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Cystathionine β-synthase 844ins68 Genetic Polymorphism in Spontaneous Abortion Susceptibility

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Abstract
Aim: Genetic polymorphisms in homocysteine-related genes are subject of a large body of research in pregnancy and newborn associated pathologies. The enzyme cystationine β-synthase (CBS) is involved in the transsulfuration pathway of homocysteine to cysteine. Our objective was to analyze the association of a common polymorphism exhibited by the *CBS* gene, 844ins68, with idiopathic spontaneous abortions (SA). *Material and Methods*: 131 patients with a history of at least one unexplained SA and 135 healthy women with at least one successful pregnancy and no SA were included in a case-control study. Simplex PCR was used to genotype the cases and control volunteers for the *CBS* 844ins68 polymorphism. Fisher's exact test was performed to obtain the odds-based parameters describing the relationship between the two variables. *Results*: The variant allele was encountered with a frequency of 0.08 in the SA group and 0.048 in controls. The dominant model analysis of risk revealed the OR 1.957, 95%CI [0.920, 4.162], Fisher's p = 0.09. *Conclusion*: The findings suggest possible effects of this polymorphism in SA risk that did not reach the significance level in this study. Future studies might validate or clarify the association between *CBS* 844ins68 and idiopathic SA.

Keywords: Cystathionine β-synthase; Genetic polymorphism; Spontaneous abortion.

Introduction

As many as 15% of clinically recognized pregnancies and up to 50% of total conceptions end up in spontaneous abortion (SA) [1]. A high proportion of these events remain of unidentified etiology [1-3]. SA is the most common pregnancy disorder affecting couples willing to conceive [1] and is defined as the loss of a pregnancy before 24 weeks of gestation, which is the time when the fetus reaches viability [1].

A series of causes responsible for SA and for the recurrence of this disorder (such as chromosomal anomalies, anatomical defects, coagulation disorders, immunological disturbances) have been described and integrated in clinical guidelines for SA management [4]. Other lines of research aim at further revealing the underlying conditions involved in the susceptibility for SA

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[2,4,5], which is currently viewed as a multifactorial pathology with a possible genetic predisposition and environmental influence [5].

The metabolic pathways of homocysteine and folate have repeatedly demonstrated their influence on human pregnancy outcome (detrimental effect of hyperhomocysteinemia and benefit of folate intake)(reviewed in [6-8]).

One of the key enzymes involved in this metabolic pathway is cystathionine β-synthase (CBS). Homocysteine disposal is performed via two pathways: remethylation to form methionine and transsulfuration to produce cysteine [9]. The latter is initiated by CBS and is the prominent way of homocysteine metabolism during the first trimester of pregnancy [10]. It becomes interesting to hypothesize that variations in CBS function could influence homocysteine levels and exert negative effects on the pregnancy outcome. Among these negative conditions associated with abnormal homocysteine levels and genetic polymorphisms in homocysteine-related genes we note the previously documented increased risk for placental vascular thrombosis (leading to various preganancy disorders such as pre-eclampsia, stillbirth, spontaneous abortion) or the higher susceptibility for fetal anomalies (neural tube defects, cleft lip, cleft palate)(reviewed in [7]).

The *CBS* gene exhibits a common genetic polymorphism consisting in a 68 bp insertion at position 844 (844ins68). This polymorphism was initially described in a homocystinuric patient by Sebastio *et al.,* 1995 [11]. Consequent studies have proven its higher prevalence [12,13]. Furthermore, it was found that a detrimental T833C *CBS* mutation cosegregated and was neutralized by the 844ins68 polymorphism. The inserion created a second splicing site downstream this mutation which has allowed for the 833C mutation to be skipped and for the mRNA transcript to be correctly generated [14]. It has been suggested that the cosegregation in *cis* of the *CBS* 833C variant with the 68 bp insertion at position 844 is the result of an unequal crossing-over that has confered a selective advantage against the deleterious 833C variant and has led to its positive selection[15].

