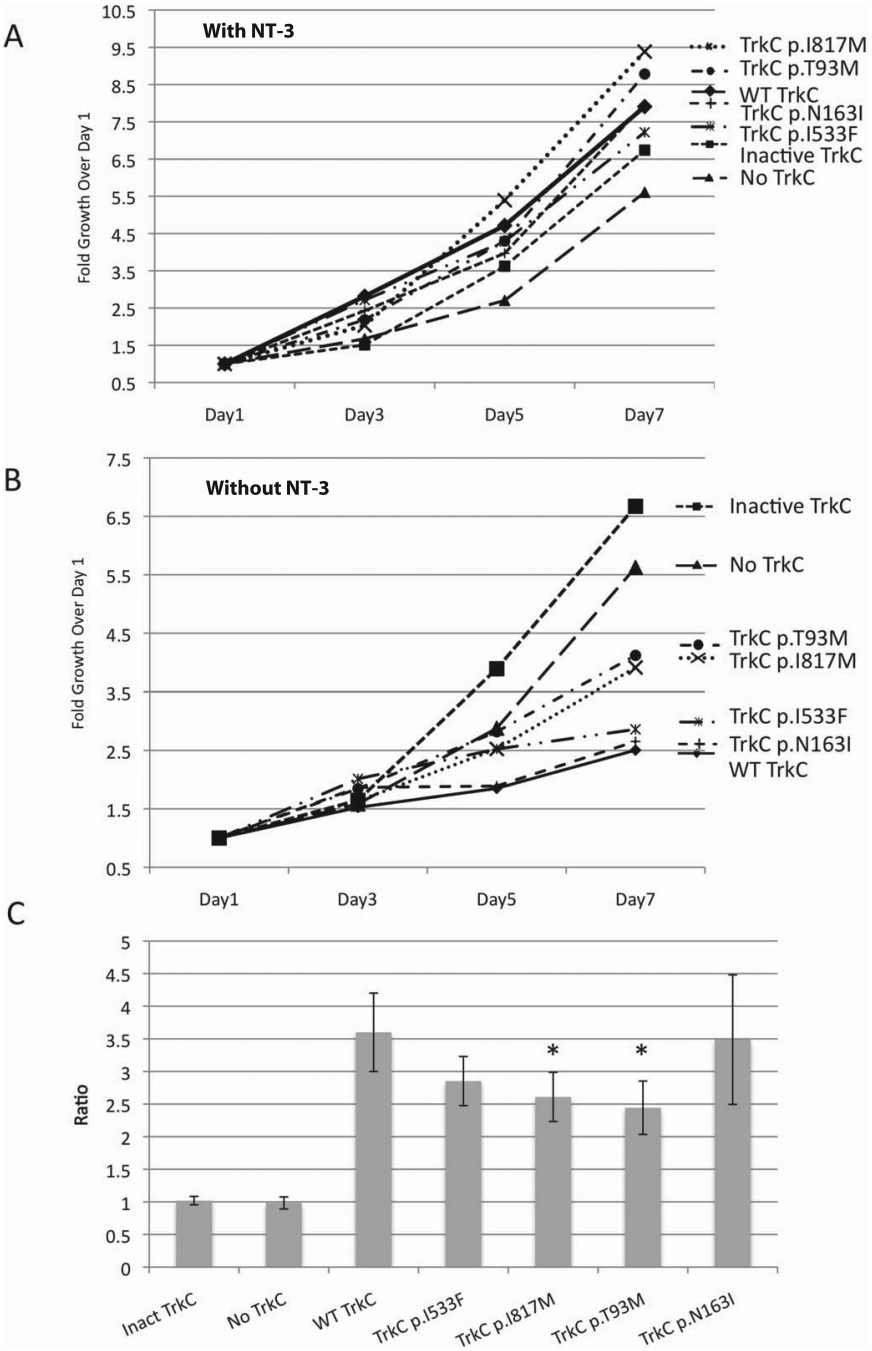

Figure 3.

