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Genome-wide CNV analysis uncovers novel pathogenic regions in cohort of five multiplex families with neurodevelopmental disorders

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A B S T R A C T

Structural reorganization of chromosomes by genomic duplications and/or deletions are known as copy number variations (CNVs). Pathogenic and disease susceptible CNVs alter gene dosage and its phenotypic expression that often leads to human genetic diseases including Neurological disorders. CNVs affecting same common genes in multiple neurodevelopmental disorders can better explain the shared clinical and genetic aetiology across brain diseases. Our study presents the novel copy number variations in a cohort of five multiplex consanguineous families with intellectual disability, microcephaly, ASD, epilepsy, and neurological syndromic features. Cytoscan HD microarray suite has revealed genome wide deletions, duplications and LOH regions which are co-segregating in the family members for the rare neurodevelopmental syndromic phenotypes. This study identifies 1q21.1 microduplication, 16p11.2 microduplication, Xp11.22 microduplication, 4p12 microdeletion and Xq21.1 microdeletion that significantly contribute to primary disease onset and its progression for the first time in Pakistani families. Our study has potential impact on the understanding of pathogenic genetic predisposition for appearance of complex and heterogeneous neurodevelopmental disorders with otherwise unexplained syndromic features. Identification of altered gene dosage across the genome is helpful in improved diagnosis, better disease management in day-to-day life activities of patients with cognitive impairment and genetic counselling of families where consanguinity is a tradition. Our study will contribute to expand the knowledge of genotype-phenotype expression and future gateways in therapeutics and precision medicine research will be open in Pakistan.

1. Introduction

Rare copy number variations (CNVs) have been reported in intellectual disability (ID), congenital microcephaly, autism spectrum disorder (ASD) and epilepsy [1]. In 2004, two separate teams, Iafrate and Sebat characterised for the first time the genome-wide presence of significant copy number modifications in the human genome [2]. Many CNVs have a minor allele frequency (MAF) greater than 5% and no discernible effect on morphology. Nonetheless, several infrequent CNVs with MAF 1% have been linked to clinical characteristics and a few of them have demonstrated diagnostic value according to their size, gene composition, or association with genes that express haploinsufficiency when CNV is CN = 1 or triplosensitivity when CNV is CN > 2 [3]. Genomic duplications, deletions or inversions range in size from several kb to Mb and even smaller [4]. CNVs occupy 4.8–9.5% of the human DNA according to publicly accessible CNV databases [5]. CNVs play key role in human genomic structure, diversity as well as in pathogenicity of various neurodevelopmental disorders, neuropsychiatric conditions, autoimmune diseases, and cancer [6]. Several shared pathogenic

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CNVs in candidate genes are identified in these diseases, interestingly. Various studies highlight the impact of chromosomal rearrangements on structural complexity and disease susceptibility of human genome [7]. Previous studies on CNV pathogenesis suggest that at some level of physiological pathway, from genes to neural circuits to the phenotypic expression, they have similar mechanisms [8]. Existing investigations, however, have mostly focused on small clinical cohorts that included a single CNV locus. For instance, it is still unclear how several CNVs increase susceptibility to the same psychiatric and developmental problems [9].

Unique CNVs affect cognitive and behavioural aspects of neurodevelopmental and neuropsychiatric disorders and increase

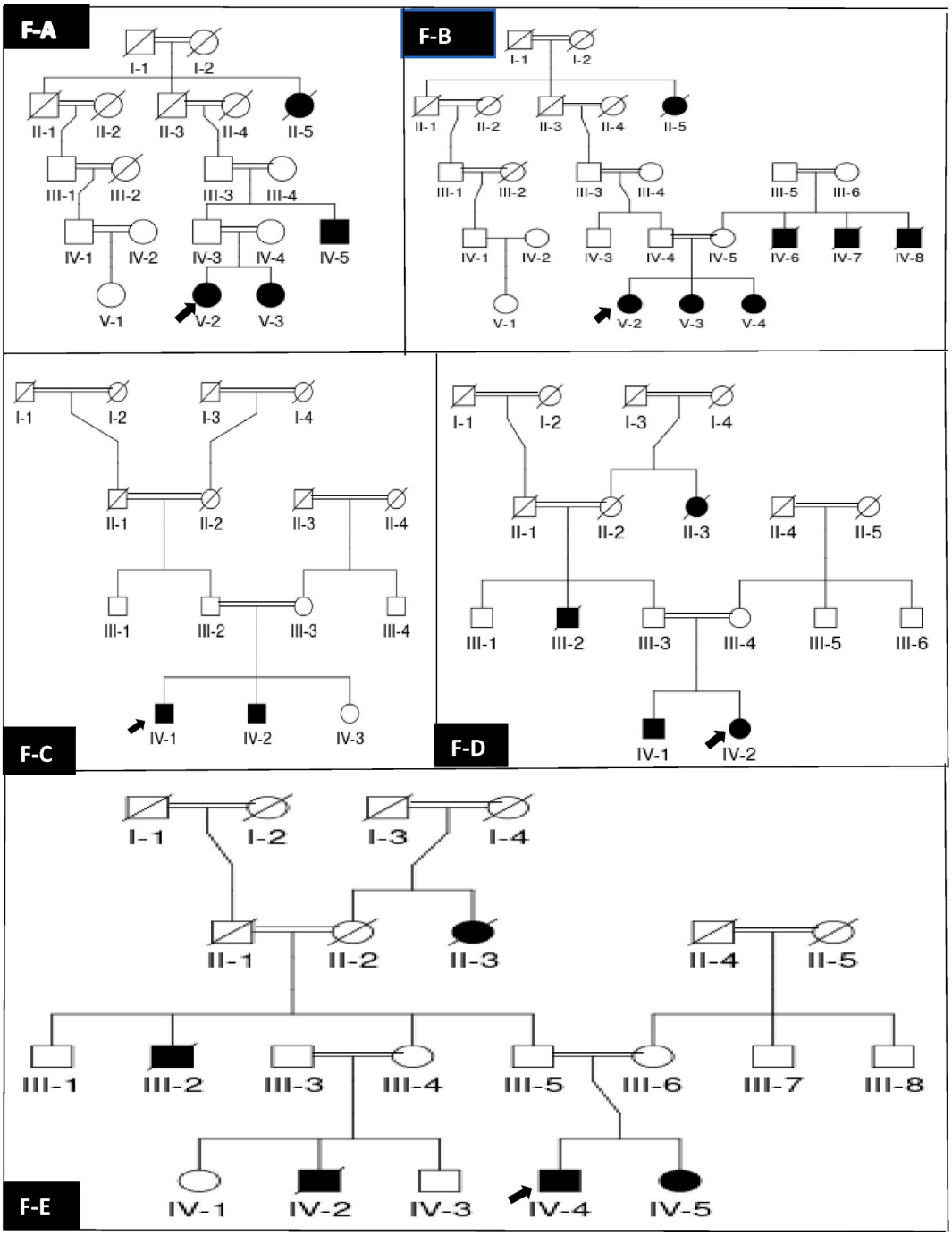

Fig. 1. Cohort of five unrelated families showing Autosomal Recessive Inheritance of Neurodevelopmental Disorders. Boxes and Circles represent males and females respectively with arrow pointed towards proband, filled shapes are affected members, diagonal lines represent deceased members, double marriage lines are consanguineous marriages.

