The Role of Molecular Karyotyping in the Genetic Etiology of Autism

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Objective: The aim of this study was to investigate the deletions and duplications with a molecular karyotyping technique and to elucidate the etiology of autism.

Method: A total of 31 patients (20 boys and 11 girls) between 4 to 18 years old with normal chromosomal analysis and no Fragile X mutation were diagnosed in the Ege University Child and Adolescent Psychiatry Clinic with autism (according to DSM-IV-TR criteria) and were enrolled in the study. Symptom severity of the patients was evaluated with a Childhood Autism Rating Scale. Blood samples (EDTA collected) were obtained in order to extract DNA. Whole genome molecular karyotyping analyses were performed with Illumina IScan system by chips, which can scan 330.000 Single Nucleotid Polymorphisms (SNPs) to detect structural anomalies with a 10-kb resolution.

Results: All patients had copy number variations (CNV) that sized between 20-kb and 3-Mb. All detected CNVs were analyzed by the help of KaryoStudio software and DGV database. The ones which might be causal and pathogenic were selected. Pathogenic CNVs (7 deletions, 2 duplications) were detected in 9 patients (29%).

Conclusion: As a result, this is the first study whereby a molecular karyotyping technique was successfully used in autism patients in Turkey. Moreover, an intermediate resolution of 330.000 SNP chips were proven to be efficient for molecular karyotyping analysis.

Keywords: autism, molecular, karyotyping, single nucleotide polymorphism, microarray

INTRODUCTION

Autism is one of many neuropsychiatric disorders of childhood. It is characterized by a delay in reciprocal social interactions and communication skills, stereotypical behaviors, and restricted activities (Bailey et al 1995). There are studies in the literature that demonstrate that Autism Spectrum Disorder (ASD) prevalance varies between 0.6 - 2.64% (Fombonne 2009, Kim et al 2011). The incidence of autism in boys is 5-fold more than girls (Fuentes et al 2014). Although the etiology is not that clear, it has been suggested that a complex interaction between environmental and genetic factors exists for the etiology of the disorder (Kim et al 2011, Levy et al 2009). Detailed studies made in ASD show that between 15 to 40% of these children may have chromosomal or Mendlian etiology or susceptibility (Schaefer et al. 2008).

It is important to investigate autism cases in two groups, such as syndromic (complex) and essential (nonsyndromic), for a better understanding of the genetics of autism (Miles et al 2005). Essential autism has been observed in 75% of cases with no dysmorphic signs. The male/female ratio is 6:1 and is higher than the normal. In this group, the recurrence ratio for siblings is higher (35%) and familial history is also higher (20%) (Gurrieri 2012). In syndromic cases with autism, definable dysmorphic signs are with the disorder and the male/female ratio is 3.5:1. The recurrence ratio in siblings are 4-6%, and 9% of cases have positive familial history (Miles 2011). It is important to distinguish these two groups, since

Received: 08.04.2016 - **Accepted:** 28.07.2016

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doi:10.5080/u18239

the prognosis and recurrence risk is marked different between both groups, (Miles 2011).

Genetic variations detected in ASD can be classified into three subgroups: 1- in up to 5% of the cases cytogenetic anomalies can be detected with conventional karyotyping; 2- in 10-35% of the cases copy number variants (CNVs) can be found with microarray analysis; and 3- in 5% of the cases single gene mutations might be found (Miles 2011). In syndromic autism, it is easier to determine the genetic etiology with the help of the dysmorphic signs. Fragile X syndrome, Angelman syndrome, or Rett syndrome are samples for the syndromic autism.