Results for the SRB cell proliferation assay. The control cell lines and cells lines expressing wild type or mutant TrkC were grown in low serum for a period of seven days. The average values for the line graphs including standard deviations are also listed in Supp. Table S8 **A:** Cell growth measured with the addition of NT-3 to the media **B:** Cell growth measured without supplemental NT-3. **C:** Bar graph showing the ratio of cell growth with vs without NT-3 for each cell line (* = $p<0.05$).

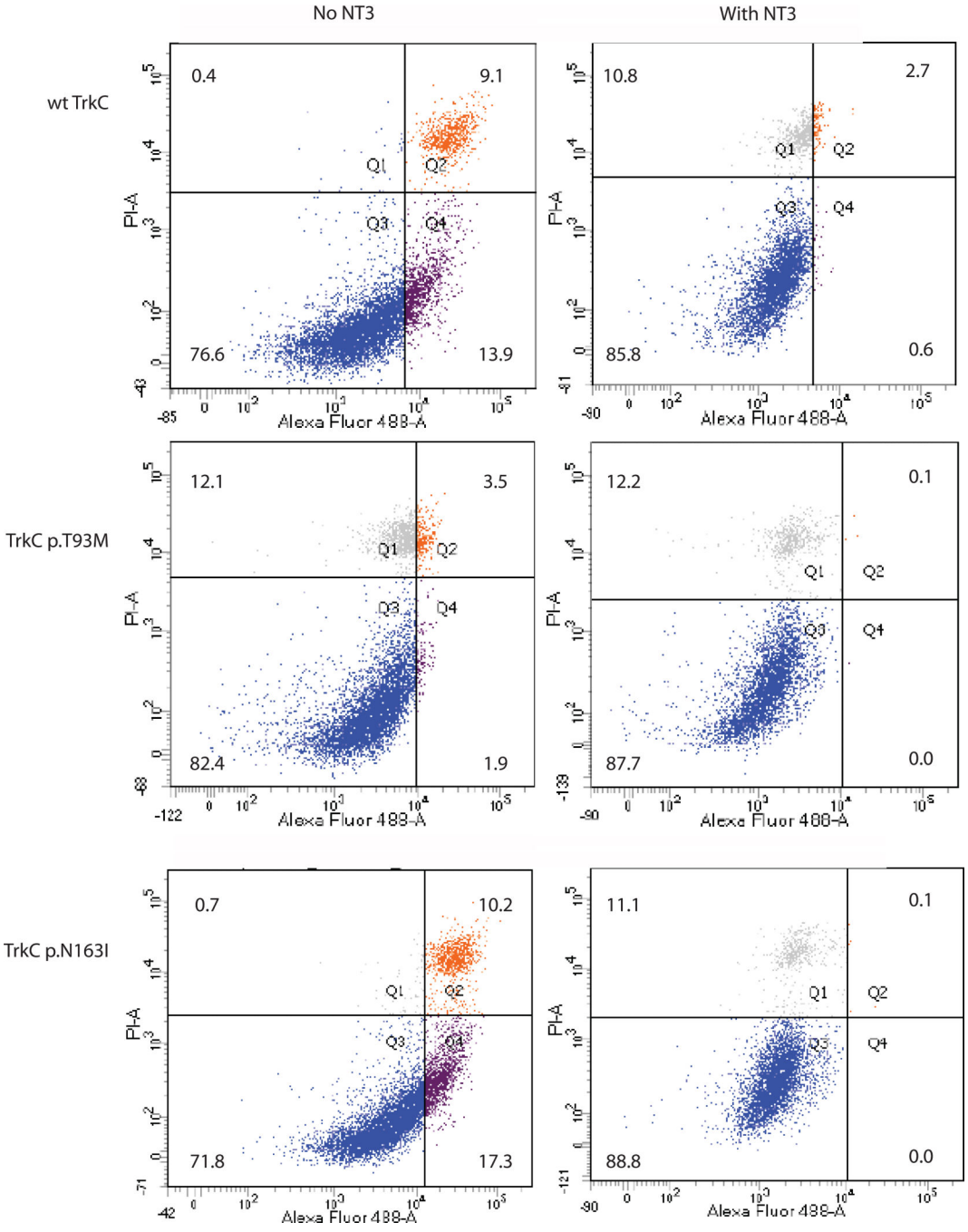

Figure 4.

