Vascular Endothelial Growth Factor Gene Polymorphism - Susceptibility Predictor for Severe Retinopathy of Prematurity?

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The aim of the present study was to determine whether there is an association between VEGF-634 G>C polymorphism, severe retinopathy of prematurity and the laser intensity. 105 subjects born preterm followed up in the Neonatology Department of the Municipal Emergency Hospital Timisoara were included in study. Allelic discrimination was use to establish the patients genotype. The association between VEGF polymorphism -634 G> C and the severe form of ROP requiring laser therapy is not statistically significant for both the CC genotype (p = 0.177) and genotype GC (p = 0.246). The CC genotype does not represent a risk factor for the severe form of ROP requiring laser therapy. There is no significant difference between premature babies with advanced retinopathy of prematurity and healthy subjects as regards of the polymorphism -634 G>C in VEGF gene. The amount of laser is not correlated with the VEGF -634 G>C polymorphism.

Keywords: retinopathy of prematurity, polymorphism, laser treatment

The retinopathy of prematurity (ROP) is a vascular proliferative disorder of the retina that can lead to visual impairment or complete visual loss [4, 18]. Retinal vascularization begins in the fetal environment, and continues until the birth at full term. The premature newborns have an incomplete vascularized retina. Fibro vascular proliferation occurs at the juncture of vascularized and avascular retina [1]. Abnormalities in the retinal architecture may lead to retinal fold and retinal detachment.

ROP is a multifactorial disorder and its risk factors have not been completely identified yet [4,16]. Associated with short gestational age and low birth weight, the risk factors include: surfactant administration, mechanical ventilation, oxygen-therapy, blood transfusion, respiratory distress syndrome, bronchopulmonary dysplasia, intraventricular hemorrhage, necrotizing enterocolitis, sepsis, multiple pregnancies [29]. It is considered that in the pathogenesis of ROP, the genetic component has an important role [4]. Recent studies suggest that VEGF may play an important role in the development of both normal retinal vascularization and in the appearance of ROP [16, 25]. The VEGF gene is located on chromosome 6 p 21.3 and genetic polymorphisms of VEGF were associated with ROP [15].

Evaluation of the immature retina is conventionally made by indirect ophthalmoscopy (in Romania it was initiated by the Institute for Protecting the Mother and Child in 2002). Information on the newborn's retina pathology can be attained through the interpretation of images obtained by using ret cam or retrieved with portable handled optical coherence tomography [14]. The criteria for screening are established at the national level, depending on the incidence of the disease. The goal of an effective screening for ROP is to identify the children that could benefit from treatment in due time [5]. Screening for ROP is uncomfortable for the new born and rather time consuming for the ophthalmologist, so that the identification of the polymorphisms of VEGF gene (central role in abnormal retinal angiogenesis) could provide an alternative [5, 27, 28]. In addition, anti-VEGF therapy is effective as a primary or adjunctive treatment for retinopathy of prematurity [10].

It is still unclear why for some newborns ROP progresses towards severe stages despite an optimal therapeutic intervention, while for others it regresses spontaneously [24, 25]. Its incidence varies between races (more frequent in white) and gender (more frequent in males).

Considering this context, the aim of this study is to evaluate the possible association of the polymorphism VEGF-634 G> C (SNP identifier rs 2010963, OMIM identification number 192240.0001) with the development and severity of ROP in Romania. We have also followed the correlation between VEGF polymorphism -634 G>C and the quantity of laser used during diode laser treatment.

Experimental part

Material and methods

Our study has enlisted 105 patients. Each patient was born with birth weight ≤ 2000 gr and gestational age ≤ 35 weeks. The selection of patients was done randomly, when evaluating and performing laser treatment in the Neonatology Clinic of the Municipal Emergency Clinical

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Hospital Timisoara, between 2014 and 2016. Informed consent was previously obtained from parents or legal tutors.

The ROP classification was made according to The International Classification of Retinopathy of Prematurity, using the staging and locating the active processes (there have been described 5 stages and three areas of location at the level of the surface of the retina) and aggressive posterior ROP [7]. All children were evaluated with the help of indirect ophthalmoscopy by an experienced ophthalmologist. The examination was performed one hour after being fed, in mydriasis (cyclopentolate 0.5%, 0.5% tropicamide or phenylephrine 2.5%). For topical anesthesia, propacaine drops 0.5% were administered.

