1 INTRODUCTION

One billion of the world's current population is projected to live in communities that favor consanguineous marriages [1].Consanguineous marriages are common and accepted in most North Africa, Near-East and West Asian cultures, where the arrangement between intrafamily groups accounts for 20-50 + % of all marriages [2]. The negative impact on children and their children in consanguine marriage and the possible social and economic advantages of enhanced genetically-based threats is recognized by healthcare providers and genetic experts [1]. Children with consanguineous relationships may be at increased risk of recessive disease in cases of autosomal recessive gene mutations acquired from a common ancestor. The stronger the parental link of the parents, the more likely they are to receive the same versions of one or more detrimental genes [3]. Autosomal recessive primary microcephaly is one of the genetic conditions inherited by parents to children.The MCPHMIM 251200 (Autosomal recessive primary microcephaly) is a brain developmental disorder, defined by congenital reduction of a head circumference of 2 minimum standard deviations (SD) smaller than the ethnically stable, age-and gender-related norm and a variable degree of non-progressive mental impairment [4]. Microcephaly is common in MCPH. It appears at the 32nd week of conception, is present during birth and non-progressive [5]. The most commonly employed diagnostic tool for MCPH is head circumference (HC) [6].

Depending on the type of demographic used to identify microcephaly the main prevalence of microcephaly ranges from 1.3 to 150 per 100,000 individuals [7]. In Asians and Arabs, the incidence of primary microcephaly is greater than in whites and more frequent in consanguineous populations [8]. It has been proposed that in India 1 in 4348 births be affected by microcephaly, indicating that 5887 microcephaly babies are born every year in India [9]. In Brazil, an average of 157 cases of microcephaly has been detected each year [10]. The prevalence of microcephaly in Europe was estimated to be 1.53 per 10,000 births [11]. Newborn infants in Pakistan reported 3,718 cases of microcephaly in 2015 [12].

For this disorder, seven genetic Locus (MCPH1-7) are identified to date, with seven associated genes recorded from various world populations (MCPH1, WDR62, CDK5RAP2, CEP152, ASPM, CENPJ and STIL). More than 50 % of MCPH in the World is developed from ASPM and WDR62 gene mutations [4]. WDR62 mutations are the second most prevalent source of MCPH among the Pakistani community in Central Punjab. Chromosome 19q13.12 maps WDR62 (NM 001083961) and human models interpret two alternative versions of WDR62 but 32 coding exons represent 1,523 amino acid proteins [13]. Nicholas et al. [14] reported mutations in WDR62 in five families in Pakistan. Kousar et al. [13] have identified mutations in the WDR63 gene affecting three central Punjab families with microcephaly. The current study explored the prevalence of microcephaly among central Punjab (Pakistan) families due to consanguineous marriages and the role of the WDR62 gene in the prevalence of microcephaly among these groups. The goal was to provide genetic counseling to impacted households.

2 MATERIALS AND METHOD

The research work was carried out at the GCUF Health Laboratory (Biochemistry and Biotechnology). In order to conduct this study, 4 families with microcephaly infected children were selected from different areas of central Punjab, Pakistan. Consanguineous marriage was also taken into account in the selection of families. Pedigree of these families was developed and blood samples were also taken for genomic studies. The blood samples obtained were used to extract the DNA. Isolated DNA samples were run using agarose gel electrophoresis and PCR to investigate the infected genes in patients.

2.1 DNA Extraction

DNA extraction from collected blood samples was performed using a protocol explained by Koh [15]. Frozen blood samples at-70oC were thawed at room temperature. Blood wash was achieved by applying 12mL of Tris EDTA buffer to 2mL of blood sample and centrifuging at 6000 rpm (20 min) and discarding the supernatant. Wash was done 3-4 times. Collective sediments (2mL) were mixed with TNE buffer 1200µL, SDS (10 percent) 40µL and Proteinase K 15µL, and samples were incubated at 37oC overnight. NaCl (6 M) 200µL was applied to the samples and placed on ice for 10-15 min. At 6000 rpms (15 min) the samples were centrifuged and the supernatant removed. Again, the

samples were centrifuged at 6000 rpm (10 min) and the pallet was collected with the same amount of isopropanol. Extracted pallet washed with 70% ethanol and stored for further analysis.

