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Whole genome sequencing of a dizygotic twin suggests a role for the serotonin receptor *HTR7* in autism spectrum disorder

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Running title: WGS of a dizygotic twin links *HTR7* and ASD

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Abstract

Whole genome sequencing of a severely affected dizygotic twin with an autism spectrum disorder and intellectual disability revealed a compound heterozygous mutation in the *HTR7* gene as the only variation not detected in control databases. Each parent carries one allele of the mutation, which is not present in an unaffected stepsister. The *HTR7* gene encodes the 5-HT₇ serotonin receptor that is involved in brain development, synaptic transmission and plasticity. The paternally inherited p.W60C variant is situated at an evolutionary conserved nucleotide and predicted damaging by Polyphen2. A mutation akin to the maternally inherited p.V286I mutation has been reported to significantly affect the binding characteristics of the receptor. Therefore the observed sequence alterations provide a first suggestive link between a genetic abnormality in the *HTR7* gene and a neurodevelopmental disorder.

Key Words

HTR7, autism, intellectual disability, dizygotic twin, serotonin receptor

Text

Introduction

Autism spectrum disorders (ASDs), characterized by a lack of social communication and interaction, in combination with restricted repetitive behaviors, interests and activities[APA, 2013], are clinically and genetically heterogeneous. According to recent estimates, one in 68 children (1.47%) in the US is affected, with a fourfold bias of boys (1 in 42) over girls (1 in 189)[Atladottir et al., 2015]. Family and population-based studies argument for a high degree of heritability and the recurrence risk for siblings of autism patients ranges from an estimated 10 to 19%[Constantino et al., 2010; Ozonoff et al., 2011; Sandin et al., 2014]. Both genetic and environmental causes of ASDs have been described[Sandin et al., 2014], but according to a recent estimate, in no more than 10-20% of patients a genetic diagnosis can be established[Carter and Scherer, 2013].

Despite this low diagnostic yield, it is expected that the actual contribution of genetic causes in ASD etiology is significantly larger. Over the last years, technical developments including next generation sequencing (NGS) technologies, have catalyzed the discovery of additional disease genes in various disorders, including ASD. So far, family based exome or genome sequencing has proven to be a very successful strategy. In this approach, the DNA of a patient is compared with that of the parents at the sequence level, to specifically identify *de novo* variants[Visser et al., 2010]. Using this so-called trio approach, sometimes supplemented with targeted resequencing of specific candidate genes in large patient cohorts, a number of novel genes associated with autism have been discovered, increasing the diagnostic yield[O'Roak et al., 2012a; O'Roak et al., 2012b; Bernier et al., 2014; De Rubeis et al., 2014; Helsmoortel et al., 2014; Iossifov et al., 2014; O'Roak et al., 2014; van Bon et al., 2015]. Remarkably, these studies mention little to no possible recessive causes of autism, while efforts specifically addressed to detect autosomal recessive ASD genes found evidence

for increased runs of homozygosity and identified some candidate genes regulating neuronal activity[Chahrour et al., 2012; Gamsiz et al., 2013; Gamsiz et al., 2015]. Hence, the search for autosomal recessive causes of autism is lagging behind.

Materials and Methods

Patients

We here report on a family with a 7 year old dizygotic twin, both diagnosed with an autism spectrum disorder, co-morbid with severe developmental delay and intellectual disability (figure 1). The probands, a boy and a girl, were born at 35^{2/7} weeks gestation. The pregnancy was only complicated by severe vomiting with weight loss requiring frequent hospitalisations. They are the only children of non-consanguineous and healthy parents. The mother has a healthy daughter from a previous relationship and lost a sister with a neural tube defect. The boy had a birth weight of 2kg 490 g (-0.2 sd) and a birth length of 48.5 cm (+0.66 sd). The girl had a birth weight of 2kg 230g (-0.72 sd) and a birth length of 47 cm (+0.22 sd). The boy was on a ventilator for the first 2 weeks after birth. Both received tube feeding the first days after birth but the boy required this for several weeks because of swallowing difficulties. He also experienced problems with the switch from milk to solid food around 4 months and did not like the introduction of any new food. After the age of 12 months, the parents started to notice behavioural problems in their twins. The girl had sleeping problems. It was difficult to get her to sleep and she also needed very little sleep. The boy had more persistent feeding problems. According to the parents, they also started to lose some motor skills such as clapping of the hands and rotating their head to indicate “no”. At the age of 2 years there was no language development. Developmental testing at 22 months revealed evidence for a psychomotor delay and an autism spectrum disorder in both twins (developmental age of 12 months for the boy and 11.5 months for the girl) (Bayley Scales of

