© Kamla-Raj 2019 Int J Hum Genet, 19(3): 152-157 (2019)
PRINT: ISSN 0972-3757 ONLINE: ISSN 2456-6330 DOI: 10.31901/24566330.2019/19.1-3.731

Linkage Analysis of Autosomal Recessive Non-syndromic Mental Retardation Locus in Pakistani Families

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KEYWORDS Intelligence Quotient. Linkage. Mardan. Markers. Mental Retardation

ABSTRACT Mental retardation (MR) is the retarded conditions of mind associated with lack of adaptive behavior such as communication and routine living skills having intelligence quotient (IQ) below70. It is a heterogeneous group of disorder caused by organic brain dysfunction that appears at the age of not later than 18-22 years. Ten families were enrolled having two or more affected individuals. Pedigrees of the enrolled families were drawn using software Cyrillic 2.1. The affected families were screened for linkage to known MR loci using STR markers. The haplotypes were made to establish the linkage of families to known MR loci. Out of 10 families, single family was linked to mental retardation locus MRT2A, which is the first report of MRT2A phenotype linkage in a family from Mardan. In the future, further efforts are needed for linkage in unlinked families of local population.

INTRODUCTION

Mental retardation is demarcated as having intelligence quotient (IQ) below70 and about one to three percent of the global population is affected by it (Curry et al. 1997; Maulik et al. 2011). It is divided into non-syndromic and syndromic mental retardation. Generally, in syndromic MR in combination with impaired cognitive functions, individuals have other clinically recognizable physical, neurological, and or other metabolicabnormalities. Autosomal Recessive Non-Syndromic Mental Retardation (ARNSMR) has only consistent impaired cognitive functions. The major causes of the disorder are chromosomal changes and several monogenic changes which accounts for up to approximately twenty five to fifty percent cases (Vissers et al. 2016). It has been estimated that more than 2500 genes are responsible for autosomal mental retardation, of them majority are involved in causing recessive MR. Due to the recessive nature they persist in the population in heterozygous form and play vital role in mental retardation. Due to the high level of consanguinity in populations, autosomal recessive mental retardation are common (Musante and Ropers 2014). Autosomal recessive non-syndromic mental retardation is caused by mutation in less than 50 genes out of the total ~700 known mental retardation genes (Vissers et al. 2016). Six genes causing the autosomal recessive non-syndromic MR, PRSS12 [MRT1 (MIM 249500)] on chromosome 4q26, CRBN [MRT2A (MIM 607417)] on chromosome 3p26, CC2D1A [MRT3 (MIM 608443)], on chromosome 19p13, GRIK2 (MIM 138244) on chromosome 6q16.1-q21, TUSC3 on chromosome 8p22 and TRAPPC9 on chromosome 8q24, have been identified (Kaufman et al. 2010).CRBN gene mutations cause mild NS-ARMR phenotype having IQ's value from 50 to 70. Recently 26 new autosomal recessive non-syndromic intellectual disability genes including PIDD1, UBA7, USP44, MAPK8, MPDZ, TBC1D23, SLAIN1, TRAPPC6B and ABI2, with loss-of-function mutations and BDNF or TET1 with missense mutations (Harripaul et al. 2017). Due to a high consanguinity rate in isolated populations autosomal recessive diseases are more common (Hey 2005).

Genetic linkage analysis is not only used for mapping new locations, but also used for refining previously mapped mental retardation loci

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intervals. In the background of the above, it was proposed to carry out linkage analysis in consanguineous families from population of Khyber Pakhtunkhwa Pakistan.

Objectives of the Study

1. Identification of Pakistani families with clinically diagnosed mental retardation.
2. Screening of affected selected families for linkage to MRT2A loci/CRBN gene using STR markers.

METHODOLOGY

Enrollment of Families

A total of 10 families containing at least two or more than two affected individuals were enrolled in the present study. Families were personally visited and family history and information were collected. Other members of the family (relatives affected with MR) were also enrolled on their willingness and availability in the study. For participating in the research a written consent was taken from each family. To check the occurrence of other defects and environmental causes for MR, complete history was obtained from each family. For confirmation of syndromic and non-syndromic MR, families were asked about the presence of other diseases like epilepsy, cerebral palsy, autism, neuronal defects. Pedigrees of the enrolled families were drawn using software Cyrillic 2.1 on the basis of interviews from different members of the family. The inheritance mode was autosomal recessive in the enrolled families because consanguineous reunions were common as family members rarely marry outside the kindred.