In essential autism, the first genetic analyses must be to investigate the copy number variants in the whole genome (Shen et al 2010). This technique has a diagnositc yield of 10-20% in essential autism cases (Miles 2011). Molecular karyotyping test is a new molecular test that detects chromosomal gain, loss, and rearrangement. However, this cannot be detected by conventioanl karyotyping. Chromosomal abnormalities, which have been detected in conventional karyotyping, are also found with this new technique. This test is now a routine test that is used as a primary test in the world in some disorders (Shen Y et al. 2010). In this method, 300.000 single nucleotide polymorphisms that are selected specifically for indicating a chromosome region are evaluated in order to decide how many chromosome copies of related region exist (there are normally two copies). In this system (with the help of the analyses of 15.000 SNPs per chromosome) chromosomal gains and losses can be detected with much higher resolution than conventional karyotyping where a light microscope is used to evaluate chromosomes.

In some disorders such as mental retardation/multiple congenital anomalies, autism spectrum disorders and in defined microdeletion syndromes, the molecular karyotyping test is accepted as the first tier test (Miller et al. 2010). In addition, it is expected to have a remarkable number of chromosomal aberrations. However, conventional karyotyping performed by light microscope does not detect all chromosomal anomalies. Molecular karyotyping studies in recent years in these disorder groups revealed diagnostic rates of 15-20% better the conventional karyotyping methods (Miller et al 2010). Diagnositc rates of the microdeletion syndromes with this technique are nearly 100%. In our country, there are limited number studies on genetics of autism and some reviews paid attention to this issue (Caglayan 2010). Additioinally, chromosomal anomalies that were evaluated with conventional karyotyping, MECP2 mutations, and triple repeat numbers for Fragile X were analyzed (Çöp et al 2015). The MTHFR C677T polymorphism, which was thought to be important in susceptibility to disease, was studied (Sener et al 2014). Nevertheless there has been no research in our country that has evaluated the molecular karyotyping that was proposed as a first tier test in autism.

In our study, deletions and duplications were evaluated with a molecular karyotyping method for the first time in our country in 31 autism cases that were nonsendromic and whose chromosome and Fragile X studies were normal. This study may be the first study to use molecular karyotyping routinely as the first tier test and may provide valuable information to the forthcoming studies in this patient group.

METHODS

This study was approved by the Ege University Ethical Commitee on 05.09.2012 with the number of 12-8/22.

In this study 31 cases, between ages of 4-18 (20 boys and 11 girls) that applied to Ege University Child and Adolescent Psychiatry Clinic were diagnosis of autism (according to the DSM-IV-TR criteria) and conventional karyotyping and Fragile X test were normal and recored (American Psychiatric Association 1994). Ege University Child and Adolescent Psychiatry Clinic have a special sub-unit like "Development Clinic" and "Ages of 0-6 Clinic". In these special sub-units, all children with autism have special and detailed records about their developmental history, intellectual levels, and if they were able to get the intelligence tests. In this study, these data records were used. A Childhood Autism Rating Scale (CARS) was applied to all children in this study and the severity level of symptoms was noted (Sucuoğlu et al 1996). According to the developmental and ability levels of the cases, Ankara Development Screening Inventory (ADSI) and Wechsler Intelligence Scale for Children-Revised (WISC-R) were used for determine their mental capabilities (Şahin and Savaşır 1995, Savaşır et al 1998).

The CARS is a behavioral evaluation scale with 15 parts developed by Schopler and collegeaus in 1980. It has the validity study in Turkish and is widely used to distinguish the autistic children from other children with developmental delay (Schopler et al. 1980, Sucuoğlu et al. 1996). The scale has 15 items as subscales. The 15 items in the scale are: relating to people; imitative behavior; emotional response; body use; object use; adaptation to change; visual response; listening response; perceptive response; fear or anxiety; verbal communication; non-verbal communication; activity level; level and consistency of intellective relations; and general impressions. The examiner assigned a score of 1 to 4 for each item. The 15th item has a different characteristic. The behavior of the child is evaluated when the 15th general evaluation is made. For each item: 1 indicates not autistic; 2 mild autistic; 3 moderate autistic; and 4 indicates severe autistic. The total score range is between 15 and 60. In the general sum, scores like 15-29 means "not autistic", 30-36 mild-moderate autistic, and 37-60 indicates severe autism.

