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ORIGINAL PAPER

Whole Genome Analysis of Dizygotic Twins With Autism Reveals Prevalent Transposon Insertion Within Neuronal Regulatory Elements: Potential Implications for Disease Etiology and Clinical Assessment

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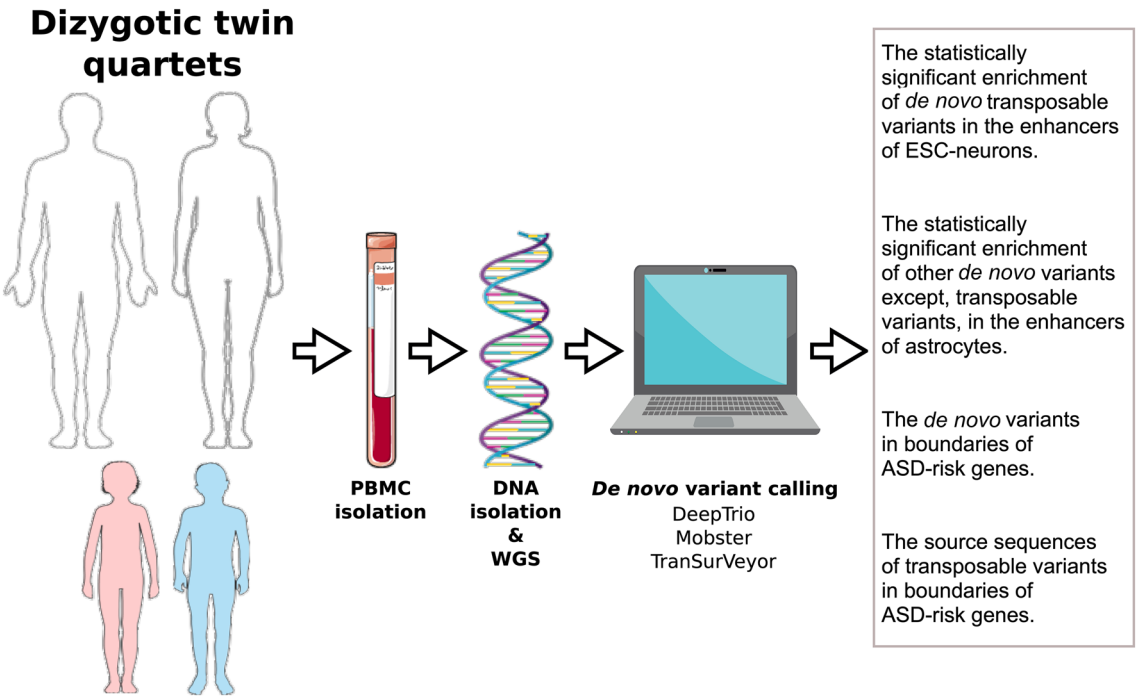
Abstract

Transposable elements (TEs) have been implicated in autism spectrum disorder (ASD). However, our understanding of their roles is far from complete. Herein, we explored de novo TE insertions (*dn*TEIs) and de novo variants (DNVs) across the genomes of dizygotic twins with ASD and their parents. The neuronal regulatory elements had a tendency to harbor *dn*TEIs that were shared between twins, but ASD-risk genes had *dn*TEIs that were unique to each twin. The *dn*TEIs were 4.6-fold enriched in enhancers that are active in embryonic stem cell (ESC)-neurons ($p < 0.001$), but DNVs were 1.5-fold enriched in active enhancers of astrocytes ($p = 0.0051$). Our findings suggest that *dn*TEIs and DNVs play a role in ASD etiology by disrupting enhancers of neurons and astrocytes.

Graphical Abstract



Keywords Autism spectrum disorder · Variant calling · Transposable element · Whole genome sequencing · Quartet families

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Introduction

Autism spectrum disorder (ASD), a neurodevelopmental disorder characterized by aberrations in reciprocal social communication and the existence of restrictive interests and repetitive behaviors, has a strong genetic basis (Anagnostou et al., 2014). ASD displays broad clinical and genetic heterogeneity with high heritability and a high risk of recurrence, with males being affected almost four times more than females.

Twin studies suggest robust heritability in ASD (Ronald & Hoekstra, 2011). Families have a high risk of recurrence of up to 18% (Ozonoff et al., 2011), suggesting that in quartet families with two affected siblings and their unaffected parents, those siblings might have the same ASD-susceptibility alleles (Zhao et al., 2007). The heritability studies of ASD underpin a crucial role for inherited variants (IVs) (Ronald & Hoekstra, 2011) but sporadic mutations are more prevalent in families having offspring with autism than in the general population (Iossifov et al., 2014). However, the approach of searching for de novo variants (DNVs) in ASD has emerged as an effective way to uncover mechanisms underlying ASD (Ronemus et al., 2014). DNVs are generally considered to be more deleterious than IVs owing to their lack of exposure to negative selection (Veltman & Brunner, 2012). In a study where 250 families were analyzed by low-coverage whole genome sequencing (WGS), DNVs including deletions, duplications, and transposable element insertions (TEIs) were characterized (Kloosterman et al., 2015). It is estimated that penetrant DNVs in genic regions contribute to ASD in ~ 11% of trio families (unaffected parents and affected child) (Sanders et al., 2015). However, best practices and tools have not been established yet in this rapidly growing area, and therefore it is likely that these numbers are underestimates.

Structural variants (SVs), including deletions and duplications, contribute to the genetic risk of neuropsychiatric disorders (Malhotra & Sebat, 2012). When deletions and duplications are larger than 1 kb in length, they are called copy number variations (CNVs) (Zhang et al., 2006). The majority of SVs are CNVs, and they can alter transcriptional activity, which may in turn result in disease predisposition (Zhang et al., 2006). Studies of CNVs have been influential in unraveling the importance of rare genetic variants in the etiology of ASD (Pinto et al., 2010; Sebat et al., 2007). Despite that, characterization of SVs in ASD genomes remains an important challenge. Advanced analytical methodologies for the discovery and genotyping of SV have scarcely been adapted in studies of ASD, and the broader detection of SVs is necessary for elucidating the genetic mechanisms underpinning ASD risk. The characterization of SVs in human disease and at the population level has been limited by the low sensitivity and accuracy of microarray and common next-generation sequencing techniques (O'Roak et al., 2012; Sanders et al., 2015); nevertheless, the latest technical advances in sequencing have permitted the uncovering of a wide diversity of SVs from WGS datasets of ASD, including deletions and duplications, inversions, translocations, and TEIs.