susceptibility for ID, ASD, schizophrenia, bipolar disorder, post-traumatic stress disorder and Depression [10]. Despite of having basic behavioural similarities, people who satisfy the criteria for a clinical assessment might also have a variety of functional restrictions, behavioural disturbances, and associated complications. This high level of clinical heterogeneity is related, in part, to the various phenotypic effects of the several known and unidentified genetic aetiologies for NDDs [11]. It has been widely accepted that individual variability in hereditary diseases is significantly influenced by genetic history. A variety of familial disorders have now been associated to probands and their closest relatives [12]. Moreover, carriers of neurological CNVs may only have clinical manifestations and show partial penetrance, that means it may be linked to differential expressivity, whereby identical CNVs are correlated to a multitude of symptoms with varying severity levels, implying amazingly complex molecular processes underlying disease risk in CNV carriers [13].

Chromosomes 1,4,16, and X contain numerous hotspots with rare and heterogenetic neurological CNVs of variable expression and penetrance in syndromic phenotypes. According to literature review 1q21.1 duplication syndrome is expressed as dysmorphic faces, developmental delay, cardiac defects, and ASD [14]. It is established by embryonic and molecular studies in animal models that 1q21 duplication results in altered calcium channel expression, neuron proliferation and function which makes it interesting region for therapeutics [15]. Recurrent deletions and duplications of GABA-receptor genes at 4p12 region are associated with various neurological phenotypes as reported previously [16]. 4p12 duplications involving GABAR genes are rarely reported phenotypes and are of great interest for the molecular and physiological research [17]. Previously 4p deletions were associated with Wolf-Hirschhorn syndrome which is a rare disorder. Neuronal pathological and functional manifestations at GABAR genes cluster make it the susceptible region for neurodevelopmental disorders phenotypic expression [18].

Human 16p11 genetic locus was reported as susceptible region for neurodevelopmental and neuropsychiatric disorders phenotypes [19]. Gene dosage alterations in this region are associated with shared and unique NDDs with more than twenty disorders reported according to DSM diagnosis [20]. 16p11.2 deletions and duplications are frequently reported for prenatal and postnatal diagnosis of patients with neurological syndromes [21]. Xq21 microdeletions and duplications are currently in lime light due to its unique penetrance mechanism in neurodevelopmental disorders like Prader-Willi syndrome [22]. Deletions at Xq21 locus explains neurodevelopmental malformations due to decrease in dosage of genes involving rare hereditary and congenital brain disorders [23]. Similarly, Xp11 locus deletions and duplications show variable expression in neurodevelopmental syndromic and non-syndromic phenotypes [24].

We present a genome wide copy number variation analysis for novel, pathogenic microdeletions and microduplications <5 Mb in size and associated with neurodevelopmental disorders phenotypes in a cohort of five consanguineous families from Islamabad, Pakistan for the first time. Our study identified three gain-of-function gene variants 1q21.1 microduplication, 16p11.2 microduplication, Xp11.22 microduplication and two loss-of-function gene alterations 4p12 microdeletion and Xq21.1 microdeletion in the five probands by Cytoscan HD microarray genetic detection which co-segregate in the affected siblings and are either paternal or maternal in origin.

Present study has strong impact on the research of human chromosomes 1, 4, 16 and X with novel structural alterations that generally are, unnoticed in resource poor societies due to scarce knowledge of genetic predisposition in aetiology of diseases but inherit in families due to consanguinity. This study will lay the foundations for proper diagnosis in patients with neurological syndromes which face partial clinical assessment and treatment. It will contribute to knowledge expansion of NDDs genetics and furnish future opportunities for strong therapeutic interventions to cure phenotypic malformations.

2. Materials and methods

2.1. Recruitment of families, cohort description and pedigree analyses

We present a cohort of five unrelated consanguineous families F-A, F–B, F–C, F-D, and F-E (Fig. 1). who were referred to Department of Neurology, Pakistan Institute of Medical Sciences, Islamabad (recruited between June 2021 and December 2021). The phenotypic features, diagnosis, sibling analysis, male to female ratio of affected and unaffected family members were recorded **(**Table 1**).**

Family A: Proband was a 7-year-old girl (V-2) with primary microcephaly, intellectual disability, short stature, absence of speech and drooling. Her 9-year-old sister (V-3) was affected with microcephaly, intellectual disability, pointed chin, wide nasal area, and eyes with skin folds in the corners. No prenatal complication or medication was reported by the mother. Pedigree analysis (Fig. 1-A) was performed for last five generations which reported autosomal recessive inheritance and significant family history (II-5; IV-5) of

Table 1
Cohort description with clinical assessment of families.

Family ID	Affected Members		Unaffected Members		Clinical Diagnosis	Phenotype Analysis		Siblings Analysis	
	Male	Female	Male	Female		Syndromic NDDs	Other NDDs	Affected	Unaffected
F-A	1	3	7	8	IDM	Syndromic	EPI, MC, DD	1	0
F–B	3	4	9	9	ID	Syndromic	ASD, EPI, DD	2	0
F–C	2	0	7	6	ID	Syndromic	ASD, DD, speech delay	1	1
F-D	2	2	8	5	ID	Syndromic	ASD, Speech delay	1	0
F-E	3	2	10	7	ID	Syndromic	ID, DD	1	0
Total (%)	11(50%)	11(50%)	41(54%)	35(46%)				6 (86%)	1(14%)

NDDs. Proband and her affected sibling were only children to their parents who were diagnosed with NDDs at the age of 6 months and 5 months respectively.

Family B: Proband was 23-year-old female (V-2) with short stature, obesity, intellectual disability, speech delay, behavioural problems, mood disorder, epilepsy, insomnia, and infertility due to absence of menstrual cycle. Her two sisters, 13-year-old (V-3) and 8-years-old (V-4) were also suffering from intellectual disability and ASD respectively. Family (Fig. 1-B) has a positive family history of abnormal prenatal development of brain and postnatal epilepsy and intellectual disability in three maternal uncles (IV-6, IV-7, IV-8). One still birth was also reported in the family due to abnormal neurodevelopment. Five generation pedigree was analyzed to interpret autosomal recessive mode of inheritance.

Family C: Proband was 10-year-old boy (IV-1) with intellectual disability, dysmorphic facial features, tooth anomaly, low set ears, deep set eyes and repetitive behaviour. His brother, 6 year 4 months old (IV-2) was diagnosed with recurrent seizures, developmental delay, intellectual disability, and bilateral cataract. Both affected children were born to normal parents who had a first cousin marriage. Their mother reported a full-term normal pregnancy and delivery. Family lacks a positive history of NDDs in previous three generations. Pedigree analysis has shown the autosomal recessive inheritance of NDDs in the family (Fig. 1-C).