Wechsler Intelligence Scale for Children-Revised (WISC-R): The WISC-R is a standardized intelligence test developed by Wechsler (1974) for children that includes two parts as verbal and performance subtests. Total IQ score was calculated by summing verbal and performance scores. The Turkish

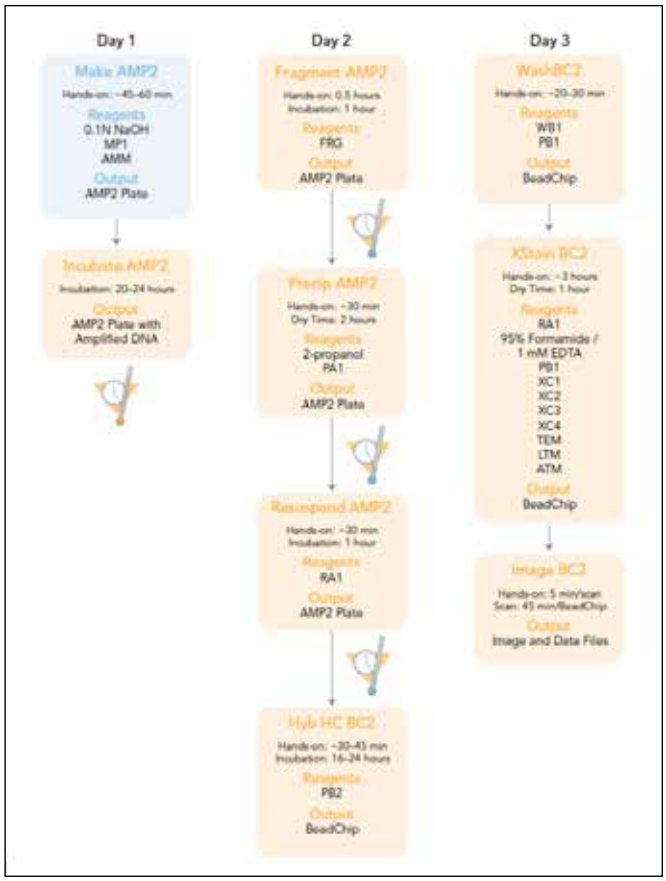

Figure 1. A flowchart of SNPs microarray studies

standardization was made by Savasir and Sahin (1995) from a 6-16 years of age group.

Ankara Development Screening Inventory (ADSI): It was developed for Turkish children by Savasir et al. (1998). The ADSI is a 154-item scale widely used in Turkey for the assessment and evaluation of social, motor, cognitive, and communicative levels in children aged between 0 and 6 years. The total development score reflects the general development level of the child, and it is obtained from the total of the four subscales (language-cognitive development, fine motor development, gross motor development, social interaction subscales). Furthermore, the reliability and validity of the inventory was reported (Savasir 1998).

The molecular karyotyping studies of all cases with autism in the study were performed in the Ege University School of Medicine, Medical Genetics Department. Genomic DNA was extracted from peripheral blood cells by using QIAcube AllPrep DNA/RNA FFPE Kit (Product No:80234; QIAGEN, Germany) with QIAcube Robotic DNA isolation machine. Whole genome molecular karyotyping analyses were performed with Illumina IScan system by chips that can scan 330.000 Single Nucleotid Polymorphisms (SNPs) to detect structural anomalies with 10-kb resolution. A flowchart of SNPs microarray studies is given below (Figure 1).

RESULTS

In this study, 31 patients with autism (according to the DSM-IV-TR criteria) between ages of 4-18 were included.

