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Molecular analysis of Candidate genes at the 22q region in Schizophrenia subjects

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22q11.2 deletion syndrome (22q11.2DS), also known as Velo-Cardio-Facial Syndrome (VCFS) or DiGeorge Syndrome, is a genetic disorder due to a micro deletion on chromosome 22q11.2. VCFS is associated with abnormalities in brain structure and with an increased risk of psychiatric disorders, particularly schizophrenia (SCZ). DNA copy number is a largely unexplored source of human genetic variation that may contribute risk for complex disease like SCZ. The aim of this study was to assess Copy number variations (CNV) at candidate genes located in 22q11 region in SCZ subjects. We report aberrations in copy number at PRODH and COMT gene loci supporting the hypothesis that dosage effects of 22q genes could lead to disruptions in neurotransmitter signaling and related neurobehavioral symptoms observed in SCZ subjects. The results support the hypothesis that the complex phenotype of 22qDS results either from the overlapping regulation of several genes within this region or from its concerted participation in a highly regulated process.

Keywords- Velo-Cardio-Facial Syndrome (VCFS), 22q deletion syndrome, Schizophrenia(SCZ), Copy number variations(CNV).

Velo-Cardio-Facial Syndrome (VCFS) is caused by a 3 Mbp hemizygous microdeletion at 22q11. VCFS patients show an unusually high rate of psychiatric disorders, particularly SCZ and bipolar disorder; in fact, VCFS and the 22q11 deletion represent the highest known risk factor for SCZ aside from having either parents or a monozygotic twin with the disease (Drew et al., 2010). Several genes located in the 22q11 chromosome regions have been studied and suggested as candidate genes for SCZ. A number of genes of the VCFS critical region on chromosome 22 are expressed in the developing and adult central nervous system, and potentially contribute to VCFS neuropsychiatric phenotypes (Maynard et al., 2003 and Arinami, 2006). Given the remarkably high incidence of comorbid diagnoses of VCFS and SCZ, these associations are important clues about both the genesis of SCZ symptoms in VCFS and the neurogenetic mechanism of non VCFS-related SCZ.

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It is proposed that the 22q11 VCFS deletion may predispose to psychotic disorders through haplo-insufficiency of critical developmental genes in this region, or perhaps through unmasking of deleterious polymorphisms in the intact copy. Recent reports on the association of copy number variations (i.e. deletions/insertions) with genetic susceptibility to autism (Sebat et al., 2007) and SCZ (Xu et al., 2010) have rekindled the interest in VCFS. The study of the molecular mechanisms causing the clinical manifestations of VCFS offers a unique opportunity to concentrate on a specific region in the genome that contains a limited number of genes, and to assess their relevance to chromosomal abnormalities as well as neuropsychiatric disorders. Several linkage studies highlight 22q12.3 as a region of interest for psychosis (Mowry et al., 2004; Fallin et al., 2005; Condra 2007). Further, meta-analyses point to chromosome 22q as harboring one or more genetic risk factors for SZC susceptibility (Badner and Gershon, 2002; Lewis et al., 2003). Also, meta-analysis of magnetic resonance imaging studies in chromosome 22q11.2 deletion syndrome supports that volumetric brain abnormalities in VCFS is associated with a greatly increased risk of psychosis and other psychiatric disorders (Tan 2009).

Copy number variations (CNVs) are deletions and duplications of DNA sequences that vary in length from few base pairs to several Mb. While these structural variations are often benign, they can disrupt vital biological functions and result in disease (Glessner et al., 2012). Studies *in vitro* and in engineered mice show that CNVs affect genes in the duplicated region and flanking gene stretching to several Mb. Several CNV induced mechanisms that include physical dissociation of the transcription unit from its cis-acting regulators, chromatin modification /positioning within the nucleus have been suggested to influence the expression of genes.