Family D: Proband was a 6-year-old girl (IV-2) diagnosed with microcephaly, intellectual disability, and seizures was born to normal couple who had a consanguineous marriage. Her sibling was 9 year 3 months old boy (IV-1) with intellectual disability, microcephaly, cataract, speech delay and short stature. During first year of post-natal development the neurological symptoms of proband and her brother appeared and diagnosed as syndromic intellectual disability. Pedigree analysis of the family for four generations showed autosomal recessive pattern of inheritance (Fig. 1-D) with positive family history (II-3; III-2) of NDDs in two consecutive generations.

Family E: Proband was 13-year-old boy (IV-4) with craniofacial dysmorphism, hypodontia, frontal bossing, short stature, bilateral cataract, and microcephaly. His sister was 8- year-old girl (IV-5) with intellectual disability, frontal bossing with wide eyes, speech delay and upper limb paralysis. They were born to normal parents with first cousin marriage. Pedigree of the family was kept for four generations (Fig. 1-E) which described positive history of intellectual disability, epilepsy, and autism spectrum disorder in previous three generations (III-2; IV-2) with autosomal recessive mode of inheritance.

2.2. Diagnosis, ethical approval, and consent of participants

Recruited families were diagnosed by consultant neurologists at Pakistan Institute of Medical Sciences, Islamabad. Informed consent of participants was taken in writing from their biological parents who were counselled to carry out genetic testing by chromosomal microarray Cytoscan HD which is a first-tier genetic diagnosis for NDDs [25]. This study was approved by ethical review board of Biosciences Department, COMSATS University, Islamabad wide notification Notif-1616/18/2/27 (June 5, 2018).

2.3. Sample collection, DNA extraction and cytoscan HD microarray experiment

2 ml peripheral blood samples were collected from proband, their parents and other affected members of family and processed for gDNA extraction by phenol-chloroform method in Translational Genomics Lab, COMSATS university, Islamabad, Pakistan. Extracted gDNA was processed for chromosomal microarray Cytoscan HD (Thermofischer sc., USA) testing at Institute of Biomedical and Genetic Engineering Division, KRL Hospital, Islamabad under the project of PCSIR developmental programme of data repository and scientific instrumentation funding (Accessed during April, 2022 to July 2022). Genome wide array data was generated by Cytoscan HD suite which is the most advanced hybrid SNP-array with 2.9 million copy number markers, 750,000 SNP probes and 1.9 million non-polymorphic biomarkers covering all OMIM and GRCh38/hg38 ref seq genes for parent-of-origin in clinical research and diagnosis. . CEL files were uploaded and converted to CYCHP files for analyses by ChASv4.2 software. Copy number loss and gain, LOH and mosaicism across whole genome were identified using HMM and BRLMM algorithms with a threshold of log2ratio (log2sample-log2reference) for novel and known pathogenic CNVs less than 5 Mb in the entire genome of probands, their affected siblings and parents. (Accessed during July 2022 to December 2022). Frequency of CNVs was determined in the data by direct count method. Every CNV was viewed in the ChASv4.2 and verified, then the filtered CNVs were validated through qPCR. Clinical significance was assessed by the databases UCSC, ClinVar, DGV, DECIPHER, OMIM, and PubMed according to ACMG criteria. ChAS v 4.2 has the provision of integrated databases ClinGen, Decipher, UCSC, ClinVar, Toronto DGV, Ensembl, and OMIM. Furthermore, segment filters for annotation types and properties, graphs for annotations, Whole genome view of CHP files, and ChAS DB information is available which aided in filtration of clinically pathogenic CNVs in the probands. (Assessed during Jan 2023 to Feb 2023).

2.4. Molecular analyses of data and identification of pathogenic regions

Rare copy number variants associated with neurologic phenotypes were filtered and prioritized by inhouse pipeline as pathogenic, likely pathogenic, uncertain, and benign according to ACMG guidelines (Fig. 3). In this cohort, no overlapping copy number variations with the same sizes and markers were discovered in the DGV Gold Standard variations database for the same phenotypes. Furthermore, using the identical break points, no disease-causing variant in HGMD or pathogenic/likely pathogenic variant in ClinVar was discovered which has concluded these CNVs as novel findings in Pakistani population. Additionally, these CNVs have been reported as clinically relevant in many peer-reviewed articles in other nations around the globe, even though the CNV's penetrance and expressivity are known to be varied in individual population. The American college of Medical Genetics and Genomics, the international Collaboration of Clinical Genomics, and the American Academy of Neurology, recommended CMA as first-line test in patients with

unexplained DD/ID/ASD and other Neurodevelopmental disorders. In this study we present only the pathogenic CNVs that segregate in the family and explain the complex phenotypic expression in probands. Results were recorded for reporting all the probands, affected siblings and parents in cohort of five unrelated families [3]. Total duplications, deletions and loss of heterozygosity regions were also documented to present the CNV burden in the cohort of neurodevelopmental disorder families. Similarly, CNV genomic attributes, copy number state and genes involved as identified by ChASv4.2software were pinned down. Mode of inheritance was identified as autosomal recessive with either paternal or maternal origin after pedigree analysis up to previous five generations due to positive family history of NDDs in either father or mother family even though both parents were normal phenotypically.

2.5. qPCR validation of CNVs in the cohort

Co-segregation and validation of CNVs in the family members was performed by quantitative real-time PCR, at IBGE lab, KRL Hospital, Islamabad. (Accessed during January 2023 to February 2023). Qiagen's real-time PCR cycler, the Rotor-Gene Q and optimized QIAGEN kits with manufacturer's protocol were used to perform pathogenic variants detection. Rotor-Gene Software was used for variant screening, data analysis and interpretation. (Accessed during February 2023) For each proband, results of qPCR validated CN variants segregating in the affected siblings and parents were obtained.

3. Results

We present a cohort of five unrelated multiplex families with syndromic autosomal recessive shared molecular transition of intellectual disability, ASD, epilepsy, and microcephaly as primary diagnosis among affected probands and their siblings. Associated syndromic characteristics in various affected members of the cohort were facial dysmorphism with short stature, deafness, delay and absence of speech, infertility, dental anomalies, bilateral cataract, cardiac manifestations, repetitive behaviour, and insomnia. Male to female ratio in affected (50%) and unaffected members (female 46% and male 54%) does not vary with either sex in the cohort which interprets lack of any association between syndromic features and the gender. Clinical diagnosis has shown that despite shared phenotypes of variety of neurodevelopmental disorders, all the affected members suffer from intellectual disability and syndromic characters that vary in severity. Sibling analysis results for number of affected and unaffected siblings were interesting since probands with affected siblings were higher (84%) than those with unaffected siblings (16%) (Table 1).

