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Original Article

Are *COL4A1* and *COL4A2* gene polymorphisms associated with cerebral palsy?

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ABSTRACT

Objectives: This study aims to investigate the association of *COL4A1* and *COL4A2* gene polymorphisms with susceptibility to risk of developing cerebral palsy (CP) and severity of CP.

Patients and methods: Between December 2016 and June 2017, a total of 176 patients with CP (101 males, 75 females; mean age 71.8±37.9 months; range, 24 to 184 months) and age-, sex-, and ethnically-matched 178 (90 males, 88 females; mean age 69.3±55.2 months; range, 24 to 214 months) controls were included. Two polymorphisms of *COL4A1* (rs1961495) and *COL4A2* (rs9521733) genes were typed from genomic deoxyribonucleic acid. Genotype distributions and allelic frequencies were compared between the patient and control groups. Gross Motor Function Classification System, the use of medical drugs, type of involvement, number of affected limbs, accompanying conditions, birth weight, gestational age, and magnetic resonance imaging (MRI) findings were used to evaluate the disease severity and their relationships with the *COL4A1* and *COL4A2* gene polymorphisms.

Results: There was no statistically significant difference between the groups in terms of genotype distribution and allele frequency of *COL4A1* and *COL4A2* gene polymorphisms (p>0.05). In addition, there was no relationship between severity of CP and two gene polymorphisms (p>0.05). A significant association was detected between the *COL4A2* polymorphism and growth retardation in CP. The TT genotype (57.1%) and T allele (76.2%) were higher, compared to CC (4.8%) and CT genotypes (38.1%) and C allele (23.8%) in patients with CP with growth retardation (p=0.03 for genotype and p=0.01 for allele frequency).

Conclusion: These findings suggest that *COL4A1* and *COL4A2* gene polymorphisms are not associated with susceptibility to CP in a group of Turkish populations, although *COL4A2* gene polymorphism may be associated with growth retardation in patients with CP.

Keywords: Cerebral palsy, *COL4A1*, *COL4A2*, gene polymorphism.

Cerebral palsy (CP) is defined as a group of non-progressive permanent diseases which occurs in the developing fetal or infant brain. It is associated with movement and postural disorders, thereby, restricting daily living activities.[1] The motor disorders of CP are often accompanied by sensory, perceptual, cognitive, communicative, behavioral, and secondary musculoskeletal problems. In many countries, CP is the most common cause of physical disability in childhood. It affects one newborn in every 500 births. The worldwide prevalence of CP is estimated at 17 million.[2] Despite the improvement in perinatal care, this rate has remained stable for more than four decades.[3]

Although CP develops due to similar damages in similar brain areas, it may differ clinically. The reason for these differences is that CP is a multifactorial disease. It is a heterogeneous disease and its etiology

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has not been fully understood, yet. Recent studies have reported that genetic factors may be responsible in CP. Some of the genetic factors are polymorphic changes in genes.[4,5] The prevalence of congenital anomalies has increased in CP patients. Previous studies have reported a significantly higher concordance rate for CP in monozygotic twins, compared to dizygotic twins. The risk of CP is more than 2.5 times in families with consanguineous marriage. It has been reported that CP cases have a familial clustering and several new single gene mutations have been detected in idiopathic CP pedigrees. All these factors strongly support the contribution of genetic factor in the etiology of CP.[6] Genotypes that may be susceptible to cerebral injury have been studied in the literature. Relationships between hereditary thrombophilia polymorphisms, cytokine polymorphisms, and cerebral palsy have been investigated in previous studies.[7,8]

Basal membranes are highly specialized extracellular matrix which can change cellular behavior to regulate tissue development, function, and repair. Type IV collagens are the main components of basal membranes and are encoded by six genes found in three different chromosomes. In humans, *COL4A1* and *COL4A2* are located on chromosome 13, COL4A3 and COL4A4 are located on chromosome 2, and COL4A5 and COL4A6 are located on chromosome X.[9] The *COL4A1*, *COL4A2* is the most important component of type IV collagen in the basal membrane and has been widely found in all tissues, including brain tissue.[10,11] Mutation of *COL4A1* is responsible for general and systemic basal membrane diseases presenting with various phenotypes, including neurological features such as stroke, infantile hemiparesis, and epilepsy.[12] In *COL4A2* mutations, similar to the *COL4A1* mutations, pseudocysts in the cortex, focal hemorrhagic necroses surrounding small blood vessels can be observed.[13]