Blood Sampling and DNA Extraction

From each normal as well as affected individuals of the family, 5 ml blood samples were obtained in 200 µl EDTA (0.5M) containing 15 ml falcon tubes. Using Sambrook and Russel's (2001) inorganic method, DNA was isolated from blood samples for PCR amplification. DNA quantification was carried out using 0.8 percent Agarose gel. The quantification analysis of all the DNA samples was done and all samples were brought to the same concentration level 50 ng/µl.

Genotyping STR Markers by PCR

Three microsatellite markers encompassing the chromosomal locations for the MRT2A locus were typed as mentioned by UCSC Genome Browser. Unlabeled primers were used for the purpose of genotyping and linkage analysis as shown in Table 1.

PCR amplification for each STR marker on the genomic DNA sample of selected 10 families were performed using thermal cycler. To produce multiple copies of STR markers, amplification was performed with final reaction volume of 25 µL, including forward and reverse primers for all STR markers, dNTPs (Deoxy Nucleoside Tri Phosphates), Taq buffer, Magnesium Chloride ($MgCl_2$), DNA Taq Polymerase, Double Distilled Deionized water and genomic DNA and Thermal Condition as shown in Tables 2 and 3.

Amplified products of all the STR markers were run on 1.2 percent agarose gel along with 100 base pair ladder to visualize the bands at 110

Table 2: Recipe for each STR markers

S. No.	*Reagents*	*Quantity*	*Concentration*
1	DNA (Sample)	2 µL	50 ng/µL
2	PCR Buffer	2 µL	10X
3	$MgCl_2$	2 µL	2.5 mM/µL
4	dNTPs	2 µL	2.5 mM/µL
5	Forward Primer	0.75 µL (each)	10 pM/µL
6	Reverse Primer	0.75 µL (each)	10 pM/µL
7	Taq Polymerase	0.1 µL	5U
8	Deionized Water	15.4 µL	

Total reaction volume = 25µL

Table 1: Markers sequence used for linkage analysis

Primer ID	*Sequence 5-3*	*Sequence 5-3*	*Product size*
D3S3630	AAGGGATAAGCTGCAAATCA	ACCAAATACAATTCATGAGACCTGA	172-188 bp
D3S3050	TGGTGGTATGCATTTGTCAG	ATTCCCTGACTTCAAGTGCA	227-242 bp
D3S1620	CCTTGGTAGGAGGTCCC	GATCAAATGATGAAGTGTATCAG	239-255 bp

Table 3: Thermal condition of PCR used for amplification of all markers

S. No.	*Step*	*Temperature*		*Time*
1.	Initial denaturation	95 °C		4 min
2.	Denaturation	94 °C		30 sec
3.	Annealing	53 °C	D3S3630	45 sec
		56.4 °C	D3S3050	
		59.6 °C	D3S1620	
4.	Extension	72 °C		45 sec
	Repeat step 1 to 3 for 30 cycles			
5.	Final extension	72 °C		10 min
6.	Temporary storage	4 °C		30 min

volts for 15 minutes, using Bio-Rad gel boxes. Amplification bands of all STR markers were visualized and compared with the standard ladder in the Bio-Rad gel documentation system.

Polyacrylamide Gel Electrophoresis (PAGE)

PCR products of STR markers were run on non-denaturing Polyacrylamide gel to examine amplified bands of STR markers for genotyping (Wang et al. 2003). Electrophoresis was performed for approximately 3 hours at 150 volts. After the complete running of sample PCR products, the gel was separated from the glass plates and dipped into Ethedium bromide solution (2mg/μL) for 10 minutes for staining of amplified DNA bands. The gels were visualized in UV light (254 nm) chamber of gel documentation system. Pictures of the gel were taken and saved by Bio-Rad gel documentation system, present in IBBt, UVAS, Lahore.

RESULTS

A total of 10 families were identified and enrolled in the study having multiple affected individuals from different areas of Khyber Pakhtunkhwa (Batkhela, Allahdand, Dargai, Swatand Mardan). To establish linkage of families to the *MRT2A* locus, D3S3630, D3S3050, and D3S1620 markers were genotyped. The mode of inheritance was checked through haplotypes (set of alleles) construction between the normal and affected individuals of each family. Only one family (MR-02) was found potentionally linked to the *MRT2A* locus while the other families remained unlinked to the *MRT2A* locus (Table 4).