3.1. Clinical diagnosis, genetic testing, co-segregation analysis and parent-of-origin assessment of the cohort

All the five families visited Department of neurology, PIMS Hospital, Islamabad from time to time for diagnosis of the rare neurological syndromic features of affected members. Age group of the affected members ranged from 6 years to 40years with congenital onset of the neurodevelopmental disorders. Clinical assessment was carried out by consultant neurologists as partial diagnosis and families were suggested to have Cytoscan HD microarray testing and CNV analysis for genetic and molecular diagnosis. The Cytoscan HD array results revealed total CNV burden in chromosomes and suggested that number of loss-of-function mutations due to deletions is higher (84%) than gain-of-function mutations due to duplications (10%) in the cohort which depicts that either fragmented or numerical, the deletions added more to the phenotype than do the duplications in our cohort **(Fig. 4, A-E)**. CNV genes overblown by these functional losses or gains are mostly expressed as an upset of neurologic, physiologic, and pathologic mechanisms

Table 2
CNV burden analysis with total number of deletions, duplications, and Loss-of-heterozygosity regions in genome of affected family members.

Family ID	Affected Member	Chromosome#	Deletions(n)	Duplications(n)	LOH(n)	Novel CNVs
F-A	Proband, IV-5, V-3	1,3,6,8,9,10	280	20	10	16p11.2 Microduplication
		11,15,16,18,20	350	42	8	
		21,22	10	2	1	
		X	19	1	10	
F–B	Proband, V-3, V-4	1.4,6,7,9,10	300	24	6	Xp11.22-q11.1
		11,12,14,15,19,20	270	29	12	Microduplication
		21,22	12	3	1	
		X	5	3	2	
F–C	Proband, IV-2	1,6,7,8,9	220	20	10	1q21.1-1q21.2
		11,15,16,17,20	15	17	4	Microduplication
		21,22	2	0	1	
		X	10	1	2	
F-D	Proband, IV-1	1,4,5,6,8,9,10	240	30	16	4p12-4p12 Microdeletion
		12,14,17,18,19,20	140	12	20	
		21,22	3	0	4	
		X	0	2	2	
F-E	Proband, IV-5	1,3,4,5,7,8,9	31	12	10	Xq21.1-Xq21.2
		11,12,14,16,17,20	13	11	18	Microdeletion
		21,22	4	0	2	
		X	5	2	4	
Total (%)			**1929 (84%)**	**231(10%)**	**143(6%)**	

Table 3
Analysis of CNVs by Clinical significance, phenotype-genotype characteristics, and mode of origin in cohort of five families.

Family ID	Affected member ID	Age	Onset	Phenotype	Cytoregion	CNV type (Size)	Genes	Significant Family History	Clinical significance	Detection Approach
F-A	Proband	7yrs	Congenital	ID, MC syndrome	16p11.2	Microduplication (0.99 Mb)	*ZNF267, TP53TG3* and 10 more genes	Paternal	Pathogenic	Cytoscan HD array
	V-3	9yrs							Co-segregation in family	qPCR
	IV-5	40yrs								
F–B	Proband	23yrs	Congenital	NDD syndrome	Xp11.22-q11.1	Microduplication (4.26 Mb)	*ARHGEF9, MTMR8, ZC4H2* and 6 more genes	Maternal	Pathogenic	Cytoscan HD array
	V-3	13yrs		ID					Co-segregation in family	qPCR
	V-4	8yrs		ASD						
F–C	Proband	10yrs	Congenital	ID, ASD, EPI, DD	1q21.1-1q21.2	Microduplication (1.34 Mb)	*SRGAP2B, FAM72D, NBPF, GPR89A* and 28 more genes	Maternal	Pathogenic	Cytoscan HD array
	IV-2	6.4yrs		ID, EPI, DD					Co-segregation in family	qPCR
F-D	Proband	6yrs	Congenital	ID, MC, Speech Delay	4p12	Microdeletion (1.43 Mb)	*GABRG1, COX7B2, GABRA2, GABRA4, GABRB1*	Paternal	Pathogenic	Cytoscan HD array
	IV-1	9.3yrs		ID syndrome					Co-segregation in family	qPCR array
F-E	Proband	14yrs	Congenital	ID syndrome	Xq21.1-Xq21.2	Microdeletion (2.89 Mb)	*HDX, APOOL, ZNF711, POF1B, CHM* and 8 more genes	Paternal	Pathogenic	Cytoscan HD array
	IV-5	8yrs		ID syndrome					Co-segregation in family	qPCR

which explains the origin of the disease phenotypes in the probands (Table 2). Genes involved mainly in neurologic expression of microduplications as indicated by Cytoscan HD array data were *ZNF267, TP53TG3 ARHGEF9, MTMR8, ZC4H2 HDX, APOOL, ZNF711, POF1B, CHM, SRGAP2B, FAM72D, NBPF,* and those for microdeletions were *GPR89A GABRG1, COX7B2, GABRA2, GABRA4, GABRB1* along with 52 more genes altered by these CNVs. After analysis of the Cytoscan HD array CNV data by ChASv4.2, we present five novel chromosomal microduplications and microdeletions that co-segregate in family as validated by qPCR analysis**.** Pedigree analysis depicted that three families have paternal significant family history of the NDDs while in two families there was maternal positive family history of NDDs in the cohort. Hence, here we can suspect the probable paternal or maternal origin of the neurological phenotypes of the probands. Autosomal Recessive CNVs can be expressed as a syndromic phenotype in multiple ways. Cytoscan HD array combines CN and SNP probes and has the added benefit of identifying long adjacent runs of homozygosity referred to as (LOH), which may indicate uniparental disomy (UPD) if limited to a just one chromosome, or identical by descent (IBD) that matches consanguinity if involves more than one chromosome. In case of isodisomy, two similar segments from one parent homologue exist, but in heterodisomy, fragments from both parental homologues are included. Both comparable parts of the isodisomy may have an autosomal-recessives mutation acquired from a carrier parent, resulting in a recessive hereditary neurological disorder in the proband. Consanguinity results in identical by descent (IBD) chromosomal segments, and the quantity and dimensions of LOH regions correspond with the degree of relatedness. A higher percentage of IBD matches with a first- or second-degree parental connection. In addition, when autosomal recessive illnesses are suspected, the examination of homozygous areas might be particularly valuable for identifying potential genes. As a result, combining LOH information with that supplied by CN state on the same device enhances the diagnostic output from array testing. There was a considerable variation in CNV size as it ranged from 0.99 Mb to 4.26 Mb in size in the cohort. All these genomic alterations were co-segregated in affected siblings which has confirmed the genetic legacy of the disease transition in the families of the cohort under study (Table 3). Genomic and chromosomal attributes of identified loss and gain CNVs with respect to location, size, copy number state and validated genes were summarised here. (Table 4).

3.2. Case presentations in the families of the cohort

Proband 1(F-A):

A 7-year-old girl with small head size, cognitive impairment, short stature, absence of speech, recurrent seizures and drooling was referred to neurologist at PIMS hospital for clinical assessment. She was clinically suspected as patient of rare neurodevelopmental syndrome. Her CNV analysis reports a unique microduplication of 0.99 Mb size in 16p11.2 region, that diagnose her as 16p11.2 duplication syndrome **(**Fig. 2-C**)**. qPCR results identified the same locus in her affected sister and is suspected to be paternal in origin after pedigree analysis and presence of affected sibling of father (F-A, IV-5). There isn't any family history for neurological impairment in maternal family, 16p11.2 microduplication syndrome has AR inheritance here.

