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Gene Expression Profile Associated with Neural Tube Defects among Infants at a Tertiary Centre in Tanzania

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Abstract

Background

Neural tube defects (NTDs) are severe congenital anomalies of the central nervous system. The specific cause is not known, though several factors including gene variants involving the folate metabolism have been implicated in the etiology. This study aimed at identifying the gene expression profile of selected genes known to be associated with NTDs among infants attending Muhimbili National Hospital (MNH) and Muhimbili Orthopaedic Institute (MOI).

Methods

We conducted a matched case control study involving infants who were attending MNH and MOI during 6 months of the study period. Each case was matched with two controls by gestational age, sex and birth weight. Whole blood samples were collected from all study participants for genetic analysis. Total RNA was isolated using Qiagen RNA blood mini kit, and reversed transcribed into complementary DNA (cDNA) using Super Script II Reverse Transcriptase cDNA Synthesis kit. Real-time polymerase chain reaction was performed on extracted cDNA by a Light Thermal Cycler 480 machine using specific primers for studied genes to determine their expression levels. Results were analysed by GraphPad Prism 5 Software, using Student *t*-test, and Bonferroni post hoc statistical tests. A p value of < 0.05 was considered to be statistically significant.

Results

The study recruited 50 cases and 100 controls. Among eight studied genes, we found significantly low expression of Methylenetetrahydrofolate reductase *[MTHFR]* gene among cases than controls (p=0.006). Expression of others genes were having variations and expressed at very low levels in both cases and controls.

Conclusion

A low expression level of *MTHFR* gene was a significant risk factor for the occurrence of NTDs amongst infants attending MNH and MOI. We recommend further DNA sequencing studies to determine exact genetic mutations that affect the low expression of *MTHR* gene amongst infants with NTDs in our setting.

Key words: *Neural tube defects, gene expression profile, Methylenetetrahydrofolate reductase [MTHFR] gene.*

Background

Neural tube defects (NTDs) are congenital malformations of the central nervous system including: the brain, spinal cord, and skull. These result from failure of the neural tube to close properly during fetal embryogenesis around the third and fourth week of conception(1). Globally, 300,000 babies are born with NTDs per year(2), with the prevalence ranging from 0.2 to 10 per 1000 live births in specific geographical locations(3). In Tanzania, a study by Kishimba *et al* in 2015, reported NTDs to have the highest incidence of 9 per 10,000 live births among selected external structural defects(4).

The majority of NTDs have unknown etiology, involving complex interactions of genetic, nutritional and environmental factors(5). Despite normal or high folate supplementation pre and post conception, NTDs are still known to occur. There is evidence that folate deficiency changes epigenetics and coding of methionine during embryogenesis and is thus implicated in the occurrence of NTDs. Studies have shown that genetic mutations involving the folate metabolism and planar cell polarity (PCP) signaling pathways may be associated with an increased risk of NTDs(6). Gene variants that reduce the 'efficiency' of folate one-carbon metabolism have also been shown to increase the risk of NTDs. The Methylenetetrahydrofolate reductase (MTHFR) polymorphism C677T (rs1801133) has also been found to be associated with a 1.8-fold increased risk of NTDs, although this predisposition was detectable only in non-Hispanic populations(7). Similarly, a study done in the Irish population, Kirke *et al* (8) also found that both the homozygous (TT) and heterozygous (CT) genotypes for *MTHFR* C677T were associated with an increased risk of NTDs (odds ratio of 1.52 and 2.56 respectively). In contrast, Hayati *et al* (9) found no association between *MTHFR* C677T gene and NTDs in the Malaysian population as the homozygous genotype was absent in both cases and control groups.

Another significant risk factor detected is the Methylenetetrahydrofolate dehydrogenase (MTHFD-1) R653Q variant (rs2236225), a trifunctional enzyme that catalyses the conversion of tetrahydrofolate to 5,10-methylenetetrahydrofolate(10). Polymorphism in the Methylenetetrahydrofolate dehydrogenase-1-like(MTHFD-1-L), the gene for mitochondrial 10-formyl-tetrahydrofolate synthetase is also associated with an increased risk of NTDs(11).