Due to the fact that CP is a multifactorial and heterogeneous disease, it is important to clarify the underlying genetic factors. In addition, the association of the characteristics of the disease with the genotype may be important for prognosis. In the present study, we hypothesized that *COL4A1* rs1961495 and *COL4A2* rs9521733 gene polymorphisms may be associated with the risk of development of CP and/or phenotype and clinical severity of CP in the light of all these data. We, therefore, aimed to investigate the association of *COL4A1* and *COL4A2* gene polymorphisms with susceptibility to risk of developing CP and severity of CP.

PATIENTS AND METHODS

The study was designed as a case-control trial. A total of 276 patients diagnosed with CP and followed at Mersin University Faculty of Medicine, Department of Physical Medicine and Rehabilitation between December 2016 and June 2017 were included in the study. The control group was composed of 200 individuals who were referred to the Children's Health Department of the institution and fulfilled inclusion criteria. Age and sex were matched with the propensity score method and 176 children (101 males, 75 females; mean age 71.8±37.9 months; range, 24 to 184 months) for the patient group and 178 children (90 males, 88 females; mean age 69.3±55.2 months; range, 24 to 214 months) for the control group were included in the study. *Inclusion criteria for the patient group were as follows:* having diagnosed with CP by the pediatric neurologist based on medical history, clinical, and imaging findings; age <18 years; and having consent from his/her parents or legal guardians to participate in the study. *Inclusion criteria for the control group were as follows:* being healthy and age <18 years; having no medical or family history of congenital musculoskeletal disease, neurological disease, metabolic/endocrine disease, hematological disease or malignancy; and having consent from his/her parents or legal guardians to participate in the study. A written informed consent was obtained from each parent and/or his/her or legal guardians. The study protocol was approved by the Ethics Committee of Mersin University. The study was conducted in accordance with the principles of the Declaration of Helsinki.

All participants were evaluated in terms of age, sex, birth weight, gestational age, medications, and history of operation. The patient group was also evaluated in term of prenatal, natal, and postnatal risk factors, presence of accompanying problems such as epilepsy, mental retardation, visual and hearing impairment in their families, and presence of consanguineous marriage among the parents. In the examination of the patients, the presence of accompanying problems such as speech disorder, dysphagia, chewing and swallowing difficulties, mental retardation, epilepsy, growth and development retardation, sleep problems, tooth and gum problems, skeletal deformities and gross motor levels based on the Gross Motor Function Classification System (GMFCS), muscle tone, type of involvement, number of affected extremities, the use

of medical drugs, and cerebral magnetic resonance imaging (MRI) findings were recorded.

Molecular analysis

A blood sample was drawn from each participant. Venous blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes. Deoxyribonucleic acid (DNA) was extracted from the whole blood. Genetic analysis was performed in Medical Biology and Genetics Department of Mersin University Faculty of Medicine.

Genotypic analysis of *COL4A1* (rs1961495) and *COL4A2* (rs9521733) polymorphisms