TEIs are a class of SVs, and they occur when TEs move to other locations in the genome via DNA transposases or reverse transcription (Hancks & Kazazian, 2012). The long interspersed nuclear element (LINE) is a type of TE and constitutes ~ 17% of the human genome (Cordaux & Batzer, 2009). Although there are many subsets of TEs in humans, only long interspersed nuclear elements-1 (LINE-1 or L1s) have an autonomous function to undergo retrotransposition (RT); however, other TEs, such as ALU elements, short interspersed element variable number tandem repeat-ALUs (SINE-VNTR-ALUs or SVAs), and U6s depend on L1s for their RT activity in the genome (Kazazian & Moran, 2017). In humans, the majority of TEs do not maintain the ability to constitute new insertions (Mills et al., 2007), but certain of them from, the L1, ALU, and SVA families, stay active and are capable of producing new insertions (Bennett et al., 2008; Brouha et al., 2003; Wang et al., 2005). The production of new insertions is achieved if the molecular structures of TEs are intact. TEs can copy themselves from many genomic loci and then integrate into other genomic loci. These loci, where TEs make copies of themselves, are called their source sequences. Nonetheless, DNA repair at the new TE sites and at the source sequences is necessary to complete transposition by reproducing intact double-strand DNA following transposase-mediated DNA breakage and joining. Removal of the TE from the source sequence by DNA double-strand breaks at TE ends creates a double-strand gap in the donor DNA that must be repaired. DNA repair mechanisms are capable of restoring the source sequence to its pre-transposon condition, resulting in unambiguous excision. Previous studies have demonstrated that non-homologous end-joining (NHEJ) and homology-dependent repair can be used to repair gaps in the source sequences (McVey et al., 2004; Yant & Kay, 2003). When the NHEJ repair of the source sequence backbones occurs, the broken DNA ends are often rejoined, but this tends to be erroneous. As a result, the majority of source sequences of TEs are not accurately restored to their pre-transposition sequence.

It is estimated that 1 in 12–14 live human births has a de novo TEI (*dn*TEI) (Gardner et al., 2019). Another study estimated that a *dn*TEI arises in every 1 out of 18.4–26.0 human births (Stewart et al., 2011). Each individual human

genome possesses ~ 1200 polymorphic TEI variants at the population-level, with ALU contributing almost 75% of total RT polymorphisms (Zhang et al., 2017).

Several studies achieved the goal of creating a broad catalog of pathogenic TEIs (pTEIs) in several genes and conditions (Hancks & Kazazian, 2016; Qian et al., 2017). Most TEs act throughout the genome with no obvious phenotypic consequences, despite being inserted into critical regions, leading to disruption of transcription, splicing, or translation at times (Hancks & Kazazian, 2016; Kazazian & Moran, 2017). However, some of the TEIs are considered pTEIs and have been associated with a number of neurodevelopmental disorders, including ASD (Cupaioli et al., 2021). The prevalence of pTEIs amongst all pathogenic variants (PVs) is estimated to be between 0.16 and 0.3% (Hancks & Kazazian, 2016). However, this number is likely an underestimation owing to challenges in their detection. Yet, several studies of ASD have been able to identify de novo and inherited pTEIs in both the coding and noncoding genomic regions (Borges-Monroy et al., 2021; Williams et al., 2019). The sequence variants in the non-coding genome, including those at or near the regulatory elements (REs), are known to affect developmental processes, as exemplified by the frequent colocalization of genome-wide association study (GWAS) hits with enhancers (Nord & West, 2020). Indeed, *dn*TEs in ASD patients were found to be enriched in fetal brain enhancers, underlining the importance of neuronal REs in brain development (Borges-Monroy et al., 2021). On the other hand, some other extensive WGS-based analyses of ASD genomes failed to discover likely causative TEI variants (Brandler et al., 2018; Werling et al., 2018). This could be due to several factors: (i) low frequency of ASD cases due to gene disruption by TEIs (Werling et al., 2018); (ii) relatively low TE mutation rate (Stewart et al., 2011); and (iii) lack of a larger sample size (Brandler et al., 2018). Despite these factors, recent technological breakthroughs in sequencing have permitted the identification of those causal TEIs in WGS datasets of ASD. Overall, it is not clearly understood what the functional effects of *dn*TEIs are, their roles in the etiology of neurodevelopmental disorders including ASD, and whether assessment of patient genomes for detrimental TEI events could improve clinical efforts.

In this study, we performed WGS in blood samples of three quartet families with dizygotic (DZ) twins with non-syndromic autism to better understand de novo SVs in ASD. To this end, we performed comprehensive in silico analyses by combining a deep-learning-based tool for standard SV detection, including deletions, insertions, and specialized methods for potential pTEI detection. This combinatorial approach provided a broader perspective and revealed unique aspects of TEIs in the ASD genomes. Overall, in this study, we tested whether pTEI analysis, even in small cohorts of ASD, could be successfully applied towards the identification of PVs. By using a DZ twin family design, we aimed to minimize the noise from non-PVs.

Materials and Methods

Samples and DNA Isolation Protocol

The ASD diagnosis was decided based on the Diagnostic and Statistical Manual of Mental Disorders, Fifth edition (DSM-5) criteria (Association, 2013). The schedule for affective disorders and schizophrenia for school-age children-present and lifetime version (K-SADS-PL) (Kaufman et al., 1997) was employed to detect other diagnoses comorbid with ASD. The childhood autism rating scale (CARS) (Schopler et al., 1980) was used to assess the diagnosis and severity of ASD. We utilized the autism-spectrum quotient (AQ), a self-reported questionnaire for quantifying autistic traits, to evaluate whether the parents had the broader autism phenotype (BAP), which reflects the phenotypic expression of the genetic liability to ASD in non-autistic relatives of autistic individuals (Wheelwright et al., 2010). Additionally, the presence of other psychiatric disorders in the parents was determined with the structured clinical interview for DSM-IV axis I disorders (SCID-I) (First et al., 2002). All diagnostic criteria are shown in Table 1, and the detailed information on samples and DNA isolation protocol can be found in Supplementary File 1.

