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Factor V Leiden Mutation as a Novel Marker in Children with Cerebral Palsy

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Abstract

Background: Gene mutations are known to play a role in the development of cerebral palsy (CP). The aim of this study was to determine the frequency of factor V Leiden (fVL) mutation as an etiological novel marker in Egyptian children with cerebral palsy.

Methods: The study included 70 children; 50 patients with cerebral palsy (Group I) and 20 healthy subjects (Group II) matched age and sex as a control group. Venous blood samples were used for DNA extraction using PCR testing. Polymerase chain reaction (PCR) primers were designed based on exon 10 sequence of human factor V gene.

Key findings: There was insignificant difference between both groups regarding comparison of demographic characteristics and risk factors except for pre-term birth (26% in study group versus 5% in control group with P = 0.04). The frequency of fVL mutation was 42% in the study group, 15% in control group with significant difference between study and control groups. There was a significant association and for the first time between homozygous fVL mutation and severe type of cerebral palsy; 60% of homozygous mutations associated with severe CP versus 9% of heterozygous mutations.

Conclusions: The fVL mutation is one of the major risk factors that may increase the likelihood of cerebral thrombo-embolism and subsequent cerebral palsy in Egyptian children.

Keywords: Cerebral palsy; Risk factors; Factor V Leiden mutation

Introduction

Cerebral palsy (CP) is a heterogeneous condition with multiple causes; multiple clinical types; multiple patterns of neuropathology on brain imaging; multiple associated developmental pathologies, such as intellectual disability, autism, epilepsy, and visual impairment; and more recently multiple rare pathogenic genetic mutations [1]. Globally, cerebral palsy is a common neurologic problem in children and is reported as occurring in approximately 1.5-3 of 1000 live births [2,3]. In Egypt, the prevalence of cerebral palsy in children was 3.6 per 1,000 live birth in another study recorded in Al-Quseir City. The currently high prevalence of cerebral palsy in our country may be attributed to an improved survival rate of preterm and low birth weight infants reported with cerebral palsy [4]. While CP was initially attributed to injuries resulting from birth asphyxia, recent studies have shown that in actuality it includes a myriad of factors. Injury to the developing brain may be prenatal, natal or postnatal. Risk factors were known to play a role in the development of cerebral palsy includes; multiple gestation, gender, infection, prematurity and low birth weight as well as genetic determinants [5]. Mutations in genes associated with the coagulation cascade trigger hypercoagulable states (hereditary thrombophilia) that, in theory, increase the risk of cerebral palsy [6]. The factor V Leiden (fVL) mutation is the most common form of hereditary thrombophilia, and heterozygosity increases the risk of thrombosis three to seven folds [7]. The aim of this study was to determine the frequency of factor fVL mutation in Egyptian cerebral palsy children In El-Minia Governorate, and to ascertain whether children with cerebral palsy have higher frequency of factor V leiden mutation compared with normal children, aiming to decrease the frequency of occurrence of cerebral palsy by diagnosing the mutation and trying to control the environmental circumstances.

Materials and Methods

The study included 70 children; 50 patients with cerebral palsy (Group I; study group) selected from the out patients' pediatric neurology clinic and pediatric in-patients' department in El-Minia University Children Hospital, in the period from November 2014 to July 2015. In addition to 20 healthy subjects (Group II; control group) matched age and gender as a control group. Children with cerebral palsy secondary to CNS infections, kernicterus, head trauma or intracranial hemorrhage were excluded. All patients and their families were interviewed in details of thorough history (demographic characteristics, consanguinity, pregnancy, delivery, perinatal events, etc.). All participants were subjected to complete general examination and full neurological examination, brain CT scan. Venous blood samples were collected from all patients using standard phlebotomy. Samples were collected from each case, in lavender-top (EDTA) tube. The samples were used for DNA extraction. Blood samples were centrifuged at 3000 rpm for 10 minutes plasma was separated and

frozen at -20°C until processed for PCR testing. The serial number of the used device is 2990212679.

Polymerase Chain Reaction for FVL

Sample preparation

1. Add 150 µl of Wizard® SV lysis buffer to the washed cells. Mix lysate by pipetting.

2. If the cell lysates will not be used immediately, they can be frozen at -70°C until needed. Purification of Genomic DNA Using a Vac-Man® Vacuum Manifold

3. For each lysate, use one Wizard® SV Minicolumn. Attach one Miniprep Vacuum Adapter with Luer-Lok® fitting to one port of the manifold. Gently press a minicolumn into the vacuum adapter. Ensure that all unused ports of the vacuum manifold are closed.