2.2 Agarose gel electrophoresis for DNA integrity

The process of electrophoresis of agarose gel was used to clarify the integrity of DNA [16]. Mix 0.8 g of agarose powder with 100 mL of T.B.E buffer (1X) to make 0.8 % gel and 5 µL of ethidium bromide added to the prepared gel. Gel was poured into the tank and allowed to solidify. Then the DNA sample (5µL) along with the bromophenol (2µL) loaded in wells and the electrical supply was provided for 35 min. The DNA was visualized using a gel documentation system.

2.3 PCR analysis for WDR62 (muted gene)

The DNA markers for WDR62 were constructed using the UCSC genome explorer [17]. PCR reaction mixture containing 10mM injection H2O 14µL, MgCl2 1.5µL, dNTPs 1µL, forward primer 1µL, reverse primer 1µL, Taq polymerase 0.2µL, Taq buffer 2.5µL and DNA sample 5µL is tested in PCR amplification machines [18]. Amplified samples were run on agarose gel (1.5 %) electrophoresis and the gel recording system was again used for visualization [16].

3 RESULTS

3.1 Pedigree/Family chart

The pattern of Mendelian inheritance sequence was used to validate inheritance in the affected families. Clinical and genealogical data obtained by personal interviews with their ancestors. The males were shown in pedigrees with a square mark and the females with a circle. The unfilled symbol represented normal people in the family and the filled symbol displayed the affected person of the chosen families. The curve between symbols showed that individuals had died. A generation of each family labeled with Roman numerals and a member of each generation labeled with numeric numbers. The outcome of pedigree has shown that the risk of microcephaly increases in consanguineous marriages generation after generation.

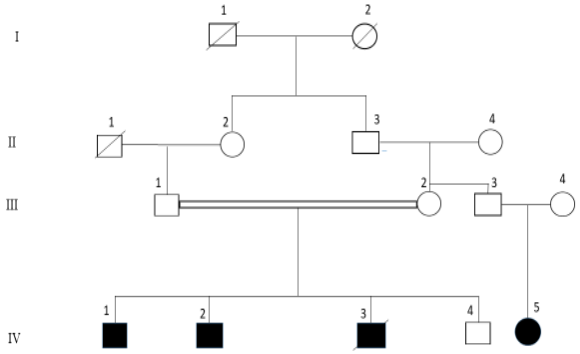

Figure 4.1: Pedigree (Family A), Black filled symbols represent affected members and unfilled represent normal members. Circles used for females and square used for males.

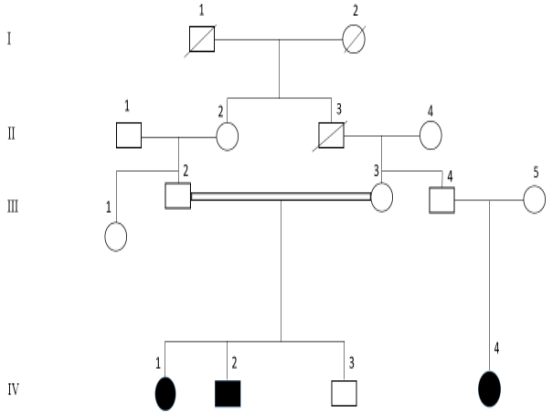

Figure 4.2: Pedigree (Family B), Black filled symbols represent affected members and unfilled represent normal members. Circles used for females and square used for males.

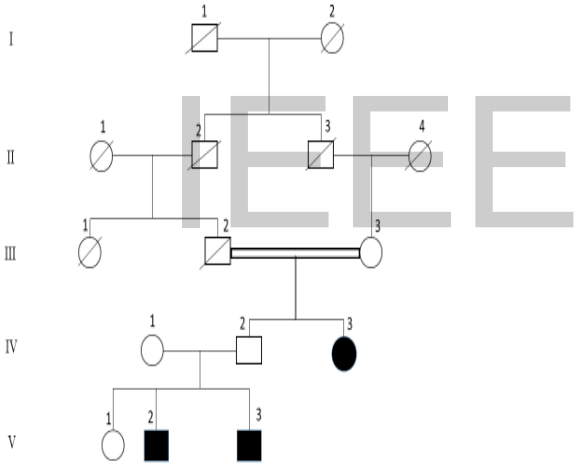

Figure 4.3: Pedigree (Family C), Black filled symbols represent affected members and unfilled represent normal members. Circles used for females and square used for males.