WGS and Pre-Processing of Reads

We have previously generated peripheral blood mononuclear cell (PBMC)-derived whole transcriptome and WGS data of twelve samples in total (Okay et al., 2021). For WGS data each library was prepared and sequenced at 150 bp paired-end read length with 30X coverage using an Illumina HiSeq instrument. Bases with < 30 quality score were filtered out using the fastp tool v0.21.0 (https://github.com/OpenGene/fastp) (Chen et al., 2018) for the downstream analysis. The alignment of reads to the hg19 human reference genome was performed with the Burrows-Wheeler Aligner v0.7.17 (BWA-mem) (Li et al., 2009b), and SAMtools v1.9.0 (Li et al., 2009a) were used to obtain binary alignment map (BAM) files.

Identification of De Novo Variants with DeepTrio

We ran trio variant calling analysis with BAM files of each sample (i.e., mother, father, and twin) to query DNVs in each DZ twin with autism. To this end, the DeepTrio tool v1.1.0 (https://github.com/google/deepvariant/blob/r1.1/docs/deeptrio-quick-start.md), a deep learning-based trio variant caller built on top of DeepVariant, was used. This

Table 1 Clinical information of the quartet families

Families	Individuals	Age	Sex	Status	Pregnancy age	CARS	ABC	BAP
F1	F1-M	44	Female	Mother	37	–	–	26
	F1-F	44	Male	Father	37	–	–	9
	F1-T1	7	Male	DZ twin	–	51	110	–
	F1-T2	7	Male	DZ twin	–	40	86	–
F2	F2-M	49	Female	Mother	39	–	–	19
	F2-F	50	Male	Father	40	–	–	26
	F2-T1	11	Female	DZ twin	–	33.5	14	–
	F2-T2	11	Male	DZ twin	–	47	48	–
F3	F3-M	30	Female	Mother	25	–	–	16
	F3-F	34	Male	Father	29	–	–	22
	F3-T1	5	Male	DZ twin	–	45.5	69	–
	F3-T2	5	Female	DZ twin	–	43	83	–

CARS childhood autism rating scale, *ABC* autism behavior checklist, *BAP* broad autism phenotype, *DZ* dizygotic

tool is a deep learning-based variant caller that takes aligned reads (i.e., BAM format files), produces pileup image tensors based on the alignments, and classifies each tensor using a convolutional neural network. Subsequently, the tool reports the results in the variant call format (VCF) or genomic VCF (gVCF) file format (https://github.com/google/deepvariant) (Poplin et al., 2018). The Ensembl variant effect predictor (VEP) (McLaren et al., 2016) was utilized for the annotation of VCFs using the GRCh37 assembly. We utilized the developmental disorders G2P ($G2P^{DD}$) plugin (https://www.ebi.ac.uk/gene2phenotype/g2p_vep_plugin) (Thormann et al., 2019) to identify potential PVs. Besides, the ClinVar annotation was added to each VCF file. After comprehensive annotation of variants, the mother, father, and twin VCF files for each family were merged using the vcf-merge executable of VCFtools v0.1.17 (https://vcftools.github.io/man_latest.html) (Danecek et al., 2011). For the detection of high quality DNVs in each twin, variants with > 10 read depth (i.e., DP header in VCF) and > 20 genotype quality (i.e., GQ header in VCF) were used. In this analysis step, the merged VCF files were utilized as input to VCFProcessor v1.0.4 (http://lysine.univ-brest.fr/vcfprocessor/index.html#). Eventually, BCFtools v1.12 (https://samtools.github.io/bcftools/) (Danecek et al., 2021) was used to identify shared variants amongst twin siblings. Of note, all VCFs were indexed through Tabix v1.10.2 (http://www.htslib.org/doc/tabix.html) (Li, 2011).

Exploring De Novo TEIs

We used Mobster v0.2.4.1 (https://jyhehir.github.io/mobster/index.html) (Thung et al., 2014) and TranSurVeyor v1.0 (https://github.com/Mesh89/TranSurVeyor) (Rajaby & Sung, 2018) tools to call TEIs in each genome. The called TEIs were kept for downstream analysis if they had at least five reads, including discordant reads for each individual. BEDTools v2.30.0 (https://bedtools.readthedocs.io/en/latest/) (Quinlan & Hall, 2010) was used to find *dn*TEIs in twins that were absent in their parents. To this end, the browser extensible data (BED) files of each parent containing TEI coordinates were merged and overlapped with the BED files of each twin. We focused on the *dn*TEIs that are common to both twin siblings and detected by Mobster and TranSurVeyor. These TEIs were intersected through BEDTools. In addition to common *dn*TEIs in twin siblings, we also explored *dn*TEIs that uniquely occurred in each twin. Additionally, TranSurVeyor output was used to inspect the source sequences from which *dn*TEIs copied themselves to be inserted into any locus in the genome. The identification of source sequences was achieved through the detection of multiple copies of TE reads in many possible loci and subsequently selecting reads that had the highest mapping quality score. The HOMER v4.11 (http://homer.ucsd.edu/homer/index.html) (Duttke et al., 2019) was utilized to annotate each *dn*TEI that was detected by both tools according to genic-contexts, including promoters, transcription start and termination sites (TSS and TTS, respectively), coding and noncoding exons, introns, and intergenic regions. Besides, BEDTools was employed for annotation of these TEIs as well as HOMER annotation to capture a more detailed annotation layer and support our results.

Inspection of De Novo Variants that Occurred in ASD-Risk Genes

The DNVs and *dn*TEIs in ASD-risk genes that are listed in the simons foundation autism research initiative (SFARI, accessed on 08 Feb 2021) (https://www.sfari.org/) and the autism database (AutDB, accessed on 08 Feb 2021) (http://autism.mindspec.org/autdb/Welcome.do) databases were

inspected. We included all genes in the SFARI database in our downstream analyses, regardless of the categories of genes. The gene scoring system in the SFARI categorizes genes into four different categories: syndromic (S), high confidence (score 1), strong candidate (score 2), and suggestive evidence (score 3) according to certain criteria, including gene-disrupting DNVs, GWAS studies, and how many times a gene has been reported. We also focused on genes that are considered ASD-risk genes in the AutDB database. The scoring system in the AutDB database considers similar criteria to categorize genes.

