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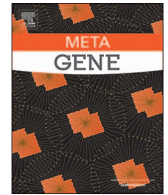

Whole exome sequencing of discordant diseases in Monozygotic twins with Down syndrome reveals mutations for Congenital Heart Defect and epileptic seizures

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ABSTRACT

Discordant phenotypes and diseases in Monozygotic twins (MZ) are always intriguing and genetics is the exclusive reason. Down syndrome is one such disorder, which shows heterogeneity, with occurrences of incidental diseases discordant between cases. When such disease discordance is noted in Down syndromic MZ twins, it becomes a true genetic case and the necessity to uncover the pathogenic variants becomes imperative. One such case of MZ twins reported by an earlier study failed to identify the pathogenic variants behind the discordance between Congenital Heart Defect (CHD) and Epilepsy. In the current study, using the Whole Exome Sequencing (WES) datasets of the original investigators along with our custom variant identification pipeline, we investigated the consequences of damaging novel and rare discordant mutations in *TGFBR2*, for CHD; *ITPR1* and *RYR2* for Epilepsy. *TGFBR2* was identified with a frameshift deletion resulting in a stop gain mutation and appeared to be dominant in function causing Ventricular Septal Defect in the older Down syndrome twin, while nonsynonymous heterozygous substitutions was identified in *ITPR1* and *RYR2*, causing epileptic seizures in the younger twin. Pathway analysis on mutant genes revealed disruption of several processes such as vascular septal formation for *TGFBR2*, and calcium signalling for *ITPR1* and *RYR2*. The present investigation identified novel discordant mutations relevant to the discordant disease presented, using appropriate analysis and pipelines.

Twin studies have long been used to measure the contribution of genetics on a trait or disease of interest. The classic twin study design relied on studying twins raised in the same family environment. Evidences gathered lately have shown significant genetic differences to exist between Monozygotic twins (MZ) disrupting the earlier assumption that MZ share the same set of genes and variations. Studies have identified somatic *de-novo* mutations that accumulate post zygote formation and/or differential epigenetic mechanisms (Castillo-Fernandez et al., 2014; Selmi et al., 2014) as the reason behind the disease discordance in MZ twins and thereby, represent true genetic cases to perform appropriate genetic tests to understand the role of genes towards phenotypes. The small genetic differences that exist between MZ twins form ideal genetic model to identify putative causal variations leading to disease (Bruder et al., 2008; Maiti et al., 2011).

The present report is based on an earlier study conducted by Chaiyasap et al. (2014) recording MZ twins with Down syndrome (DS) born at the King Chulalongkorn Memorial Hospital, Bangkok and clinically diagnosed to have Trisomy 21 showing disease discordance. The older 'Twin A' had a Ventricular Septal Defect (VSD) with no other disease conditions and was operated at one year and two months of age. Younger 'Twin B' had a healthy heart, but developed seizures when he was six months old that was untreatable with common infantile convulsants and showed improvements with Vigabatrin at one year of age. Performing Whole Genome Sequencing (WGS) and Whole Exome Sequencing (WES) on these twins, the authors failed to identify the causal DNA variations leading to disease discordance. Details to the techniques used to perform WES are described in the original paper (Chaiyasap et al., 2014). In this study, we reanalyzed the WES data using a combination of robust alignment programmes, variant callers, and several variant annotators combined with a stringent pipeline for variant identification, to report the plausible disease causing variants that went unidentified earlier.

Raw FASTA files were obtained from National Center for Biotechnology Information, Sequence Read Archive (NCBI-SRA) database using accession numbers SRX522555 and SRX522556 for Twin A and Twin B respectively. We made use of Strand NGS for genome alignment and variant calling because of its high reported accuracy (Gupta et al., 2014). The VCF files with Single Base Variants (SBVs) and Multi Base Variants (MBVs) were exported and annotated on programmes such as webANNOVAR (wANNOVAR) (Chang and Wang,

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2012), Ingenuity Variant Analysis (IVA) (QIAGEN, n.d.), Scripps Genome Annotation and Distributed Variant Interpretation Server (SG-ADVISER) (Pham et al., 2015) and Elsevier Pathway Studio Genotyping Suite (Nikitin et al., 2003) based on position, gene, amino acid change, zygosity and mutation effects. We collated the data from all the four annotators. The variant calls agreeing with all annotator programmes along with those variants which had higher read depth (> 15) but were still found to be missing in some programmes were included in the study. Non synonymous variants designated as pathogenic, damaging and deleterious on predictors like MutationTaster and FATHMM programmes, with population frequency < 0.05 were chosen for further pathway and networking analysis. CNV calling was performed by aligning the Twin A and Twin B exome FASTA files across the HapMap NA21112 normal exome sequence dataset. CNVs were called based on the length, position, overlapping genes, copy numbers and were assessed based on neutral, amplification and deletion effects. Furthermore, disease pathway was created using Ingenuity Pathway Analysis (IPA) and Elsevier Pathway Studio (PS) on mutation datasets. Upstream and downstream genes for the mutant genes were looked into and the pathway enrichment was performed by means of z-score, p-value and Jaccard similarity testing to identify enriched disease pathways that could be disrupted/blocked due to mutations.