3.2.1. Proband 2(F–B)

A female (V-2) with short stature, obesity, intellectual disability, speech delay, behavioural problems, mood disorder, epilepsy, insomnia, and absence of menstrual cycle was presented to neurologic consultant for diagnosis of her phenotype. She was clinically diagnosed as suspect of Turner's syndrome phenotype which was not confirmed after CMA test. Her CNV analysis across the genome diagnosed her with Xp11.2-q11.1 microduplication syndrome due to a novel 4.26 MB duplication in X chromosome (Fig. 2-D) which is pathogenic, co-segregating in her two affected sisters, and is maternal in origin as evident from careful pedigree analysis. Three of her maternal uncles (F–B, IV-6, IV-7, IV-8) were affected with pre-natal neurological defects and perinatal seizures. Her father family didn't show any NDDs significant history, F–B shows AR-CNV inheritance and Xp11.2-q11.1 microduplication syndrome.

3.2.1.1. Proband 3(F–C). Absence of any previous reports of the NDDs in the family, proband was 10-year-old boy (IV-1) diagnosed clinically with intellectual disability, dysmorphic facial features, tooth anomaly, low set ears, deep set eyes and repetitive behaviour who visited neurologist with his parents and affected sibling. Due to absence of any significant family history in previous generations, CNV analysis for proband genome was performed for genetic and molecular diagnosis of his syndromic phenotype. We report 1.34 Mb pathogenic microduplication at 1q21.1-1q21.2 locus for the first time in Pakistani cohort (Fig. 2-A). CNV validation analysis in parents' genomes has revealed mother as carrier with normal phenotype. No such CNV has been validated in father genome, while co-segregation analysis data supports that 1q21.1-1q21.2 microduplication is co-segregating in affected sibling. AR-CNV mode of inheritance has been indicated after careful pedigree analysis of family (F–C).

3.2.1.2. Proband 4(F-D). 6-year-old girl (IV-2) with microcephaly, intellectual disability, and seizures was diagnosed clinically as

Table 4
Chromosomal attributes of microduplications and microdeletions showing co-segregation in family members of the NDDs cohort.

Family ID	Member ID	Identified CNV	CNV Location	CNV Type	CN state	Validated Genes in family
F-A	Proband 1	16p11.2-p11.2	31,871,490–32,862,089	Microduplication	**3**	*ZNF267, TP53TG3*
F–B	Proband 2	Xp11.22-q11.1	50,388,622–50,607942		**3**	*ARHGEF9, MTMR8, ZC4H2*
F–C	Proband 3	1q21.1-1q21.2	14,499,3069–14,968,9394		**3**	*SRGAP2B, NBPF, FAM72D, GPR89A*
F-D	Proband 4	4p12-p12	45,767,244–47197,116	Microdeletion	1	*GABRG1, GABRA2, GABRA4, GABRB1*
F-E	Proband 5	Xq21.1-Xq21.2	87,900,051–88,104,754		1	*HDX, APOOL, ZNF711*

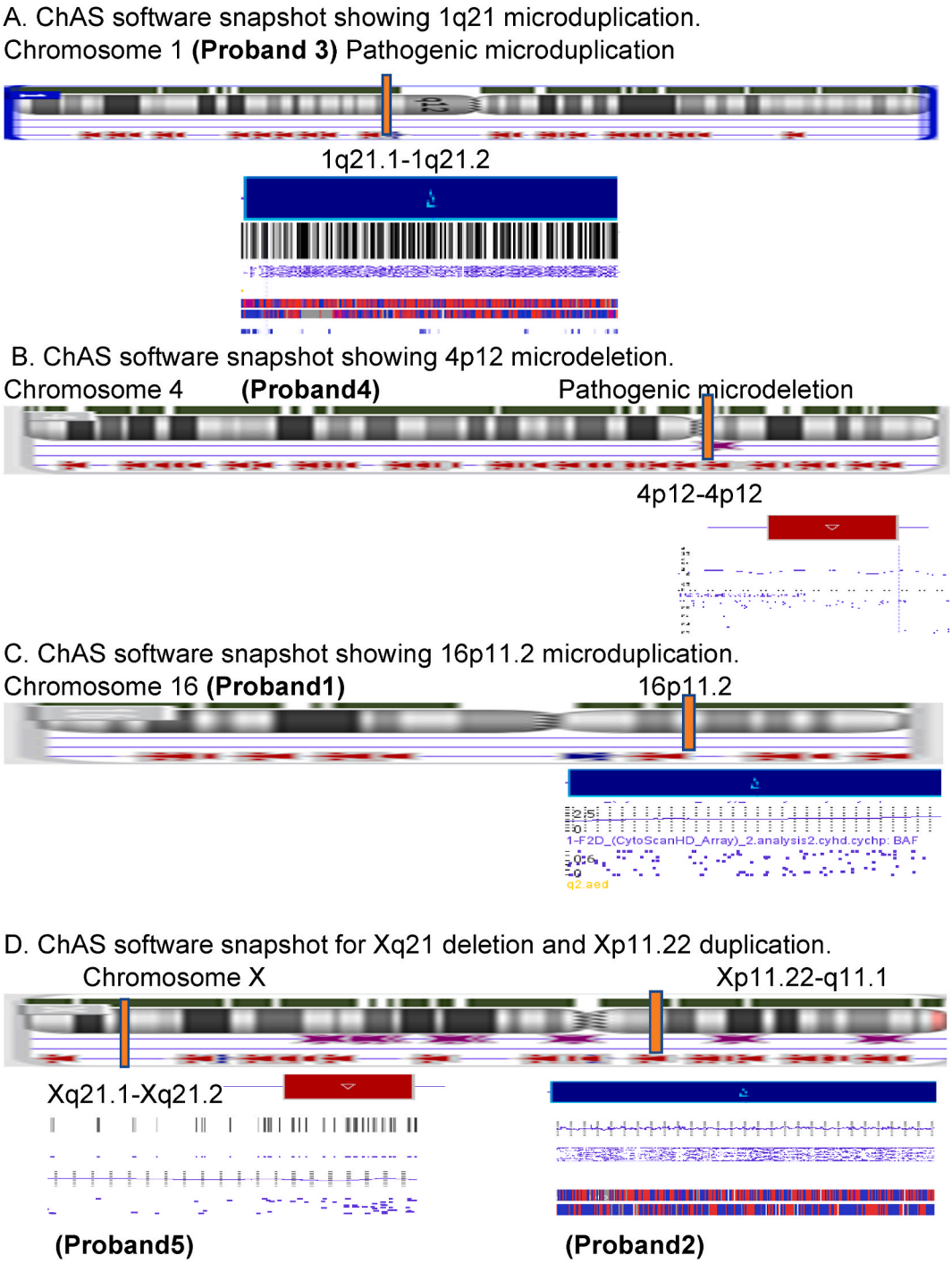

Fig. 2. **(A–D)** Karyotype view of chromosomes 1,4,16 and X with pathogenic CNVs identified by ChAS v4.2 software.

neurological syndrome. Her genome wide CNV analysis shows a unique 1.43 Mb sized microdeletion at 4p12 locus which affects GABA-receptor genes (Fig. 2-B). This deletion co-segregated in her affected brother. Pedigree analysis has revealed the affected individuals in previous generations of father family (III-2) thus the 4p12 microdeletion has a probable paternal origin with AR mode of inheritance.