In the present study we carried out Copy number variation analysis of eight SCZ candidate region genes in the VCFS critical region viz., *COMT* (Catechol-Omethyl transferase), *PRODH1* (Proline Dehydro-genase (Oxidase)1, *ZDHHC8* (The zinc finger and DHHC domain-containing protein 8) and *CACNG2*(calcium channel, voltage-dependent, gamma subunit 2) and flanking genes in the distal breakpoint region viz., GGT2 (gamma-glutamyl transpeptidase 2). HIC2 (hypermethylated in cancer 2) and UBE2L3 ubiquitin-conjugating enzyme E2L 3.

The gene coding catechol-Omethyl transferase (*COMT*) encodes two distinct *COMT* isoenzymes; membrane- bound *COMT* (MB-*COMT*) and soluble *COMT* (S-*COMT*) (Bertocci et al., 1991). MB-*COMT* is primarily found in the brain. *COMT* is a plausible candidate susceptibility gene for SCZ given its positional association with 22q11.2DS and its functional role cortical synaptic dopamine catabolism (Williams et al., 2007).

The *ZDHHC8* gene, codes for proline dehydrogenase, a mitochondrial membrane enzyme that catalyzes the first step in the proline degradation pathway (Bender et al., 2005). Proline is a nonessential amino acid, which may have a role in brain function; as a modulator/ precursor of neuronal glutaminergic activity (Murphy and Scambler, 2005) and the regulation of cortical acetylcholinesterase function (Delwing et al., 2003). Gogos et al. (1999) found that mice, which were homozygous *ZDHHC8* mutants, had increased brain proline levels and had a significant decreased prepulse inhibition (PPI) of the startle reflex, suggestive of a defect in sensorimotor gating, an endo-phenotype of scz. In addition, mice with

deletions in the 22q11.2 region showed severe sensorimotor gating defects, learning, and memory impairment (Paylor et al., 2001).

The zinc finger and DHHC domain-containing protein 8 (*ZDHHC8*) encodes for a transmembrane palmitoyltransferase, which is required for protein palmitoylation. Palmitoylation is a posttranslational modification of proteins with the lipid palmitate, a process which is required for protein interactions, signaling, neural development, neurotransmitter receptor function, and synaptic transmission (El-Husseini and Bredt, 2002). *ZDHHC8* is expressed in the adult human brain (Mukai et al., 2004) and is found predominantly in the cortex and hippocampus of the mouse brain (Karayiorgou and Gogos, 2004). *ZDHHC8* has been proposed as a plausible candidate gene contributing to the behavioral phenotype of 22q11.2.2DS, given its functional role and its association with PPI (Karayiorgou and Gogos, 2004).Furthermore, *ZDHHC8* female knock-out mice showed significantly lower levels of PPI compared with wild types (Mukai et al., 2004). However, several studies have found no association with *ZDHHC8* variants and scz in the Japanese and European populations (Otani et al., 2005; Saito et al., 2005; Glaser et al., 2006b).

The *CACNG2* gene is the γ subunit of neuronal voltage activated L-type calcium channels, which might stabilize the calcium channel in an inactive state (Tomita et al., 2010). The *CACNG2* protein is similar to the mouse stargazin protein (Bedoukian et al., 2006), which is also implicated in _-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking.

The AMPA receptor mediates fast excitatory synaptic transmission in the brain and underlies aspects of synaptic plasticity (Yamasaki et al., 2011). Protein interaction networks suggests that the *CACNG2* mediated AMPA receptor recruiting may act in the glutamatergic signaling process(Hsu 2008).

Gamma-glutamyl transpeptidase 2 gene is involved in the degradation of glutathione. The encoded enzyme also acts as part of a GSH pumping gamma-glutamyl cycle in this tissue and may also be involved in gamma-glutamyl amino acid formation.
HIC2(hypermethylated in cancer 2) the protein encoded by the gene has a histone deacetylase activity.

UBE2L3 ubiquitin-conjugating enzyme E2L 3. The gene encodes a protein member of the E2 ubiquitin-conjugating enzyme family part of the cellular mechanism for targeting abnormal or short-lived proteins for degradation.

