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Original scientific paper

ANALYSIS OF T-786C And 4a/b ENDOTHELIAL NITRIC OXIDE SYNTHASE GENE POLYMORPHISMS IN RETINOPATHY OF PREMATURITY

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Retinopathy of prematurity (ROP) is a vascular proliferative disorder of retina, that causes visual impairment in premature children. Beside well known risk factors such as short gestational age, low birth weight and early oxygen exposure, genetic susceptibility is considered as a risk factor for development of the disease. The aim of our study was to explore the association of T-786C and 4a/b *eNOS* gene polymorphisms with the development of severe ROP. Study included 174 preterm infants, 84 with ROP and 90 as a control group. No differences have been observed in genotypes and alleles distributions of *eNOS* T-786C and *eNOS* 4a/b polymorphisms between two analyzed groups. There was significant difference in female infants by dominant model for 4a/b genotypes (4bb/4ba+4aa). Namely, female infants in ROP group were more frequently carriers of 4ba and 4aa genotypes than female infants in control group (p=0.037). Analysis of association between 4a/b e*NOS* polymorphism and ROP among preterm infants have not shown statistically significant association (p=0.288). Gestational age values by recessive model (4bb+4ba/4aa) were significantly lower in infants with 4aa genotype (t=2.034

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p=0.044). Almost all detected 4aa genotypes were present in the group of infants with gestational age under 30 weeks (p=0.032), but multivariate linear regression analysis does not show association of 4a/b genotypes with gestational age of premature infants. According to results of the present study T-786C and 4a/b polymorphisms of the eNOS gene may not be the risk factors for the manifestation of severe ROP in Serbian infants.

Key words: T-786C polymorphism, 4a/b polymorphism, *eNOS* gene, SNP, retinopathy of prematurity.

INTRODUCTION

Retinopathy of prematurity (ROP) is a vascular proliferative disorder of the retina that can lead to visual impairment or complete vision loss in premature infants (American Academy of Pediatrics 2001). Higher risk of developing ROP is expected in infants who have low birth weight and are born at a lower gestational age (KARNA *et al.* 2005).

Normal retinal vascularization occurs in the fetal environment and it is characterized by a relatively low oxygen level (GU *et al.* 2002). After birth, premature neonates are exposed to a higher concentration of oxygen, which is required for their survival. In hyperoxic extrauterine environment normal vascularization in retina of premature infants is arrested and developed vessels undergo regression. Further development of retina results in maintaining avascular areas that become relatively hypoxic and abnormal retinal neovascularization is induced (GU *et al.* 2002).

It is well known that nitric oxide (NO) mediates angiogenesis through vascular endothelial growth factor (VEGF) (PAPAPETROPOULOS *et al.* 1997). Several studies described that long term VEGF exposure stimulates endothelial nitric oxide synthase (eNOS) to produce NO. eNOS plays a predominant role in hypoxia-induced angiogenesis and vascular permeability (MUROHARA *et al.* 1998 and FUKUMURA *et al.* 2001). Deficiency of eNOS is responsible for retinal neovascularization (QAZI *et al.* 2009).

eNOS is expressed primarily in endothelial cells and at low level in platelets (SASE *et al.* 1995). Studies evaluating polymorphisms in specific candidate genes have demonstrated possible association between sequence variations and severity of ROP (COOKE *et al.* 2004). Well known functional *eNOS* gene polymorphisms are T-786C in the promoter region and the variable 27-bp tandem repeat (VNTR) in intron 4 (*eNOS* 4a/b). These polymorphisms are responsible for the reduction of the endothelial NO synthesis and therefore alteration of the NO plasma levels.

The association between *eNOS* gene polymorphisms and the risk of severe ROP development has not been evaluated in the Serbian premature infants. Additionally, there has been no report dealing with the association between polymorphisms of *eNOS* gene and clinical risk factors of severe ROP expression, such as low birth weight and gestational age, low Apgar score and long period of oxygen therapy.

The aim of our study was to investigate whether T-786C and 4a/b *eNOS* gene polymorphisms are associated with severe ROP development in Serbian premature infants and to evaluate their influence on clinical risk factors of ROP.

MATERIALS AND METHODS

Study included 174 preterm infants borne at the Clinic of Obstetrics and Gynecology, Clinical Center of Serbia, Belgrade. Selected babies had less than 2000 gr. at birth and gestational age below 35 weeks or higher birth weight and gestational age if they have had additional risk

factors such as respiratory distress syndrome, anemia, intracranial hemorrhage and treatment with mechanical ventilation.