Investigation of Tissue-Specific Regulatory Elements Harboring De Novo Variants

To investigate DeepTrio DNVs and *dn*TEIs that occurred in REs, including promoters, enhancers, and open chromatin regions (OCRs), ENSEMBL (https://grch37.ensembl.org/Homo_sapiens/Info/Index), GeneHancer (Fishilevich et al., 2017), the open regulatory annotation (ORegAnno) (Lesurf et al., 2016), and the EnhancerAtlas v2.0 (http://www.enhanceratlas.org/indexv2.php) (Gao & Qian, 2020) databases were used. In particular, we focused on the EnhancerAtlas database to test the hypothesis of whether *dn*TEIs and DNVs were enriched in enhancers of certain tissues and/or cells that have been associated with ASD etiology, including ESC-neurons, fetal brain, cerebellum, astrocytes, and cortex (Donovan & Basson, 2017; Petrelli et al., 2016; Velmeshev et al., 2019). The hg19 RE coordinates were intersected with TE coordinates through the *intersect* function of BEDTools. The enrichment analysis of *dn*TEIs and DNVs within enhancer coordinates was performed by the *fisher* function of BEDTools to see whether those *dn*TEIs and DNVs were randomly distributed throughout the genome according to Fisher's Exact test.

Results and Discussion

We performed standard and TE variant calling analyses on the WGS data of PBMC samples from three families, consisting of DZ twins with autism and their parents. In doing so, we determined certain kinds of de novo ASD-relevant variants through DeepTrio, Mobster, and TranSurVeyor. For TE variants, only those that were detected by both Mobster and TranSurVeyor are discussed.

DeepTrio and dnTEI Variants Across Samples

Our analysis with the DeepTrio pipeline found 4,440,443 variants per genome, with the following distribution: insertions (~7.5%), deletions (~8%), single nucleotide variants (SNVs) (~83.5%). Total DNVs per genome of twins was 189,656. The removal of low-quality variants permitted us to determine 38 DNVs with high quality (> 10 DP and > 20 GQ) per genome, most of which are located in the intronic and intergenic parts of the genome. All de novo high quality variants can be found in VCF format in Supplementary File 2.

Besides, the transposition callers, Mobster and TranSurVeyor, detected 267 and 519 TEIs per genome on average, respectively. According to the predictions by Mobster, there were 214 ALU (80.1%), 47 L1 (17.6%), and 6 SVA (2.3%) insertions per genome on average. We were not able to calculate the total number of TEIs per genome with TranSurVeyor, as this caller is not capable of classifying TE families by default. The Mobster pipeline, in each twin, determined 167 *dn*TEIs on average; however, this number was found to be 442 by the TranSurVeyor tool. In a family-specific context, 140 (83.8%), 23 (13.7%), and 4 (2.4%) of these 167 TE variants belonged to the ALU, L1, and SVA families, respectively. Further analysis of the TEI predictions by both callers revealed that there were 60 *dn*TEIs per genome on average, 52 (86.6%), 6 (10%), and 1.1 (1.8%) of which belonged to the ALU, L1, and SVA families, respectively. The detailed information on TEs provided by Mobster and TranSurVeyor in all samples can be found in Supplementary File 3.

De Novo Variants in ASD-Risk Genes

We focused on DeepTrio DNVs within genes that were listed as ASD-risk genes by the SFARI and AutDB. F1-T1 child had a de novo insertion downstream of the *SOX5* gene (score 1), which encodes a transcription factor involved in the regulation of embryonic development and chondrocyte differentiation. F1-T2 child had a substitution in intron 1 of the *ERBB4* gene (score 2), which induces a variety of cellular responses to neuregulins, including mitogenesis and differentiation. An insertion in intron 1 of the *UBE2H* gene (score 3) was identified in the F1-T2 child and the protein encoded by this gene is critical for protein ubiquitination and immune response. In the F2-T1 child, a deletion in intron 3 of the *PLCB1* gene (score 2), which plays an important role in Apelin signaling, was identified. Lastly, we detected DNVs in two SFARI genes in the F3-T2 child. One of them was *BRINP3* (score 3), with an insertion in intron 2, which inhibits neuronal cell proliferation by negative regulation of the cell cycle transition. Another gene, *NRXN3* (score 1), with deletion and insertion in intron 12, functions in the vertebrate nervous system as a cell adhesion molecule and receptor. Detailed information on all detected variants can be found in Table 2.

In addition to the DeepTrio variants, ASD-risk genes with *dn*TEIs were examined, and their source TEs were identified, as well. We observed that the F1-T1 child had *dn*TEIs in *ANK3* (score 1, 15 bps of ALU in intron 1), *SASH1*

Table 2 SFARI and AutDB genes with de novo DeepTrio variants in the dizygotic twins

DZ twins	Chr	Coordinates (hg19)	Gene	REF allele	ALT allele(s)	Variant class	HGVS annotation	Annotation	Genotype			dbSNP	SFARI score
									Proband	Mother	Father		
F1-T1	12	23683429	SOX5*	C	CAAT,CAAACAAT	Insertion	NM_006940.6:c.*3723_*3724insATTGTTT	Downstream	0/2	0/1	0/1	rs10649079	1
F1-T2	2	212998134	ERBB4	T	TG,G	Substitution	NM_005235.3:c.83-8507_83-8506A > C	Intron 1	0/2	0/1	1/1	rs142020967	–
	7	129584555	UBE2H*	AACACACAC	AACACACACACAC,A,AACACACACACAC	Insertion	NM_003344.4:c.53 + 7780_53 + 7787dupGTGTGT	Intron 1	0/1	0/2	0/3	rs57825154	3
F2-T1	20	8560519	PLCB1*	GAA	G,GA	Deletion	NM_015192.4:c.247-48417delAA & NM_015192.4:c.247-48417delA	Intron 3	1/2	0/2	0/2	rs11087810	2
F3-T2	1	190308135	BRINP3*	AAAAT	A,AAAATAAAT	Insertion	NM_199051.3:c.237-57259_237-57256dupATTT	Intron 2	0/2	0/1	0/1	rs144106726	3
	14	79500692	NRXN3*	TAC	T,TACACACACACACACACAC	Insertion	NM_004796.6:c.2143 + 46232_2143 + 46237delCA & NM_004796.6:c.2143 + 46232_2143 + 46237dupCACACACACACACACA	Intron 12	1/2	0/1	1/1	rs56226324	1