Table 4: Status of pedigrees analyzed by linkage analysis for *MRT2A*

S. No.	*Family No.*	*Status of family*
1	MR-01	UNLINKED TO *MRT2A*
2	MR-02	LINKED TO *MRT2A*
3	MR-03	UNLINKED TO *MRT2A*
4	MR-04	UNLINKED TO *MRT2A*
5	MR-05	UNLINKED TO *MRT2A*
6	MR-06	UNLINKED TO *MRT2A*
7	MR-07	UNLINKED TO *MRT2A*
8	MR-08	UNLINKED TO *MRT2A*
9	MR-09	UNLINKED TOMRT2A
10	MR-10	UNLINKED TO *MRT2A*

The family MR-02 was collected from Arabi kaly, Mardan. This family consisted of seven members from which blood samples were taken having three affected individuals (IV:1, IV: 2 and IV: 3), two normal sibling (IV:4 and IV: 5) mother and father (III: 1 and III: 2). The affected individuals were of 13 to 18 years age. All the affected individuals appeared in the 4th generation. After DNA isolation the markers D3S3630, D3S3050, and D3S1620 straddling in the region of *MRT2A* were amplified. The amplified products were run on seven percent Polyacrylamide gel at 150 volts for 3 hours. Ethedium bromide was used for staining the Polyacrylamide gel. Pictures of the gel were taken and saved by Bio-Rad gel documentation system. The gel was analyzed larger allele was denoted by 2 and smaller allele with 1. The normal sibling, father and mother were heterozygous while affected individuals were homozygous with STR markers D3S3630, D3S3050, and D3S1620 at the *MRT2A* candidate interval, except two affected individual (IV: I, IV: 3) which were heterozygous with marker D3S3050 (Fig. 1). Family MR-02 haplotypes showed linkage to the *MRT2A* locus.

DISCUSSION

Regardless of current advances in molecular technologies the main approach for the identification of molecular defects causing ARNSMR are positional cloning and homozygosity mapping in single and consanguineous families

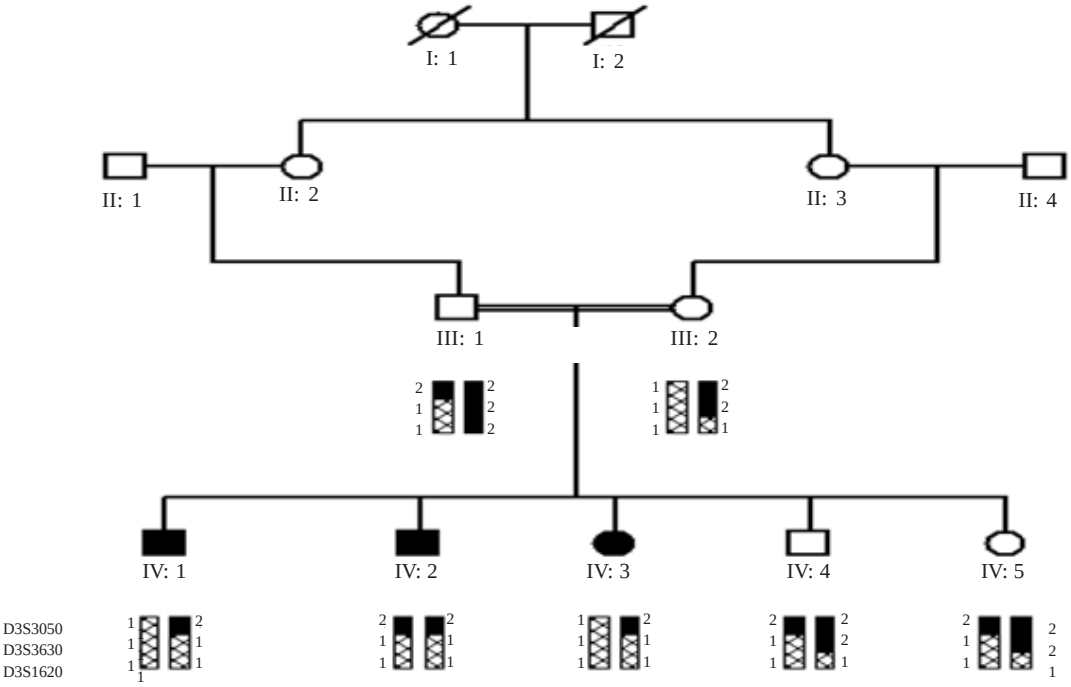

Fig. 1. Pedigree of family MR-02. The three STR markers D3S3050, DD3S3630 and D3S1620 in the candidate region of *MRT2A* showed linkage. Mental retardation phenotype in this family was linked to *MRT2A* locus