Patients were divided into two groups, based on requirement for the ROP treatment:

- he treated group: 59 children with ROP stage 3 in area I or II or posterior aggressive form that have required laser treatment. The 810 nm indirect diode laser (IRIS Medical Instruments Inc., California, USA) was used. This was done within 24-72 h after the diagnosis. Laser ablation was focused on the avascular retina between the fibro vascular ridge and the ora serrata, and was applied on the entire circumference of the eye globe [23]. Medium intensity (grey-white burn) scattered burns were applied. The distance between burns was half of the diameter.

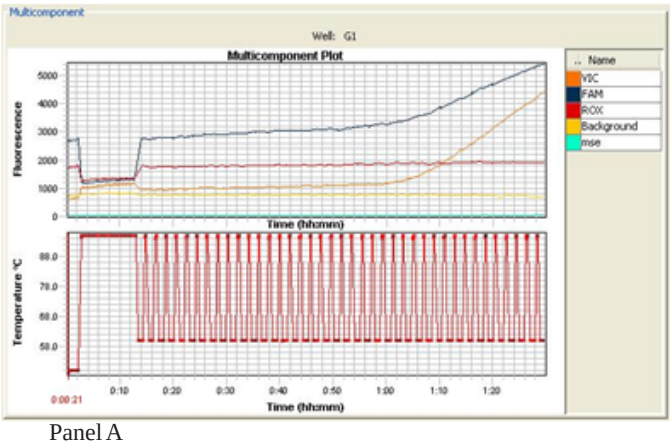
-the control group: 46 children with similar characteristics of weight and age, who have not reached the threshold stage, with self-regressed ROP without treatment.

Genotyping

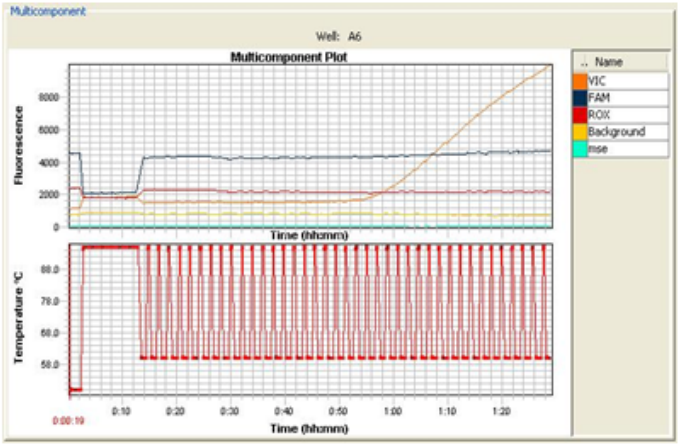
EDTA blood samples were collected from each patient (0.3 mL), after obtaining the informed consent. The extraction of the genomic DNA was performed out of 200 ìL of fresh blood, by using PureLink® Genomic DNA Mini Kit (Invitrogen TM). The extracted DNA was stored at -20 °C. Genotyping VEGF was performed by using TaqMan® Genotyping Master Mix (Applied Biosystems) and Genotyping Assay (Applied Biosystems), according to the protocol described by the producer. For the allelic discrimination of VEGF polymorphism -634 G->C the experiments were carried out on Applied Biosystems 7900HT Fast Real-Time PCR System in a reaction volume of 25 µL, containing TaqMan Drug Metabolism Genotyping Assay and TaqMan® PCR Master Mix and DNA probe. VEGF genotyping was done in the presence of two controls, AL-1 (wild type) and AL-2 (rs 2010963). The AL1 probe corresponding to the wild type was VIC dye-labeled while the AL2 probe corresponding to the mutant type (rs 2010963) was FAM dye-labeled. Genotypes were determined by measuring allele-specific fluorescence using the software for allelic discrimination (Applied Biosystems). The fluorometric reading allowed the distinction of three possible situations: the presence of both alleles (the wild and the polymorphic ones) the patient being genotyped as a carrier of the unfunctional polymorphic allele (heterozygote), the presence of only the wild allele or the presence of only the polymorphic variant (fig. 1).

Ethics Statement

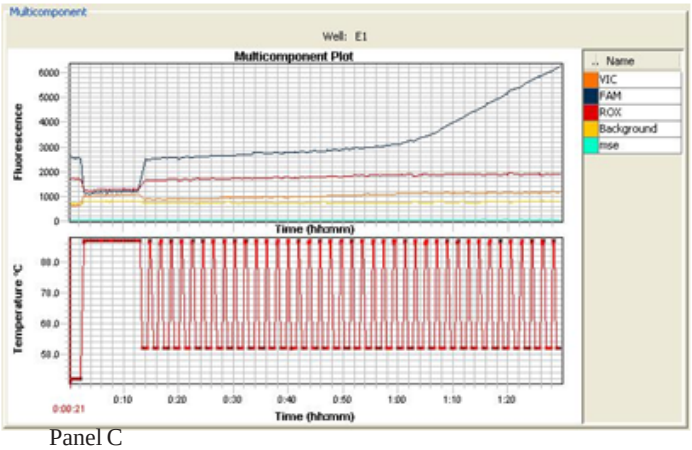
The Human Research Ethics Committee of Municipal Clinical Emergency Hospital Timisoara approved this study. The study adheres to the Declaration of Helsinki. Written informed consent was attained from all participants' parents or legal guardians.