The genotyping of analysis of the *COL4A1* (rs1961495) and *COL4A2* (rs9521733) polymorphisms were performed using a full-blood commercial kit, High Pure PCR Template Preparation Kit (Roche Applied Science, Penzberg, Germany). The polymorphisms of the *COL4A1* gene (rs196149) and *COL4A2* gene (rs9521733) were performed using a pre-designed LightSNIP assay (TIB Molbiol, Berlin, Germany). Real-time polymerase chain reaction method (RT-PCR) was used for single nucleotide polymorphism (SNP) amplification experiment. The SNP amplification assays were performed according to the manufacturer's instructions. In brief, 20 µL of reaction solution containing 30 ng of DNA was mixed with 10 µL LightCycler Probe Master Mix (Roche Applied Science, Penzberg, Germany), 1 µL of the pre-synthesized LightSNIP kit, 4 µL of water and 5 µL of DNA. The reaction conditions are composed of three steps: Denaturation, amplification, and melting curve. It was pre-denatured at 95°C for 10 min. The amplification was, then, subjected to amplification at 95°C for 10 sec, 60°C for 10 sec, and 72°C for 15 sec for 45 times. Finally, the melting curve stage (30 sec at 95°C, 120 sec at 40°C, and 75°C for 0 sec) was analyzed in the LightCycler 480 II RT-PCR System (Roche Applied Science, Penzberg, Germany). Melting temperatures for melting curve analysis of the *COL4A1* variant were evaluated as a melting heat (TM) of 55.64°C for the CC genotype, a melting heat (TM) for the TT genotype of 61.00°C, and heterozygous for both genotypes. For the *COL4A2* variant, the melting temperature (TM) for the TT genotype was 56.47°C, the melting temperature (TM) for the CC genotype was 63.92°C, and heterozygous for both genotypes.

Statistical analysis

In the reference study, the incidence of *COL4A1* (rs1961495) T allele in the control group was given as 44.8% with an odds ratio of 1.387.[3] Accordingly, it was planned to enroll 173 individuals in the patient group, 173 individuals in the control group, and a total of a minimum of 346 individuals with 80% study power and 5% type I error. In this study, 200 children were included in each group, and age and sex were matched with the propensity score method. Finally, 176 children for the patient group and 178 children for the control group were included in the study.

Statistical analysis was performed using the Statistica version 13.3 software (StatSoft Inc., OK, USA). Descriptive data were expressed in mean ± standard deviation (SD) and median (min-max) or number and frequency. The Shapiro-Wilk test was used to check the normality of continuous variables. Accordingly, the Student's t-test was used to compare the mean of two independent groups. The chi-square test was used to analyze the relationship and distribution of categorical variables. The Cochran-Armitage trend test was used to determine the relationship of *COL4A1* and *COL4A2* gene polymorphisms with categorical variables. In case of significant correlation, two ratio comparisons were applied according to each genotype. For the *COL4A1* and *COL4A2* gene polymorphisms, the Hardy-Weinberg equilibrium (HWE) was investigated separately in the patient and control groups. A p value of <0.05 was considered statistically significant.

RESULTS

Both groups were similar in terms of age and sex ($p>0.05$, Table 1). The birth weight, gestational age, and maternal age at birth were smaller in the patient group ($p<0.001$, $p<0.001$, and $p=0.02$, respectively). The distribution of birth weight of the patient group is given in Figure 1. There was no statistically significant difference in the consanguineous marriage between the groups ($p=0.16$).

According to the conditions accompanying CP, 57 (32.4%) patients had speech disorder, 37 (21%) had chewing difficulty in swallowing, 53 (30.1%) had salivation problem, 53 (30.1%) had mental retardation, 27 (15.3%) had visual impairment, nine (5.1%) had hearing impairment, 37 (21.1%) had epilepsy, 62 (35.2%) had strabismus, 21 (11.9%) had growth retardation, 11 (6.3%) had sleep problem, and 15 (8.5 %) had dental problems.

Of the patients with CP, 81% had spastic type, 12.5% had dyskinetic type, and 6.3% had hypotonic