Clinical evaluation

Shown Apgar score was determined in the 5th minute. Intracranial hemorrhage (ICH) was determined by ultrasound of the central nervous system and graduated I-IV degrees according Papile (PAPILE *et all*. 1978). Infants with stage III and IV were in ICH group. The degree of respiratory distress syndrome (RDS) was based on the assessment of Chest X-rays according to Bomsel (I-V degree) (BOMSEL 1970). Hemoglobin less than 90 gr./L was the criterion for the anemia.

Ophthalmological screening for ROP was performed in all preterm infants by indirect binocular ophthalmoscope at the moment of maximum mydriasis. Preterm infants were divided into two groups according to the degree of ROP. The ROP group included infants with active form of the disease that required therapy. The control group included infants without signs of retinopathy and infants with retinopathy forms that did not require therapy.

Genotyping

All molecular genetic analyses were conducted at the Institute of Human Genetics, Faculty of Medicine, University of Belgrade. Genomic DNA was extracted from buccal swabs by phenol-chloroform method. Genotypes for *eNOS* T-786C polymorphism were detected by a polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method. Sequences of forward and reverse primers were 5′ GTG TAC CCC ACC TGC ATT CT 3′ and 5′ CCC AGC AAG GAT GTA GTG AC 3′, respectively. The products of PCR were digested with *Nae*I (*Pdi*I) endonuclease. Results of digestion were: DNA fragments of 225 and 81 bp were present in case of CC genotype, 306, 225 and 81 bp long DNA fragments in CT genotype and in TT genotype a single undigested 306-bp long DNA fragment.

Genotypes of the *eNOS* intron 4 VNTR polymorphism were detected by PCR using forward 5′ CTA TGG TAG TGC CTT GGC TGG AGG 3′ and reverse 5′ ACC GCC CAG GGA ACT CCG CT 3′ primers. The DNA fragment 210 bp long corresponds with allele b (five 27-bp repeats) and 183-bp DNA fragment corresponds with allele a (four 27-bp repeats).

Statistical analysis

Differences in genotypes and alleles frequencies between ROP group and control group were analyzed by Chi-square test and Fisher exact test. Mean values were compared between genotype groups by ANOVA or Kruskal–Wallis test, depending on variable distribution. After grouping of genotypes (by dominant or recessive model) Student's *t* test and Mann– Whitney *U* test were used. Association between analyzed polymorphisms and ROP requiring treatment among preterm infants was explored by logistic regression analysis after the adjustment for gender, BMW, gestational age, Apgar score and days of oxygen therapy. Association analysis between analyzed polymorphisms and gestational age was performed by multivariate linear regression analysis with gender, BMW and Apgar score as covariates. Statistical analyses were performed using SPSS statistical package, version 16.0 (SPSS Inc, Chicago, IL, USA).

RESULTS

We have analyzed 174 premature infants divided in two groups. ROP group included 84 preterm infants with severe ROP stage that required therapy. Control group included 90 preterm infants who did not require any ophthalmological treatment.

Table1. Clinical data of two premature infants groups

Clinical characteristics	ROP group n=84	Control group n=90	P values
Gender M/F	44/40	42/48	0.451
GA weeks*	29.8±1.9	31.1±1.9	<0.001
BMW grams*	1212±234	1377±279	<0.001
Apgar score *	4.7±1.7	6.3±1.6	<0.001
PA **	65 (77.4%)	39 (43.3%)	<0.001
RDS**	71 (84.5%)	54 (60%)	<0.001
ICH**	16 (19.0%)	7 (7.8%)	0.028
Anemia**	76 (90.5%)	65 (72.2%)	0.002
MV**	45 (53.6%)	23 (25.6%)	<0.001
O2 therapy days*	25.6±18.1	13.99±10.0	<0.001

*mean values ± standard deviation, ** No (%), Gestational age (GA), Birth weight mass (BWM), Perinatal asphyxia (PA) Respiratory distress syndrome (RDS), Intracranial hemorrhage (ICH), mechanical ventilation (MV)

Table 1 shows clinical data recognized as clinical risk factors for severe ROP development. Statistically significant differences were observed between ROP group and control group in mean values of gestational age, weight mass at birth, Apgar score and days of O2 therapy. Infants with gestational age bellow 32 weeks were present in group of ROP (89.2%) significant more frequently than in control group (74.4%) p=0.013. Also, frequencies of respiratory distress syndrome expression, broncho-pulmonary dysplasia, intracranial hemorrhage and requirement for mechanical ventilation were statistically significantly different between two analyzed groups of infants.