HGVS human genome variation society, *SFARI* simons foundation autism research initiative

*Available in both SFARI and AutDB databases

(score 3, 10 bps of ALU in intron 7), and *TSHZ3* (score 1, 15 bps of ALU in intron 3) genes. Their source TEs originated from an intergenic region on chromosome 1, intron 2 of the *LINC00327* gene, and intron 2 of the *SLBP* gene, respectively. The F1-T2 child had *dn*TEIs in *GRIP1* (score 2), *PCDH9* (score 3), and *RPS6KA2* (score 3) genes. These genes had 19, 8, and 16 bps of ALU in their introns 1, 2, and 3, respectively. Their source TEs were located in the first introns of *GTDC1*, *RP11-946L20.4*, and *RP11-22P4.1* genes. Of note, *GTDC1* was reported among the mutated genes in an ASD study (Aksoy et al., 2017). We discovered a 15 bp-long ALU insertion in the intron 15 of the *ACE* gene (score 3) in F2-T2 child, which originated from an intergenic region of chromosome 6. In the F3-T1 child, we identified 10–19 bp-long ALU insertions in the first introns of *GPC6* (score 3) and *POMGNT1* (score 1), and their source TEs were located in an intergenic region of chromosome 6 and intron 7 of the *AC007682.1* gene, respectively. The detailed information on these variants is summarized in Table 3. Moreover, the HOMER and the BEDTools annotations of all *dn*TEIs are available in Supplementary Files 4 and 5, respectively.

De Novo Variants Within Regulatory Elements

Next, we focused on the REs to determine whether DNVs are enriched in these regions. Considering the effects of REs such as promoters, enhancers, and OCRs on their target genes through alterations in expression and other mechanisms, we postulated that target genes of these regions with DNVs could have a relationship with ASD. To determine potential target genes of ENSEMBL REs, we used GeneHancer (Fishilevich et al., 2017) and the open regulatory annotation (ORegAnno) database (Lesurf et al., 2016) tracks in the UCSC genome browser, as well as the EnhancerAtlas (Gao & Qian, 2020). We detected an OCR (*ENSR00001028261*) with a deletion in the F3-T2 child. This OCR was also reported as an enhancer (*GH02J018522*) in the GeneHancer database and was associated with the *KCNS3* SFARI gene (score 2), whose protein product mediates the voltage-gated potassium channel activation. DNVs in this gene were previously identified in ASD probands (De Rubeis et al., 2014). Another gene, *AKAP7*, which regulates signaling cascades downstream of D1-like dopamine receptors (Cantrell et al., 1999), was associated with an enhancer (*ENSR00001376701*) with de novo deletion. This gene has been associated with a weighted co-expression module containing ASD-risk genes (Konopka et al., 2012) and has been reported as differentially expressed in lymphoblastoid cells of ASD patients (Ghahramani Seno et al., 2011). Also, it was reported that a splice variant of this gene interacts with sodium channels in the brain (Tibbs et al., 1998). We annotated a deletion within the promoter-flanking region (PFR)

Table 3 SFARI and AutDB genes with de novo transposable element insertions in the dizygotic twins

DZ twins	Chr	Coordinate (hg19)	Gene	SFARI score	Type	Annotation	IS (bp)	Source element		
								Chr	Coordinate (hg19)	Annotation
F1-T1	10	62124844–62124858	ANK3	1	ALU	Intron 1	15	1	120873149-NA	Intergenic
	6	148802151–148802160	SASH1	3	ALU	Intron 7	10	13	24057937-NA	Intron 2 of LINC00327 gene
	19	31690670–31690684	TSHZ3	1	ALU	Intron 3	15	4	1709023-NA	Intron 2 of SLBP gene
F1-T2	12	67011570–67011588	GRIP1	2	ALU	Intron 1	19	2	145003853-NA	Intron 1 of GTDC1 gene
	13	67725849–67725856	PCDH9	3	ALU	Intron 2	8	8	112168571-NA	Intron 1 of RP11-946L20.4 gene
	6	167124797- 167124812	RPS6KA2	3	ALU	Intron 3	16	11	28375188-NA	Intron 1 of RP11-22P4.1 gene
F2-T2	17	61565890–61565904	ACE	3	ALU	Intron 15	15	6	85503972-NA	Intergenic
F3-T1	13	94120721–94120730	GPC6	3	ALU	Intron 1	10	6	126590325-NA	Intergenic
	1	46679029–46679047	POMGNT1	1	ALU	Intron 1	19	2	52199450-NA	Intron 7 of AC007682.1 gene

IS insertion size (base pair), *Chr* chromosome number, *SFARI* simons foundation autism research initiative

(*ENSR00001720880*) that interacts with the *ADAP1* gene, which has a developmentally regulated expression pattern in the rat brain (Stricker & Reiser, 2014). Moreover, a de novo missense variant in this gene, potentially disruptive to its interaction with the neuronal polarity and axon specification gene *KIF13B,* was identified in an ASD patient (Chen et al., 2020). We identified a PFR (*ENSR00001737393*) with an insertion that interacts with the *MTUS1* gene, involved in neuronal differentiation. However, an exonic nonsense variant in this gene has been reported in an ASD study (Krupp et al., 2017). There was a PFR (*ENSR00000979145*) with an insertion that interacts with the *ZNF365* gene, which is responsible for DNA integrity maintenance. A GWAS study of ASD suggested that this gene may play a role in maintaining genomic integrity in the pathophysiology of ASD (Chaste et al., 2015). Lastly, we detected another deletion in PFR (*ENSR00001685628*). This element regulates the Ras activator *RAPGEF2* gene, which is thought to control developmental neuronal migration in the cerebral cortex (Ye et al., 2014). Furthermore, genetic studies in individuals with schizophrenia (SCZ) demonstrated the robust genetic association of rare inherited CNVs involving *RAPGEF2* (Xu et al., 2008). More detailed information about these variants is available in Table 4.