Infant Development BSID-II-NL, N-CDIs, NNST). The girl had one episode of febrile seizures. Brain MRI of the boy at the age of 3 years was normal. Clinical evaluation of both twins at the age of 3 years and 2 months revealed normal values for weight (boy at -0.16 sd; girl at +0.5 sd), length (boy at +0.85 sd; girl at +1.57 sd) and head circumference (boy at mean; girl at -0.54 sd). The boy was very anxious upon examination and did avoid eye contact. He was walking in circles and tightening himself up with increased muscle tone. He did not communicate and was not responsive. The girl was asleep during the consultation.

The twins were re-evaluated at the age of 6.5 years. Both were still not speaking any words, they were only producing certain sounds to make clear what they want. They were able to walk independently, the girl was even able to jump and walk backwards. They could eat with a spoon and drink from a cup. The boy was still more restless than his sister and by times aggressive with injuring himself and others. Both were not very responsive to their names. They looked for physical contact and protection by their parents and liked to be cuddled. Both seemed to have a disturbed circadian rhythm and this was most pronounced in the girl. She could be awake for more than 24 hours. Sometimes she waked up in the middle of the night and wanted to start the usual daily activities. The boy needed more sleep. Sometimes the parents noticed, especially in the girl, sudden increases in body temperature. Both could also have unusual habits with biting on batteries and eating of glass wool. Clinical examination of the boy did not reveal major dysmorphic features. Mild plagiocephaly, relatively large ears, broad face, full lips and small, widely spaced teeth were the most relevant craniofacial findings. The thorax was rather narrow with pectus carinatum. Organomegaly was absent. A mild glandular hypospadias was present. Hyperlaxity was found in the finger and elbow joints. The girl was also not dysmorphic. She was resembling her brother but the ears were smaller and the teeth larger. Both had a normal height (boy at +1.2 sd; girl at +0.3 sd) and head circumference (boy at mean; girl at +0.5 sd).

Molecular analysis

Conventional karyotyping and SNP array (Cyto-SNPv2 DNA Analysis BeadChip, Illumina, San Diego, CA, USA) revealed no pathogenic abnormalities. Fragile X testing was negative. To identify a potential genetic cause, all five family members (affected male II.2 and female II.3, both unaffected parents I.2 & I.3 and the unaffected stepsister II.1) were subjected to whole genome sequencing (WGS) with informed consent. Written permission for the presentation of clinical data has been obtained. Whole Genome Sequencing was performed by Complete Genomics, now a BGI Company (Mountain View, CA), using a sequencing-by-ligation method described previously[Drmanac et al., 2010]. Briefly, the human genome sequencing procedures include DNA library construction, DNA Nano-Ball (DNB) generation, DNB array self-assembling, cPAL-based sequencing, imaging, image data analyses including base calling, DNB mapping, and sequence assembly. Reads were mapped to the NCBI reference genome, build 36.3. Mapped sequence of the five samples varied in size between 194 and 325 Gigabytes, resulting in an average coverage between 68- and 114-fold per genome. Confident variant calling could be performed for 96–97% of the reference genome in all samples.

Variants were called by local *de novo* assembly according to the methods previously described[Carnevali et al., 2012], and annotated using NCBI build 36.3 and dbSNP build 130. Data were provided as VAR files. See table 1 for details regarding the quality metrics and numbers of variants per sample.

Results

In total 7,374,455 variants were detected in one or more family members, including single nucleotide variants and small insertions, deletions and substitutions of up to about 50 bp. The VAR files were analyzed using the Complete Genomics program “CGA tools”

version 1.6.0.43 and TIBCO Spotfire. 2,003,441 variants remained after filtering out those present in dbSNP130.