Other genes such as Aminomethyltransferase (*AMT*) and Glycine dehydrogenase (*GLDC*) that encode enzymes of the glycine cleavage system have also been found to harbour a number of missense mutations resulting into NTDs. In the study done by Narisawa *et al* (12) in 2011, two *AMT* gene variants *c.589G>C(D197H)* and *c.850G>C(V284L*) were identified in anencephaly and spinal bifida patients respectively, but they were absent in controls.

Planar cell polarity (PCP) genes such as *VANGL1&2,Dvl2&Dvl3* which are essential for proper gastrulation in the neural tube formation in humans, have also been implicated as predisposing factors of isolated non-syndromic NTDs. Lei *et al* (13), detected 3 missense mutations in *VANGL 2* (*S84F (737C→T*), *R353C (1543C→T*) and *F437S (1796T→C*) from studying 163 stillborn babies and miscarried Chinese fetuses with NTDs, and these mutations were found to be absent in the control group. Similarly, Kibar *et al* (14) identified three Italian patients with sporadic and familial NTDs to have missense mutations in *VANGL1*(*V239I, R274Q, and M328T*) and these mutations were not identified in controls. Correspondingly, in a genetic study of a cohort of 473 NTD patients, De Marco *et al* (15) found 8 rare mutations (5 in *Dvl2* and 3 in *Dvl3*) in eight patients which were not present in the controls. Therefore, the evidence is highly suggestive of a genetic component as a contributory risk factor for NTDs.

There is limited data on genetic etiology of NTDs in sub Saharan Africa of which Tanzania is part and where the incidence of NTDs remains high. Based on the evidence, a genetic causation cannot be excluded, which prompted this study. Therefore, we conducted this study to determine gene expression profile for eight genes which have been implicated in NTDs causation from previous studies. The aim was to determine the genetic contribution to NTDs in our setting, which may serve as a basis for genetic counseling of couples, so as to reduce the incidence of NTDs.

Methods

A hospital-based matched case control study was conducted over a period of six months from November 2016 to April 2017 at the Muhimbili National Hospital and Muhimbili Orthopaedic Institute in, Dares Salaam Tanzania. All infants who were receiving neonatal, general paediatrics, and neurosurgical services, during the study period were eligible.

Sampling procedure and sample size

A total of 50 cases and 100 controls were enrolled using a 1: 2 ratio in order to reduce the chances of random phenotypic variance in genetics. A case was defined as infant with any type of NTDs and controls were those without detectable birth defects who met matching criteria. NTDs are a relatively rare condition and the genetic cause is not fully known. Hence we enrolled all cases who presented consecutively until the sample size was reached. Two controls who matched a case by gestational age, birth weight and sex and who met the inclusion criteria were also consecutively enrolled among infants attending MNH/ MOI who

had no obvious birth defects. Infants who met inclusion criteria but, were very sick or whose mother did not consent were excluded.

Outcome variable

In this study, the dependent variable was the presence of the NTDs among the infants whilst for the independent variables; we selected eight genes which have been implicated from previous studies in NTDs etiology as shown in the Table 1.

Blood collection and laboratory procedures

We collected whole blood samples from both cases and controls. The blood samples were taken under aseptic technique using a 2 milliliter syringe and 1 milliliter volume of blood was collected into an EDTA vacutainer tube. The vacutainer tubes were clearly labeled with the study identification number, time and date when the sample was drawn. The samples were placed in a cooler box and sent for genetic analysis within 2 hours of drawing the blood; to the MUHAS Genetic Laboratory. In the laboratory, the samples were stored in a freezer at -80ºC to ensure preservation of genetic materials before analysis**.**

RNA extraction and RT-PCR analysis

Total RNA for all 150 infants were prepared from the blood samples using QIAamp RNA Blood Mini Kit (Qiagen) following the manufacturer's recommendations, 1µg of the total RNA was reverse transcribed into cDNA using Super Script II Reverse Transcriptase First-Strand cDNA Synthesis kit in accordance to the manufacturer's protocol (Invitrogen). A real-time polymerase chain reaction(RT-PCR) was performed on extracted cDNA by a Light Cycler 480 Real Time PCR system (Roche) according to the manufacturer's protocol using primers indicated in the Table 1, and 2x SYBR Green Master Mix (Roche). *GAPDH* was used as the house-keeping gene (16) and the results were analysed using the delta-delta Ct method ($2^{-\Delta\Delta}$Ct method) (17) and presented as relative gene expressions. The PCR reaction was performed using standard RT-PCR conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 30seconds and 60°C for 1 minute.

