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Brief Communication

Polymorphisms in the endothelial nitric oxide synthase gene in thalidomide embryopathy

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ABSTRACT

Thalidomide is one of the most potent teratogens known to humans. It is currently used for many clinical situations such as treatment of leprosy reactions and multiple myeloma. However, the teratogenic mechanisms by which it produces morphological defects still remain unclear. One of the hypotheses is the blockage of angiogenesis by reduction of nitric oxide (NO). In this study, we evaluated two functional polymorphisms of the endothelial nitric oxide synthase (eNOS) gene which is a constitutively expressed enzyme responsible for production of NO. The promoter −786T > C exon 7 (896G > T) polymorphisms were genotyped using real-time PCR for 28 individuals with thalidomide embryopathy (TE), 27 first-degree relatives of these individuals, and 68 individuals from the general population. Their allele, genotypic, and haplotypic frequencies were compared. A significant difference was observed in the −786T > C polymorphism genotypes ($p = 0.03$) between the groups affected by TE and those unaffected (non-relatives). The TT genotype of the 896G > T polymorphism was observed in 10.7% of those affected and 2.9% of those unaffected, but the difference was not statistically significant ($p = 0.09$). The haplotypic analysis indicated that the wild haplotype −786T/896G was distributed differently in the affected and unaffected groups ($p = 0.004$). These results indicate that the individuals with TE have a higher frequency of alleles associated with lower expression of eNOS, indicating that this may be a genotype susceptible to TE.

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Introduction

Thalidomide was marketed worldwide in the late 1950s as a safe sedative in comparison with barbiturates, but it was quickly withdrawn from the market due to the birth of children with congenital defects [1,2]. The acknowledgment of the teratogenicity of thalidomide did not mark the end of the drug's use, since it returned to being used in the 1990s for the treatment of other conditions such as immune reactions to leprosy, multiple myeloma, and lupus, due to its antiangiogenic [3] and immunomodulatory [4–6] properties.

To date, the molecular mechanisms by which the drug causes teratogenicity have not yet been fully explained. Studies with animal models have provided evidence of the role of oxidative stress [7,8], the antiangiogenic mechanism [3,9] and binding with the Cereblon (Crbn)[1] protein [10] for the appearance of embryopathy.

Some studies have shown that the antiangiogenic mechanism of teratogenicity is involved at the cellular level in the decrease of nitric oxide (NO) by blocking its signaling [11,12]. NO is produced by three types of NO synthase (NOS): neuronal (nNOS), inducible (iNOS), and endothelial (eNOS). The eNOS is constitutively expressed in vascular endothelial cells and is involved in several cellular processes, including angiogenesis, because it is a potent vasodilator [13]. The gene of the eNOS, *NOS3*, is located in chromosome 7 and has 26 exons [14,15]. Polymorphisms in *NOS3* have been associated with various clinical situations; the two most studied are the substitution of T for C at position 786 of the promoter of the gene (rs2070744), and a variation in exon 7 from G

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[1] *Abbreviations used:* Crbn, cereblon protein; CRBN, gene encode Crbn protein; TE, thalidomide embryopathy; ABPST, brazilian association of people affected by thalidomide syndrome; VEGF, vascular endothelial growth factor; bFGF, the basic fibroblast growth factor.

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to T at position 894, resulting in the replacement of glutamic acid to aspartic acid at codon 298 (Glu298Asp) (rs1799983). These variants are associated with cardiovascular disorders and cancer, among other conditions which are dependent on angiogenesis [16–18].

In this study, two polymorphisms of the *NOS3* gene, −786T > C and 894G > T (rs2070744 and 1799983, respectively), were evaluated in: people with thalidomide embryopathy (TE), their first-degree relatives, and in individuals without malformations, in order to identify the frequencies of these variants in these groups.