Table 1. Sociodemographic characteristics, mental capabilities, CARS levels of the cases

Features of the cases		n	%
Sex	Girls	10	32.3
	Boys	21	67.7
Educational level	Special education	10	32.3
	Special education and formal education	21	67.7
Familial status	Core	24	77.4
	Divorced-seperate	7	22.6
CARS levels	Mild autism	14	45.2
	Severe autism	17	54.8
Mental capability	Normal mental level	7	22.6
	Mild mental retardation	11	35.4
	Moderate mental retardation	13	41.9
Regression history	Yes	2	6.5
	No	29	93.5

Table 2. Pathogenic variants detected in the patients

Case No	Deletion Duplication	Zygosity	Chromosome	CNV Size	CARS Scores	Mental capability	Regression
AUT-1	Deletion	Hemizygous	Xq11	2 Mb	33,5	Moderate MR	No
AUT-9	Deletion	Homozygous	17q12	153 kb	31	Normal	No
AUT-11	Deletion	Heterozygous	12q23	126 kb	32,5	Mild MR	No
AUT-12	Deletion	Heterozygous	16p11.2	350 kb	39	Mild MR	No
AUT-15	Deletion	Hemizygous	Xq11	2 Mb	35	Moderate MR	No
AUT-19	Duplication	Heterozygous	16p11.2	620 kb	34.5	Moderate MR	No
AUT-21	Deletion	Heterozygous	16p11.2	1.1 Mb	32.5	Moderate MR	No
AUT23	Deletion	Homozygous	16p11.2	417 kb	30	Normal	No
AUT-24	Duplication	Heterozygous	15q13.3	494 kb	37	Normal	No

