Mutational analysis of FOLR1 and FOLR2 genes in children with Myelomeningocele

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Myelomeningocele (MMC) is a congenital disease. For a long time, molecular mechanism of MMC, the role of folate receptor and transporter proteins remain unclear. Folate from maternal lumen to developing embryo is carried out with the help of folate transporters (SLC46A1, SLC19A1, FOLH1 and SLC25A32) and folate receptor (FOLR1, FOLR2 and FOLR3). Due to the loss of function of these important genes, complications can facilitate the risk of MMC. This study focused on the mutational analysis of FOLR1 and FOLR2 genes in children suffering from MMC. Myelomeningocele is a rare disorder so twenty blood samples from the children were collected. Primers of selected exons for FOLR1 and FOLR2 genes were designed with the help of PrimerFox software. Extracted DNA was amplified, and PCR based mutational analysis was done to check any type of mutation/SNPs in these genes. Sanger sequencing method was performed to confirm mutation in FOLR1 and FOLR2 genes. The results showed that certain environmental factors (smoking, low socio-economic status of mother bearing MMC fetus) were found to be significantly (P<0.05) associated with MMC but no mutation in the selected exons of FOLR1 and FOLR2 genes was detected. Thus, genetic variations in the folate transporter gene may have no role in the progression of MMC in the studied population.

Keywords: FOLR1, FOLR2, Congenital, Myelomeningocele, phenotype, mutation

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Abbreviations: CSF, Cerebrospinal fluid; FOLR1, Folate Receptor Alpha; FOLR2, Folate Receptor Beta; MMC, Myelomeningocele

INTRODUCTION

One of the most important and congenital forms of spina bifida is Myelomeningocele (MMC). In this disease, two sides of backbone become unable to fuse with one another and forms a gap between them (Hassan et al., 2022). This usually happens after about 2–4 weeks of gestation. Due to the failure of fusion of both backbone side nerves, spinal cord and meninges remain uncovered. This can lead to the partial or complete paralysis of the body. Partial paralysis happens in the lower portion of the body from the point of opening (Moldenhauer & Adzick, 2017). The evidence of this disease in first-degree relatives and second-degree relatives is about 3–4% and 1–2%, respectively (Farmer et al., 2018). Genomic and environmental factors are responsible for this situation. Folic acid plays a vital role in the organ development of fetus in placenta (O'Byrne et al., 2010). Genes involved in folate transport play an important contribution in the development of MMC. Other environmental factors such as deficiency of vitamins, certain medications, tobacco usage, drug addiction, maternal infections, maternal hyperthermia and exposure to some metals play vital role in tube defects in developing fetus (Naveed et al., 2023; Muhammad et al., 2023; Naveed et al., 2022; Tauheed et al., 2017).

Studies on animal models illustrate involvement of about 200 genes in tube defects (Cavalheiro et al., 2021). It is proposed that certain genes and their variants make an interaction with each other and, being affected by environmental factors, cause MMC (Mazumdar et al., 2015). Certain studies highlighted certain mutations and/or SNPs i.e. rs223622 and rs180113 in the MTHFR gene, MTHFD1 gene, rs13908 in FOLR2 and rs792687, rs792554 and rs792698 in FOLR3 gene (Ntimbani et al., 2020). These mutations have been reported in different ethnic groups, such as Dutch, Italian, and British population. The role of folate transporter genes has already been documented worldwide (Wolujewicz et al., 2021). Unfortunately, little work has been done on this among the Pakistani population so far. The main objective of this study is to check the role of environmental factors and to perform the mutational analysis of FOLR1 and FOLR2 genes with reference to Myelomeningocele.

METHODOLOGY

All the patients in this study had less than 2 years of age. Before the procedure, informed consent of the parents was taken. The skin of the patients was sterilized with alcohol swabs. About 2 to 3cc blood of MMC patients was collected with the help of sterilized syringes and stored in refrigerator at 4°C in an EDTA vacutainer. The sample of mothers of MMC patients was also collected when it was possible. DNA extraction was done by using the standard method (Albertsen et al., 2015). Forward and reverse primers were prepared for exons 3–4 and 5 for FOLR1 gene, and exons 3–4, 5 of FOLR2 gene. Primers were designed by using PrimerFox Software (Table 1).
The standard PCR procedure was followed for the amplification of FOLR1 and FOLR2 genes (Lorenz, 2012). The denaturation temperature was 95°C for both FOLR1 and FOLR2 genes and it was done for 4 minutes. The annealing temperature for FOLR1 gene was 58°C for 45 seconds and 51–59°C for FOLR2 gene and it was done for about 45 seconds. Elongation temperature was 72°C and it was done for one minute and finally for 4 minutes. To get the appropriate amount of PCR product, 35 cycles of PCR were run. Holding temperature of the PCR vials was 4°C. Afterward, the PCR product was loaded on agarose gel and visualized with the help of UV light. To rule out mutation sequencing of the amplified products Sanger Sequencing method was done. Statistical analysis was done with the use of GraphPad Prism software v13.