Supplementary tables 1 and 2 respectively describe the *de novo* and inherited CNVs and SVs. The variants are classified as deletion, distal-duplication, interchromosomal, probable-inversion and tandem-duplication. MatePairCounts and FrequenciesInBaselineGenomeSet are provided as well. Although unlikely in dizygous twins, *de novo* variants were identified in both affected siblings and not in the other family members, and are listed in supplementary table 1). This results in 58 variants, from which most are located in non-coding areas. The coding variants show either low mate pair counts (≤ 10) or high frequencies in control samples, which is not expected for causal variants involved in such an extremely rare condition. The second table describes the inherited CNVs and SVs, present in both affected siblings and in one of the parents. Filtering resulted in a list of 332 variants based on the following criteria: variants present in all family members were removed, as well as variants present in both parents, and variants present in the unaffected daughter of the first marriage as well as in the father. Detailed analysis of all 332 hits did not result in clear candidates based on allele frequencies and positioning in coding regions. Moreover, all variants detected within genes (present in both parents) seemed to have a high frequency (≥ 1 per 100) in control samples or turned out to be homozygous in both parents.

Next, 10,452 variants located in the consensus coding sequence (CDS) or in splice donor/acceptor sites were retained. Of these, 6,924 variants were potentially affecting the encoded protein, i.e. non-synonymous variants and variants present at splice sites. To detect potential disease causing variants (not including no-call variants), further filtering was then performed for (1) heterozygous variants present in both patients, but absent from any parent, (2) homozygous variants and (3) compound heterozygous variants. Five genes, including *LNPI*, *ADCY1*, *LRRC32*, *NLRP1* and *CCDC123*, showed identical and Sanger sequencing

confirmed variants in both patients, which were not detected in either parent (see table 2A). Since the patients formed a dizygotic twin, the presence of identical, apparently *de novo* variants is likely due to a mutation in the germline of one of the parents. Each of the variants in these five genes was present in the control datasets HuVariome database[Stubbs et al., 2012], Public54[Drmanac et al., 2010] (the frequency table for the Public54 (Hapmap Coriell and ATCC Repository number(s)) can be downloaded from <ftp.completegenomics.com>, the identity of the 54 samples can be found in the header of the table), 1000 Genomes[Siva, 2008], and/or the Welllderly database (490 control samples sequenced by Complete Genomics)[Study] (frequencies see table 3). Hence, we considered their pathogenicity less likely and excluded them from further analysis. No homozygous variants were detected that conformed to a recessive mode of inheritance.

In only a single gene (*HTR7*, GenBank: NM_000872.4, MIM: 182137) a compound heterozygous variation was identified (table 2A). The two variants, p.W60C and p.V286I, were not present in control databases from the 1000 Genomes Project[Siva, 2008], Exome Sequencing Project[ESP], HuVariome[Stubbs et al., 2012], Welllderly[Study] and dbSNPv137[Sherry et al., 2001] (table 3). The sole database listing both variants was the ExAC database[ExAC], albeit at a very low frequency (p.W60C: 9.111×10^{-5} ; p.V286I: 8.266×10^{-5}). The first variant (p.V286I), predicted to be maternal by the WGS data, was confirmed in both patients II.2 and II.3 and the mother I.2, using Sanger sequencing. The second variant (p.W60C), predicted to be of paternal origin, could however not be confirmed under PCR conditions routinely used in our laboratory. We hypothesized that this was a consequence of mismapping of reads from the *HTR7* pseudogene, *HTR7P1*, which is 83.5% homologous on the sequence level (see figure 2). To exclude this, we re-mapped the NGS reads manually for accuracy and confirmed that the mapping was correct. However, mFold analysis[Zuker, 2003] suggested a preference for secondary structures in the mutated, but not

in the wild type sequence (see figure 3). Hence, we repeated Sanger sequencing in the presence of betaine. Under these denaturing reaction conditions, we were able to confirm the p.W60C variant in both patients II.2 and II.3 and in the father I.3. In accordance to the WGS data, neither variant was present in the unaffected stepsister (individual II.1).

