Turkish Journal of Molecular Biology & Biotechnology (2016) 1: 28-34

ORIGINAL ARTICLE

Turkish JMBB

Lack of association between plasminogen activator inhibitor-1 4G/5G polymorphism and retinopathy of prematurity in premature neonates

Didem ARMANGİL[1], İbrahim AKALIN[2,3], Yakup ASLAN[4], Duygu ÖZEL DEMİRALP[5]

[1] Department of Pediatrics, Neonatology Unit, Koru Hospital, Ankara, Turkey
[2] Trabzon Kanuni Education and Research Hospital, Genetic Diseases Diagnosis Center, Trabzon, Turkey
[3] Department of Medical Genetics, İstanbul Medeniyet University, Faculty of Medicine, İstanbul, Turkey
[4] Department of Pediatrics, Neonatology Unit, Karadeniz Technical University, Faculty of Medicine, Trabzon, Turkey
[5] Ankara University Biotechnology Institute, Besevler, Ankara, Turkey

Keywords
Retinopathy of prematurity, plasminogen activator inhibitor, 4G/5G gene polymorphism, neonate

Received: 06 July 2015
Accepted: 21 August 2015

ABSTRACT • Background and aims: Retinopathy of prematurity (ROP) is a proliferative vascular disorder in premature neonates. Due to differences in individual responses to the treatment, various genetic factors have been investigated in the etiology of ROP. We investigated the gene polymorphism of plasminogen activator inhibitor (PAI-1) 4G/5 as a risk factor of ROP development. **Materials and Methods:** 73 neonates with ROP and 101 controls were enrolled to study. Genotyping was analyzed using real time PCR. **Results:** The proportion of 4G/4G, 4G/5G and 5G/5G genotypes did not differ statistically between the ROP and control groups ($p>0.05$). Having PAI-1 4G/4G genotype polymorphism seems to develop the risk of ROP (OR =0.702; 95% CI: 0.300-1.639) less than PAI-1 4G/5G polymorphisms (OR =1.064; 95% CI: 0.469-2.410). 4G/4G genotype frequency was decreasing as the stages of ROP were increasing though there was no statistically significant difference between proportion of genotypes and ROP stages. **Conclusion:** This study showed that PAI-1 4G/5G genotype which is known as a risk factor for angiogenesis is not a predisposing factor for ROP development.

INTRODUCTION

Retinopathy of prematurity (ROP) is the chief proliferative vascular disorder leading to visual impairment or complete vision loss in premature neonates as a result of incomplete and abnormal retinal vascular development (1). The main pathology in ROP is abnormal angiogenesis. During angiogenesis extracellular proteinases and their inhibitors play important roles in the regulation of endothelial cell matrix (ECM) remodeling. The restructuring of ECM by secretion of proteolytic enzymes is one of the well-characterized steps of the angiogenic program (2) and interconnected with neonatal dis-

Correspondence: Duygu ÖZEL DEMİRALP, Ankara University Biotechnology Institute Central Laboratory Tandoğan Campus 06110 Beşevler, Ankara/ TURKEY • Tel: +90 505 403 22 07 • Fax: +90 312 222 58 72 • E-mail: ozeldemiralp@gmail.com

eases such as respiratory distress syndrome (RDS) (3), brochopulmonary dysplasia (BPD) (4), as well as ROP[5] by responses to tissue injury (6).

Despite decades of researches on the pathogenesis of ROP, we still lack adequate knowledge to predict in which infants ROP will regress spontaneously or progress in spite of reasonable treatment. Because of differences in individual responses to the treatment, various genetic factors have been looked into to understand the etiology of ROP (7). The alterations in the regulation or expression of the responsible genes might contribute to a genetically determined susceptibility to ROP as shown in the monozygotic twin studies (8). Plasminogen activator inhibitor (PAI) is a serine protease inhibitor (serpins) (9) and play a central role in retinal angiogenesis. Intravitreal injection of human recombinant PAI-1 significantly reduced retinal neovascularization in ROP rat model and found to inhibit pathologic angiogenesis (10). The PAI-1 gene contains a common 4G/5G insertion/deletion polymorphism that plays an important role in the regulation of PAI-1 gene expression (9).

The aim of this study was to investigate the gene polymorphism of PAI-1 4G/5G as an important risk factor of tissue remodeling and angiogenesis within ROP pathophysiology. To our knowledge, this study is the first report investigating the association between PAI-1 4G/5G polymorphisms and ROP in premature infants.