Post application of our stringent variant filtering pipeline, we identified plausible causal variations in *Transforming Growth Factor B Receptor 2* (*TGFBR2*) for Twin A with CHD and in genes *Inositol 1,4,5-Trisphosphate Receptor Type 1* (*ITPR1*) and *Ryanodine Receptor* 2 (*RYR2*) for Twin B with Epilepsy (Table 1). The CNV analysis performed on Strand NGS using the exome sequence data showed no discordant CNVs between twins. *TGFBR2* gene on chromosome 3 of Twin A was found to carry a novel heterozygous frameshift deletion mutation that caused a frameshift at 23rd amino acid (aa) position and truncated the protein through STOP codon at 54th aa resulting in the synthesis of only 9% of the protein (Table 1) (Supplementary Fig. 1). The mutation was novel since the mutation was not found in the 1000 Genomes Project, dbSNP 147, or the NHLBI Exome Variant Server. *ITPR1* in chromosome 3 of Twin B carried a novel heterozygous nonsynonymous substitution mutation replacing Glycine with Glutamic acid at 1152th aa position (Supplementary Fig. 1) and *RYR2* showed a heterozygous mutation in chromosome 1, replacing Alanine to Valine at 1365th aa position (Table 1) (Supplementary Fig. 1). Although, previous studies have reported discordance in CNV status between MZ twins, we did not find any in this study; however there have been reports on absence of discordant CNVs in studies conducted on MZ twins showing discordance for Autism Spectral Disorder (ASD) and Schizophrenia (Laplana et al., 2014; Bloom et al., 2013).

Ventricular Septal Defect (VSD) is one form of Congenital Heart Defects (CHD) formed due to incomplete closing of the wall separating the left and the right chambers of the heart. *TGFBR2* is deeply involved in cardiogenesis and forms a receptor to ligands TGFB1, TGFB2, TGFB3, BMP2, BMP4, BMP6, and BMP7 (Bobik, 2006). In normal cells, *TGFBR2* is a homodimer exhibiting constitutive kinase activity (Hart et al., 2002; Shi and Massagué, 2003) and TGFB ligand binding triggers the activation of TGFB-SMAD signalling (Shi and Massagué, 2003; Massagué, 1998). *TGFBR2* kinase forms a heterodimer with TGFBR1, phosphorylating TGFBR1 at its Glycine/Serine-rich domain (Fig. 1a). This activated TGFBR1 further phosphorylates SMAD2 and SMAD3 transcription factors to form SAMD2/3 complex. Further, it allows SMAD4 to associate with itself and translocate into the nucleus to transcriptionally regulate cardiac epithelial to mesenchymal transition and cell differentiation genes (Fig. 1a). Mutations in TGFB2 have been shown to cause developmental anomalies such as, atrioventricular (AV) cushion defects, VSD, aortic dilation, and ventricular hypoplasia (Frutkin et al., 2006; Carvalho et al., 2007; Langlois et al., 2010; Huang et al., 2015) (Fig. 1b). The role TGFB signalling in cardiogenesis is enhanced in EMT during the formation of the cardiac cushions (Arthur and Bamforth, 2011) and its fusion during closure of embryonic interventricular communication and development of the tricuspid valve (Robson et al., 2010). Therefore, mutation in *TGFBR2* results in a truncated TGBR2 protein, preventing TGFB ligands binding to *TGFBR2* receptor disrupting the downstream cascade leading to VSD. Further, *TGFBR2* knock out studies in mice have shown embryonic lethality (ED) at approximately ED 10.5, when the heart is at an early stage of development and VSD was observed in most mutant embryos (Langlois et al., 2010; Oshima et al., 1996; Larsson et al., 2001). In the current study, a novel frameshift deletion (A/−) mutation in *TGFBR2* truncated the protein at the 186th aa through a stop codon position resulting in the production of only 31% of the protein, lacking the Glycine/Serine-rich domain, kinase domain and the cytoplasmic domain failing to initiate downstream cascade of signalling (Supplementary Fig. 1).

ITPR1 regulates intracellular calcium concentration and signalling (Berridge, 1993; Hirota et al., 2003). Phospholipase C with the help of G proteins releases Inositol Triphosphate 3 (IP3) as an intracellular secondary messenger. This triggers calcium release from endoplasmic reticulum by promoting the binding of the IP3 gated calcium channel-*ITPR1* to other receptors coupled to calcium channels (Fig. 2). *ITPR1* receptor molecules are expressed in neuronal and non-neuronal tissues therefore plays a vital role in brain function. Mice models have established that *ITPR1*-deficient mice die *in utero* and those born die after a weaning period by exhibiting severe ataxia and tonic or tonic-clonic seizures (Matsumoto et al., 1996). Twin B suffering from Epilepsy showed a substitution mutation in *ITPR1* resulting in a change from aa Glycine to Glutamic acid at 1152th position located within the cytoplasmic region of the receptor molecule (Supplementary Fig. 1). Glycine is known to be evolutionarily conserved in the cytoplasmic domain of certain receptors and is found to play essential roles in kinase activations and coupling. We believe that mutation from evolutionarily conserved Glycine to Glutamic acid could severely affect the functionality of the protein, thereby disrupt the signalling cascade leading to Epilepsy (Betts and Russell, 2003).

RYR2 forms the α-subunit of the tetrameric intracellular calcium

Table 1
List of heterozygous mutations identified in Twin A for CHD and Twin B for Epilepsy based on gene, position, mutation type, change and functional prediction.

Twin ID	Genes	Chromosome coordinates	Type of mutation	Coding position	Amino acid change	Mutation taster/FATHMM	Read depth
Twin A	*TGFBR2*	3:30691872–30691872	Frameshift deletion	exon3: c.374delA/ exon4: c.449delA	Glutamate to STOP Codon (p.E125fs p.E150fs)	Damaging	20
Twin B	*ITPR1*	3:4725969–4725969	Nonsynonymous substitution	exon28: c.G3458A exon28: c.G3431A exon29: c.G3476A	Glycine to Glutamate (p.G1153E p.G1144E p.G1159E)	Damaging	53
	RYR2	1:237754226–237754226	Nonsynonymous substitution	exon31: c.C4094T	Alanine to Valine (p.A1365V)	Damaging	11

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