3.2.1.3. Proband 5(F-E). 13-year-old boy (IV-4) with craniofacial dysmorphism, hypodontia, frontal bossing, short stature, bilateral cataract, and microcephaly was clinically diagnosed as intellectual disability and microcephaly syndrome. His chromosomal

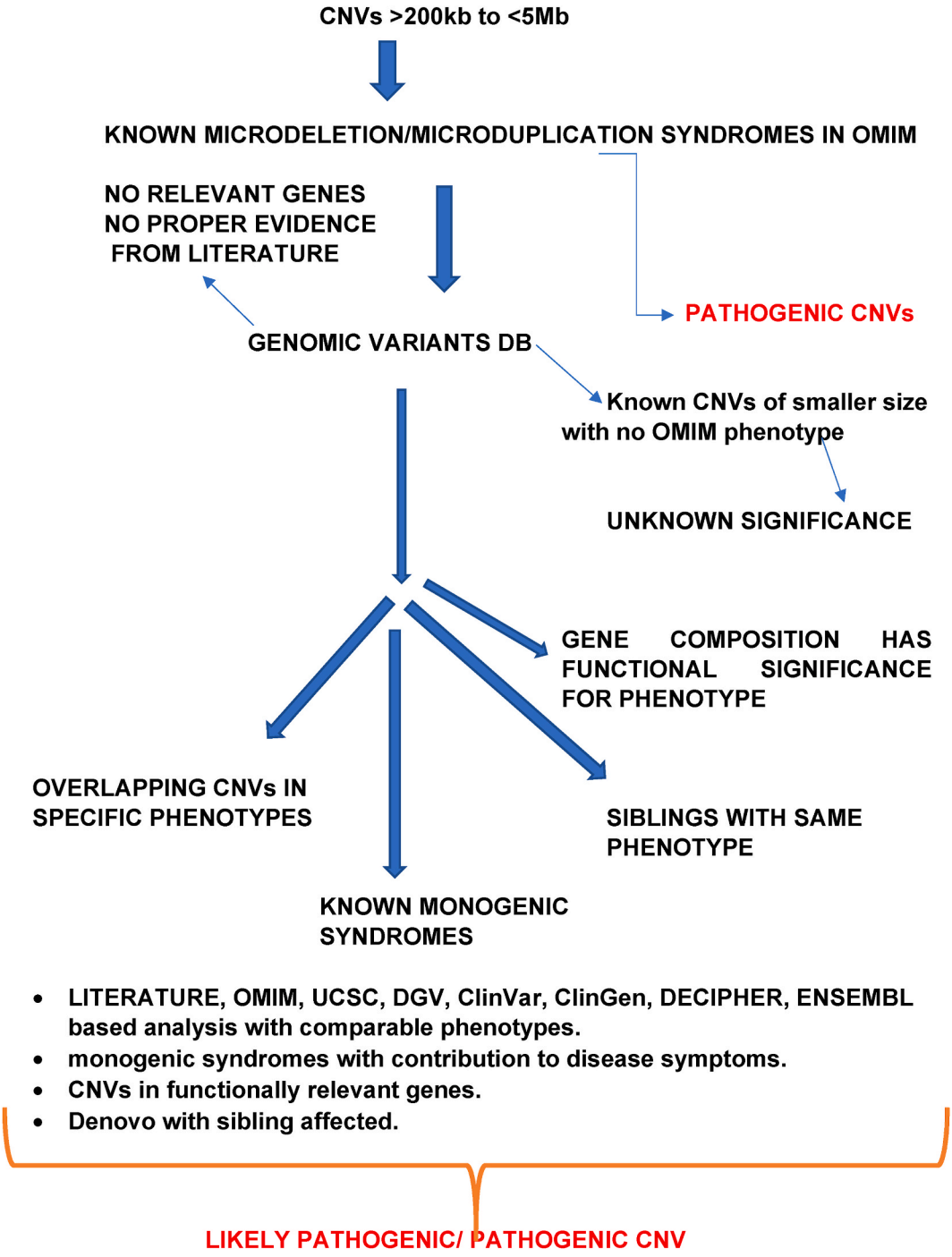

Fig. 3. In-house pipeline for CNV filtration to identify novel pathogenic CNVs in cohort of five multiplex families.

microarray Cytoscan HD test and subsequent CNV analysis reports novel pathogenic Xq21.1-Xq21.2 microdeletion of 2.89 Mb (Fig. 2-D) which has maternal family history in the pedigree (III-2) while its validation and co-segregation analysis confers it in father and affected sibling genome. Both the parents were normal in phenotypes and pedigree shows AR-CNV inheritance.

Our results have contributed to the solution of the partial clinical diagnosis by validation and confirmation of the underlying genetic and molecular pathways. The probands with neurological syndromes phenotypes have complete diagnosis now and we can head to the possible ways for eradication of their illness in future.