4. Transfer the prepared sample lysate to the Wizard® SV Minicolumn.

5. Apply a vacuum until the lysate passes through the minicolumn. After the lysate has passed through the column, close the Luer-Lok® stopcock.

6. Add 800 µl of Column Wash Solution (CWA; with 95% ethanol added) to the minicolumn. Apply a vacuum until the solution has passed through the column, then close the port. Repeat this step for a total of 4 washes.

7. Open each port and continue to pull a vacuum for 4 minutes to dry the binding matrix.

8. Close each port. Turn off the vacuum source and break the vacuum.

9. Remove the Wizard® SV minicolumn and place in a new 1.5 ml tube. Add 250 µL of room temperature nuclease-free water to the minicolumn. Incubate for 2 minutes at room temperature.

10. Place the minicolumn/tube assembly into the centrifuge and spin at 13,000 x g for 1 minute. Total solution volume will be approximately 250 µL.

11. Remove the minicolumn. Store the purified DNA at -20°C to -70C.

PCR reaction

According to this protocol, PCR primers were designed based on Exon 10 sequence of human factor V gene. The primers used in the PCR amplification reactions include a common primer 5'-ACTCTTAGAGTTTGATGA-3', a normal allele specific Primer 5'-GGACCAAAATACCTGTATTCCGC-3' and a mutation specific primer a 5'-GGACAAAATACCTGTATTCCCT-3'.

For a 20 µL reaction volume

Component	Volume
Intron Master Mix, 2X	10 µL
upstream primer, 10 µM	1 µL
downstream primer, 10 µM	1 µL
DNA template	3 µL
Nuclease free water	5 µL

Amplification was done for 36 cycles of 94°C for 2 minutes, 55 C for 1 minute, 72 for 1 minute, followed by extension at 72 C for 5 minute, the PCR product (220 base pair) was resolved at 1.5 % agarose gel. Accordingly all subjects were categorized as homozygous normal (GG), heterozygous (GA) or homozygous with mutant allele (AA).Venous blood samples were collected from all participants using standard phlebotomy. The samples were used for DNA extraction. Blood samples were centrifuged at 3000 rpm for 10 minutes; plasma was separated and frozen at -20°C until processed for PCR testing. PCR primers were designed based on Exon 10 sequence of human factor V gene. The primers used in the PCR amplification reactions include; a common primer 5'-ACTCTTAGAGTTTGATGA-3', a normal allele specific Primer 5'-GGACCAAAATACCTGTATTCCGC-3' and a mutation specific primer a 5'-GGACAAAATACCTGTATTCCCT-3'. Factor V Leiden mutation was looking for is Arg 506Gln, located in exon 10 in chromosome number one. It works as missense substitution of the nucleotide bases.

Statistical analysis

Statistical analyses were performed using SPSS software, version 18.0 (SPSS, Chicago, IL, USA). Data were presented as mean and standard deviation (SD) for quantitative variables, or as number and per cent for categorical (qualitative) variables. For univariate analysis, two-sided t-student used was used to compare independent groups of quantitative data, and Chi-square test was used to compare independent groups of qualitative data. The statistical significance of the used tests for analysis was considered when P-value was less than 0.05.

Results

The mean age of children with cerebral palsy was 3.6 ± 2.4 years (58% male and 42% female), while the control group included 20 children with a mean age of 4.7 ± 3.7 years (60% male and 40% female). There was insignificant difference regarding comparison of demographic characteristics (age, gender and cesarean delivery) and risk factors (consanginuity, maternal risk factors) except for pre-term birth (26% in study group versus 5% in control group with P = 0.04) between the studied groups as shown in table 1. The neurological motor disorders in children with cerebral palsy were spastic in 45 (90%), atonic in 3 (6%) and athetotic in 2 (4%) as shown in figure 1. The frequency of fVL mutation was 42% in the study group, 15% in the control group with significant difference between study and control groups as shown in table 6. All 3 cases of fVL mutations in the control group were heterozygous, while in the study group there were 10 cases of homozygous mutations and 11 cases of heterozygous mutations, with insignificant difference between both groups. There was insignificant difference regarding demographic characteristics and risk factors between children with and without fVL mutation as shown in table 2. There was a significant association between homozygous fVL mutation and severe type of cerebral palsy; 60% of homozygous mutations associated with severe cerebral palsy versus 9% of heterozygous mutations as shown in table 3 and figure 2. Moreover the number and percentage of the brain atrophy and neurological motor disorders in the studied patients with cerebral palsy are shown in tables 4 and 5.