Although the main conclusion regarding the consequences of this polymorphism is that it does not affect CBS function nor homocysteine levels, higher homocysteinemia was also reported in 844ins68 carriers [16]. It has been described that, in those conditions where the *CBS* 844ins68 variant is inherited from the mother rather then from the father, there is an 18 fold higher risk of cleft lip and palate [17]. This could be due to genomic imprinting or to the multiplication of risk when the variant *CBS* allele is present in both maternal and the embryonic genotypes [17]. A previous study on a limited group of patients showed no association between *CBS* 844ins68 and SA with fetal chromosomal aneuploidy [18]. In the present study, we aimed at assessing the possible association of the *CBS* 844ins68 polymorphism with spontaneous pregnancy loss of undetermined etiology.

Material and Method

Selection and Description of Participants

A total of 266 Caucasian women of self-declared Romanian descent were included in our case-control study. The participants were recruited from the patients visiting the IInd Genetic Explorations Office, Ist Gynaecology Clinic, Cluj-Napoca during the timeframe March 2007 - December 2009, for physical examination, laboratory investigations or genetic counseling. The case group consisted of 131 women of fertile age with a history of at least one first trimester idiopathic SA. The SA was declared idiopathic after clinical and paraclinical investigations: ultrasonography, karyotype analysis, hormonal dosage, autoantibody testing (for anticardiolipin and anti-b2-microglobulin antibodies), TORCH serologic tests. The control group included 135 women that had experienced at least one uneventful term-delivery and presented negative history of SA. Patients presenting vascular, hormonal, immunological, chromosomal pathologies, obesity or diabetes as comorbidities were not included in the study. The participation in the study was voluntary, informed and written consent was obtained from all participants. This study was

approved by the Ethics Committee of the "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca, and was led in compliance with the Helsinki Declaration.

Blood Sample Collection and Molecular Analysis

Blood samples were withdrawn from a peripheral vein of the volunteers and immediately stored at 4°C until further use. Genomic DNA was extracted out of white blood cells from 300µl peripheral blood using a commercial kit (Wizzard Genomic DNA Purification Kit, Promega®).

For genotype identification the Simplex Polymerase Chain Reaction (PCR) was used as previously described [19, 20], with minor modifications. Briefly, the PCR reaction was performed in a final volume of 25µl containing: 12.5µl PCR Mix–Taq DNA-polymerase 0.05U/µl, MgCl2 4mM, dNTP mix 0.4mM each (Fermentas MBI, Vilnius, Lithuania®); 1µl BSA (bovine serum albumin, Fermentas MBI, Vilnius, Lithuania®) 2mg/ml solution; 8 pmoles of each primer (Eurogentec, Seraing, Belgium®); approximately 100 ng genomic DNA; nuclease-free water. The reaction was set up on a MastercyclerGradient thermal cycler (Eppendorf®, Hamburg, Germany) and the program consisted of initial denaturation of 5min at 95°C followed by 35 cycles of denaturation 30s at 94°C, annealing 1min at 57°C and elongation 1min at 72°C, with a final elongation of 10min at 72°C. Primer sequences were as follows: Forward-5'CTGGCCTTGAGCCCTGAA3' and Reverse-5'GGCCGGGCTCTGGACTC3'.

Wild-type samples showed a single band of 184bp when tested on a 2% agarose gel electrophoresis, whereas the insertion allele yielded a 252bp fragment (Figure 1.)