TABLE 1
Demographic and clinical characteristics of patient and control groups

	Patient group			Control group			
	n	%	Mean±SD	n	%	Mean±SD	*p*
Age (month)			71.8±37.9			69.3±55.2	0.62
Sex							0.2
Male	101	57.4		90	50.6		
Female	75	42.6		88	49.4		
Gestational age							<0.001
<28 Week	20	11.4		0	0.0		
28-31 Week	62	35.2		0	0.0		
31-37 Week	47	26.7		14	7.9		
>37 Week	47	26.7		164	92.1		
Birth weight (g)			2080.8±952.2			3049.8±470.3	<0.001
Maternal age at birth (year)			27.0±4.7			27.1±4.3	0.02
Relative marriage rate	38	21.6		28	15.7		0.16
Type of CP							-
Spastic CP	143	81.3		-	-		
Dyskinetic CP	22	12.5		-	-		
Hypotonic-ataxic CP	11	6.3		-	-		
GMFCS							-
Level 1	16	9.1		-	-		
Level 2	36	20.5		-	-		
Level 3	55	33		-	-		
Level 4	52	29.5		-	-		
Level 5	14	8		-	-		
Magnetic resonance imaging							-
Normal	31	17.6		-	-		
Abnormal	145	82.4		-	-		
Accompanying							-
Epilepsy	37	21.1		-	-		
Speech disorder	57	32.4		-	-		
Defect of vision	27	15.3		-	-		
Hearing disorder	9	8.1		-	-		
Mental retardation	53	30.1		-	-		
Growth retardation	21	11.9		-	-		

SD: Standard deviation; CP: Cerebral palsy; GMFCS: Gross Motor Function Classification System.

- ataxic type. Sixteen (9.1%) patients were in the GMFCS Stage 1, 36 (20.5%) patients were in the GMFCS Stage 2, 58 (33%) patients were in the GMFCS Stage 3, 52 (29.5%) patients were in the GMFCS Stage 4, and 14 (8%) patients were in the GMFCS Stage 5.

Genotype frequencies of rs1961495 and rs9521733 showed no deviations from HWE either in patients or in controls. The frequencies of alleles and genotypes of rs1961495 of *COL4A1* and rs9521733 SNPs of *COL4A2* are listed in Tables 2, respectively. The *COL4A1* and *COL4A2* genotype distributions and allele frequencies were not significantly different between the groups (p=0.12 and p=0.08, respectively).

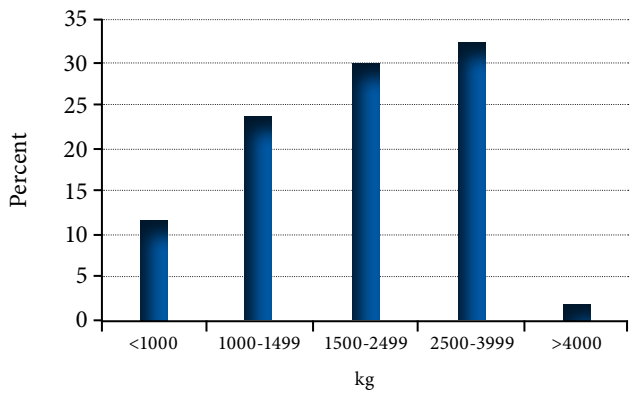

Figure 1. The distribution of birth weights of the patient group.

TABLE 2
Genotype distributions and allele frequencies of gene polymorphisms in patient and control groups

Gene polymorphism	Genotype and allele	Patient group n	Patient group %	Control group n	Control group %	*p*
COL4A1	C/C	133	75.6	124	69.7	0.12
	C/T	42	23.9	48	27.0	
	T/T	11	0.6	6	3.3	
	C	308	87.5	296	83.1	0.1
	T	44	12.5	60	16.9	
COL4A2	C/C	32	18.2	39	21.9	0.08
	C/T	85	48.3	95	53.4	
	T/T	56	33.5	44	24.7	
	C	149	42.3	178	49.0	0.06
	T	203	57.7	183	51.0	
COL4A1 and *COL4A2*	CCCC	24	13.6	27	15.2	0.44
	CCCT	65	36.9	66	37.1	
	CCTT	44	25	31	17.4	
	CTCC	8	4.5	12	6.7	
	CTCT	19	10.8	24	13.5	
	CTTT	15	8.5	12	6.7	
	TTCC	0	0	0	0	
	TTCT	1	0.6	5	2.8	
	TTTT	0	0	1	0.6	

The distribution of genotypes coexistence of these two gene polymorphisms were examined between the groups and no statistically significant difference was found (p=0.44) (Table 2).