Details on these variants along with their pathogenicity scores (CADD, SIFT, PolyPhen2, MutationTaster, PhyloP and Grantham matrix) are listed in table 2A. The paternal variant (p.W60C) lies within the first extracellular domain of the HTR7 receptor protein (see figure 4). This variant is a tryptophan to cysteine substitution at an evolutionary conserved position. Since tryptophan has a hydrophobic side chain and cysteine is uncharged, this variant may affect receptor function. The maternal variant (p.V286I) is located in the cytoplasmic domain after the fifth transmembrane domain (see figure 4). This variant is a valine to isoleucine substitution. Both amino acids have a hydrophobic side chain and only differ from each other by one C bound.

Discussion

The *HTR7* gene encodes the G protein coupled serotonin type 7 receptor (5-HT₇) which is expressed in the central nervous system and peripheral tissues[Gellynck et al., 2013]. Upon stimulation by serotonin (5-hydroxytryptamine, 5-HT), the G α subunit dissociates from the G $\beta\gamma$ subunits and activates adenylate cyclase (AC), which then catalyzes the formation of 3',5'-cyclic AMP (cAMP) from adenosine triphosphate (ATP). Subsequent activation of several pathways (reviewed by Gellynck *et al.* [Gellynck et al., 2013]) implies the 5-HT₇ receptor in the regulation of brain development and synaptic transmission and plasticity. Several observations suggested a role for the serotonin receptors in autism [Ciranna and Catania, 2014]. First, serotonin synthesis in the brain of autistic children during early childhood (2-5 years) is significantly lower when compared to non-affected

children[Chugani et al., 1999]. Second, abnormal morphology of the serotonergic fibers has been reported in postmortem brains of young autistic children, potentially as a consequence of the lowered serotonin levels[Azmitia et al., 2011]. Finally a reduced 5-HT receptor density was observed in the postmortem brains of young autistic adults, a difference that was not detected in individuals with Asperger syndrome[Oblak et al., 2013].

Genetic ablation of the 5-HT₇ receptor in animal models revealed a specific role for the receptor in physiological functions[Ciranna and Catania, 2014]. In a loss of function C57BL/6 mouse model, in which exon 2 of the *Htr7* gene is replaced by a neocassette[Hedlund et al., 2003], a specific deficit in fear-related learning as measured in the contextual fear conditioning test was observed[Roberts et al., 2004]. As other forms of hippocampal learning, without a significant emotional component, were unaffected, it was suggested that the receptor has an important role in complex integrative learning mechanisms[Roberts et al., 2004]. These mechanisms seem to involve long-term synaptic plasticity, namely, knockout mice show reduced ability to induce LTP[Roberts et al., 2004]. From the differential performance in the different protocols of the location/novel object tests and the Barnes maze it was concluded that the striatum-dependent, egocentric memory is relatively spared in the knockout mice, as opposed to the hippocampus-dependent allocentric spatial memory[Sarkisyan and Hedlund, 2009], an observation in line with the distribution of the receptor in the brain, as the 5-HT receptors are well represented in the hippocampus, but nearly absent in the striatum. In human, 5-HT receptors have been found in the caudate nucleus and the putamen, forming the striatum [Martin-Cora and Pazos, 2004].

Many observations in the knockout mice were validated using pharmacological treatment of wildtype mice. Three different splice variants of the *HTR7* gene exist, and their pharmacology was found to be essentially identical[Heidmann et al., 1997; Jasper et al., 1997; Stam et al., 1997; Gellynck et al., 2013]. Pharmacological blockade of the receptor

with the 5-HT₇-antagonist SB-269970 for instance led to the same deficit in the tail suspension, forced swim task and novel object recognition test[Hagan et al., 2000; Ballaz et al., 2007; Bonaventure et al., 2007; Gasbarri et al., 2008; Monti et al., 2012]. Pharmacological activation with the 5-HT₇ receptor agonists 5-CT led to improvements in the novel object recognition protocol when the drug was administered with a 2-hour delay after training[Freret et al., 2014], an effect blocked by SB-269970[Ballaz et al., 2007].