We tested the same postulation for *dn*TEIs to seek whether they are enriched in the REs, as well as the detection of source sequences of those *dn*TEIs that existed in loci of ASD-risk genes. For instance, a 19 bp-long ALU insertion in the cerebellum-specific enhancer (*GH14J073717*) regulating the *ELMSAN1* gene (also known as *MIDEAS*) stemmed from intron 7 of the *SOX5* SFARI gene in the F1-T1 child. In mouse embryonic stem cells (mESCs), Mondal et al. identified a set of neurodevelopmental genes involved in neurogenesis, axon guidance, and neurotransmitter receptor signaling that were regulated by *ELMSAN1* (Mondal et al., 2020). We discovered a 14 bp-long ALU insertion in the enhancers of the *MBOAT1* gene (*GH06J020325* and *GH06J020497*), originating from intron 3 of the *NCKAP5* SFARI gene (score 3). The enzyme encoded by the *MBOAT1* gene is involved in arachidonic acid (AA) recycling (Gijón et al., 2008). AA-derived eicosanoids, regulating immune and inflammatory responses, have recently emerged as key players in neuropsychiatric disorders (Yui et al., 2015).

Besides, we examined whether DNVs were randomly distributed across the genome or whether REs could preferentially be targeted in twins with autism. We focused on REs that are active in certain tissues and/or cells. For this purpose, we performed Fisher's Exact test and discovered that DNVs were enriched in the active enhancers of astrocytes (enrichment ratio = 1.518, $p = 0.0051$) but they were not statistically significant enriched in other tissues/cells (Supplementary File 2). Considering that astrocytes influence synapse formation, function, and pruning and that they are closely coupled with neurons in the early stages of development (Petrelli et al., 2016), our results suggest that targeting of active enhancers in astrocytes by DNVs might contribute to ASD pathogenesis.

Likewise, we examined whether *dn*TEIs were enriched in REs. We found that enhancers active in ESC-neurons showed a 4.6-fold enrichment with statistical significance ($p < 0.001$); however, those active in the fetal brain had depletion with borderline significance (enrichment ratio = 0.3, $p = 0.056$). We did not observe any significance for enhancers active in the cerebellum ($p = 0.274$) or in other tissues/cells. The non-random insertion of *dn*TEIs into enhancers that are active in human ESC-neurons could possibly be explained by the accessibility of these regions at a developmental stage when TEs are less repressed due to hypomethylation during embryogenesis (Yandım & Karakülah, 2019). To sum up, the active enhancers in human ESC-neurons might be considered "*hotspots*" for *dn*TEIs in ASD. The mentioned enhancers with *dn*TEIs are listed in Table 5, and the information about other enhancers that are tested can all be found in Supplementary File 6, along with Fisher's Exact test results.

De Novo Variants Shared Between DZ Twin Siblings

We found a shared DeepTrio variant that was an insertion between the F2-T1 and the F2-T2 DZ twin siblings, located in intron 1 of the *MPPED2* gene, which encodes a metallophosphoesterase and is expressed in the fetal brain. This gene is not listed in the SFARI or the AutDB databases, but deletions harboring the *MPPED2* gene were previously identified in patients with ASD, intellectual disability, and mental retardation (Davis et al., 2008; Toral-Lopez et al., 2020).

Next, we focused on shared TE variants between DZ twin siblings. Most of these variants were located in the intergenic and intronic parts of the genome, but none of them were linked with the coding regions of the genome. Genes associated with these variants were predominantly non-coding, and none was an ASD-risk gene in the SFARI or the AutDB databases. However, there were several source TEs localized to the SFARI genes. For example, in F1-T1 and F1-T2 twins, an 18 bp-long L1 insertion in intron 2 of the *LINC01340* gene originated from intron 9 of the *CDH13* SFARI gene (score 2); in the F2-T2 twin, a 19 bp-long ALU insertion in an intergenic region of chromosome 13 close to the *LINC00392* gene originated from intron 2 of the *RNF38* SFARI gene (score 3). Interestingly, the same insertion in the F2-T1 twin originated from an intergenic region of chromosome 12. In the F3-T1 twin, a 16 bp-long ALU insertion in an intergenic region of chromosome 2 close to the *MIR4268* gene stemmed from intron 1 of the *CTNND2* SFARI gene (score 2), whereas in the F3-T2 twin, the same

Table 4 De novo DeepTrio variants overlapping with ENSEMBL regulatory elements

DZ twins	Chr	Coordinates (hg19)	Target gene(s)	REF allele	ALT allele(s)	Variant class	Regulatory type (ENSEMBL ID)	Genotype			dbSNP
								Proband	Mother	Father	
F1-T2	17	13632588	N/A	GT	G,GTT	Insertion	Open chromatin region (ENSR00001592382)	1/2	0/1	1/1	rs11364444
F2-T1	6	131704846	AKAP7 gene	AGAAGGAAG	A,AGAAG	Deletion	Enhancer (ENSR00001376701)	1/2	0/1	0/1	rs145950392
	7	979966	ADAP1 & COX19 genes	TCACACA	T,TCA	Deletion	Promoter flanking region (ENSR00001720880)	0/1	0/2	0/2	rs56715852
	7	139968546	RNU1-58P gene	T	TA,A	Substitution	Promoter flanking region (ENSR00001733964)	2/2	1/1	1/1	rs780132409
	8	17521118	MTUS1 & PDGFRL genes	T	TGTTTTG,TG	Insertion	Promoter flanking region (ENSR00001737393)	0/1	0/2	2/2	rs11463390
F2-T2	4	74473451	RASSF6 & MTHFD2L & AFM genes	AACCCTCC	A,ATCC	Deletion	Promoter flanking region (ENSR00001678323)	1/1	2/2	2/2	rs74266874
	10	64214402	ZNF365 gene	T	C,TC	Insertion	Promoter flanking region (ENSR00000979145)	1/2	0/1	1/1	rs4746123
F3-T1	4	160205633	RAPGEF2 gene	GACACACACACACACAC	G,GACACACACACAC	Deletion	Promoter flanking region (ENSR00001685628)	1/2	0/1	1/1	rs36232973
	11	34364467	ABTB2 gene	CATTTATTTATTT	C,CATTTATTT,CATTT	Deletion	Promoter flanking region (ENSR00001533732)	1/2	0/3	1/1	rs3035434
F3-T2	2	8038203	LINC00299 gene	A	AGAAG,AGAAA	Insertion	Promoter flanking region (ENSR00000112333)	2/1	0/1	0/1	rs5829130
	6	5735656	N/A	T	TCTAC,TC	Insertion	Enhancer (ENSR00001711233)	0/2	0/1	0/1	rs11396607
	12	33523295	KCNS3	CATAT	C,CAT	Deletion	Open chromatin region (ENSR00001028261) & Enhancer (GeneHancer ID = GH02J018522)	1/2	0/1	1/1	rs71948243
	15	20859148	N/A	ATTAT	A,ATTATTTATTTATTTATTTATTTAT	Insertion	Promoter flanking region (ENSR00001573419)	0/2	0/1	0/1	rs35140687