Flow cytometry analysis of cell lines grown in low serum condition with or without addition of NT-3. Annexin-V/propidium iodide double staining was performed to determine the rate of early apoptosis (Q4) and apoptotic cells (Q2). The data shown is representative for results in cell lines expressing WT TrkC, TrkC p.T93M, and TrkC p.N163I on day 7.

Table 1

Cardiac phenotype of study cohort

Cardiac Lesion	Patients (n)
Ventricular septal defect (VSD)*	284
Tetralogy of Fallot (TOF)	104
Truncus arteriosus (TA)	44
Interrupted aortic arch (IAA)‡	35
Total	467

* includes conoventricular, malalignment and conoseptal hypoplasia type VSDs

‡ IAA type A: 8 patients, IAA type B: 27 patients

Table 2

Mutations identified in *NTRK3*

Patient ID	Amino Acid Change	Nucleotide Change (based on cDNA)	Conserved Domain	Published SNP	Parents		Gender	Controls	DX			Extracardiac Findings
					Father	Mother			CA	AA	Asian		
1047	p.I71V	c.211 A>G	None	rs200923715:A>G ClinSeq Project, allele freq 0.0007	Normal	Normal	Male	CA	0/150	0/47		IAA/pmVSD	Von Willebrand Disease
2809	p.T93M	c.278 C>T	None	rs147992979:C>T JPT cohort of 1000 Genomes, allele freq 0.0005	Carrier	Normal	Male	Asian (Indian) paternal	0/83		0/93	cVSD	Undescended testis, bilateral inguinal hernias
571	p.N163I	c.488 A>T	LRRCT		NA	NA	Male	AA	0/100	0/100		cVSD	none
327	p.I533F	c.1597 A>T	PTKc_Trk-C		NA	NA	Female	AA	0/100	0/100		cVSD	none
2010	p.I817M	c.2451 C>G	Pkinase_Tyr		Normal	Carrier	Male	CA	0/200			cmVSD RAA	ptosis, abnormal ears (pixie-like), undescended testes, dermoid cyst, crossed-over toes, developmental delay with unremarkable brain MRI

NTRK3 based on isoform precusor a (Acc#: NM_001012338.2), NA: Not available, AA: African American, CA: Caucasian, LRRCT: Leucine-rich repeat C terminal domain {cl1530}, PTKc_Trk-C: Catalytic domain of the Protein Tyrosine Kinase, Tropomyosin Related Kinase C, Pkinase_Tyr [pfam07714], Protein tyrosine kinase (based on CDD, Marchler-Bauer, et al., 2009), IAA: Interrupted aortic arch; pmVSD: posterior malalignment type ventricular septal defect; cVSD: conovetnricular ventricular septal defect, cmVSD: conoventricular plus muscular ventricular septal defect, RAA: Right aortic arch.

Table 3

Nomenclature of *NTRK3* (NM_001012338.2) constructs

***NTRK3* construct**	**Location of mutation (*)**	**AA-Change**	**mRNA position, bp change**
NTRK3-wt	Wild type	-	-
NTRK3-Inact	ATP binding site of kinase domain	p.K572N	r.2022G→C
NTRK3-2809	Extracellular domain	p.T93M	r.584C→T
NTRK3-571	LRR_RI; Leucine-rich repeats (LRRs), ribonuclase inhibitor (RI)-like subfamily	p.N163I	r.794A→T
NTRK3-327	Beginning of catalytic domain of the protein tyrosine kinase	p.I533F	r.1903A→T
NTRK3-2010	Protein tyrosine kinase domain	p.I817M	r.2757C→G

* Domain location determined by CDD (Marchler-Bauer. et al., 2009)