In addition, EEG activity recordings showed a reduction in REM sleep in *Htr7*^{-/-} mice[Hedlund et al., 2005], linking the receptor to sleep/wake cycle disturbances. Both activation and blockade of the HTR7 receptor has been demonstrated to cause a reduced duration of the REM sleep[Hagan et al., 2000; Thomas et al., 2003; Bonaventure et al., 2007; Monti et al., 2008; Monti et al., 2012; Monti and Jantos, 2014]. Additionally, a phase-shift in the sleep/wake cycle was observed after administration of agonists[Adriani et al., 2012]. Finally, a role for the receptor in thermoregulation was found after observing hypothermia as a consequence of the addition of agonists to wildtype mice and guinea pigs[Hagan et al., 2000; Guscott et al., 2003; Hedlund et al., 2003].

The dizygotic twins presented here carry a compound heterozygous variant in the *HTR7* gene. No other coding sequence abnormalities (CNVs and SVs) were observed in their fully sequenced genomes consistent with either dominant or recessive inheritance. As the *HTR7* receptor has been implicated in autism and cognition based on functional animal studies, it can be hypothesized that the identified compound heterozygous variant may contribute to the intellectual disability of the patients. Furthermore, disturbances observed during sleep/wake tests might link a less functional 5-HT₇ receptor to the sleeping problems and disturbed biological clock of the twins. Finally the receptor appears to play a role in thermoregulation. In that respect, it is interesting to note the deregulation of the body temperature in the female patient. Despite the limited predicted functional effect of the

maternal V286I *HTR7* variant, it should be noted that a similar variant (p.P279L) located close to the maternal variant was demonstrated to severely reduce signal transduction, likely due to altered binding to the G protein [Kiel et al., 2003]. Furthermore, a rare mutation in the *VPS13B* gene with pathogenicity prediction scores in the range of the maternal variant, was reported as causative in a patient with mild features of the Cohen syndrome (MIM: 216550, table 2B)[Yu et al., 2013]. To test the specific effect of the identified variants to the protein, functional analyses on the mutated protein are an interesting next step forward. Although we recognize the need to identify more patients with similar variants in order to firmly establish the relationship between mutations in the *HTR7* gene and autism, the presented family provides a first suggestive genetic link between the 5-HT₇ receptor and ASD in patients.

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The authors declare that there are no conflicts of interest.

Ethical statement

Informed consent was obtained for the patients and the study was approved by the authors' hospitals ethical committees.

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Web Resources

CGA tools version 1.6.0.43: <http://www.completegenomics.com/sequence-data/cgatools/>

Spotfire (Tibco): <http://spotfire.tibco.com/>

ExAC database: <http://exac.broadinstitute.org/>

Welllderly database: stsi-ftp.sdsc.edu

Public54: ftp.completegenomics.com

Table Captions

Table 1 - Details on Whole Genome Sequencing results of all five family members.

Table 2 – A) Molecular details of the *de novo* and compound heterozygous variants identified in this family. B) For comparison, pathogenicity scores of a missense mutation in the *VPS13B* gene causing Cohen syndrome[Yu et al., 2013].

Table 3 - Allele frequencies in various datasources (Welllderly, Public54, HuVariome and 1000 Genome Project) of the *de novo* and compound heterozygous variants.

Supplementary Table 1 - *De novo* CNV/SVs (58)

Supplementary Table 2 - Inherited CNV/SVs (332)

Figure Legends

Figure 1 - Pedigree of the family with the affected dizygotic twin

Figure 2 - ClustalW multiple alignment of the *HTR7* gene and its pseudogene (*HTR7P1*).

Primers used for PCR and Sanger sequencing are highlighted in green, differences with the pseudogene in the primersequences in red and the paternal mutation (W60C) in yellow.

Figure 3 - Mfold predictions[Zuker, 2003] of the secondary structures of the A) wildtype and

B) mutated *HTR7* amplicon strands surrounding the paternal (W60C) mutation, using

standard conditions except for temperature and ionic conditions: 60°C, 30mM Na⁺, 1.5mM

Mg⁺⁺, mimicking the PCR master mix conditions.

Figure 4 - Schematic representation of the 5-HT₇ receptor protein together with the subunits

of the coupled G protein. Both mutations identified in the reported family are shown in yellow.