MATERIAL and METHODS

Patients and controls

The study was performed at the Neonatal Intensive Care Unit (NICU) of the both Trabzon Women's and Children's Hospital and Karadeniz Technical University Children's Hospital, Trabzon, Turkey, between June 2010 and June 2011. To investigate the genotype distribution of PAI -1 4G/5G gene polymorphism, 73 preterm infants with ROP and 101 controls were enrolled in the study. Our study group included neonates with a birth weight of less than 1.500 g or gestational age of 32 weeks or less and/or selected neonates with a birth weight between 1.500 g and 2.000 g or gestational age of more than 32 weeks with an unstable clinical course, including those requiring cardio-respiratory support. Infants were submitted to fundus examination using indirect ophthalmoscopy. Before ocular examination, 2.5% phenylephrine and 0.5% cyclopentolate drops were administered for pupil dilatation. The first screening examination was conducted on all infants when their chronological age was 4-6 weeks. When premature retinopathy was not detected, they were re-evaluated within 2-week intervals until the retinal vascularization was complete. Serial eye examinations were performed according to a standard schedule suggested by the American Academy of Ophthalmology, the American Academy of Pediatrics, and the American Association for Pediatric Ophthalmology and Strabismus. ROP stages were determined according to the International Premature Retinopathy Classification Committee. Stages of retinopathy of prematurity are defined by vessel appearance at the interface between the vascular and avascular retinal areas. This interface resembles a line for stage 1, a three-dimensional ridge for stage 2, and a ridge with neovascularization extending into the vitreous gel for stage 3 (Plus disease — as in stage 3+ — indicates that two or more quadrants of the eye have dilated veins and tortuous arteries near the optic disk). The neovascularization can progress to form fibrous bands that cause partial retinal detachment (stage 4), and ultimately, total retinal detachment (stage 5). When ROP was diagnosed, patients were examined every week starting from the beginning of the disease until it regressed. Control groups were defined as premature neonates with no diagnosis of ROP. Neonates with congenital anomalies were excluded. All blood samples for hematological testing were obtained from a peripheral vein during lifetime before any blood product transfusion.

DNA extraction and genotyping of the 4G/5G polymorphism

We obtained blood samples and genomic DNA with informed consent as overseen by the Local Ethical Committee of Trabzon Numune Training and Research Hospital. EDTA-blood was collected for DNA extracted and genotyping was performed using a primer probe set of PAI-1 PCR system (Dr. Zeydanli Life Sciences, Ankara, Turkey) having 5'-3' exonuclease activity. After that, PCR reaction was set according to the manufacturer's instructions.

Statistical methods

Using Statistical Package analyzed the data for the Social Sciences (SPSS) 11.5 for Windows (Chicago, Illinois). Values were expressed as mean, ±SD. Differences between groups were analyzed using $\chi 2$, Mann-Whitney U, and Kruskal-Wallis tests. Unmatched Odds ratio (OR) and 95% confidence intervals (CI) as estimates of the relative risk of the allele frequency were calculated in the entire study population. The 95% CI were calculated from a conditional logistic regression algorithm using the maximum likelihood method. A p value <.0.5 was considered statistically significant.

RESULTS

Of 174 infants included in the study, 73 (41.9%) were infants with ROP and 101 (%58.1) were controls with gestational age ranging between 26 and 35 weeks. The mean birth weight was 1322±431 g and 1414±313 g in infants with ROP and control group, respectively. The mean gestational age was 29.4±0.8 weeks and 30±1.4 weeks, respectively and the difference was statistically significant ($p<0.01$). There was no statistical difference between two groups with respect to percentages of males, infants undergoing cesarean section, mean birth weight, or percentage of infants who were born with premature rupture of membranes, chorioamnionit and preeclampsia, except their 5th minute Apgar score (Table 1).