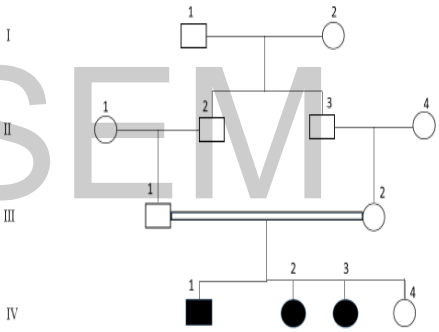

Figure 4.4: Pedigree (Family D), Black filled symbols represent affected members and unfilled represent normal members. Circles used for females and square used for males.

3.2 DNA isolation

DNA extracted from four selected families using the standard inorganic method was used for further analysis. The integrity of DNA was monitored with an 0.8 % agarose gel electrophoresis. Figure 4.5 showed conformational results for concentration and integrity of DNA.

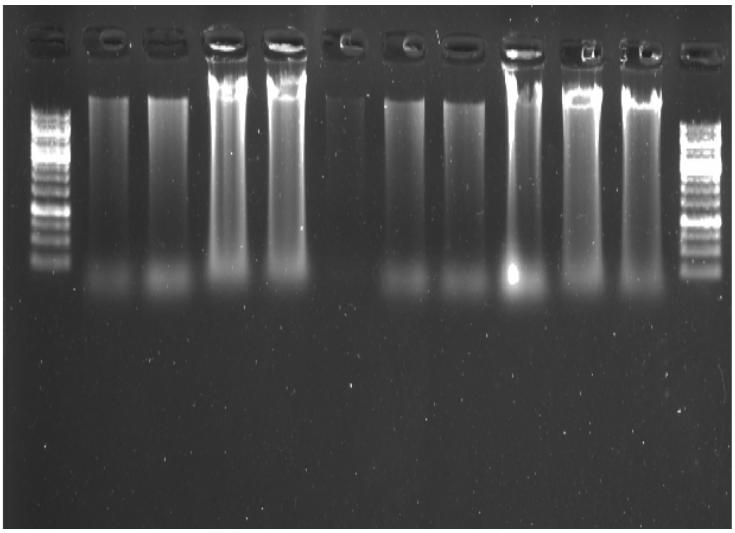

Figure 4.5: DNA integrity confirmation through gel electrophoresis

3.3 Linkage analysis

All four families investigated the identification of the WDR62 mutation in all affected individuals with DNA markers of the WDR62 gene. The DNA (microsatellite) markers used for identification were: D19S224, D19S876, D19S220 for the WDR62 gene. Results showed that three Pakistani families linked to the WDR62 gene (family A, family B and family D) while one family showed exclusion for the WDR62 gene (family C) as shown in fig. 4.6, 4.7, 4.8 and 4.9.

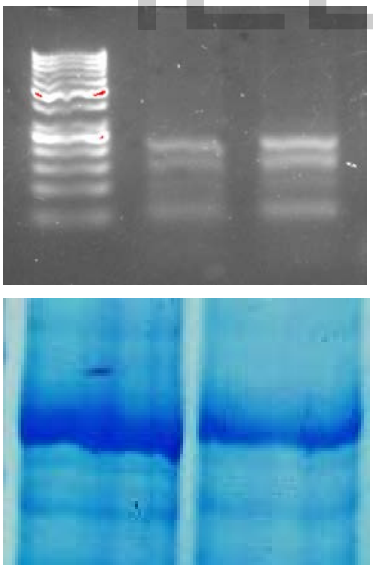

Figure 4.6: DNA amplification and Polyacrylamide gel for marker D19S224 showing family A linkage to WDR62 gene

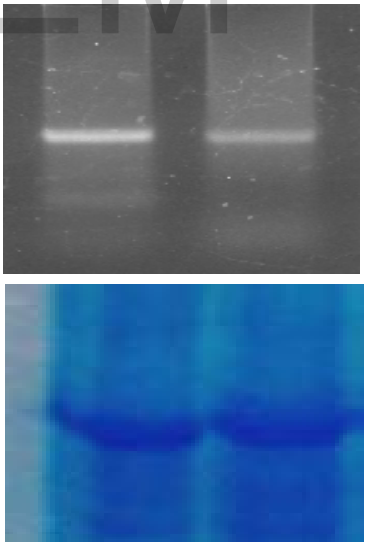

Figure 4.7: DNAamplification and Polyacrylamide gel for marker D19S224 showing linkage of family B to WDR62 gene