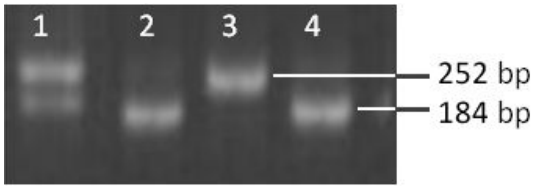

Figure 1. Agarose gel electrophoresis for *CBS* 844ins68. (lane 1: heterozygous genotype; lanes 2 and 4 : wild type homozygous genotype; lane 3: *CBS* 844ins68 homozygous genotype)

Statistical Analysis

Data were collected and analyzed using GraphPad Prism for Windows Version 5, GraphPad InStat Version 3.05 and Epi Info Version 3.5.3. Quantitative variables are summarized using centrality and dispersion parameters, whereas qualitative variables are depicted as percentages with 95% Confidence Intervals. The age distribution was tested for normality using the Kolmogorov-Smirnov (KS) test. Kruskal-Wallis and Mann-Whitney tests were used to compare the age variance between cases and controls. Differences in genotype distribution between cases and controls were estimated using Fisher's exact test. Results were considered statistically significant at p-value < 0.05. The approximate risk is presented as Odds Ratio with 95% Confidence Interval.

Because the current management of SA in clinical practice indicates that serious attention should be given to patients after 3 consecutive SA, we provide separate analyses for this subgroup of patients experiencing recurrent miscarriage. Also, for comparison, we show the data for the remaining patients in the case group that, until their recruitment in this study, have had 1 or 2 sporadic pregnancy losses.

Results

Descriptive Statistics of the Study Groups

A set of main parameters of cases and controls are presented in Tables 1 and 2.

Table 1. Age-related parameters for spontaneous abortion (SA) patients and control volunteers

	Mean [95%CI]	Standard deviation	Median	Range	KS test p-value
All SA cases (n=131)	31.2 [30.3, 32.1]	5.22	31	24	>0.10
One or two sporadic SA (n=85)	30.02 [28.94, 31.1]	5.02	29	22	0.041
Recurrent SA (at least three SA) (n=46)	33.46 [32, 34.91]	4.9	33	24	0.082
Controls (n=135)	40.62 [39.14, 42.1]	8.73	39	45	0.0011

As shown in Table 1, the age variable did not follow a normal distribution in all groups and subgroups, therefore, Kruskal-Wallis test was used to test for age variance yielding a p-value<0.001. Mann-Whitney testing has revealed a significant difference in age median between controls and each of the case subgroups (controls *vs.* all cases, p<0.0001; controls *vs.* sporadic SA cases, p=0.0001; controls *vs.* recurrent SA cases, p<0.0001)

Table 2. Number of spontaneous abortions (SA) in the case group and subgroups

	Mean [95%CI]	Standard deviation	Median	Range
All SA cases	2.40 [2.18, 2.61]	1.25	2	6
One or two sporadic SA	1.67 [1.56, 1.77]	0.49	2	1
Recurrent SA (at least three SA)	3.78 [3.47, 4.09]	1.07	3	4

Association Analysis of CBS 844ins68 Polymorphism with SA

The findings revealed by the molecular analyses are summarized in Table 3. The genotype frequencies observed in controls were in compliance with the Hardy-Weinberg equilibrium, as the comparison with the expected values yielded a p-value of 0.19.

Table 3. Summary of *CBS* 844ins68 genotype and allele frequency in cases and controls

	Genotypes, number (%) [95%CI]*			Alleles, no (%)#	
	844ins68 homozygote	844ins68/wild-type	wild-type homozygote	844 ins 68	wild-type
All SA cases	0 (0) [0, 2.8]	21 (16) [10.2, 23.5]	110 (84) [76.5,89.8]	21 (8)	241 (92)
One or two sporadic SA	0 (0) [0, 4.2]	13 (15.3) [8.4, 24.7]	72 (84.7)[75.3,91.6]	13(7.6)	157(92.4)
Recurrent SA (at least three SA)	0 (0) [0, 7.7]	7 (15.2) [6.3, 28.9]	39 (84.8) [71.1, 93]	7 (7.6)	85 (92.4)
Controls	1 (0.7) [0,4.1]	11 (8.1) [4.1, 14.1]	123 (91.1) [85,95.3]	13(4.8)	257 (95.2)

*Genotype frequencies depicted as: absolute numbers (percentage) [95% Confidence Interval]
#Allele frequencies depicted as: absolute number (percentage)

In order to evaluate the association between the *CBS* 844ins68 polymorphism and SA risk we performed Fisher's exact test using several hypothetical models. Table 4 shows the results applying for the dominant model in which we hypothesised that an effect of the studied polymorphism could be manifested in both homozygous and heterozygous status. Thus, in this setup, the risk was attributed to 844ins68 homozygotes and heterozygotes.