(Lander and Botstein 1987). Identification of new genes through linkage analysis has been proven to be successful (Deardorff et al. 2007; Roberts et al. 2007). Genetic analysis in autosomal recessive nonsyndrmic ID (ARNSID) has mapped 51 disease loci (Khan et al. 2016). More than 40 genes linked to autosomal-recessive non-syndromic intellectual disability (ARNS-ID) have been identified, eight of which (PRSS12, CRBN, CC2D1A, GRIK2, TUSC3, TRAPPC9, ZC3H14, MED23) have been found by examining consanguineous families with members affected by ARNS-ID (Sheereen et al. 2017). The associated genes play diverse physiological roles in various molecular processes, including methylation, proteolysis, glycosylation, signal transduction, transcription regulation, lipid metabolism, ion homeostasis, tRNA modification, ubiquitination and neuromorphogenesis (Khan et al. 2016). Cereblon (CRBN) is located at locus 3p26.3 and is highly expressed in human tissues such as the hippocampus, cerebral cortex, cerebellum and striatum (Sheereen et al. 2017). The protein encoded by the CRBN gene have adenosine-triphosphate-dependent Lon protease N-terminal domain, a four putative protein kinase C phosphorylation sites, a leucine zipper motif, and a G-protein-signaling-like domain regulator (Jo et al. 2005). CRBN is known to play a role in memory and learning by regulating the assembly and neuronal surface expression of large conductance calcium-activated potassium (BKCa) channels. These ion channels are known to regulate membrane excitation, and mutations of ion channels often cause severe neurological disorders including seizures and epilepsy (Lupas et al. 2015). Compelling evidence suggests that BKCa channel loss-of-function mutations contribute to neuronal hyperexcitability that leads to enhanced seizure susceptibility (Wang

et al. 2016). Kyle and Braun explain that CRBN, as a substrate of the CRL4ACRBN complex, interacts with BKCa in the central nervous system (CNS), leading to the ubiquitylation of the BKCa á subunit and retention of the channels in the ER. Any changes that affect the CRL4ACRBN complex could prevent ubiquitylation of the BKCa channels, which increases trafficking of these channels to neuronal cell membrane and increases the incidence of seizures and epilepsy in mice. Hence, the elevated BKCa channel expression in the CNS is closely linked with epilepsy, strongly suggesting that increased BKCa current density can lead to neurological disorders and possibly synaptic dysfunction/degeneration (Liu et al. 2014; Kyle et al. 2014). Literature data also showed that the mutation in CRBN gene results in mild mental retardation having no physical anomalies and dysmorphic features (Higgins et al. 2000; Higgins et al. 2004).

Only one family (MR-02) out of 10 families showed potential linkage to the *MRT2A* locus, mapped to chromosome 3p26.2 (Higgins et al. 2004). *MRT2A* was mapped from 6.71-cM region. Markers D3S3525 and D3S1560 flanked the region of homozygosity. A maximum two-point LOD score of 9.18 (theta = 0) was found with a marker D3S3050 at chromosome, 3p25-pter (Higgins et al. 2000).

Out of ten families nine were found unlinked to MRT2A a genetic candidate interval. Mental retardation is a complex disorder as having above 900 different genetic disorders associated with it (Rajeb et al. 2009) so the probability of linkage with a single locus is very low. Thirty percent of severe mental retardation is due to chromosomal abnormalities while mild mental retardation with identifiable causes account for upto four to eight percent (Schaefer and Bodensteiner 1992). Thapar et al. (1994) reported that approximately 210 disorders caused by single gene disorder have been identified and more are yet to be identified. So the probability of finding linkage with a single gene is very low. There is only a small fraction of the total number of cases of autosomal recessive non-syndromic mental retardation caused by mutations in the currently known genes (Basel-Vanagaite 2007). Similarly in another study conducted by Khan et al. (2017) consanguinity and autosomal recessive mental retardation in South Waziristan Agency was studied. They found a linkage in family PKMR438 to the MRT4 locus, showing that the candidate gene GRIK2 in this region was responsible for MR. The researchers' findings and literature data on genetic study of ARNSMR should be helpful in developing gene panels for whole genome and whole exome sequencing.

CONCLUSION

This was first report of MRT2A phenotype linkage in a family from district Mardan. In future, there is a need to explore new linkage in unlinked nine mentally retarded families and in the local population, which will help in prenatal diagnosis of mental retardation and also in genetic counseling strategies for the Pakistani population.

RECOMMENDATIONS

From the researchers' findings it is recommended that sequence analysis is required to find out the possible mutation in the linked family and the unlinked families should be screened for other ARNSMR genes.

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Paper received for publication in June, 2017
Paper accepted for publication in September, 2019