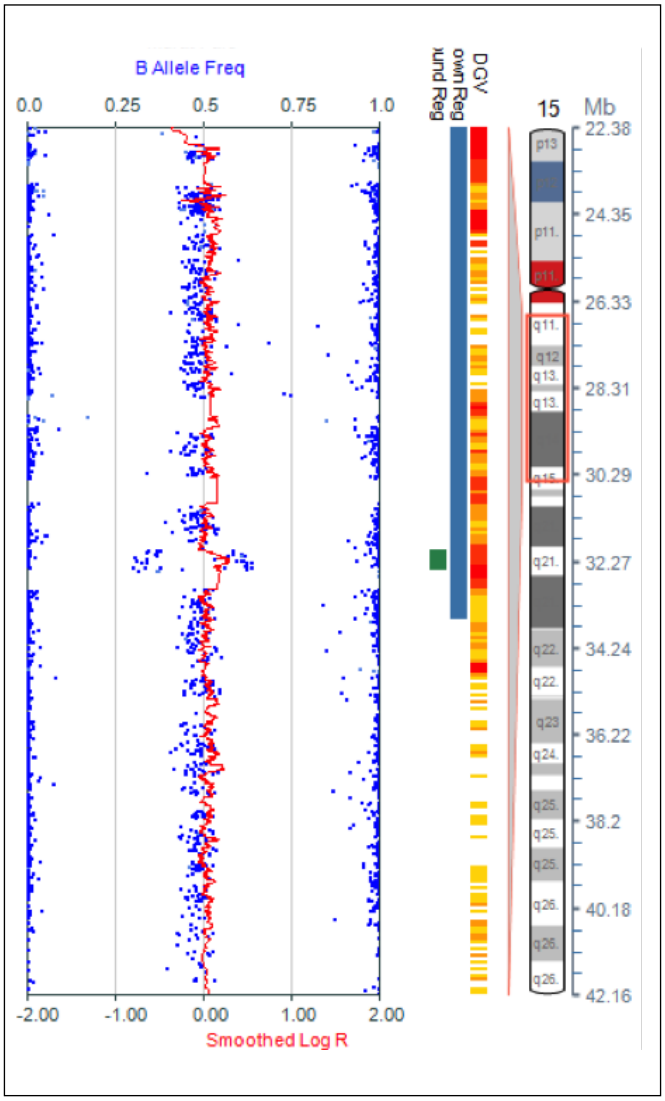

Figure 2. Duplication in case AUT-24

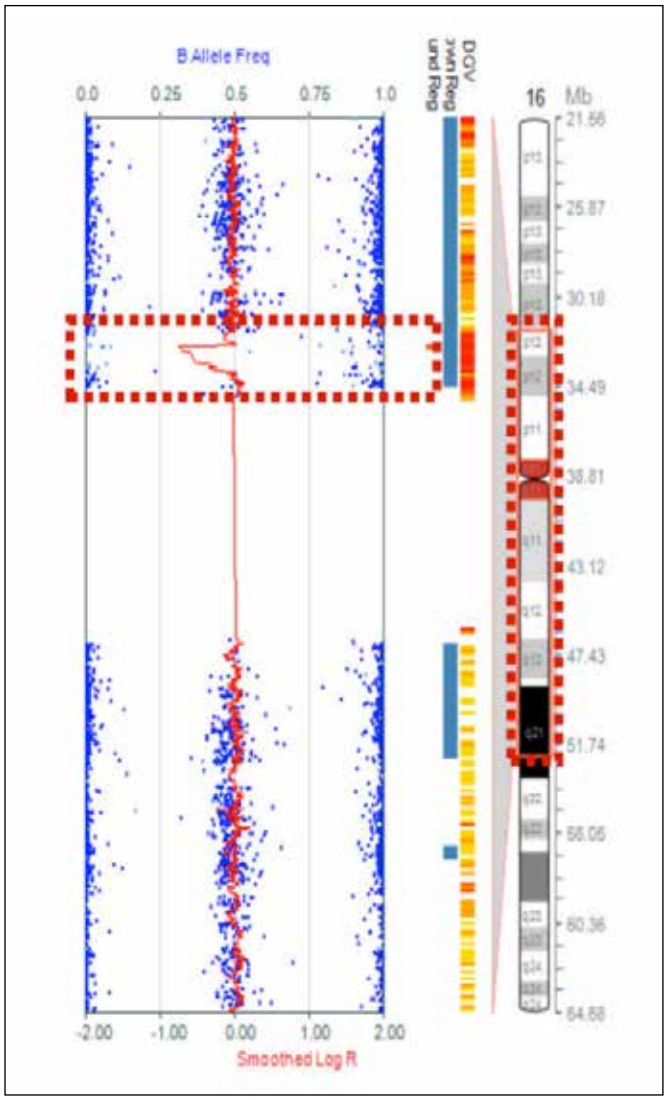

Figure 3. Deletion in case AUT-23

Cases with Asperger Syndrome, Pervasive Develomental Disorders, and Desintegrative Syndrome among others were excluded. The age mean was 8.52 (SD: 3.87) and the male/female ratio 2.1/1. None of the cases had comorbid disesases. Dysmorphological evaluations of the cases were normal. Neurological evaluations, EEG's, MR's, conventional karyotyping, and Fragile X results were normal. In Table 1, the educational level, family status, sosiodemoghraphic characteristics, regression history, entelluctual levels and CARS scores are shown.

All patients had copy number variations (CNV) that sized between 20-kb to 3-Mb. All detected CNVs were analyzed with the help of KaryoStudio software and DGV database, and the ones that might be causal and pathogenic were selected. In 9 patients (29%), copy number variants were defined and evaluated by the clinic. Seven of them were deletions and 2 duplications. (Table 2) (Figure 2 ve 3).