We investigated the relationship between the phenotype, severity of CP and genotype distribution, allele frequency in patients. To evaluate this, sex, gestational age, birth weight, type of CP, number of affected extremities, MRI findings, medical treatment, risk factors, GMFCS, positive family history, problems associated with CP (speech disorder, chewing dysphagia, drooling, mental retardation, visual impairment, hearing impairment, epilepsy, growth retardation, sleep problem, dental gingival problems) were used. No statistically significant correlation was found (p>0.05) (Table 3). There was a statistically significant relationship between growth retardation and *COL4A2* polymorphism (p=0.03). TT genotype distribution and T allele frequency were significantly higher in CP children with growth retardation (p=0.02 and p=0.01) (Table 4).

TABLE 3
The relation of gene polymorphisms with clinical and radiological findings

	COL4A1 *p*	*COL4A2* *p*
Gestational age	0.48	0.22
Birth weight	0.84	0.66
Sex	0.25	0.34
Cerebral palsy type	0.11	0.59
Number of limbs held	0.83	0.32
MRI findings		
White matter lesion	0.72	0.09
Gray matter lesion	0.95	0.80
Vascular/ventricular disorders	0.63	0.71
Developmental disorder	0.57	0.49
Medical treatment	0.78	0.64
GMFCS	0.54	0.68
Risk factors		
Prenatal	0.69	0.21
Natal	0.87	0.35
Postnatal	0.70	0.42
Accompanying		
Speech disorder	0.34	0.81
Chewing swallowing difficulty	0.63	0.98
Salivation	0.20	0.19
Mental retardation	0.55	0.96
Vision disorder	0.69	0.89
Hearing disorder	0.94	0.28
Strabismus	0.35	0.34
Epilepsy	0.71	0.99
Sleep problem	0.91	0.73
Dental gingival problems	0.85	0.44

MRI: Magnetic resonance imaging; GMFCS: Gross motor function classification system.

DISCUSSION

Cerebral palsy is a neurological syndrome which is a combination of symptoms, but not a disease.[13] Although prematurity, hypoxia-ischemia, placental insufficiency, and prenatal infections are well-known causes of CP, for other patients, particularly those born at term and/or without a clear etiology identifiable by MRI, its etiology is still unclear. Some researchers

TABLE 4
Relationship between growth retardation and polymorphisms, alleles distribution of *COL4A1* and *COL4A2* alleles

		Growth retardation (+)		Growth retardation (-)			
Gene polymorphism	Genotype	n	%	n	%	P1	P2
COL4A1	CC	14	66.7	119	76.8	0.52	-
	CT	7	33.3	35	22.6		
	TT	0	0	1	0.6		
	C	35	83.3	273	88.1	0.38	-
	T	7	16.7	37	11.9		
COL4A2	CC	1	4.8	31	20.0	0.03	0.09
	CT	8	38.1	77	49.7		0.32
	TT	12	57.1	47	30.3		0.02
	C	10	23.8	139	44.8	0.01	-
	T	32	76.2	171	55.2		

believe that genetic and epigenetic factors may be the cause of CP. Previous studies have shown that 30% of CP cases may be genetically inherited.[5]

There are studies investigating the association of collagen gene polymorphisms with many diseases. Apart from our study, the only study examining the relationship between CP and collagen gene polymorphisms was conducted in the Chinese population by Bi et al.[3] They investigated only the relationship between the CP and six SNPs of the *COL4A1* gene in their study including 351 CP and 220 healthy controls. In this study, a statistically significant relationship was found between rs1961495, rs1411040 polymorphisms and CP. For the rs1961495 polymorphisms, the frequency of the C allele was found to be 63% in the CP group and 55% in the control group. The frequency of the T allele was 37% in the CP group and 45% in the control group (p=0.008). In the CP group, genotype distributions were found to be 40% for the CC genotype, 46% for the CT genotype, and 14 % for the TT genotype. In the control group, genotype distributions were found to be 29% for CC genotype, 51% for the CT genotype, and 19% for the TT (p=0.02). Similar to our study, in the aforementioned study, CC genotype and C allele frequencies were found to be higher in patients, and CT, TT genotypes and T allele frequencies were found to be higher in the controls. Unlike our study, these rates were statistically significant. Bi et al.[3] found that *COL4A1* rs1961495, rs1411040 gene polymorphisms are associated with CP. However, they did not examine the relationship between the severity and phenotypic features of CP. We believe that this is one of the limitations of their study. When we examined the relationship between the phenotypic features and severity of CP with *COL4A1* and *COL4A2* gene polymorphisms, we found no statistically significant relationship between genotype distributions of each two gene polymorphisms and sex, gestational age, birth weight, risk factors, type of CP, GMFCS, MRI findings, family history of neurological diseases, and accompanying problems with CP (p>0.05). We only found a statistically significant relationship between growth retardation and *COL4A2* rs9521733 gene polymorphism (p=0.03). We found that TT genotype and T allele frequency were statistically significantly higher in patients with growth retardation (p=0.02 and p=0.01, respectively).