Panel A



Panel B



Panel C

Fig. 1. Amplification curve indicating the presence of both alleles (panel A), the presence of wild type alleles (panel B) and the polymorphic alleles (panel C)

Statistical analysis

Statistical data processing has been done with the SPSSv17 program. For numerical variables indicators of central tendency (mean and median) and dispersion (standard deviation, standard error of the mean minimum and maximum values, respectively) have been calculated, and for nominal variables, relative frequencies have been calculated. Comparisons between two independent sets of numeric values have been conducted with the help of the non-parametric Mann-Whitney test. Comparisons between nominal variables were conducted with Chi-square test and the risk analysis was applied, to determine the variant of gene responsible for the appearance of the diagnosis. In this manner the OR risk index has been calculated and 95% confidence interval for OR. In all cases (interpretation of the meaning, i.e. the width of the confidence intervals) the significance threshold of 0.05

(5%) was used, corresponding to the level of confidence of 95%.

Results and discussions

The study lot included 105 children from which 59 presented ROP and needed laser treatment (Group 1), and 46 represented the control group (Group 2). All three genotypes (GG, GC, CC) were present in patients with advanced ROP at a rate of 23.73, 33.90 and 42.37%, while the patients in the control group at a rate of 13.04, 36.96 and 50.00%.

The data analysis shows that both gestational age and birth weight were significantly higher for the control group, compared to the treatment group -nonparametric Mann-Whitney test (table I).

The results of our study show that the association between VEGF polymorphism -634 G> C and severe form of ROP requiring laser therapy is not statistically significant for both the CC genotype (p = 0.177) and genotype GC (p = 0.246).

Therefore, our results show that the CC genotype does not represent a risk factor for the severe form of ROP, requiring laser therapy in relation to the wild type genotype GG (OR = 0.47), 95% CI = (0.153, 1.416). Moreover, the heterozygote genotype GC also does not increase the risk for severe form of ROP requiring laser therapy (OR = 0.504), 95% CI = (0.159, 1.599). In our study the heterozygous and homozygous carrier state of the VEGF -634C alleles is not an independent risk factor for severe ROP.

Diode laser treatment was made in a time period that has ranged between 2.5 and 11.5 weeks after birth, with an average 7.2 ± 1.84 weeks (7.4 ± 1.99 for the genotype CC, 7.1 ± 1.67 for GC and 7.0 ± 1.79 weeks for genotype GG respectively). It was applied to a total of 118 eyes (59 children). 78 of them had stage 3 ROP (66.10%) at the time of treatment and 40 eyes aggressive posterior form of the disease (33.90%). In 28 cases, ROP has been located in area I (23.73%), and in 90 of the cases in the area II (76.27). The number of burns applied ranged between 300 and 3689, energy used between 250 mW and 650 mW, and the time of exposure was 0.2 ms in all cases (table 2).

Laser energy used is significantly increased in the genotype GC related to GG genotype (p =0.035). The number of impacts is significantly increased for genotype GC related to CC genotypes (p = 0.019) and GG (p = 0.040) - Mann-Whitney nonparametric correlation test.

VEGF has an important role in vascular permeability, growth of endothelial cells and physiological and pathological angiogenesis [17, 21]. It is widely accepted the involvement of endothelial growth factor in the pathogenesis of ROP [2]. Therefore, by inhibiting VEGF, it is aimed to stop the pathological neovascularization and finally the regression of ROP [26]. This objective is achieved either by laser ablation of peripheral avascular retina (retinal cells that produce VEGF are destroyed), or by the use of anti-VEGF agents (pharmacological inhibition of VEGF) [11, 22]. Currently, the golden standard treatment for proliferative ROP is considered the laser photocoagulation [20].

In our study we have evaluated the relationship between VEGF -634 G>C polymorphism (previously denoted + 405 G/C), severe ROP and the amount of laser. The results obtained by us prove that the heterozygous and homozygous carrier state of the VEGF-634C alleles is not an independent risk factor for advanced ROP. The amount of the laser is not correlated with the VEGF -634 G>C polymorphism.