DISCUSSION

In our study for elucidating the genetic etiology of autism, deletions and duplications were investigated in cases with autism using a whole genome molecular karyotyping technique. All patients had CNV that sized between 20-kb to 3-Mb. All detected CNVs were analyzed with the help of KaryoStudio software and DGV database, and the ones that might be causal and pathogenic was selected. In 9 patients (29%) (7 deletion, 2 duplication), CNV were defined, evaluated by the clinic, and were found to be related. To date, there is no published report in this area within our country. Studies on the autism genetics in our country were about conventional karyotyping (Çöp et al 2015) and also about certain polymorphisms (Sener et al 2014). In the literature, there are studies on molecular karyotyping in ASD. In the study of Capuccio and colleagues, they detected copy number variants in 17.3% of the patients with molecular karyotyping methods in ASD (Cappuccio et al 2016). Kanduri and colleagaues

evaluated 83 families with ASD and found CNVs in 22.5% of the cases (Kanduri et al 2016). In a study that evaluated 195 cases with ASD, CNVs were detected in 26.1% of the cases (Oikonomakis et al 2016). When we reviewed these studies on molecular karyotyping in literature our results are consistent.

The prevelance of ASDs rises continuously and genetic factors are the most important factors in the etiology (Xu et al. 2012, Gurrieri 2012). Because of its heterogenous nature, it is hard to diagnose ASD; however it is also necessary to clarify the underlying etiological mechanism because phenothypical differences and comorbidities (epilepsy, mental retardation etc.). The most important etiological factor is about genetics. There are very important evidences for the genetic origins of autism. The first of these were twin studies. In twin studies, it was found that the heritability coefficient for autism was between 0.85-0.92. In monozygotic twins, the concordance rate has been suggested to be 64% and in dizygotic twins 9% (Xu et al 2012). Family studies have been shown to be the second most important study group for determining the genetic origin (Schaaf et al 2011). In studies with large families, it was found that when one child was affected, the autism risk was 8.6% for the sibling; when two or more children were affected the risk may increase to 35% (Dhillon et al. 2011). Third, detailed studies in ASD suggest that a chromosomal or Mendelian etiology can be determined in 15-40% of these children. Although all these evidences suggest a strong relation between genetics and autism, the genetics of autism has still been a difficult issue for experts and an open area for fortcoming studies. Due to significant differences in prognosis and recurrence risk between two groups, it's important to examine the autism cases in two groups like syndromic (complex) and essential for better understanding the genetics of autism (Miles 2011).

In our study, it was found that none of the cases with mutation had a regression history. The mental capability level was mild or moderate retardation in cases with mutation. When these results compared between cases with mutation and no mutation, there was no significant clustering of the data. These results may be interpreted that the genetic mutation effect was limited on data distribution.

ASD genetics has been shown to be extremely heterogeneous like in other psychiatric disorders. When the alterations in this disorder group were reviewed, it may be stated that molecular karyotyping has a 10-35% diagnostic success. This ratio was approximately 5% in conventional karyotyping methods (Miles 2011). There was an approximate 1000-fold difference between the two methods in terms of resolution. Conventional chromosome karyotyping methods have been evaluated with the lightmicroscope. Chromosomes in molecular karyotyping methods were analyzed in more detail with the help of 330.000 SNPs located on chromosomes. Our study results are important because this is the first study with this method to study ASD in Turkey to our knowledge. Molecular karyotyping studies with microarray analysis can be used with different resolution chips. Studies with very high resolution chips (1 million SNPs and above) can detect many changes and their clinical relevance is very compelling. Studies with low resolution chips (100,000 SNPs and below) cannot determine variations that may cause disease. In this study, preferred 330.000 SNPs using a medium resolution chip rate of detection for copy number variation appears to be very convenient. These results may facilitate the forthcoming studies.

As a summary, this study is the first study in our country performed with molecular karyotyping using microarray system in ASD cases. The copy number variant detection rate of the study is compariable with other studies from other countries.

LIMITATIONS OF THE STUDY

The main limitation of the study is the limited number of cases and increasing the number of cases may expand the data. The other limitation of the study is that the previous regression history of cases is noted according to the family anamnesis instead of using a standardized scale. Future studies should be established to collect data with standardized scales. Another limitation is that the parents were not evaluated because of the restricted funds of the study. Evaluation of the parents may be of interest to determine the novelity of the mutations.

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