Type IV collagen is encoded by three paralog gene pairs, which encode six unique alpha chains to form heterodimers. The *COL4A1* and *COL4A2* are the most abundant component of type IV collagen.[3] Therefore, we believe that our study would have a special place in the literature, since it is the first study to examine the coexistence of these polymorphisms, playing an important role in the synthesis of *COL4A1* and *COL4A2* peptides.

Yoneda et al.[14] detected that *COL4A1* mutations in patients with porencephaly, intracerebral calcification, focal cortical dysplasia, pontocerebellar atrophy, ocular abnormalities, myopathy, and high serum creatine kinase levels. Furthermore, in another study, it was reported that heterotrimer abnormalities of type IV collagen α1α1α2 caused porencephaly.[15] The importance of screening for *COL4A1* was also emphasized.[14,15]

Jeanne et al.[16] reported that the mutations of *COL4A1* and *COL4A2* disrupted the secretion of

proteins and caused cytotoxicity, and these mutations were associated with spontaneous intracerebral hemorrhage. Similar to the aforementioned studies, Shah et al.[17] reported that *COL4A1* mutations could occur in children with infantile hemiplegia, quadriplegia, epilepsy and motor impairment, and that white matter changes on MRI might be helpful in the diagnosis in childhood. Rannikmäe et al.[18] showed that there was a common relationship between the *COL4A2* gene and symptomatic small vessel disease, particularly deep intracerebral hemorrhage. They also reported that these findings should be studied in non-European ethnic groups. Vahedi and Alamowitch[19] observed that *COL4A1* mutations could cause multisystemic phenotypic features such as retinal arteriolar deposits, retinal hemorrhages, anterior segment dysgenesis, intracranial aneurysms, porencephaly, infantile hemiparesis, muscle cramps, optic nerve dysgenesis, and secondary glaucoma. In contrast to the aforementioned studies, in patients with CP, we found no statistically significant difference in the relationship between both *COL4A1* and *COL4A2* gene polymorphisms with cerebral MRI findings. We believe that this may be due to the fact that our study was conducted in a small group of patients and our study population was in a different ethnic group than previous studies.

Nonetheless, there are some limitations in our study. Our study was conducted in a small population and a single center in southern Turkey. On the other hand, we believe that our study would contribute to the literature, as it is the first study to investigate the effects of *COL4A1* and *COL4A2* gene polymorphisms on phenotypic features of CP, clinical severity, and risk of developing CP in our country and the world. Therefore, it can be speculated that this study would make significant contributions to the effect of *COL4A1* and *COL4A2* gene polymorphisms on the etiopathogenesis and clinic of CP.

In conclusion, in this study, we found no significant relationship between gene polymorphisms of *COL4A1* rs1961495 and *COL4A2* rs9521733 and the risk of developing CP. Apart from growth retardation, we found no significant correlation between *COL4A1* rs1961495 and *COL4A2* rs9521733 gene polymorphisms and phenotypic characteristics and disease severity of patients with CP. However, our study results showed a statistically significant relationship between growth retardation and *COL4A2* rs9521733 gene polymorphism in patients with CP. We believe that TT genotype and T allele frequency are higher in patients with growth retardation and *COL4A2* rs9521733 gene polymorphism may increase susceptibility to growth retardation in patients with CP. Of note, further studies are needed to confirm these results in CP patients. These studies should be conducted in a large number of polymorphic genes in large sample groups and in similar and different populations.

Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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