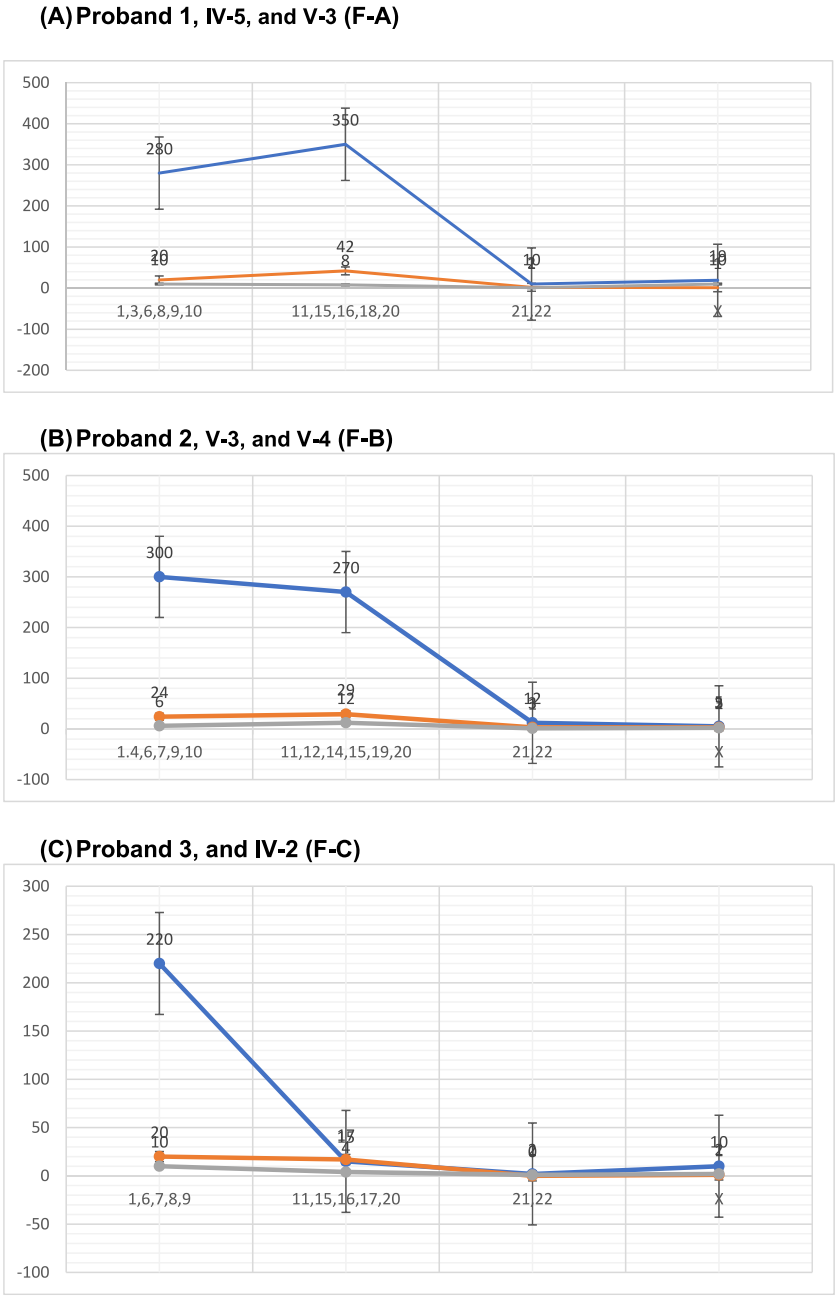

Fig. 4. Distribution plots of identified CNVs in all chromosomes of five probands and affected family members of the cohort. (A) Proband 1, IV-5, and V-3 (F–A).

4. Discussion

We present five novel regions with microduplications and microdeletions on chromosome 1,4,16 and X, in a cohort of five multiplex consanguineous families residing in suburbs of Islamabad, Pakistan. This study reports three novel microduplications 16p11.2, Xp11.22-q11.1,1q21.1-1q21.2 and two novel microdeletions 4p12-4p12., Xq21.1-Xq21.2 associated with the neuro-developmental syndromes of probands in the cohort. This is the first Cytoscan HD array-based NDD-CNV analysis in cohort of multiplex Pakistani families. The aim of this study was to identify the NDD-CNVs and their possible role for observed clinical symptoms in our study cohort. The probands and their affected siblings of our cohort tested positive for pathogenic NDD-CNVs. Literature review with variety of case presentations across the globe by clinical and molecular genetics research groups have suggested the possible

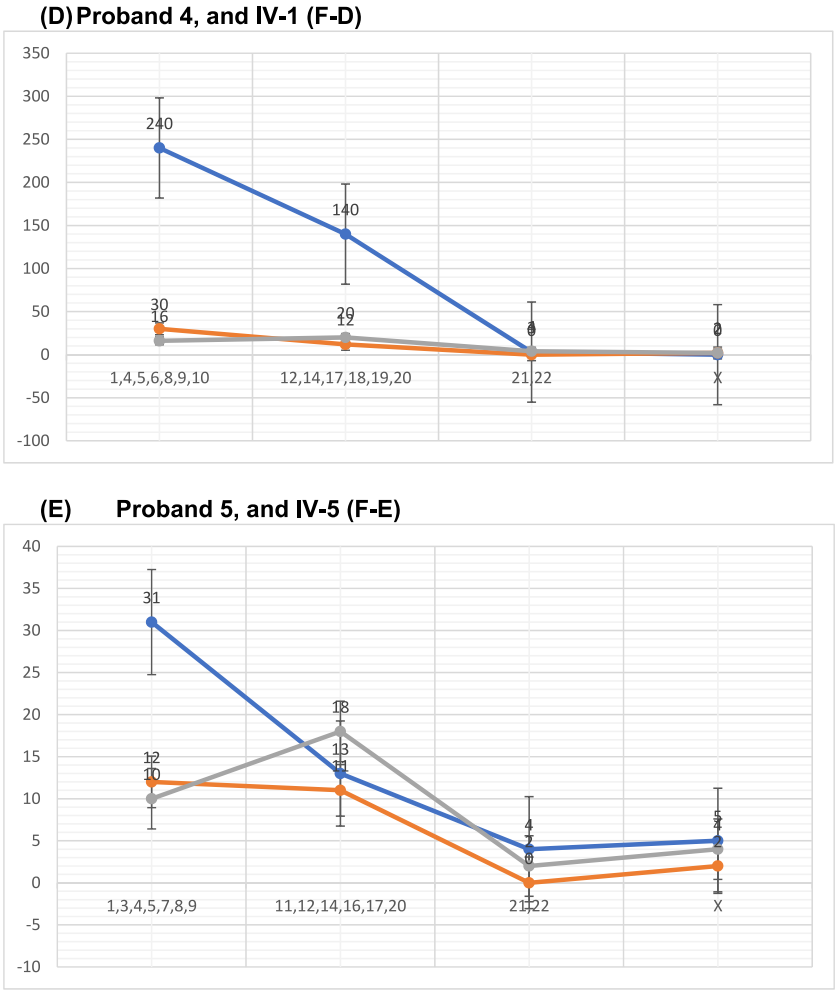

Fig. 4. (*continued*).

neurologic and pathogenic role of CNVs on various loci of chromosome 1,4,16 and X in human genome [26]. These alterations in genes impact the expression by addition or removal of gene function and result in malformation during neurodevelopment which results in unexplained phenotypes of neurodevelopmental disorders [27]. Previous findings agree strongly with our cohort that has five multiplex families with 22 affected (23%) and 76 unaffected (77%) members which shows variable prevalence and penetrance as reported in larger clinical cohorts [28]. The affected members age group 6–40 years have congenital origin of the respective neurological syndromic phenotype in our cohort which is in accordance with other reported NDD-CNV cohorts studied. Genome wide arrays uncover the underlying pathogenic chromosome segmental microduplications and microdeletions to explain the pathways related to prenatal neurodevelopment and dysmorphic phenotype [29].

Among the affected members proband1 is a girl with dysmorphic features of microcephaly, bulging eyes, small forehead, broad implantation of ears, thick eyebrows, scarce hair, and absence of speech. She used to remain her mouth open and constantly drooling. She had mental retardation and seizures. Her CNV analysis pathogenic microduplication of 0.99 Mb on 16p11.2 (31.871,490–32,862,089) harbouring many genes including *ZNF267*and *TP53TG3*. Duplication of 16p11.2 locus increase the function of neuron connectivity in the brain by upregulating the gene expression [30]. Familial background of 16p11.2 syndrome lowers the cognitive capabilities and social skills in the probands showing deleterious effects of these duplications with variable phenotypes [31]. Diagnosis in such patients before complete manifestation can help in proper clinical care.