Table 2. Genotype and allele distribution of eNOS T-786C and eNOS 4a/b polymorphisms in infants with retinopathy of prematurity

eNOS gene polymorphism	Genotype	ROP group n (%)	Control group n (%)	*p value
T-786C	TT	24 (40.0)	26 (38.2)	0.978
	CT	31 (51.7)	36 (52.9)	
	CC	5 (8.3)	6 (8.8)	
Allele %	T	65.8	64.7	0.850
	C	34.2	35.3	
4a/b	bb	50 (60.2)	61 (70.1)	0.384
	ba	29 (34.9)	24 (27.6)	
	aa	4 (4.9)	2 (2.3)	
Allele %	b	77.7	83.9	0.146
	a	22.3	16.1	

*Chi-square test or Fisher exact test

Both analyzed genotypes in premature infants were in Hardy-Weinberg equilibrium. The differences in *eNOS* T-786C and *eNOS* 4a/b polymorphisms genotypes and alleles frequencies between two analyzed groups of premature infants were not statistically significant (Table 2). Genotype frequencies between ROP and control groups by dominant 4bb/4ba+4aa, p=0.921; TT/ TC+CC, p=0.838) or recessive (4bb+4ba/4aa, p=0.435; TC+TT/CC, p=0.176) model did not show significant differences. Statistically significant difference was observed only in female infants by dominant model (4bb/4ab+4aa) (Fig. 1). Female infants in ROP group were more often carriers 4ba and 4aa genotypes than female infants in control group (X2=4.333, p=0.037; Risk ratio 1.6849, CI 0.95, 0.962- 2.951). We analyzed association between 4a/b e*NOS* polymorphism and ROP requiring treatment among preterm infants by logistic regression analysis. After the adjustment for gender, BMW, gestational age, Apgar score and days of oxygen therapy, logistic regression does not show statistically significant association between 4a/b genotypes and development of severe ROP in premature infants (β=0.346, p=0.288).

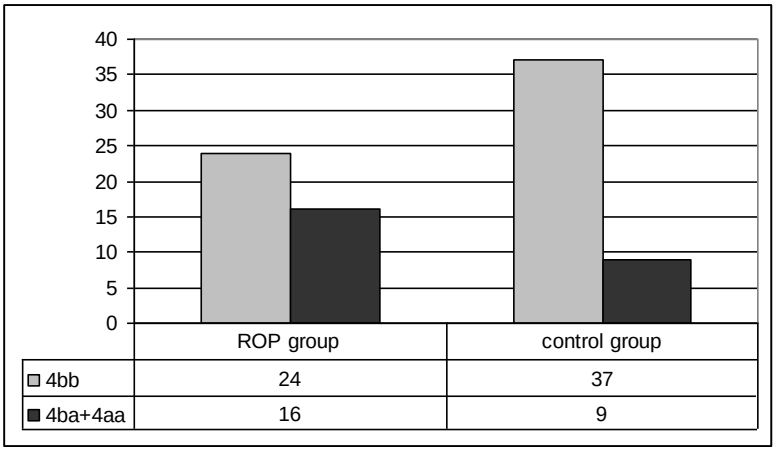

Fig 1. *eNOS* 4a/b genotypes frequencies in female infants

Table3. Clinical parameters depending of eNOS T-786C and eNOS 4a/b genotype

eNOS	T-786C			4a/4b		
polymorphism	TT	CT	CC	4bb	4ba	4aa
Gender M/F	20/30	39/28	5/6	50/61	30/23	4/2
GA weeks*	30.5±1.8	30.5±1.9	30.4±1.4	30.4±1.9	30.6±2.1	28.8±1.4
BMW grams*	1300±295	1285±255	1305±266	1286±268	1316±257	1220±231
Apgar score *	5.7±1.8	5.2±1.8	6.1±2.2	5.5±1.8	5.6±1.9	5.2±1.6
Therapy O2 days*	19.2±13.7	20.1±14.2	14.5±10.3	20.1±16.3	18.5±13.9	27.2±18.9

*mean values ± standard deviation, Gestational age (GA), Birth weight mass (BWM)

Table 3 shows clinical risk factors according *eNOS* T-786C and *eNOS* 4a/b genotypes. Carriers of 4aa genotype of *eNOS* 4a/b polymorphism had lower gestational age compared to other genotypes groups (4bb and 4ba) (p=0.114). Gestational age values analyzed by recessive model were significantly lower in infants with 4aa genotype (t=2.034 p=0.044). Fig. 2 illustrate that genotype 4aa has been ten times more often observed in group of infants with gestational age lower than 30 weeks compared with group of infants with gestational age higher than 30 weeks (p=0.032, by recessive model p=0.0144, Risk ratio 10.3636, CI 1.2405-86.5849) (Odds Ratio 11.3, 1.2867-99.239). Multivariate linear regression analysis with gender, BMW and Apgar score as covariates does not show association of 4a/b genotypes with gestational age of premature infants.