DZ dizygotic, *N/A* not applicable

Table 5 EnhancerAtlas regulatory elements overlapping with de novo transposable element insertions in the dizygotic twins

DZ twins	Tissue/cell sources	Chr	Coordinate (hg19)	Target gene	Type	Annotation-ID	IS (bp)	Source element			
								Chr	Coordinate (hg19)	MES (bp)	Annotation
F1-T1	Fetal Brain	7	157448772–157448787	PTPRN2	ALU	Promoter/enhancer—(GH07J157612)	16	6	47726643-N/A	N/A	Intergenic
	ESC neuron	12	12933679–12933694	APOLD1	L1	Promoter/enhancer—(GH12J012784)	16	6	153031759-N/A	N/A	Intron 1 of MYCT1 gene
	ESC neuron	17	72620206–72620217	CD300E	ALU	Promoter/Enhancer—(Gh17j074620)	12	6	168059236-N/A	N/A	Intergenic
	Cerebellum	14	74186531–74186549	ELMSAN1 or MIDEAS	ALU	Enhancer—(GH14J073717)	19	12	23795739–23795854	115	Intron 7 of SOX5 gene
F1-T2	ESC neuron	14	95024605–95024621	SERPINA4	ALU	Promoter/enhancer—(GH14J094560)	17	1	234386417-N/A	N/A	Intron 2 of SLC35F3 gene
	ESC neuron	17	72620206–72620217	CD300E	ALU	Promoter/enhancer -(GH17J074620)	12	6	168059236-N/A	N/A	Intergenic
F2-T1	Fetal brain	7	157448772–157448787	PTPRN2	ALU	Promoter/enhancer -(GH07J157612)	16	6	47726919-N/A	N/A	Intergenic
F2-T2	Fetal brain	7	157448772–157448787	PTPRN2	ALU	Promoter/Enhancer -(GH07J157612)	16	6	47726919-N/A	N/A	Intergenic
	ESC neuron	13	78584663–78584671	LINC00446	ALU	Promoter/Enhancer -(GH13J077974 & GH13J077980)	9	Y	16601315-N/A	N/A	Intergenic
	ESC neuron	15	55702233–55702249	CCPG1	ALU	Promoter/enhancer -(GH15J055401)	17	6	69339687-N/A	N/A	Intron 3 of AL391807.1 gene
	ESC neuron	17	72620205–72620217	CD300E	ALU	Promoter/enhancer -(GH17J074620)	13	4	53476270–5347089	181	Intron 5 of USP46 gene
	ESC neuron	6	2972589–2972629	SERPINB6	ALU	Enhancer – (GH06J002973 & GH06J002975)	41	15	75867323-N/A	N/A	Intron 1 of PTPN9 gene
F3-T1	ESC neuron	12	12933679–12933694	APOLD1	L1	Promoter/Enhancer—(GH12J012784)	16	2	4785584-N/A	N/A	Intergenic
	ESC neuron	14	65015015–65015055	PPP1R36	ALU	Promoter/Enhancer—(GH14J064549)	41	6	145918011–145918329	318	Intron 2 of EPM2A gene
	ESC neuron	6	20242969–20242982	MBOAT1	ALU	Enhancer -(GH06J020325 & GH06J020497)	14	2	134066290-N/A	N/A	Intron 3 of NCKAP5 gene

Table 5 (continued)

DZ twins	Tissue/cell sources	Chr	Coordinate (hg19)	Target gene	Type	Annotation-ID	IS (bp)	Source element		MES (bp)	Annotation
								Chr	Coordinate (hg19)		
F3-T2	ESC neuron	10	88513411–88513427	BMPR1A	ALU	Promoter/enhancer—(GH10J086752)	17	13	96633649-N/A	N/A	Intron 10 of UGGT2 gene
	ESC neuron	12	11179023–11179038	TAS2R19	ALU	Promoter/enhancer -(GH12J011169)	16	4	69960145-N/A	N/A	Intron 2 of UGT2B7 gene
	ESC neuron	14	32669217–32669233	RNU6-2	ALU	Promoter/enhancer—(GH14J032200)	17	4	86564402-N/A	N/A	Intron 2 of ARHGAP24 gene
	ESC neuron	4	76993809–76993824	ART3	ALU	Enhancer -GH04J076087	16	9	31353740-N/A	N/A	Intergenic
	ESC neuron	9	112261383–112261393	PTPN3	ALU	Promoter/enhancer—(GH09J109494)	11	6	142117356-N/A	N/A	Intron 3 of AK097143 gene

IS insertion size (base pair), *TES* transposable element size (base pair), *Chr* = chromosome number, *ESC* embryonic stem cell, *N/A* not applicable

ALU insertion originated from an intergenic region of chromosome 1. Details of these aforementioned TE variants can be found in Table 6, and all *dn*TEIs in each twin are available in *BED* format in Supplementary file 7.