Genotype distributions of PAI-1 4G/5G polymorphisms and allele frequency in preterm infants with ROP and control groups are shown in Table 2. There are no differences between the ROP and conrtol groups for the proportion of 4G/4G, 4G/5G, and 5G/5G genotypes and 4G, 5G allele frequencies ($P >0.5$). Having PAI-1 4G/4G genotype polymorphism appears to developed the risk of ROP (OR =0.702; 95% CI: 0.300-1.639) less than PAI-1

Table 1 Demographic characteristic of the study group

Clinical data	ROP group (n=73)	Control group (n=101)	*p* values
Sex (M/F)	38/35	55/46	.754
Gestational age (weeks ± SD)*	29.4±0.8	30±1.4	<.001
Birth weight (g ± SD)*	1322±431	1414±313	.103
PROM	12 (16.4%)	13 (13%)	.508
Apgar score <7 (5^{th} minute)	31 (42.5%)	14 (14%)	<.001
Cesarean delivery	50 (68.5%)	88 (87.1%)	.003
Chorioamnionitis	3 (4.1%)	0 (0%)	.041
Preeclampsia	10 (13.7%)	8 (9%)	.318

ROP, retinopathy of prematurity; PROM, premature rupture of the membranes. *Mean.

4G/5G polymorphisms (OR =1.064; 95% CI: 0.469-2.410), though it was not statistically significant. The percentages of 4G/4G genotype frequency were decreasing as the stages of ROP were increased although there was no statistically difference between proportion of 4G/4G, 4G/5G and 5G/5G genotypes and ROP stages (p=6.25) (Table 3) (Figure 1).

Table 2 Distribution of PAI-1 4G/5G polymorphisms and allele frequency in preterm infants with ROP and control groups

PAI genotype	ROP group (n=73)	Control group (n=101)	OR (95% CI)	p
PAI-1 4G/4G	25 (34.2%)	30 (29.7%)	0.702 (0.300-1.639)	>.05
PAI-1 4G/5G	27 (37.0%)	49 (48.5%)	1.064 (0.469-2.410)	>.05
PAI-1 5G/5G	21 (28.8%)	22 (21.8%)	1	
PAI 4G allel	77 (52.7%)	109 (54.0%)	0.952 (0.621-1.459)	>0.05
PAI 5G allel	69 (47.3%)	93 (46.0%)	1	

ROP, retinopathy of prematurity; PAI, plasminogen activator inhibitor; OR, odds ratio; CI, confidence interval.

Table 3 Distribution of PAI-1 4G/5G polymorphisms among stages of ROP

ROP stage (n)	4G/4G (n=25)	4G/5G (n=27)	5G/5G (n=21)	p
Stage 1 (18)	8 (44%)	7 (38.9%)	3 (16.7%)	.374
Stage 2 (29)	9 (31%)	11 (37.9%)	9 (31%)	.829
Stage 3 (23)	7 (30.4%)	9 (39.1%)	7 (30.4%)	.897
Stage 4 (3)	1 (33.3%)	0 (0%)	2 (66.7%)	.257

ROP, retinopathy of prematurity; PAI, plasminogen activator inhibitor; OR, odds ratio; CI, confidence interval.