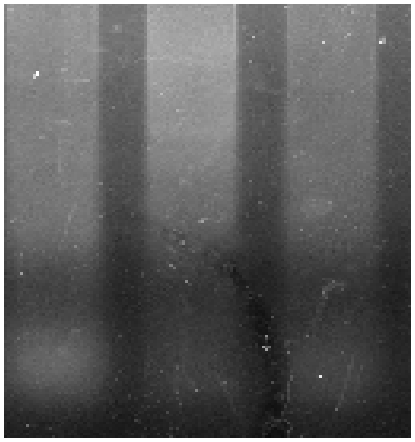

Figure 4.8: Family C, WDR62 not amplified

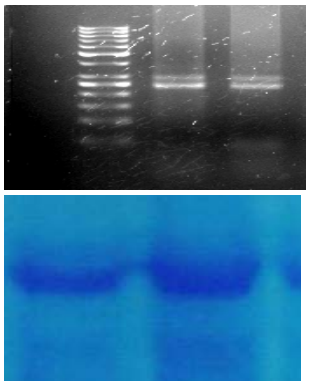

Figure 4.9: DNA amplification and Polyacrylamide gel for marker D19S224 showing linkage of family D with WDR62 gene

4 DISCUSSION

Microcephaly is more prevalent in Pakistan due to consanguineous marriages. The primary objective of this research was to raise awareness among the population of the less advanced regions of central Punjab (Pakistan). Another objective was to provide genetic counseling to households. The family history of infected families was obtained and pedigree was used to obtain the estimated prevalence of microcephaly among newborns due to consanguineous marriages. Pedigree estimates that the risk of microcephaly increases to a ratio of 2:3 due to consanguineous marriage after 3rd or 4th generations. Indeed, consanguineous marriages have played a harmful role in causing high rates of malformation, one of which is microcephaly [7].

Microcephaly mutations in the WRD62 gene have been reported [19]. The gene WDR62 has a genomic size of 50,230 and exons 32, located on chromosome 19q 13.12 inside the nucleus of MCPH2 [20]. This gene is a repetitive WD40 protein that is produced in an increased mind and mitotic position in the neuron on the neural ancestry. It was discovered in the dividing cells of the spindle poles. Variations in the structure of this gene indicate its significant function in the distinctive characteristics of cerebral cortical development, which is the cause of serious brain malformations [21]. Our results also found a muted WRD62 gene in patients of three microcephalic-infected families. The family C genomic analysis showed no relationship between microcephaly and the WRD62 gene that provides information that other loci may be involved in the prevalence of microcephaly in these patients.

5 CONCLUSIONS

Autosomal primary recessive microcephaly is a common genetic disorder of consanguineous marriage in Pakistan. Consanguineous marriages increase the risk of microcephaly generation after generation. The mutation of the WDR62 gene is one of the causes of microcephaly. Our study concludes that the WDR62 gene is the main cause of microcephaly in families belonging to central Punjab (Pakistan).

Declaration of conflicting interests

The first two author(s) declared no potential conflicts of interests with respect to the research authorship and/or publication of this article.

REFEERENCES

[1] H. Hamamy, "Consanguineous marriages," *Journal of community genetics,* vol. 3, pp. 185-192, 2012.
[2] A. Bittles, "Commentary: the background and outcomes of the first-cousin marriage controversy in Great Britain," *International Journal of Epidemiology,* vol. 38, pp. 1453-1458, 2009.