De novo Variants Within Coding Regions

While we could not identify coding variants in DZ twins through DeepTrio, we were able to determine a few genes with exonic *dn*TEIs in each twin, except the F2-T1 twin. However, we focused on only two potential ASD-risk genes that were found to harbor coding insertions by both transposition callers. One of these genes, *RYR3*, is located in the autism susceptibility region (15q14-q15) and is a family of intercellular Ca2+ release channels that play a pivotal role in the regulation of intercellular Ca2+ homeostasis in neurons. *RYR3* had a 413 bp-long ALU insertion in its coding exon 78 that stemmed from an intergenic region of chromosome 8 in the F1-T1 child. *RYR3* was reported as an ASD-risk gene (Lu et al., 2012; Schmunk et al., 2015) and Nguyen et al. found missense variants in the coding exons of this gene in ASD probands (Nguyen et al., 2017). Another gene with exonic TE insertion in the same child was *IPO11*, which is also an ASD-risk gene (Kousoulidou et al., 2013). It mediates the nucleocytoplasmic transport of protein and RNA cargos and is involved in embryonic development. This gene had a 39 bp-long ALU insertion in its coding exon 27, originating from an intergenic region of chromosome 3.

Conclusion

TEIs have been associated with many complex disorders as well as Mendelian diseases, albeit at a much lower frequency compared to SNVs or CNVs. It is uncertain whether this seemingly low rate truly reflects the rarity of TEIs or is actually the result of the inability of conventional computational approaches to dissect RT events. Our findings suggest that the use of specialized pipelines, despite increased computational time and resources, could be justified by the increased detection of potentially disease-associated TEIs. Herein, we successfully detected ALU and L1 insertions into exonic and regulatory regions by using two specialized pipelines for TE transposition, Mobster and TranSurVeyor, in three quartet families, each with DZ twins. Whereas previous findings needed large cohorts to detect enrichment of TEIs at critical regulatory regions, we were able to do so in a much smaller group of three families with six patients. This study corroborates previous findings in the literature and further demonstrates the applicability of TEI analysis in ASD. Testing for TEIs should be considered as an option in clinical settings, in addition to the standard variant analysis, particularly in cases where a clear pathogenic variant cannot be identified.

Table 6 Shared de novo transposable element insertions and their sources among the dizygotic twin siblings

DZ twins	Chr	Coordinate (hg19)	Gene	Type	Annotation	IS (bp)	Source element			
							Chr	Coordinate (hg19)	TES (bp)	Annotation
F1-T1 & F1-T2	10	9510981–9510994	LOC101928272	ALU	Intergenic	14	2 & 4	130173255-N/A & 19643414-N/A	N/A & N/A	Intergenic & intron 2 of RP11608O21.1 gene
	10	21192817–21192834	NEBL	ALU	Intron 4	18	1 & 10	52384958-N/A & 49091938-N/A	N/A & N/A	Exon 5 of RAB3B gene & intron 1 of FAM35EP gene
	12	88338159–88338170	C12orf50	ALU	Intergenic	12	6 & 10	20936780–20936481 & 9258932–N/A	299 & N/A	Intron 9 of CDKAL1 gene & intron 4 of LOC101928272 gene
	17	72620206–72620217	CD300E	ALU	Promoter-TSS (GH17J074620)	12	6	168059236–N/A	N/A	Intergenic
	3	31881382–31881398	OSBPL10-AS1	ALU	Intron 3	17	8 & 2	13778387–N/A & 197614948–197614629	N/A & 319	Intergenic & intron 2 of CCDC150
	5	96866406–96866423	LINC01340	L1	Intron 2	18	16	83676916–N/A	N/A	Intron 9 of CDH13 gene
F2-T1 & F2-T2	13	73964677–73964695	LINC00392	ALU	Intergenic	19	12 & 9	96546762–N/A & 36423664-N/A	N/A & N/A	Intergenic & intron 2 of RNF38 gene
	6	120568246–120568262	MIR3144	ALU	Intergenic	17	9 & 20	110432891–N/A & 48772779–N/A	N/A & N/A	Intergenic & intergenic
	7	157448772–157448787	MIR153-2	ALU	intron 13	16	6	47726919–N/A	N/A	Intergenic
F3-T1 & F3-T2	12	106890946–106890961	POLR3B	ALU	Intron 25	16	15 & 13	43660748–43660441 31175835–31175600	307 & 235	Intron 2 of ZSCAN29 gene & intron 1 of HMGB1 gene
	13	59396879–59396895	LINC00374	ALU	Intergenic	17	12 & 3	85130776-N/A & 26734539–26734829	N/A & 290	Intergenic & intron 1 of LRRC3B gene
	2	220937913–220937928	MIR4268	ALU	Intergenic	16	5 & 1	11738208-N/A & 181829153–N/A	N/A & N/A	Intron 1 of CTNND2 gene & intergenic
	5	8749514–8749528	LINC02199	L1	Intergenic	15	1 & 8	56833520–N/A & 80083974-N/A	N/A & N/A	Intergenic & intergenic
	8	138295183–138295197	LOC101927915	ALU	Intergenic	15	7 & 7	128309532–N/A & 120881115–N/A	N/A & N/A	Intron 1 of RP11274B21.5 gene & intron 17 of CPED1 gene

IS insertion size (base pair), *Gene* te inserted or nearest gene, *TES* transposable element size (base pair), *Chr* chromosome number, *N/A* not applicable

However, standardized guidelines are lacking and interpretation of TEIs into non-coding regions and REs could be less straightforward compared to exonic variants. Incomplete annotation of non-coding regions makes it difficult to assign pathogenicity to individual TEIs. Nevertheless, with improved annotation, this shortcoming will be overcome in the coming years.

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Author contributions KO conceptualized and designed the study, analyzed and interpreted the results, drafted the initial manuscript, revised the manuscript, and approved the final manuscript as submitted. PUV collected data and acquired the financial support for the project, and approved the final manuscript as submitted. SM collected data, acquired the financial support for the project leading to this publication, and administered the project, and approved the final manuscript as submitted. AP analyzed and interpreted the results, reviewed manuscript, and approved the final manuscript as submitted. YO supervised the publication, conceptualized, and designed the study, interpreted the results, drafted the initial manuscript, revised the manuscript, and approved the final manuscript as submitted. GK supervised the publication, conceptualized and designed the study, analyzed and interpreted the results, drafted the initial manuscript, revised the manuscript, and approved the final manuscript as submitted. KO, GK, and YO had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest with the contents of this article.

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