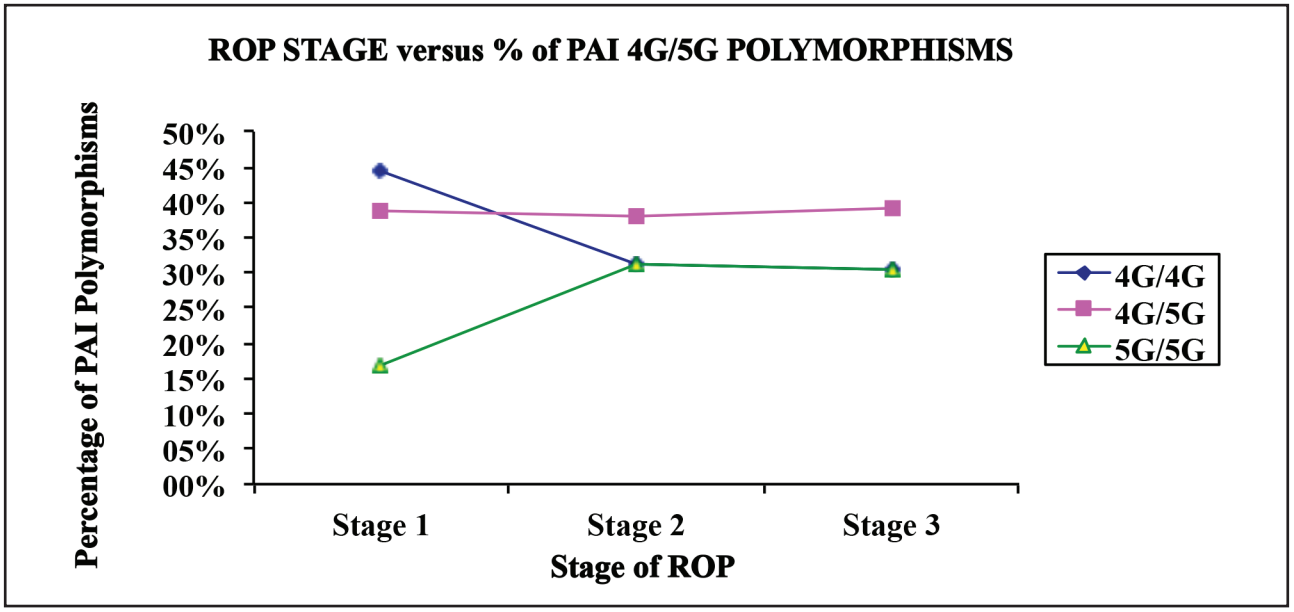

Figure 1 Percentages of PAI 1 4G/5G gene polymorphisms according to stage of ROP.

DISCUSSION

Retinopathy of prematurity remains a significant cause of morbidity among preterm infants worldwide. Even in developed countries screening programs and interventions aimed to prevent and treat the ROP, it still accounts for 3% to 11% of blindness in children (11). Therefore, in this study we aimed to investigate promising underlying effect of PAI-1 4G/5G gene polymorphism within ROP infants.

Of the numerous risk factors that have been investigated, gestational age and birth weight remain the most important. Taking into account the effect of gestational age and the duration of supplemental oxygen use, the investigators showed that 70% of the variance in susceptibility to ROP was the result of genetic factors alone (13). In our study, we found statistically significant lower mean gestational age within ROP infants compared to control group in consistent with the literature that it's expected to have a higher risk of developing ROP as infants who were born at a lower gestational age (14,15) (Table 1). Studies evaluating polymorphisms in specific candidate genes, such as vascular endothelial growth factor (VEGF), have also demonstrated an association between sequence variations and severity of ROP (12,16). However, the literature was lack of study which investigates the genetic susceptibility in the pathogenesis of ROP in association with PAI-1 gene polymorphism. Premature neonates are exposed to higher oxygen levels in the early stages of retinal vascular development. This hyperoxia down regulates the essential angiogenic factors those are necessary for the growth of the vasculature. This results in a vaso-obliterative phase and has seen in neonates born at early gestational ages about 30-32 weeks (1,17). The mean gestational age of our ROP group was 29.4±0.8 week.

The activity of urokinase-type plasminogen activator (uPA) and the surface expression of the uPA/uPA receptor (uPAR) complex is tightly regulated by PAI-1. PAI-1 a member of the serpin superfamily whose primary function is the inhibition of plasminogen activator by internalizing uPA/uPAR complex and thought to play a role in the regulation of angiogenesis (18,19). Recently, it has been speculated that genetic factors may affect in ROP development either by changing expressions of critical genes who were mentioned above (12, 20, 21) by acting as accomplice at pathogenesis and it is possible that the combined effects of those polymorphisms may differ in susceptibility to development of severe ROP. However, scientist has failed to show significant association between VEGF, IGF-1 or Norrie disease gene polymorphisms with ROP, respectively[13]. Since the PAI-1 is a critical control protein in ECM remodeling and angiogenesis in ROP pathogenesis, the absence of association between the gene polymorphisms of previous candidate genes and ROP might be because of the probable diverse expression of PAI gene in the angiogenesis cascade of the individuals.

The 4G/5G polymorphism at 675th basepairs of the 5' start site of transcription in the PAI-1 promoter has been identified, which relates to the assorted circulating PAI-1 concentrations (22). The functional nature of this mutation is that the 5G allele binds both to a transcription and to a repressor factor, whereas the 4G allele only binds the transcription factory (23). In vitro experiments have shown that the 4G allele produces more PAI-1 RNA than the 5G allele and presence of the homozygous 4G state was associated with higher PAI-1 level (24, 25) which could lead to inhibition of pathologic angiogenesis (19,26). We expected inhibition of pathologic angiogenesis due to higher PAI levels would be leaded by decreased ROP risk in 4G/4G homozygote neonates. In our study, we found PAI-1 4G/4G genotype was less likely to develop ROP compared to PAI-1 4G/5G genotype, though it was not statistically significant. To our knowledge, our study is the first report to investigate the association of PAI-1 gene polymorphisms on retinal an-

giogenesis demonstrating decreased risk of ROP development within the 4G homozygous patients.