Data analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA) using the standard two-tailed Student's *t*-test for two data sets and ANOVA followed by Bonferroni post hoc tests for multiple data sets. A *p*-value of < 0.05 was considered to be statistically significant.

Results

Socio- demographic characteristics of infants in the study population

A total of 50 cases and 100 controls participated in this study. Amongst the cases, 42 had spinal bifida, 4 encephalocoele and 4 had anencephaly. Most of the infants were <1month old (74%) with almost equal male to female ratio (52% male versus 48% female). The mean age of the participants was 1.78 ±3.2 months with an age range of 1 to 11 months. Majority of the infants (92%) were delivered at term, with 66% having normal birth weights (Table 1).

Table 1: Socio-demographic characteristics of 150 infants attending MNH and MOI

Child characteristic	Case(N=50)	Control(N=100)	Total (N=150)
Gender			
Male	26(52.0%)	52(52.0%)	78
Female	24(48.0%)	48(48.0%)	72
Gestational age			
Preterm	4(8.0%)	8(8.0%)	12
Term	46(92.0%)	92(92.0%)	138
Birth weight(kgs)			
< 2.5	5(10.0%)	10(10.0%)	15
2.5-3.5	33(66.0%)	66(66.0%)	99
>3.5	12(24.0%)	24(24.0%)	36
Mean age(months)	1.78 ± 3.259	1.78 ± 3.243	

Gene expression profile for eight (8) genes analysed in the study population

We included eight genes of folate and planar cell polarity pathways which were selected from previously published research on NTDs (Table 2). After gene expression analysis, we found that there was a significantly low level of expression of the *MTHFR* gene amongst the NTDs cases than amongst the controls (p=0.006). On the other hand, there were a lot of variations in the expression levels of other genes and these were expressed at very low levels in both controls and cases (Figure 1).

Table 2: Primer sequences used for RT PCR

Gene	Forward5'→3'	Reverse5'→3'
AMT	GTGTTGGATAATGCCCTGCT	ACCGAGATCTCCACACCATC
DVL-2	GCTTTTGCAGGTGAATGACA	GAGGAGGAACCTGGATAGGC
DVL-3	GCTAAATGGAACTGCGAAGG	CCGCTTGTGTCTTCTCATCA
GAPDH*	GTCAGTGGTGGACCTGACCT	TGCTGTAGCCAAATTCGTTG
MTFHD-1	TTCATCCCATGCACACCCAA	ATGCATGGGTGCACCAACTA

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MTFHD-1L	GGACCCACTTTTGGAGTGAA	ATGTCCCCAGTCAGGTGAAG
MTFHR	AACCTGCCACTCAGGTGTCTTG	TGACAGTTTGCTCCCCAGGCAC
VANGL-1	TTACCTCCGATCCTGTGGAG	AGGCCTTCATCCACATTCAG
VANGL-2	CCAAACAGTGGACATTGGTG	GACTGCAGCCTCATGACAAA

******** *GADPH [Glyceraldehyde-3-Phosphate Dehydrogenase] was used as an internal control to ensure similar amounts of cDNA in the PCR mix*