Family F–B has proband 2 as an interesting case presentation, a 23-year-old female with no menstrual cycle, obesity, short stature, intellectual disability, frequent seizures, absence of speech, flat and broad face, insomnia, and depressive mood. She was previously misdiagnosed clinically as suspect of Turner's syndrome. Her CNV analysis reported her with a 4.26 Mb deleterious microduplication at chromosome Xp11.22-q11 position (150,388,622–50,607942) with *ARHGEF9, MTMR8, ZC4H2* and six more genes. Xp11.22 duplications in literature have long been reported and may involve inactivation of X-chromosomes in female suspects or have Turner's syndrome phenotype [32]. X-p11.22 duplication has variable expression and may explain multiple shared syndromic features in the affected [33]. Families with a positive history of NDDs must be counselled for a prenatal diagnosis to arrange better postnatal clinical

care.

Microduplication of 1.34 Mb involving mainly *SRGAP2B, FAM72D, NBPF, GPR89A* and 28 more genes at 1q21.1-1q21.2 (14,499,3069–14,968,9394) was identified in a family F–C of our cohort. This family was negative for any NDDs in previous generations. The proband and his brother both bear NDD syndromic features of variable severity with craniofacial dysmorphism, teeth anomalies, intellectual disability and dyscephaly. 1q21.1 locus has been reported for multiple deletions and duplications in recent studies that suggests to relate it to NDDs [34]. Cognitive regression, neurodevelopment, dental anomalies, multiple sclerosis, cardiac manifestations are reported to be associated with 1q21.1 deletions and duplications in recent data [35]. In the absence of any family history, appearance of dysmorphic features in two brothers confers the gain-of-function in underlying genes. These human genome duplications play a key role in neurodevelopment [36].

GABA-receptor genes have always been a focus in the research of neurodevelopmental and neuropsychiatric conditions. In our cohort, Proband 4 of family F-D presents 4p12 (45,767,244–47,197,116) microdeletion syndrome characteristics. *GABRG1, COX7B2, GABRA2, GABRA4, GABRB1* genes were downregulated by 1.43 Mb microdeletion which was expressed as recurrent seizures, microcephaly, bilateral cataract, and intellectual disability as main phenotype landmarks. This paternally inherited deletion was indicated in two siblings in a family of positive history of multiple NDDs. Deletions and duplications at chromosome 4p12 locus have been associated with NDDs and *GABRA4* gene is designated as Autism susceptibility gene in case reports of 4p12 duplication syndromes [16]. Clinical and genetic studies review the gene dosage decrease effects on the neurological phenotypes in familial inheritance [37]. 4p12 mutations involving variety of genes have been a novel evidence for neurodevelopment mechanisms [38].

2.89 Mb microdeletion Xq21.1 with loss-of-function deleterious mutations in *HDX, APOOL, ZNF711, POF1B, CHM* and eight more genes show expression of NDD syndromic phenotype in Proband 5 of family F-E. The CNV was paternal in origin and was expressed in two siblings' syndromic phenotype and cranio-facial and mandibular dysmorphism and ophthalmic manifestations. The X chromosome deletions and duplication loci not only account for normal disease features like other autosomes but they also influence sex linked disorders [39]. *ZNF711* gene is one of the zinc-finger genes on X chromosome that shows expression of X-linked intellectual disability [40]. X-linked microdeletions and duplications are reported in various NDDs studies and reviews which in strong agreement with our genetic findings [41].

Copy number variations (CNVs) have become an increasing concern in the neuro-genetics research and medical diagnosis. Gain or loss of sub microscopic areas might result in clinical symptoms which are thought to be linked to neuro-disorders. AR-CNV are genetic basis of neurodevelopmental syndromes and parents' genomes are characterized and analyzed genetically for specific CNVs to ascertain the parent-of-origin. The mechanism by which CNVs alter size when passed on from generation to generation, i.e., whether DNA-regions within a CNV are deleted or amplified, is not well known. Unequal crossing over instances, complex repair steps, non-allelic homologous recombination, microhomology-mediated break-induced repetition, as well as chromothripsis have been studied. Another explanation for CNV formation relies on the observation that parts of the genome involved in microdeletion/microduplication syndromes can be accompanied by identical recurrent attributes, as first described by Jim Lupskis group for a 1.4 Mb region in 17p12 containing the PMP22 gene [42]. The determination of the parent of origin for CNVs or areas of lack of heterozygosity (LOH) is crucial in clarifying the medical implications of specific genetic variations, especially about neurodevelopmental diseases with varying expressiveness. Several studies have demonstrated that de novo CNVs have a paternal origin bias, that can assist biological comprehension as well as sorting [43].

Neurodevelopmental disorders have variable phenotype expression and heterogenetic pathways which make it challenging for clinicians and geneticists to diagnose. With advent of modern technology involving CMA and genome-wide CNV analysis, Cytoscan HD array is considered as first-tier test for diagnosis of neurodevelopmental disorders with more than 99% accuracy [44]. Despite these facts, a large number of neurodevelopmental and neuropsychiatric families genetic outlook is missing. In Pakistan, genetic research groups are working robust for the expansion of knowledge of genetic basis of neurodevelopmental phenotypes for proper diagnosis and clinical cure to ease the difficulties of the patients and their families in daily management of syndromic phenotypes. Our study is a valuable contribution which adds five rare and novel CNVs in gene pool of neurodevelopmental syndromes. We hope that future research in human molecular genetics will help discover remedy to eliminate the pathways that lead to genomic deletions and duplications.

5. Conclusion

We discovered five novel CNVs in probands and siblings of the neurodevelopmental syndrome families cohort. Our study explains the complex neurological symptoms and phenotypes in families with identified genetic basis. We hope that our findings will help clinicians and parents to take proper care of probands and their siblings. This study emphasizes prenatal diagnosis in families with significantly reported NDDs in previous generations. It also explains the necessity of early genetic testing to prevent the difficulties after full manifestation of the NDDs. Lastly, we elaborated personalised molecular diagnosis by Cytoscan HD array which will open the ways for further investigation in establishing clinical utility of this test for NDDs.

Author contribution statement

Behjat Ul Mudassir: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Mashael Alhumaidi Alotaibi; Nadeem Kizil bash; Anwar Alruwail; Modhi Alenezi: Contributed reagents, materials, analysis tools or data.

Daliyah Alruwaili: Conceived and designed the experiments.

Zehra Agha: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data will be made available on request.

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Ethical review board statement

This study was approved by the Institutional Review Board of Department of Biosciences, The COMSATS University, Islamabad (CUI-Reg/Notif-1616/18/2/27 dated 05th June 2018).

Written informed consent statement

Written informed consent for clinical and genetic testing, publication of information and sequencing details were provided by parents of affected family members.

Diagnosis criteria statement

Diagnosis was carried out in collaboration with the neurological consultants in their OPD clinics Department of Neurology, PIMS Hospital, Islamabad.

Declaration of competing interest

The authors declare that they have no competing financial interests or relationships that could influence the work presented in the study.

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Abbreviations

CNV	Copy number variation
NDD	Neurodevelopmental Disorder
ASD	autism spectrum disorder
LOH	Loss of heterozygosity
CMA	Chromosomal Microarray
ID	Intellectual Disability
MC	Microcephaly
AR	Autosomal Recessive

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