Evaluation of maternal serum folate, vitamin B12, and homocysteine levels and factor V Leiden, factor II g.20210G>A, and MTHFR variations in prenatally diagnosed neural tube defects

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1. Introduction

Neural tube defects (NTDs) are a spectrum of defects that arise as a result of nonclosure of the neural tube until 6 weeks of gestation (1). They are frequent congenital malformations affecting 0.5–2 per 1000 pregnancies (2). Several types of NTDs are recognized. Major types include acrania, anencephaly, spina bifida, craniorachischisis, encephalocele, and menencephaly. These defects are caused by multiple factors involving genetic predisposition and by environmental factors such as nutritional deficiencies and drug exposure (2).

Despite the fact that more than 200 genes are known to cause NTDs in rats, human studies have been limited in identifying the underlying molecular genetic basis. An association has been found between NTDs and 5,10-methylene-tetrahydrofolate reductase (MTHFR) variations, which lead to defective homocysteine metabolism. It has been shown that supplementation of folate and vitamin B12, which are coenzymes in the homocysteine metabolic pathway, is effective in reducing the risk of NTDs (3). Similar to MTHFR variations, factor V Leiden and factor II g.20210G>A variations also predispose to thrombophilia. However, these variations have not been associated with NTD risk.

Intake of folate during pregnancy is widely advised in Turkey. In order to demonstrate the importance of predisposing factors in the Turkish population, we investigated the effects of previously known and suspected factors in the etiology of NTDs. For this purpose, serum levels of folate, vitamin B12, and homocysteine, as well as genetic variations including factor V Leiden, factor II g.20210G>A, MTHFR c.677C>T, and MTHFR c.1298A>C, were determined in pregnant Turkish women with fetal NTDs and in healthy pregnant women in order to point out significant differences.
2. Materials and methods

2.1. Subjects

Pregnant women with fetal NTDs and the controls were retrospectively evaluated at the Zeynep Kamil Women's and Children's Diseases Training and Research Hospital, Istanbul, Turkey. Thirty-five pregnant women with fetal NTDs between 14 and 24 weeks of gestation constituted the NTD group. The diagnosis of NTD was confirmed after termination or delivery for each subject. The NTD group was made up of women who had isolated fetal spina bifida or craniorachischisis and no maternal chronic diseases. Pregnant women were included in the NTD group regardless of their folate usage during pregnancy.

The control group consisted of 40 unrelated individuals applying for different reasons. Women in the control group were observed throughout their pregnancies and did not have any maternal chronic diseases, fetal karyotype anomalies, or multiple gestations, and their pregnancies were not complicated by intrauterine growth retardation or polyhydramnios. The children born to women in the control group had a birth weight between the 10th and the 90th percentile.

Each woman was followed with regular ultrasound evaluations, which were obtained transabdominally using an abdominal 2–5 MHz curvilinear transducer (Voluson, General Electric, Milwaukee, WI, USA). In each ultrasound evaluation, biparietal diameter, head and abdominal circumference, and femur and humerus length were measured in the standard planes for fetal biometric evaluation, and a routine scan of fetal anatomy was performed.

Serum homocysteine levels (Abbott IMx Fluorescence Polarization Immunoassay, USA), serum folate (Abbott Architect Folate, USA), and vitamin B12 (Abbott Architect B12, USA) were quantified according to the manufacturer's protocol. Measurements were made with the Abbott Architect I1000 Immunoassay Analyzer.

Homocysteine levels in 10 women, vitamin B12 levels in 1 woman, and folate levels in 2 women from the NTD group were not present in their medical records. In addition, homocysteine levels in 7 women, vitamin B12 levels in 3 women, and folate levels in 3 women from the control group were not present in their medical records.

2.2. Genotyping

Genomic DNA was isolated from peripheral blood leukocytes using the Magnesia DNA Blood Kit (Anatolia Inc., Turkey). Factor V Leiden, factor II g.20210G>A, MTHFR c.677C>T, and MTHFR c.1298A>C between pregnant women with fetal NTDs and the control group. The Wilcoxon W and Mann–Whitney U tests were employed to compare serum homocysteine, folate, and vitamin B12 levels between groups. Statistical significance was established at P < 0.05.