**RESULTS**

Current study includes 20 MMC patients. Most of the patients lie within the age group of (1–2 years). Patients usually belong to Northern and Southern Punjab and most of the mothers were malnutritional due to poverty as shown in Fig. 1. The statistical analysis was done to check the association of different environmental factors (Smoking, weight/age of the mother, Folic acid intake, history of abortion and family socio-economic status) with MMC. Smoking and poor socio-economic status of

![Figure 1. Age groups, geographical location and economic background of subjects](image-url)
the mother was found to be significantly associated with MMC as the \( P \)-value is less than 0.05 (Table 2).

If we look at the clinical symptoms, 70% (\( n=14 \)) of the patients were hydrocephalus. The position of Myelomeningocele varies among patients, 35% (\( n=7 \)) had in cervical region, 60% (\( n=12 \)) covered lumbar region while 5% (\( n=1 \)) showed in both cervical and lumbar regions (Table 3).

Primer was designed for exon 5 of FOLR1 gene. After performing PCR, PCR product was visualized on 1.5% agarose gel. There was a clear bright band on 494bp location which indicates no mutation in it (Fig. 2). Further, sequencing was done, and the chromatogram also showed no mutations (Fig. 3). Exons 3-4 of FOLR1 gene primers were also designed and the band of interest was of 679bp. This clearly indicates no mutation in these exons as well (Fig. 4). This was also confirmed by the chromatogram of these exons in FOLR1 gene (Fig. 5).

### Table 3. Location of MMC and hydrocephalus common in the patients \( (n=20) \)

<table>
<thead>
<tr>
<th>Location</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumber</td>
<td>12 (60%)</td>
</tr>
<tr>
<td>Cervical</td>
<td>7 (35%)</td>
</tr>
<tr>
<td>Both</td>
<td>1 (5%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>20 (100%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hydrocephalus</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>14 (70%)</td>
</tr>
<tr>
<td>Yes</td>
<td>6 (30%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>20 (100%)</td>
</tr>
</tbody>
</table>

![Figure 2. Amplified DNA band of Exon 5 FOLR1 gene in patients](image)

![Figure 3. Chromatogram of Exon 5 FOLR1 gene in MMC patients](image)

![Figure 4. Amplified DNA Band of exon 3–4 of FOLR1 gene in patients](image)

![Figure 5. Chromatogram of Exon 3–4 FOLR1 in patients](image)

![Figure 6. Amplified DNA Band of exon 3–4 of FOLR2 gene in patients](image)

![Figure 7. Amplified DNA Band of exon 5 of FOLR2 gene in patients](image)
DISCUSSION

Myelomeningocele is an important congenital disorder. It usually occurs after the 2–4 weeks of pregnancy. Folic acid deficiency can lead towards the exaggeration of MMC disease. Folate is needed for fetal organ development (Mazumdar et al., 2015). Its demand increases significantly during pregnancy and is found to be significantly associated with MMC. Due to pediatric neurodegeneration resulting from neural folate deficiency, the role of FR can significantly inhibit folate uptake in the CSF (Canfield et al., 2009). In the present study, various environmental factors like folic acid intake, body weight and age of mother, and history of abortion were found to be insignificantly associated with MMC (P>0.05). However smoking habit in mother in the form of hookah, cigarettes, sheesha and burning of fossil fuels was found to be significantly associated with MMC. Similarly, a poor family socio-economic status was found to be significantly associated with MMC (P<0.05). (Nageen et al., 2023; Hussain et al., 2023).

Defects in folic acid receptor or transport protein can lead towards the MMC disorder. Many studies have been done for the mutational analysis of FOLR1 and FOLR2 genes but only a few studies have reported some mutations in these genes. Studies done in 2014 and 2020 indicated some novel mutations in these genes. (Tempeny et al., 2014; Steele et al., 2020). In 2017, a study was done in the United States of America and it showed the down regulation of FOLR1 gene in MMC patients (Findley et al., 2017). In 2020, a study confirmed twelve new mutations in Folate receptor genes (Hillman et al., 2020). This proved the direct link of FOLR1 protein in the disease. Just like Boyles et al., the present study also showed no mutation in FOLR1 and FOLR2 genes (Boyles et al., 2006).

In 2022, another study showed the low level of FOLR1 protein (known as glycosyl-phosphatidylinositol-anchored plasma membrane protein) in MMC patients as compared to healthy people. FOLR2 gene is present on chromosome 11 and belongs to the folate receptor family, producing folate receptor beta protein. FRβ, is a member of this family of reduced folate receptor family, producing folate receptor beta protein. FRβ, is a member of this family of reduced folate receptor protein (Han et al., 2022). In the present study, MMC patients showed no alteration in the cluster of Folate receptor protein FOLR2 (Fig. 10).

CONCLUSION

This study showed no mutation in FOLR1 gene exons 3–4 and 5, and FOLR2 gene exons 3–4 and 5. This study emphasises the significance of maternal health and dietary consumption in determining the trajectory of these receptor genes, especially FOLR1 and FOLR2, given the critical role of folate in satisfying the developmental demands of a growing fetus. The results of this study indicate that genetic variations in the folate receptor genes may not significantly contribute to the development of MMC (Myelomeningocele), but environmental factors are more important in this situation. The limitation of this study includes small sample size, as it is a rare disease. Furthermore, this study lacks ultrasound report of the infants or toddlers, maternal folic acid levels and other blood profile data during and before conceiving.

Declarations


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