Variable	Control group (n = 20)	Study group (n = 50)	P-value
Age (years)	4.7 ± 3.7	3.6 ± 2.4	0.15
Male gender No (%)	12 (60%)	29 (58%)	0.87
Consanguinity No (%)	2 (10%)	14 (28%)	0.1
Maternal thyroid disease	0	1 (2%)	0.52
Preeclampsia	1 (5%)	3 (6%)	0.87
Maternal infection	1 (5%)	4 (8%)	0.66
Multiple pregnancy	0	2 (4%)	0.36
Cesarean delivery	2 (10%)	7 (14%)	0.67
Pre-term birth	1 (5%)	13 (26%)	0.04*

Table 1: Demographic characteristics and risk factors in the both control and study groups;*Means significant difference.

Variable	(+ve) fVL mutation (n = 24)	(-ve) fVL mutation (n =46)	P-value
Age (years)	3.5 ± 1.9	4.1± 3.4	0.39
Male gender	14 (58%)	27 (59%)	0.97
Consanguinity	7 (29.2%)	9 (19.6%)	0.36
Maternal thyroid disease	0	1 (2%)	0.46
Preeclampsia	2 (8%)	2 (4%)	0.49
Maternal infection	1 (4%)	4 (9%)	0.48
Multiple pregnancy	1 (4%)	1 (2%)	0.63
Cesarean delivery	4 (17%)	5 (11%)	0.49
Pre-term birth	5 (21%)	9 (20%)	0.9

Table 2: Demographic characteristics and risk factors in relation to presence of factor V Leiden (fVL) mutation.

Severity of CP	Type of fVL mutation		P-value
	Homozygous (n = 10)	Heterozygous (n = 11)	
Mild	2 (20%)	4 (36%)	0.4
Moderate	2 (20%)	6 (55%)	0.1
Severe	6 (60%)	1 (9%)	0.01*

Table 3: Relation of the severity of cerebral palsy (CP) to the type of factor V Leiden (fVL) mutation;*significant difference.

Variable	Number (N = 50)	Percentage (%)
Normal CT	5	10%
Brain atrophy on CT	45	90%
Spastic	45	90%
Atonic	3	6%
Athetotic	2	2%

Table 4: Brain atrophy and neurological motor disorders in the studied patients with cerebral palsy.

Reference	Study type	Cases (n)	Control (n)	Analyzed genes	Results
[15]	Case-Control	31	65	fVL	Significant increase
[16]	Case-Control	49	118	fVL, MTHFR C677T, G20210A	Non-significant difference
[17]	Case-Control	61	62	fVL, PT20210, MTHFR C677T	Non-significant difference
[18]	Case-Control	57	167	fVL	Significant difference
[19]	Case-Control	138	165	fVL, PT20210, MTHFR C677T	Non-significant difference
[20]	Case-Control	94	120	fVL	Non-significant difference

Table 5: Summary of the studies evaluated the direct association between cerebral palsy and hereditary thrombophilias.

Variable	Control group N = 20	Study group N = 50	Total N = 70	P-value
FVL mutation	3 (15%)	21 (42%)	24 (34.3%)	0.03*
Homogenous/ heterogeneous FVL mutation	0 / 3	11-Oct	14-Oct	0.11

Table 6: Incidence of factor V Leiden (fVL) mutation in the studied groups.

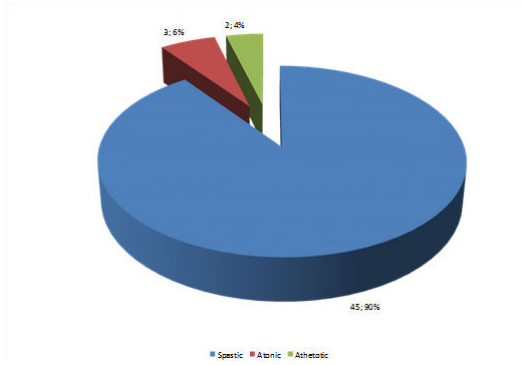

Figure 1: Distribution of neurological disorders in 50 children with cerebral palsy.