Materials and methods

The sample studied consisted of three groups: those affected by embryopathy thalidomide, relatives of those affected by embryopathy (siblings or mother), and members of the general population not affected by embryopathy. Affected individuals were recruited from Brazilian Association of People Affected by Thalidomide Syndrome (ABPST). These volunteers had their phenotype further analyzed independently by three authors of this paper (FSL, MTVS and LS-F), and it was found to be consistent with what is described as typical for TE [1,2,19]. For comparison purposes, first-degree relatives were also invited to participate. Moreover, DNA samples from anonymous people were used as controls. Inclusion criteria for recruitment were Brazilian geographical regions matched to those affected by TE, and absence of congenital anomalies. Characteristics of this control sample were previously described [20–22].

The DNA was extracted from saliva samples using the Oragene DNA extraction kit (DNA Genotek®), in accordance with the manufacturer's instructions. The genotypic determination of the rs2070744 and rs1799983 polymorphisms was performed by allelic discrimination using specific probes previously designed and validated (Real Time PCR, Applied Biosystems, USA). All the assays were performed in accordance with the protocol recommended by the manufacturer.

The Hardy–Weinberg equilibrium (HWE) was tested in all sample groups and the differences in the allele and genotypic frequencies between groups were compared by Fisher's exact Test. A bicaudal value of $p < 0.05$ was considered to be significant. The tests were performed using the SPSS® version 20 program (SPSS, www.spss.com, IBM, USA). Linkage Disequilibrium (LD) of the two single-nucleotide polymorphisms (SNPs) was calculated using the Haploview version 4.2 program [23], and haplotypes were inferred using the Bayesian algorithm implemented in the Phase 2.1.1 program [24,25].

This study was approved by the Ethics Committee at the Hospital de Clinicas in Porto Alegre (number 10-0244).

Results

The allele and genotypic frequencies of the 28 individuals affected by TE, the 27 relatives, and the 68 unaffected subjects are shown in Table 1. The distributions of both polymorphisms were in HWE for all the sample groups. The *NOS3* promoter polymorphism showed a significant difference in the allelic and genotypic frequencies ($p = 0.03$) between individuals with TE and those unaffected and unrelated (non-relatives). The wild TT genotype was more common in individuals unaffected by TE (TT = 48.5%) than in subjects affected by TE (TT = 28.6%). Similarly, the individuals with the CC genotype were more frequently affected (35.7%) compared to the group of non-relatives (13.2%). For the polymorphism in exon 7, the TT genotype was observed more frequently in those affected by TE (10.7%) than in the unaffected group (2.9%), but with no significant difference ($p = 0.09$) (Table 1).

Table 1
Allele and genotype frequencies of the studied variants in *NOS3* gene in thalidomide embryopathy affected individuals and in unaffected individuals.

Polymorphism	Genotype/allele	Affected		Unaffected		*p*
		n	(%)	*n*	(%)	
−786T>C	TT	8	(28.6)	33	(48.5)	
(rs2070744)	TC	10	(35.7)	26	(38.2)	
	CC	10	(35.7)	9	(13.2)	0.031
	T	26	(46.4)	92	(67.6)	
	C	30	(53.6)	44	(32.4)	0.009
894G>T	GG	7	(25.0)	30	(44.1)	
(rs1799983)	GT	18	(64.3)	36	(52.9)	
	TT	3	(10.7)	2	(2.9)	0.098
	G	32	(57.1)	96	(70.6)	
	T	24	(42.9)	40	(29.4)	0.103

The allelic and genotypic frequencies of those affected by TE and their first-degree relatives were very similar and, therefore, the family members were excluded from further analyses.

These two SNPs were not in linkage disequilibrium in the investigated sample ($D' = 0.229$, LOD score = 0.77). Four haplotypes were identified within the studied sample (Table 2), distributed differently among the groups ($p = 0.009$). Residual analysis indicated that the haplotype containing the wild alleles (−786T and 894G) was more frequent in the unaffected group than in the affected group. The differences in distribution of the other haplotypes among the groups were not statistically significant.