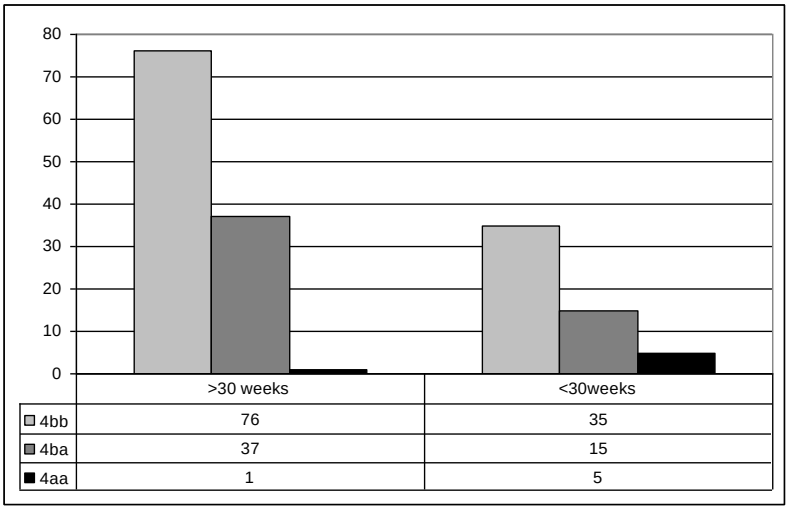

Fig 2. *eNOS* 4a/b genotypes frequencies in two groups of gestational age (> 30 weeks and <30 weeks).

DISCUSSION

ROP occurs because abnormal blood vessels grow and spread throughout the retina. These blood vessels are fragile and can lead to retinal scarring and detachment. Retinal detachment is the main cause of visual impairment in ROP. Clinical characteristics contributing to the risk of ROP include gender, low birth weight, breathing difficulties, respiratory distress, anemia, blood transfusions, intracranial hemorrhage and the long period of oxygen therapy given to premature infants (KIM *et al.* 2004), but the most important is gestational age at the moment when a baby was born. Prematurity is the reason why retina does not finish complete development and instead undergoes to neovascularisation typical for ROP (QAZI *et al.* 2009).

According to our study all clinical data, except gender, recognized as postnatal clinical risk factors, are statistically significantly different between two analyzed groups of infants. This result was expected and described previously in our population (KNEŽEVIĆ *et al.* 2011). It is observed that the severe stage of ROP progresses in some and regresses spontaneously in others

infants with similar clinical findings. It has been reported that genetic factors may influence the progression of the severe stage of ROP among infants with similar clinical findings (VANNAY *et al.* 2005). BIZZARRO *et al*. (2006) reported that genetic factors accounted for 70.1% of the variance in liability for retinopathy of prematurity. Genetic polymorphisms may alter the function of the genes that normally contribute to angiogenesis in retina, such as *VEGF* and *eNOS*. Published data in different populations described conflicting results considering genetic polymorphisms in *VEGF* gene. Some of them support the hypothesis that *VEGF* gene polymorphisms are associated with progression of ROP to the advanced stage (VANNAY *et al.* 2005 and LIU *et al.* 2012), but others did not find any association with ROP development (DUNAI *et al.* 2008, KALMEH *et al.* 2013, and MALIK *et al.* 2014).