Paterlini et al. (2005) reported an up-regulation of *COMT* mRNA in the frontal cortex of *ZDHHC8* mutant mice. In addition, when tolcapone (a *COMT* inhibitor) was administered to the *ZDHHC8* mutants, there was a greater disruption in PPI and working memory compared with the non-treated *ZDHHC8* mutants suggesting a gene–gene interaction between *ZDHHC8* and *COMT*. This is consistent with the proposal that 22q DS is a contiguous gene syndrome, in which deficiency in more than one gene contributes to the increased risk.

Wilson et al. (2008) reported two patients with interstitial deletion of the 22q13 region with intact SHANK3 indicates that haplo in sufficiency for other 22q13 genes could also have major effects on cognitive and language development. Above studies are consisted with the hypothesis that the phenotypic expression in VCFS in terms of cognition and co-morbidity can be affected by each gene alone and by interaction between genes that affect the same pathway (e.g., dopaminergic, glutamatergic). Cumulatively, these observations suggest the possible interaction of genes at the 22q locus and their role in the observed neuropsychiatric phenotype in VCFS.

Further, Boolean network model of the biochemical pathway (neuro-

transmitters) shows that, deletion/ knockout of certain biologically important nodes cause significant perturbation in networks (Gupta et al., 2007)

The molecular nature of the rearrangements responsible for 22q11 microdeletions are related to the genomic structure which contains long stretches of repeated sequences clustered together, known as low copy repeats (LCRs) (Molina et al., 2011). LCRs contain highly repetitive elements such as short interspersed nuclear elements (SINEs) and long interspersed nuclear elements (LINEs). These elements, particularly SINEs have been implicated in chromosome rearrangements and disease. Alu elements, part of the SINE family of transposable elements, have also been established as having a role in modulating the architecture of the human genome in association with human disorders and in mediating gene rearrangements has been established (Batzer 2002). Further, Low-copy repeats (LCRs) on 22q11 have been suggested to mediate non-allelic homologous recombination (NAHR), resulting in rearrangements of 22q (Shaikh 2000).

We have previously reported breakpoint associated with a novel 2.3 Mb deletion in the VCFS region of 22q11 and the role of *Alu* (SINE) in recurring microdeletions (Uddin 2006). Interestingly, not all LCRs appear to equally effect in causing microdeletions and they differ with respect to some of their sequences. Region-specific LCRs, repeat sequences, are susceptible to such genomic rearrangements resulting in CNVs. Factors such as size, orientation, percentage similarity and the distance between the copies renders them susceptible (Lee 2006). CNVs can be limited to a single gene or include a contiguous set of genes. This observation necessitates the assessment of the nature of individual LCRs elements around the candidate gene/s. CNVs can result in either too many or too few of the dosage sensitive genes, which may be responsible for a substantial amount of human phenotypic variability, complex behavioral traits, and disease susceptibility (Redon 2006).

In the present study we report CNV analysis of SCZ candidate genes in the VCFS critical region involved in dopaminergic and glutamatergic neurotransmission. Further we characterized genomic sequences flanking the candidate genes to assess the nature of CNV formation.

2. Materials and methods

2.1 Ethical approval and Recruitment

The study was conducted following approval by *The* University *of* Western Ontario Committee on Research Involving Human Subjects and all participating members provided informed consent for this research. The patients were identified and recruited by Dr. Richard O'Reilly and Dr. Jay Rao Psychiatrists. Clinical assessment was carried out using APA Diagnostic and Statistical Manual (DSM-IV) and their medical records.

DNA extraction from whole blood of study cohort was carried out using perfect pure DNA blood kit (5prime.com)following manufacturers protocol and DNA quantified using a spectrophotometer using the 260/280 nm ratio.

2.2 CNV analysis

CNV analysis was carried out using RealTime PCR with an internal control (RNASEP gene) using TaqMan detection chemistry and the ABI Prism 7300 Sequence Detection System (Applied Biosystems). Individual CNV PCRs of 18 µl were set up in triplicates in 48 well plates containing 20 ng genomic DNA (2 µl), 2× TaqMan® Genotyping Master Mix(10 µl), TaqMan® Copy Number Assay, 20× working Stock 1µl, TaqMan® Copy Number Reference Assay 1µl, 20×, Nuclease-free water 4 µl. Cycling conditions Hold 95^{o}C 10 min,

Cycle(40 Cycles) 95 °C 15 sec, 60 °C 60 sec. The copy number of the test locus in each case was defined as 2T-ΔΔC where ΔCT is the difference in threshold cycle number for the test and reference loci.