A recent study by Dammann et al. (27) has shown that even the genetic markers were not associated with ROP occurrence, the were found to be associated with the progression to high grade disease. In parallel, we examined the severity of ROP with staging; the percentages of 4G/4G genotype frequency were decreasing as the stages of ROP were increasing although there was no statistically difference between proportion of 4G/4G, 4G/5G and 5G/5G genotypes and ROP stages (Table 3). Since our cases were less in account, future studies with large groups to investigate PAI gene polymorphism with ROP stages would be helpful in the design of screening programs for ROP, and may also be a tool in discriminating infants at high risk of ROP, and therefore in need of treatment, from those at lesser risk.

In conclusion, in view of the key role of PAI-1 in ECM remodeling and angiogenesis the association of PAI-1 gene polymorphisms in neonatal diseases as IVH (28), BPD (4), RDS (3), and this study is the first report investigating the association between PAI-1 4G/5G polymorphisms in the pathogenesis of ROP in premature neonates. We found no significant difference in the genotype distribution of PAI-1 gene nor in the frequency of PAI-1 4G and 5G alleles between the ROP and control groups; however having PAI-1 4G/4G genotype appears to developed the risk of ROP less than PAI-1 4G/5G polymorphism. It would be interesting to know whether a larger dataset of ROP patients can confirm our initial findings. Further identification of new polymorphisms and mutations that may influence susceptibility to ROP could help in the development of new and innovative treatments and allow targeting reducing unnecessary exposure to potentially harmful therapies.

REFERENCES

1. Chen J, Smith LE. Retinopathy of prematurity. Angiogenesis 2007;10:133-40.
2. Mignatti P, Rifkin DB. Plasminogen activators and matrix metalloproteinases in angiogenesis. Enzyme Protein 1996;49:117-37.
3. Armangil D, Yurdakok M, Okur H, Gurgey A. Plasminogen activator inhibitor I 4G/5G polymorphism in neonatal respiratory distress syndrome. Clin Appl Thromb Hemost 2011;17:352-7.
4. Ince DA, Atac FB, Ozkiraz S, Dilmen U, Gulcan H, Tarcan A, Ozbek N. The role of plasminogen activator inhibitor-1 and angiotensin-converting enzyme gene polymorphisms in bronchopulmonary dysplasia. Genet Test Mol Biomarkers 2010;14:643-7.
5. Le Gat L, Gogat K, Bouquet C, Saint-Geniez M, Darland D, Van Den Berghe L, Marchant D, Provost A, Perricaudet M, Menasche M, Abitbol M. In vivo adenovirus-mediated delivery of a uPA/uPAR antagonist reduces retinal neovascularization in a mouse model of retinopathy. Gene Ther 2003;10:2098-103.
6. Matrisian LM. Metalloproteinases and their inhibitors in matrix remodeling. Trends Genet 1990;6:121-5.
7. Mohamed S, Schaa K, Cooper ME, Ahrens E, Alvarado A, Colaizy T, Marazita ML, Murray JC, Dagle JM. Genetic contributions to the development of retinopathy of prematurity. Pediatr Res 2009;65:193-7.
8. Bizzarro MJ, Hussain N, Jonsson B, Feng R, Ment LR, Gruen JR, Zhang H, Bhandari V. Genetic susceptibility to retinopathy of prematurity. Pediatrics 2006;118:1858-63.
9. Ryan MP, Kutz SM, Higgins PJ. Complex regulation of plasminogen activator inhibitor type-1 (PAI-1) gene expression by serum and substrate adhesion. Biochem J 1996;314 (Pt 3):1041-6.
10. Penn JS, Rajaratnam VS. Inhibition of retinal neovascularization by intravitreal injection of human rPAI-1 in a rat model of retinopathy of prematurity. Invest Ophthalmol Vis Sci 2003;44:5423-9.
11. Gilbert C, Fielder A, Gordillo L, Quinn G, Semiglia R, Visintin P, Zin A; International NO-ROP Group. Characteristics of infants with severe retinopathy of prematurity in countries with low, moderate, and high levels of development: implications for screening programs. Pediatrics 2005;115:e518-25.
12. Cooke RW, Drury JA, Mountford R, Clark D. Genetic polymorphisms and retinopathy of prematurity. Invest Ophthalmol Vis Sci 2004;45:1712-5.
13. Holmstrom G, van Wijngaarden P, Coster DJ, Williams KA. Genetic susceptibility to retinopathy of prematurity: the evidence from clinical and experimental animal studies. Br J Ophthalmol 2007;91:1704-8.