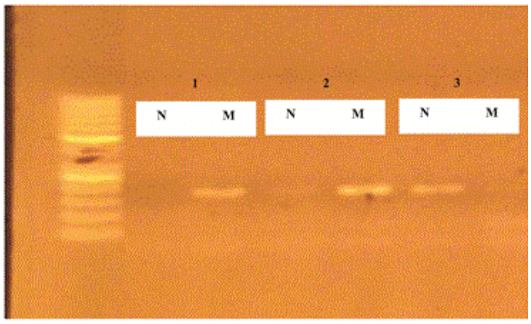

Figure 2: The normal, homogenous and heterogeneous mutant reaction as detected by PCR; The normal Specific (N) reaction on the left, the homogenous mutant Specific (M) reaction on the right (1,2) and Heterogeneous mutant (3).

Discussion

It was of interest to evaluate the etiological factors associated with cerebral palsy, in respect to the growing era of the genetic determinants of thrombophilia, in our developing country with a considerable prevalence of cerebral palsy. In the present study, cerebral palsy was more common in males than in females (58% versus 42%; male: female ratio of 1.3:1). These findings were in agreement with other studies, where males were more at risk of cerebral palsy than females with a ratio of 1.3:1 [8]. Recessive X-linked chromosome variants may contribute to this difference and males may be more vulnerable to genetic mutation [9]. In the present study, the consanguinity was reported in 14 (28%) in the cerebral palsy group and 2 (10%) in control group, with insignificant difference. This finding was supported by the fact that consanguineous marriage in developing countries is highly frequent and encountered among the risk factors of cerebral palsy [10]. In literature, there were many probable maternal and antenatal causes of white matter damage and risk factors for cerebral palsy [8,11]. In the present study, children with cerebral palsy showed higher frequencies of maternal and perinatal risk factors including maternal infection, pre-eclampsia, multiple pregnancy, and maternal thyroid disease, but with insignificant difference between both groups. These frequencies may not be accurate as it depends on obstetric history given by mothers in absence with data extraction from hospital maternal records or database, thus under estimated or over-estimated data may be given by mothers. In the present study, the frequency of children born pre-term in the cerebral palsy group was significantly higher than that in control group (26% versus 5%, $P < 0.05$). This finding was in agreement with other studies in literature which confirmed that preterm delivery is a major risk factor for cerebral palsy [12,13]. The increased frequency of pre-term children with cerebral palsy may be attributed to the effect of improved neonatal intensive care management during recent years, leading to increasing survival of children born extremely pre-term [4]. In the present study, fVL mutations present in 42% of cerebral palsy children, 15% in control group, with a statistically significant difference between cerebral palsy and control groups. Our analysis indicates a high prevalence of fVL (15%) in the normal controls however it is constant with the prevalence of fVL in related Arab general population which was reported to be 15% in Egyptians, 16% in Syrians, 23.5% in Jordanians and 25% in Palestinians [14]. The high frequency of fVL mutation in both groups (cerebral palsy and control) may be explained by high frequency of consanguinity in our study (10% in control, 28% in cerebral palsy group and 23% in all), which constant with the reported high frequency of consanguineous marriage in Egypt up to 35.3% [10]. In addition, our findings of the high frequency of fVL in children with cerebral palsy may reflect the primary role of thrombophilia in the etiology of this neurological problem. In literature, there are few case-control studies (Table 4) aimed to describe a direct correlation between hereditary thrombophilia with regard to fVL mutation and cerebral palsy but the results were controversial [15-20]. However, the findings of our study were in agreement with Nelson et al. [15] and Reid et al. [18] which suggest that fVL mutation be considered as a risk factor for CP, in addition to other risk factors that are likely associated with brain injury. Limitations in our study were in the number of the controlled cases, therefore, in the future studies we need to increase the number of the control group in a randomized control studies to establish this finding. In conclusion, the fVL mutation is one of a number of potential factors that may increase the likelihood of cerebral thrombo-embolism and subsequent cerebral palsy in children. Further understanding of the risk factors involved in the development of cerebral palsy may help in creating treatment modalities, such as anticoagulant treatment for mothers, in order to prevent this disability.

Declaration of Interest

All the authors have declared that this research has been done at El-Minia University, faculty of medicine, departments of paediatrics and clinical pathology, Egypt. There was no sponsorship. The authors declared that there is no conflict of interest. They also declared that this manuscript was not accepted or published in any Journal worldwide.

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