2.3 Bioinformatics

Further, to identify putative repeat elements in the flanking regions of CNVs the sequence features around the CNV, which could promote break points, deletion and duplication events was carried out using the http://genome.ucsc.edu/ (Build 37.1) and repeat masker http://www.repeatmasker.org/

3. Results

The physical map of the 22Q region and genes under study are represented in figure-1.The results of the Copy number analysis are summarized in the table-1a.

Table-1a Copy number analysis of candidate genes of 22q candidate genes in SCZ subjects

Sl.No	Gene	Copy Number (Loss/Gain) Cases(n=39)				
		Zero copy	One copy	Two copy	Three Copy	>3copy
1	CACNG2	0	0	29	0	0
2	**PRODH**	**0**	**16**	**16**	**0**	**0**
3	**COMT**	**0**	**6**	**33**	**0**	**0**
4	**UBE2L3**	**0**	**1**	**32**	**0**	**0**
5	**HIC2**	**0**	**1**	**32**	**0**	**0**
6	GGTL4	0	0	33	0	0
7	ACTBL2	0	0	33	0	0
8	**ZDHH4**	**0**	**5**	**32**	**0**	**0**
	Gene	Copy Number (Loss/Gain) Controls(n=96)				
		Zero copy	One copy	Two copy	Three Copy	>3copy
1	CACNG2	0	1	29	1	0
2	PRODH	3	29	62	2	0
3	COMT	0	0	93	3	0
4	UBE2L3	0	0	33	0	0
5	HIC2	0	0	31	0	0
6	GGTL4	0	0	30	0	0
7	ACTBL2	0	0	31	0	0
8	ZDHH4	0	3	92	1	0

Note-All samples have not worked for all genes. Bolded cells/genes indicate genes in Loss

We found deletions at PRODH, COMT and ZDHHC8 genes. SNP analysis of PRODH gene is summarized in table 1b.

Analysis of the repeat elements abutting the candidate genes suggests a constellation of repeats including the Short interspersed nuclear elements (SINE), which include Alu, Long terminal repeat elements (LTR), and L1 retrotransposons and repetitive DNA/RNA elements.

The instability of 22q11 has been demonstrated by the high frequency of pathological rearrangements of this region. There is overwhelming evidence that hemizygosity of a large region on 22q11.2 greatly increases the risk of SCZ and there is also evidence that VCFS is over represented in largely unselected SCZ populations. As hemizygosity changes the dosage of the genes in the region, one hypothesis is that this factor is related to susceptibility to SCZ.

In the absence of hemizygosity, either a null mutation or a sequence variant that lowers transcriptional activity would have similar effects.

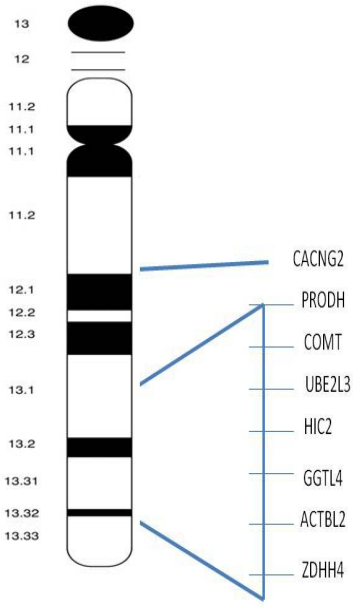

Figure 1. Candidate genes in the 22q region genotyped for copy number variation (CNV)

Table-1b SNP analysis of PRODH candidate genes in SCZ subjects

SNP	Contig Position on ENSEMBL/Mutation	Patients (n=10)	Controls (n=6)
1	2053870/E428G	0	1
2	2053864/A430V	1	0
3	2053858/R432H	0	1
4	2053861/R431H/ rs2904552	3	1
5	2053739/A472T/ rs2870983	1	0

PRODH, and *ZDHHC8* genes in the 1.5 Mb region of chromosome 22 encode mitochondrial proteins. They are expressed in the brain, and maximal expression coincides with peak forebrain synaptogenesis shortly after birth. Furthermore, their protein products are associated with brain mitochondria, including those in synaptic terminals. Distinct expression patterns and dynamic expression levels of these genes in the developing and adult brain suggest they contribute to construction and maintenance of neural circuits across synapses.