eNOS gene polymorphisms were determined as risk factors for retinopathy with similar pathogenesis like ROP. Published data suggest that genetic polymorphisms Glu298Asp, 4b/a and T-786C (EZZIDI *et al.* 2008) and C774T (TAVERNA *et al.* 2005) in the *eNOS* gene are genetic risk factors for diabetic retinopathy. These polymorphisms may be useful markers of increased susceptibility to severe diabetic retinopathy. Only two studies analyzed *eNOS* gene polymorphisms in etiology of ROP. One study identified minor -786C and 894T alleles of *eNOS* gene as significant risk factors in the development of ROP, and found strong association between those *eNOS* gene polymorphisms and the severity of disease in Caucasians and African Americans (YANAMANDRA *et al.* 2010). RUSAI *et al*. (2008) observed that the genotype distribution of eNOS 4a/b polymorphism was significantly different in preterm infants with severe ROP compared to preterm infants with ROP stage that did not require treatment. They emphasized eNOS 4aa genotype as an independent risk factor for severe ROP. In the same study association between eNOS T- 786C and ROP has not been observed. Results of our study show that no differences in genotypes and alleles distributions of *eNOS* T-786C and *eNOS* 4a/b polymorphisms between two analyzed groups of premature infants. We found that only female infants in ROP group were more often carriers of 4ba and 4aa genotypes than female infants in the control group. RUSAI *et al.* (2008) described mail gender as risk factor for severe ROP development, but our results did not show differences in gender distribution between ROP and control group. Also, logistic regression analysis did not show association between 4a/b genotype and severe ROP development after adjustment for significant clinical risk factors (gender, BMW, gestational age, Apgar score and days of oxygen therapy).

Recent studies show significant associations between the eNOS (Glu298Asp) polymorphism and idiopathic recurrent pregnancy loss (SU *et al.* 2011) and found significantly higher frequencies of mutant allele in spontaneously aborted fetal materials (YALCINTEPE *et al.* 2015). GIBSON *et al.* (2007) reported that polymorphisms of nitric oxide are associated with prematurity and suggest that they may be contributors to the risk of spontaneous preterm birth. Our results show that almost all detected 4aa genotypes (5 of 6) were present in the group of infants with gestational age under 30 weeks. This polymorphism was not investigated as a risk factor for spontaneous preterm birth in the literature. Multivariate linear regression analysis in our study did not show association of 4a/b genotypes with gestational age of premature infants.

We can conclude that in Serbian premature infants T-786C and 4a/b *eNOS* gene polymorphisms were not associated with development of severe ROP. Some association was detected for 4a/b genotypes in female gender but was not sufficient to conclude that minor allele was the risk factor for severe ROP development in female infants. Also, according to our study, T-786C and 4a/b *eNOS* gene polymorphisms are not relevant to the prediction of prematurity. In

mulifactorial trait such as ROP, expression of phenotype depends on interaction of many genes and influence of the environment. According to all this, to predict severe ROP development it may be necessary to analyze more genes in a larger cohort of ROP patients.

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ISPITIVANJE POLIMORFIZAMA T-786C I 4A/B U GENU ZA ENDOTELNU AZOT OKSID SINTETAZU KOD DECE SA RETINOPATIJOM PREMATURITETA

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Izvod

Retinopatija prematuriteta (ROP) je vazoproliferativno oboljenje retine koje dovodi do oštećenja vida. Smatra se da je genetska predispozicija, pored poznatih faktora rizika kao što su kratko vreme gestacije, mala telesna težina na rođenju i terapija kiseonikom, jedan od uzroka nastanka ROPa. Cilj naše studije bio je da se ispita asocijacija T-786C i 4a/b polimorfizama *eNOS* gena sa nastankom ROPa. U studiju je uključeno 174 prematurusa od čega je 84 bilo sa ROPom, a 90 je predstavljalo kontrolnu grupu. Rezultati analiza nisu ukazali na razliku u distribuciji alela i genotipova T-786C i 4a/b polimorfizama *eNOS* gena između analiziranih grupa. Jedina statistički značajna razlika je uočena kod prematurusa ženskog pola po dominantnom modelu za 4a/b genotipove (4bb/4ba+4aa). Naime, prematurusi ženskog pola iz grupe sa ROPom češće su imali 4ba i 4aa genotipove u poređenju sa prematurusima istog pola u kontrolnoj grupi (p=0.037). Ipak, statistički značajna asocijacija ovog polimorfizma i ROPa nije potvrđena (p=0.288). Po recesivnom modelu (4bb+4ba/4aa) dužina trajanja gestacije bila je statistički značajno kraća kod prematurusa sa 4aa genotipom (t=2.034 p=0.044). Gotovo svi 4aa genotipovi detektovani su kod prematurusa sa dužinom gestacije ispod 30 nedelja (p=0.032), ali naknadna multipla regresiona analiza nije pokazala asocijaciju 4a/b polimorfizma i trajanja gestacije. Na osnovu svih navedenih rezultata možemo pretpostaviti da T-786C i 4a/b polimorfizmi *eNOS* gena ne predstavljaju faktore rizika za nastanak ROPa kod prematurusa u populaciji Srbije.

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