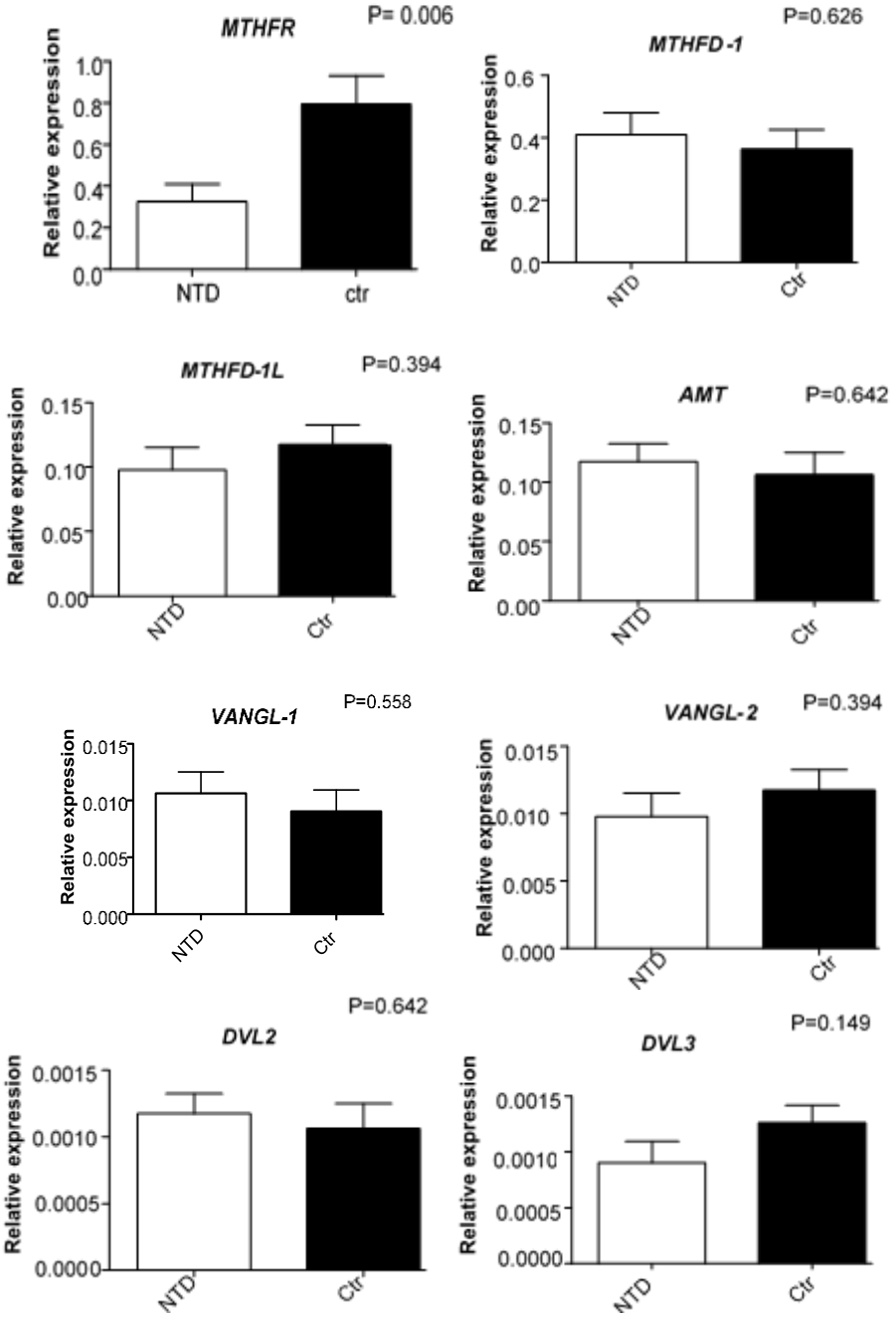

Figure 1: Relative gene expression profile for 8 selected genes analysed among 150 infants (NTD cases and controls) attending MNH and MOI

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Discussion

This study was conducted to determine the gene expression profile associated with NTDs among infants attending MNH and MOI. Findings revealed a significant low expression of *MTHFR* gene among cases than controls.

NTDs result from failure of closure of the neural tube during fetal embryogenesis. Evidence from studies done in different population groups strongly suggest that *MTHFR* polymorphism (*MTHFR C677T*) is associated with an increased risk of NTDs(7,8). In this study, DNA sequencing was not done, which is required for detecting specific gene mutation. However, the significantly low expression of *MTHFR* gene in the cases group could suggest the possibility of having variants with low enzyme activity amongst the cases with NTDs**.** Yan *et al* (18) in a meta-analysis of 2429 cases and 3570 controls, conducted in 2012 demonstrated that maternal MTHFR C677T polymorphism was associated with a 2-fold increased risk factors of NTDs in their offspring. The *MTHFR* gene plays a key role in folate and methylation cycles which are required for essential processes including; DNA synthesis, DNA/protein methylation, cell division, and tissue growth; especially in the rapidly developing cells(5). Thus, a defective gene variant could result in an impaired DNA synthesis or DNA methylation involved in the neurulation process which is essential during neural tube closure. Folate pathway genes have been studied for NTD causation, due to the role of folate in primary prevention**.** The folate one-carbon metabolism (FOCM) supports the biosynthesis of purines and pyrimidines for DNA synthesis during cell replication and the donation of methyl groups for regulation of gene, protein, and lipid function. Hence, genetic variants that reduce the efficiency of FOCM might increase the risk of NTDs either by compromising cell proliferation or the regulation of gene expression, or both (19). As a result, genetic factors are said to play a role in occurrence of NTDs. This was shown when fibroblast cells derived from fetuses with NTDs were subjected to deoxyuridine suppression test (dU), and NTD cell lines exhibited a lower suppression values of thymidine biosynthesis than control cell lines, indicative of inborn errors of folate metabolism (20).