3. Results

There were no known factors in patient history associated with NTDs, such as exposure to radiation, maternal anticonvulsant or chemical substance usage, and diabetes mellitus. The mean age of the pregnant women was 28 years (range: 20–39) and 30 years (range: 22–35) in the NTD group and control group, respectively. The mean gestational week of women in the NTD group was 18 weeks (range: 14–24), whereas that of women in the control group was 19 weeks (range: 16–23). There was no significant difference in maternal age or gestational week between groups (P = 0.207).

According to our data, factor II g.20210G>A, MTHFR c.677C>T, and MTHFR c.1298A>C genotype frequencies in the NTD group were similar to those of the controls (P = 0.521, P = 0.706, and P = 0.405, respectively) (Table 1). However, factor V Leiden genotype frequency was significantly higher in the NTD group than in the control group (P = 0.029, 95% CI = [0.014137–1.086414], odds ratio = 0.123932) (Table 1).

Additionally, there was a significant difference in serum vitamin B12 levels between groups (P = 0.006) (Table 2). On the other hand, serum folate and homocysteine levels were not significantly higher in the NTD group compared to the controls (P = 0.223 and P = 0.115, respectively) (Table 2).

4. Discussion

NTDs are among the most common birth defects. They are defects of the spinal cord or brain that result from failure of neural tube closure in the early stages of fetal development (2).

NTDs are multifactorial disorders with many genetic and environmental factors defined in their etiology. Chromosomal abnormalities can cause NTDs, although these represent only 2%–16% of isolated NTDs (4). Women bearing fetuses with chromosomal abnormalities were excluded from this study in order to prevent differences between NTD and control groups that may arise as a result of different chromosomal backgrounds.

Folate cycles between molecules in certain biological reactions carry one-carbon groups from other molecules.
to homocysteine in order to form methionine. This folate cycle is a vital biochemical reaction required for proper DNA synthesis, repair, and methylation. Thus, low folate levels can directly limit its availability to cells or indirectly disrupt methionine metabolism, thereby increasing homocysteine. MTHFR can influence serum homocysteine levels, especially in inadequacy of folate. Increased homocysteine and MTHFR variations play significant roles in NTD. Additionally, both folate and vitamin B12 insufficiency can contribute to increased risk of NTDs. As previously shown, folate given before and during the first 4 weeks of pregnancy can prevent 50% or more of NTDs. Folate availability is thought to play an important role in phenotypic expression of MTHFR mutations (5–7).

Vitamin B12 is also an important factor in folate metabolism. A previous study has shown that vitamin B12 deficiency also contributes to the risk of NTDs (8). When vitamin B12 levels are low, the resulting decrease in methylation activity is thought to account for some of the deleterious effects of vitamin B12 deficiency in fetal development (9). In a previous study, lower levels of maternal vitamin B12 were linked to higher NTD occurrence, and authors argued that supplementing vitamin B12 with folate during pregnancy may further decrease NTD risk in the fetus (10).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>FV c.1691GG</td>
<td>29/35 (82.9%)</td>
<td>39/40 (97.5%)</td>
</tr>
<tr>
<td>FV c.1691GA</td>
<td>6/35 (17.1%)</td>
<td>1/40 (2.5%)</td>
</tr>
<tr>
<td>FV c.1691AA</td>
<td>0/35 (0%)</td>
<td>0/40 (0%)</td>
</tr>
<tr>
<td>P-value</td>
<td>0.029</td>
<td></td>
</tr>
<tr>
<td>FII g.20210GG</td>
<td>35/35 (100%)</td>
<td>37/40 (97.5%)</td>
</tr>
<tr>
<td>FII g.20210GA</td>
<td>0/35 (0%)</td>
<td>1/40 (2.5%)</td>
</tr>
<tr>
<td>FII g.20210AA</td>
<td>0/35 (0%)</td>
<td>0/40 (0%)</td>
</tr>
<tr>
<td>P-value</td>
<td>0.521</td>
<td></td>
</tr>
<tr>
<td>MTHFR c.677CC</td>
<td>16/35 (40.0%)</td>
<td>15/40 (39.5%)</td>
</tr>
<tr>
<td>MTHFR c.677CT</td>
<td>16/35 (42.5%)</td>
<td>21/40 (52.6%)</td>
</tr>
<tr>
<td>MTHFR c.677TT</td>
<td>3/35 (7.5%)</td>
<td>3/40 (7.9%)</td>
</tr>
<tr>
<td>P-value</td>
<td>0.706</td>
<td></td>
</tr>
<tr>
<td>MTHFR c.1298AA</td>
<td>16/35 (45.7%)</td>
<td>13/36 (36.1%)</td>
</tr>
<tr>
<td>MTHFR c.1298AC</td>
<td>15/35 (42.9%)</td>
<td>17/36 (47.2%)</td>
</tr>
<tr>
<td>MTHFR c.1298CC</td>
<td>4/35 (11.4%)</td>
<td>6/36 (16.7%)</td>
</tr>
<tr>
<td>P-value</td>
<td>0.405</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.** The distribution of genotype frequencies and P values between NTD mothers and controls.