Diminished dosage of the genes deleted in the 1.5-megabase 22q11 minimal critical deleted region in a mouse model of 22q11DS demonstrate aberrations in neurogenesis and subsequent differentiation in the cerebral cortex. Such developmental disruption may alter cortical circuitry and establish vulnerability for developmental disorders, including SCZ and autism (Meechan et al., 2009). Further it is documented that CNVs can also result in a position effect (Kleinjan and Heyningen 2005). Bottom-up approaches to define gene–brain relationship investigating the relationship between genes and the neural substrates have provided essential insight into the pathophysiology of mental disorders in VCFS. Dosage-dependent Copy-number gain resulted in a discrete clinical phenotype of MECP2 duplication syndrome in an individual gene present in the larger genomic aberration (Vandewalle et al., 2009). As evidenced from table-1a, loss of CNV was observed at several genes at the 22q locus, however; the CNV copy number varied for each gene.

We found high preponderance of Alu family of repetitive DNA viz., AluY, Alujo, Alujr, Alujb, Alusc, Alujy around the candidate genes. The presence of Alu repeats suggests proclivity for secondary-structure formation and serve as hotspots for chromosomal rearrangement.

Alu elements, part of the SINE family of transposable elements have been shown to modulate the architecture of the human genome, gene rearrangements and associated with human disorders (Kolomietz 2002). We

find evidence for both, microhomology based nonallelic homologous recombination (NAHR) and microhomology mediated break induced replication (MMBIR) and fork stalling and template switching (FoSTeS) mechanisms for CNV loss. Different class of repeats viz., the endogenous retroviruses (*HERV-K* (also known as *ERVK*), L1 retrotransposons and family of low complexity repeats (GC, AT rich) were also enriched flanking the candidate genes. Repetitive elements DNA-(MER1B, hAT-Charlie) repeat-a class of transposable elements (TEs) which are interspersed repetitive DNA families and RNA (U3 family) repeats were found flanking the *ZDHHC8* gene providing further credence to the hypothesis that these repeats could predispose genomes to CNV through replication slippage or unequal crossing-over.

Functional analysis of polymorphisms in the promoter regions of genes on 22q11 suggests that the *ZDHHC8* gene showed activity differences between haplotypes of greater than 1.5-fold.This implies that structural variations that modulate the rate of transcription of a gene may be found in the upstream of the gene (Hoogendoorn et al., 2004).

Discussion

The 22q region continues to attract interest in schizophrenia research by virtue of its involvement in common micro deletions (e.g. deletion in 22q11), presence of unusual repeats, and as a potential site responsible for a number of clinically overlapping phenotypes. Our results are consistent with the hypothesis that genetic contribution from more than one gene at 22q11 region increase the risk for SCZ. Genomic lesions associated with significant risk for SCZ and other behavioral disorders have been shown to map to specific chromosomal segments (Girirajan et al., 2010). Recent CNV data (Babatz 2009 and data from our lab corroborate this observation (Maiti et al. 2011). Our study has a few limitations as we have done this analysis on a small cohort of patients. Further replication in a larger cohort is required to confirm the results. Also given RealTime-PCR has not worked for all samples is a reflective of the sensitivity of the methodology.

Further scrutiny of the aberrations and their functional consequences including the role of miRNA will lead to a better understanding of the etiology of psychiatric diseases in VCFS syndrome. Future, functional studies assessing the stoichiometry of gene products resulting from the gene imbalance would enable functional elucidation of the role of genes in SCZ.

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