Knowledge on the inherited factors for the genetic basis of NTDs remains incomplete; even with information on the genetic requirements for neural tube closure and neurulation. Genetic counseling for NTDs is still based on population recurrence risk values, and there are no genetic tests for prospective parents. Genotyping for the C677T polymorphism of *MTHFR* and other folate-related genetic variants is available, but interpretation of parental MTHFR genotype in terms of NTD risks in a future pregnancy in not yet defined. Therefore, full genome-wide assessment is required for potential risk variants including coding,

regulatory, and epigenetic modifications, in individuals with NTDs and their parents. However, this may still not explain the occurrence of sporadic NTDs or predict future risk of NTD in families. A research linking folate, vitamin B12 and other factors, with the genetic risk factors for NTDs is crucial so that a test with a predictive value for NTDs can be developed.

The study found no association of NTDs and the expression levels of other genes such as *VANGL1*, *VANGL2*, *Dvl2* and *Dvl3*. This is contrary to the studies done in China and Italy, which found a significant association between the incidence of NTDs and other genes in addition to *MTHFR* gene(10–15). This could be partly explained by the fact that none of these studies were done in Africa and also the other studies included aborted fetuses. Therefore, there is the likelihood that, gene expressions can be influenced by genetic diversity among different population groups and specific fetal developmental stages.

Strengths and limitations

This was a preliminary study, a first of its kind in Tanzania, whose objective was to screen for differences in the expression of implicated genes for NTDs between cases and controls, which has shown a genetic basis for NTDs; though the data is limited in functionally defining the genotypes and phenotypes which should be further investigated.

In this study, due to limited funding, further elucidation through DNA sequencing which is required for identifying specific genetic mutation/polymorphism could not be performed. Therefore, we cannot link the gene expression profile with specific gene variant/mutation(s) which might be a risk factor among the NTDs cases at the time of embryogenesis.

Conclusions

In conclusion, our findings suggest that a low expression level of *MTHFR* gene was a significant risk factor associated with NTDs amongst infants attending MNH and MOI. We recommend further DNA sequencing studies to determine exact genetic mutations that affect the low expression of *MTHR* gene amongst infants with NTDs in our setting.

Declarations

Ethical approval and consent to participate

Ethical clearance was obtained from the Institutional Review Board of Muhimbili University of Health and Allied Sciences (Ref.No.MU/PGS/SAEC/Vol.XVII/ and permission was sort from the Muhimbili National Hospital (Ref.No.MU/PGS/SAEC/Vol.XVII/ and Muhimbili Orthopaedic

Institute authorities. All mothers signed informed consent forms prior to recruitment and received counseling on prevention of the future NTDs. The results from the genetic analysis were given through mobile phones for those who could be accessed. However, the results have been stored in a password secured folder whereby mothers who may need their individual information in future can be informed.

Consent for publication

Not applicable.

Availability of data and materials

The database supporting the conclusion of this article is available with the author within reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SM, AEN, KPM and PC conceived and designed the study. SM and PC jointly analysed the data, SM and AEN developed the paper; PC and KPM contributed to the writing of the manuscript and all authors reviewed the final manuscript.

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List of abbreviations

cDNA	Complimentary Deoxyribonucleic Acid
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
FOCM	Folate One-Carbon Metabolism
MTHFD	Methylenetetrahydrofolate dehydrogenase
MTHFD	Methylenetetrahydrofolate reductase
MNH	Muhimbili National Hospital
MOI	Muhimbili Orthopaedic Institute
MUHAS	Muhimbili University of Health and Allied Sciences
NTDs	Neural Tube Defects
PCP	Planar Cell Polarity
RNA	Ribonucleic Acid
RT-PCR	Real- time Polymerase Chain Reaction

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