Table 2. The means and standard deviations of homocysteine, folate, and vitamin B12 levels and P-values between NTD mothers and controls.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Homocysteine (µmol/L)</th>
<th>Folate (ng/mL)</th>
<th>Vitamin B12 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>6.7 ± 1.7 (25 cases)</td>
<td>14.7 ± 7.0 (33 cases)</td>
<td>220 ± 67 (34 cases)</td>
</tr>
<tr>
<td>Control</td>
<td>6.0 ± 1.6 (33 cases)</td>
<td>18.1 ± 9.9 (37 cases)</td>
<td>260 ± 64 (37 cases)</td>
</tr>
<tr>
<td>P-value</td>
<td>0.115</td>
<td>0.223</td>
<td>0.006</td>
</tr>
</tbody>
</table>
In accordance with their common involvement in the same metabolic pathway, many previous studies have also found a relationship between maternal serum folate, vitamin B12, and homocysteine levels and NTD risk in humans. In a study of 107 pregnant women with NTDs and 275 controls, Cech et al. reported that median folate and vitamin B12 levels for the NTD group were significantly lower than for the controls (11). In another study by Zhang et al. comparing 82 women with fetal NTDs and 110 controls, a relation between increased risk of NTDs and lower serum levels of folate and vitamin B12 was shown (12). In a study of a different group, homocysteine and vitamin B12 levels were significantly different in the NTD group compared to the controls, whereas the median serum folate levels were similar. In this study, lower maternal serum vitamin B12 levels were associated with an approximately 2- to 3-fold increase in NTD risk (13). According to Gu et al. (14), homocysteine levels were significantly higher in the NTD group than in the control group, with an accompanying lower serum folate and vitamin B12 level in the NTD group. In a study of homocysteine in 103 cases and 139 controls, high serum levels were associated with NTD pregnancies. According to this study, high homocysteine levels have a deleterious effect on proper neural tube closure, even when serum vitamin B12 or folate levels are high (15).

On the contrary, several other studies have questioned this relationship. In two different investigations, Greene et al. (16) and Bennett et al. (17) reported that an increase in NTD incidence was not observed in mouse embryos exposed to high levels of homocysteine. Mobasheri et al. (18) reported that levels of folate and vitamin B12 did not significantly affect NTD risk in 23 women with NTD fetuses and 23 healthy controls. According to this study, high homocysteine levels have a deleterious effect on proper neural tube closure, even when serum vitamin B12 or folate levels are high (15).

In this study, significantly lower maternal serum vitamin B12 levels (220 ± 67 vs. 260 ± 64; P = 0.006) was found in pregnancies with fetal NTDs compared to controls. However, we did not observe a significant difference between serum folate and homocysteine levels. Previously, many studies reported a significant difference in maternal folate and homocysteine levels between NTD pregnancies and controls, which was not the case in our study. The reason for this may be the supplementation of folate before pregnancy in our study population.