14. Hartnett ME. Studies on the pathogenesis of avascular retina and neovascularization into the vitreous in peripheral severe retinopathy of prematurity (an american ophthalmological society thesis). Trans Am Ophthalmol Soc 2010;108:96-119.

15. Shastry BS. Genetic susceptibility to advanced retinopathy of prematurity (ROP). J Biomed Sci 2010;17:69.

16. Watson CJ, Webb NJ, Bottomley MJ, Brenchley PE. Identification of polymorphisms within the vascular endothelial growth factor (VEGF) gene: correlation with variation in VEGF protein production. Cytokine 2000;12:1232-5.

17. Ashton N, Ward B, Serpell G. Effect of oxygen on developing retinal vessels with particular reference to the problem of retrolental fibroplasia. Br J Ophthalmol 1954;38:397-432.

18. Nykjaer A, Conese M, Christensen EI, Olson D, Cremona O, Gliemann J, Blasi F. Recycling of the urokinase receptor upon internalization of the uPA:serpin complexes. Embo J 1997;16:2610-20.

19. Basu A, Menicucci G, Maestas J, Das A, McGuire P. Plasminogen activator inhibitor-1 (PAI-1) facilitates retinal angiogenesis in a model of oxygen-induced retinopathy. Invest Ophthalmol Vis Sci 2009;50:4974-81.

20. Rasmussen SK, Lautier C, Hansen L, Echwald SM, Hansen T, Ekstrom CT, Urhammer SA, Borch-Johnsen K, Grigorescu F, Smith RJ, Pedersen O. Studies of the variability of the genes encoding the insulin-like growth factor I receptor and its ligand in relation to type 2 diabetes mellitus. J Clin Endocrinol Metab 2000;85:1606-10.

21. Hutcheson KA, Paluru PC, Bernstein SL, Koh J, Rappaport EF, Leach RA, Young TL. Norrie disease gene sequence variants in an ethnically diverse population with retinopathy of prematurity. Mol Vis 2005;11:501-8.

22. Franco RF, Reitsma PH. Gene polymorphisms of the haemostatic system and the risk of arterial thrombotic disease. Br J Haematol 2001;115:491-506.

23. Eriksson P, Kallin B, van 't Hooft FM, Bavenholm P, Hamsten A. Allele-specific increase in basal transcription of the plasminogen-activator inhibitor 1 gene is associated with myocardial infarction. Proc Natl Acad Sci U S A 1995;92:1851-5.

24. Grant PJ. Polymorphisms of coagulation/fibrinolysis genes: gene environment interactions and vascular risk. Prostaglandins Leukot Essent Fatty Acids 1997;57:473-7.

25. Panahloo A, Mohamed-Ali V, Lane A, Green F, Humphries SE, Yudkin JS. Determinants of plasminogen activator inhibitor 1 activity in treated NIDDM and its relation to a polymorphism in the plasminogen activator inhibitor 1 gene. Diabetes 1995;44:37-42.

26. McGuire PG, Jones TR, Talarico N, Warren E, Das A. The urokinase/urokinase receptor system in retinal neovascularization: inhibition by A6 suggests a new therapeutic target. Invest Ophthalmol Vis Sci 2003;44:2736-42.

27. Dammann O, Brinkhaus MJ, Bartels DB, Dordelmann M, Dressler F, Kerk J, Dörk T, Dammann CE. Immaturity, perinatal inflammation, and retinopathy of prematurity: a multi-hit hypothesis. Early Hum Dev 2009;85:325-9.

28. Heineking B, Riebel T, Scheer I, Kulozik A, Hoehn T, Buhrer C. Intraventricular hemorrhage in a full-term neonate associated with sinus venous thrombosis and homozygosity for the plasminogen activator inhibitor-1 4G/4G polymorphism. Pediatr Int 2003;45:93-6.