Table 4. Fisher's exact test analysis of risk for SA compared to control volunteers

Model	Outcome variable	OR	95%CI	p-value
844ins68 homozygotes + heterozygotes *vs.* wild-type homozygotes	All SA cases	1.957	0.920, 4.162	0.094
	One or two sporadic SA	1.851	0.801, 4.274	0.190
	Recurrent SA (at least three SA)	1.840	0.677, 4.999	0.265

Discussion

The findings generated by our study could not prove a direct association between the *CBS* 844ins68 polymorphism and SA. In our groups, the inheritance of at least one allele which presents the 68 bp insertion at position 844 seems to be associated with the occurence of SA. However, these results did not reach statistical significance, and this limits our interpretation of the possible influence that this 68 bp insertion could exert in SA predisposition. The additional analyses performed in the case subgroups have a more limited strength in sustaining the hypothesis that this polymorphism influences SA susceptibility.

Because of the small frequency of the 844ins68 homozygous genotype (found in only one of the subjects tested), the dominant model, where the risk is attributed to the homozygous genotype alone, is not conclusive. A separate analysis has been performed considering the allele frequencies instead of the genotype frequencies (data not shown). This analysis has provided very similar results to those presented in Table 4.

The age analysis in our study groups revealed differences between controls and SA patients. It should be stated that this difference lies in the selection of controls. With the aim of gathering the suitable controls in our study, we have encouraged the inclusion of postmenopausal women, as in their cases, the likelyhood of having an SA after their acceptance as controls would be reduced to a minimum.

The relationship of this 68bp insertion at position 844 in the *CBS* gene with hyperhomocysteinemia is still a largely unclear matter. This polymorphism is extensively investigated in a lot of studies dealing with homocysteine and folate related pathogenesis without a clear-cut result proving its involvement [12-16, 18-20]. This comes in contrast with the very important role the CBS enzyme has in the metabolization of homocysteine. We could only assume that this polymorphism's putative deleterious effects might become validated in the presence of other common variations with similar function.

Other than the functional role of the 68bp insertion on homocysteine metabolism, this polymorphism has been an interesting point of study from the evolutionary point of view. This was due to the peculiar position of this insertion [14] and the molecular events further related to the processing of the gene (the neutralization of the cystinuria-associated *CBS* 833C variant). After several investigations in the late nineties that have tracked the evolution of the 833C and 844ins68 cosegregation, this *cis* association has become an „anthropological marker" [21].

To the best of our knowledge, this is the first study that evaluates this polymorphism in relation to idiopathic spontaneous abortions. Compared to the data obtained in other control populations, the frequency observed in our study for this polymorphism falls into the range associated with European populations 1.5-25 % (as described and summerized in [21] or [22]). The very low frequency of the homozygous genotype for the insertion has also been previously noted.

Conclusions

Our results suggest a slight involvement of the CBS 844ins68 polymorphism in idiopathic spontaneous abortion susceptibility. In our scenario, the results did not reach the level of statistical significance. Whether this finding is due to chance alone or is likely to be validated in other scientific approaches is subject to further research.

List of abbreviations

bp = base pairs
CBS = cystathionine β-synthase
DNA = dezoxyribonucleic acid
mRNA = messenger ribonucleic acid
PCR = polymerase chain reaction

SA = spontaneous abortion
TORCH = Toxoplasma, Rubella, Cytomegalovirus, Herpes virus

Ethical Issues

The participation in the study was voluntary, informed and written consent was obtained from all participants. This study was approved by the Ethics Committee of the "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca, and was led in compliance with the Helsinki Declaration.

Conflict of Interest

The authors declare that they have no conflict of interest.

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