There is a heterogeneity of data regarding the relationship between VEGF -634 G>C polymorphism and risk of ROP (Table III) in the current studies. Cooke, Vannay and Huang found a significant difference in the distribution of the VEGF -634 G>C/ +405G/C genotypes in infants who were treated for proliferative ROP, compared to preterm infants with self-regressed ROP without treatment [3, 6, 16,].

By contrast with these data, the results obtained in studies led by Shastry, Kaya, Kalmek, Kwinta and Kusuda do not support the association between this polymorphism and the risk of advanced ROP [8, 9, 12, 13, 25]. Fluctuating results of polymorphisms of gene studies must be analyzed in conjunction with other related factors and not as independent risk factors.

As an alternative to laser treatment for reducing the pathological neovascularization in the retina, the intravitreal injection with anti-VEGF agents is used. Up to the present, it has been used for the treatment of severe ROP ranibizumab, aflibercept and avastin [19]. There is a large heterogeneity in the dosage for the treatment of ROP. The

		genotype CC	genotype GC	genotype GG
gestational age (weeks)	Group 1	(29.4±2.84)	29.7±2.58	28.29±1.68
	Group 2	(31.13±2.7)	31.24±2.22	30.83±2.64
		p=0.024	*p=0.022*	*p=0.051*
birth weight (grams)	Group 1	1324.4±380.01	1244.5±283.46	1161.43±288.39
	Group 2	1489.04±370.8)	1655.29±386.12	1573.29±281.33
		p=0.204	*p=0.001*	*p=0.009*

Table 1
GESTATIONAL AGE AND BIRTH WEIGHT IN RELATION TO GENOTYPE OF VEGF -634 G>C POLYMORPHISM

Table 2
LASER TREATMENT PARAMETERS (ENERGY, NUMBER OF IMPACTS) IN RELATION TO GENOTYPE OF VEGF -634 G>C POLYMORPHISM

	Genotype	N	Mean	Standard deviation	Standard error of the media	95% confidence interval for the media		Minimum	Maximum
						Inferior limit	Superior limit		
Laser energy (mW)	CC	50	375.0	106.19	15.02	344.8	405.2	250	600
	GC	40	404.4	133.00	21.03	361.8	446.9	250	650
	GG	28	332.1	76.64	14.48	302.4	361.9	250	550
	Total	**118**	**374.8**	**112.73**	**10.38**	**354.2**	**395.3**	**250**	**650**
No. of burns	CC	50	1312.9	715.51	101.19	1109.5	1516.2	300	3689
	GC	40	1550.3	620.15	98.05	1352.0	1748.6	350	3200
	GG	28	1353.1	533.16	100.76	1146.3	1559.8	550	2710
	Total	**118**	**1402.9**	**647.83**	**59.64**	**1284.8**	**1521.0**	**300**	**3689**

Table 3

GENOTYPE FREQUENCIES OF VEGF -634 G>C POLYMORPHISM AND RETINOPATHY OF PREMATURITY (TREATED vs. NOT TREATED)

Study	Total Patients		Genotype						Association polymorphism-ROP
			Cases			control			
	Cases	Control	GG	GC	CC	GG	GC	CC	
Cooke et al (2004)	91	97	44	39	8	31	53	13	yes
Vannay et al (2005)	86	115	30	41	15	55	53	7	yes
Shastry et al (2007)	61	61	27	27	7	28	26	7	no
Huang (2008)	20	20							yes
Kwinta et al (2008)	60	101	32	18	10	55	36	10	no
Kusuda et al (2011)	127	77	49	60	18				no
Kaya et al (2013)	42	31	25	11	6	20	8	3	no
Kalmeth et al (2013)	15	66	6	6	3	24	31	11	no
our study (2016)	59	46	14	20	25	6	17	23	no

clinical benefit and safety of administration in infant are not fully supported by clinical trials.

Thus, the involvement of VEGF polymorphisms in susceptibility and in aggressiveness of ROP represents a challenge for further clinical trials.

Conclusions

Our data suggest that there is no significant difference between any of the allelic variants of the polymorphism -634 G>C in VEGF gene between premature babies with advanced retinopathy of prematurity and healthy subjects. The amount of laser is not correlated with the VEGF -634 G>C polymorphism.

We consider that further investigations of the association between VEGF gene polymorphisms and severe ROP may have great practical importance for clinicians, for diagnosis and the choice of the optimal treatment plan (laser, anti-VEGF).

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