This study shows that folate supplementation alone is not sufficient for the prevention of NTDs. In addition to folate, supplementation of vitamin B12 may lower NTD risk more efficiently. However, vitamin B12 levels were not low in the entire NTD group, which suggests that factors other than lack of vitamin B12 are also important for increased NTD risk. Future investigations to determine the contribution of other coenzymes and cofactors affecting methionine-homocysteine metabolism to NTD risk might help us understand NTD pathogenesis in more detail and provide us with additional dietary tools to allow for a decrease in NTD risk.

Among folate metabolism-related genes, MTHFR has been the principal focus of attention. Previous studies have shown that the c.677C>T and c.1298A>C variations are associated with increased risk of NTDs (20,21). Studies also suggest that the c.677TT genotype confers a 2- to 3-fold increased risk of NTDs (20,22). The c.1298A>C variation is also a risk factor for NTDs, though with a smaller contribution compared to c.677C>T variation. Indeed, the frequency of the c.1298A>C variation in children with NTD tends to be increased, which also suggests that this mutation is a genetic risk factor (23).

Another study by Liu et al. (24) showed that MTHFR c.677C>T variation was significantly higher in the tissue or blood samples of 609 aborted fetuses with NTD compared to 1106 parental blood samples as controls. However, this study failed to show any significant effect of folate supplementation and gestational diabetes mellitus on NTD occurrence (24). In a similar study, 51 women who had children with NTDs had a significantly different genotype distribution for MTHFR c.677C>T variation compared to 51 age-matched women with healthy children (25).

In a study from Turkey, Boduroğlu et al. (26) reported that the MTHFR c.1298AA/c.677TT genotype was significantly more frequent in 95 children with NTDs than in the 93 healthy controls.

On the other hand, Candito et al. (27) reported that MTHFR c.677C>T and c.1298A>C variations were not associated with increased NTD risk in fetuses, following the comparison of 77 women with severe fetal NTDs and 61 women with normal pregnancies.

In this study, frequencies of MTHFR c.677C>T and c.1298A>C variations did not differ significantly between NTD and control groups. This shows that these variations in pregnant women do not confer an increased fetal NTD risk in our study population.

Factor V Leiden variation is an important risk factor for hereditary thrombophilia. Thrombophilia-related conditions such as thromboembolism and deep vein thrombosis have also been associated with factor V Leiden. However, Ceyhan et al. showed no relationship between factor V Leiden or factor II g.20210G>A variations and spina bifida. On the other hand, they suggested that the presence of MTHFR c.677C>T variation increased the risk of spina bifida (28). In our study, factor II g.20210G>A, MTHFR c.677C>T, and c.1298A>C are not significantly related with NTDs, whereas factor V Leiden variation significantly increased the risk of NTDs.
To the best of our knowledge, this is the first study showing a possible relationship between factor V Leiden variation and NTD risk. A possible mechanism that can explain the increased risk of NTDs in factor V Leiden-carrying mothers may be insufficient nourishment of the fetus during critical stages of development and closure of the neural tube due to placental insufficiency. It has been shown previously that genetic thrombophilia causes placental insufficiency resulting in intrauterine growth retardation (29). In addition, it has been reported that mothers with factor V Leiden variation are more prone to having children with fetal ischemia-related conditions such as cerebral palsy or perinatal arterial ischemic stroke (30,31). However, further investigation is needed to show that increased NTD risk for factor V Leiden variation carriers found in this study is not coincidental, and to establish a causal link explaining this relationship. Lack of association between factor II and NTDs may be due to the very small number of patients with factor II g.20210G>A in the study.

As the result of this study, we suggest that intake of folate alone is not sufficient in the prevention of NTDs; instead, folate intake of vitamin B12 is also important. A dietary supplement combining folate and vitamin B12 might be an effective measure to decrease the NTD incidence in these areas. Moreover, further investigations are required for identifying the interaction between factor V Leiden and NTDs.

References

7. [No authors listed.] Recommendations for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects. MMWR Recom Rec 1992: 41: 1–7.