[3] R. L. Bennett, A. G. Motulsky, A. Bittles, L. Hudgins, S. Uhrich, D. L. Doyle*, et al.*, "Genetic counseling and screening of consanguineous couples and their offspring: recommendations of the National Society of Genetic Counselors," *Journal of genetic counseling,* vol. 11, pp. 97-119, 2002.
[4] S. Mahmood, W. Ahmad, and M. J. Hassan, "Autosomal recessive primary microcephaly (MCPH): clinical manifestations, genetic heterogeneity and mutation continuum," *Orphanet journal of rare diseases,* vol. 6, p. 39, 2011.
[5] A. Saadi, G. Borck, N. Boddaert, M. C. Chekkour, B. Imessaoudene, A. Munnich*, et al.*, "Compound heterozygous ASPM mutations associated with microcephaly and simplified cortical gyration in a consanguineous Algerian family," *European journal of medical genetics,* vol. 52, pp. 180-184, 2009.
[6] J. D. M. Abramowicz and Y. Tunca, "Novel mutations in prenatal diagnosis of primary microcephaly," *Prenat Diagn,* vol. 26, pp. 989-994, 2006.
[7] A. Saki-Malehi, G. Seddigh-Rad, A. Sayyahi, F. Mousavi-Far, M. Veysi, and F. Rahim, "The pattern of inherited microcephaly and role of the consanguineous marriage: A study from Southwestern Iran," *Ethiopian Journal of Health Development,* vol. 31, pp. 119-123, 2017.
[8] G. K. Thornton and C. G. Woods, "Primary microcephaly: do all roads lead to Rome?," *Trends in Genetics,* vol. 25, pp. 501-510, 2009.
[9] P. Bhide and A. Kar, "Birth prevalence of microcephaly in India," *Bull World Health Organ,* vol. 23, 2016.
[10] W. K. de Oliveira, J. Cortez-Escalante, W. T. G. H. De Oliveira, G. M. I. d. Carmo, C. M. P. Henriques, G. E. Coelho*, et al.*, "Increase in reported prevalence of microcephaly in infants born to women living in areas with confirmed Zika virus transmission during the first trimester of pregnancy—Brazil, 2015," *Morbidity and Mortality Weekly Report,* vol. 65, pp. 242-247, 2016.
[11] J. K. Morris, J. Rankin, E. Garne, M. Loane, R. Greenlees, M.-C. Addor*, et al.*, "Prevalence of microcephaly in Europe: population based study," *bmj,* vol. 354, p. i4721, 2016.
[12] M. Khalid, S. A. Khan, and A. A. Ahmed, "Zika Virus in Pakistan: A Looming Adversity?," *Journal of the College of Physicians and Surgeons--Pakistan: JCPSP,* vol. 26, p. 943, 2016.
[13] R. Kousar, M. J. Hassan, B. Khan, S. Basit, S. Mahmood, A. Mir*, et al.*, "Mutations in WDR62 gene in Pakistani families with autosomal recessive primary microcephaly," *BMC neurology,* vol. 11, p. 119, 2011.
[14] A. K. Nicholas, M. Khurshid, J. Désir, O. P. Carvalho, J. J. Cox, G. Thornton*, et al.*, "WDR62 is associated with the spindle pole and is mutated in human microcephaly," *Nature genetics,* vol. 42, pp. 1010-1014, 2010.
[15] K. K. Ganta and B. Chaubey, "Endoplasmic reticulum stress leads to mitochondria-mediated apoptosis in cells treated with anti-HIV protease inhibitor ritonavir," *Cell biology and toxicology,* vol. 35, pp. 189-204, 2019.
[16] P. Y. Lee, J. Costumbrado, C. Y. Hsu, and Y. H. Kim, "Agarose gel electrophoresis for the separation of DNA fragments," *J Vis Exp,* Apr 20 2012.
[17] W. Li, X. Zheng, L. Ren, W. Fu, J. Liu, J. Xv*, et al.*, "Epigenetic hypomethylation and upregulation of GD3s in triple negative breast cancer," *Ann Transl Med,* vol. 7, p. 723, Dec 2019.
[18] M. R. Green and J. Sambrook, "Polymerase Chain Reaction," *Cold Spring Harb Protoc,* vol. 2019, Jun 3 2019.
[19] V. Bhat, S. C. Girimaji, G. Mohan, H. R. Arvinda, P. Singhmar, M. R. Duvvari*, et al.*, "Mutations in WDR62, encoding a centrosomal and nuclear protein, in Indian primary microcephaly families with cortical malformations," *Clin Genet,* vol. 80, pp. 532-40, Dec 2011.
[20] K. Bilguvar, A. K. Ozturk, A. Louvi, K. Y. Kwan, M. Choi, B. Tatli*, et al.*, "Whole-exome sequencing identifies recessive WDR62 mutations in severe brain malformations," *Nature,* vol. 467, pp. 207-10, Sep 9 2010.
[21] T. W. Yu, G. H. Mochida, D. J. Tischfield, S. K. Sgaier, L. Flores-Sarnat, C. M. Sergi*, et al.*, "Mutations in WDR62, encoding a centrosome-associated protein, cause microcephaly with simplified gyri and abnormal cortical architecture," *Nat Genet,* vol. 42, pp. 1015-20, Nov 2010.