Discussion

It is known that the occurrence of malformations after exposure to a teratogen depends on several variables, such as the exposure period during the embryo-fetal development, the exposure dose, the mechanism by which the teratogenicity occurs, and the maternal-fetal genotype, among other variables. These characteristics are common to any teratogenic agent and its consequences in the organism [26,27]. In the case of thalidomide, the factors related to the susceptibility period and susceptibility dose are widely known, due to the large number of babies born with TE in the 1960s. It is estimated that about 20% of the embryos exposed to a dose of at least 50 mg during the teratogenicity window (3–8 weeks of gestation) are born with some consequence of thalidomide, with the most known consequences being limb reduction defects, the eyes and ears defects, and heart defects [1,2,19]. However, little is known about susceptibility genotypes that may be involved in the approximately 80% of people exposed to thalidomide that do not exhibit TE.

In this investigation, the objective was to evaluate polymorphisms in the *NOS3* gene. The eNOS gene is constitutively ex-

Table 2
Haplotype frequencies of the *NOS3* polymorphisms in affected by thalidomide embryopathy and in unaffected individuals groups.

Haplotypes	eNOS[a]	Affected		Unaffected		*p*[b]
		n	(%)	*n*	(%)	
1	−786T/894G	17	(30.3)	77	(57.5)	0.004
2	−786T/894T	10	(17.9)	15	(11.2)	0.95
3	−786C/894G	14	(25.0)	19	(14.2)	0.36
4	−786C/894T	15	(26.8)	25	(18.7)	0.96

Affected × Unaffected fisher exact test $p = 0.009$.

[a] SNPs composing *NOS3* haplotypes are disposed as follow: rs2070744 (−786T > C) and rs1799983 (894G > T).

[b] Fisher exact test *p*-values with Bonferroni correction obtained from residual analysis.

pressed in cells and is one of three isoforms responsible for NO production. NO is involved in a variety of cellular processes such as vasodilation, angiogenesis, platelet aggregation, apoptosis, and gene regulation, which are the fundamental mechanisms during embryo-fetal development [13,28]. The polymorphisms evaluated here are the two most studied of this gene and their functional activity in the enzyme is different. The polymorphism of the promoter is associated with the decreased expression [29], whereas the amino acid change caused by the SNP in exon 7 (Glu298Asp), there is evidence of a decrease in NO production in carriers of the allele T [30]. The data presented here show that the individuals with TE have a higher frequency of the variants of these sites when compared to the unaffected group, although without statistical differences between the TT genotype distribution of the polymorphism of exon 7 between these two groups. The comparison with the first-degree relatives of those affected was as expected and did not show differences.

The frequencies observed in this sample are similar to those described in other Brazilian population studies [16,31,32]. Although we did not find these SNPs to be in LD in our study – possibly due to the low sample size – other studies with these polymorphisms showed a high degree of LD in the same population [16,31,32],. Studies of diseases linked to angiogenesis show that the rarest alleles of these polymorphisms are associated with the occurrence of diseases, especially cardiovascular and cancer, or with greater severity in these clinical conditions [16–18,33].

Other studies evaluating the role of NO in embryo-fetal development have suggested that both the excess and the deficiency of NO may underlie, among other causes for teratogenesis occurrence [9,12,28,34]. Knockout rodent studies of the *NOS3* gene showed cardiovascular and limb deficiencies. These deficiencies are different among the species studied, thus demonstrating the role of this gene in embryo-fetal development [28,35–38].

In 1994, through inhibition of the vascular endothelial growth factor (VEGF) and the basic fibroblast growth factor (bFGF), D'Amato et al. identified the antiangiogenic action mechanism of thalidomide and hypothesized that this was also the teratogenicity mechanism [3]. Consistent with this line, other studies have shown that the antiangiogenic property of thalidomide is fundamental for the occurrence of malformations, through the blocking of vasculogenesis which is a key process in limb development [9,11,12,34,39]. Besides this, Siamwala et al. recently showed, *in vitro*, that the teratogenicity of thalidomide is associated with the blocking of angiogenesis and that administration of NO can avoid the malformations caused by the drug [34]. Thus, it is possible that this scenario of blocking the angiogenesis induced by exposure to thalidomide is stronger in individuals with eNOS polymorphisms that affect NO production. The carriers of these alleles could be considered the most susceptible to TE, which is consistent with what was observed in the present investigation.

Clearly, the number of subjects evaluated in this study is too small to confirm this hypothesis. However, the sample number was conditioned to the possibility of including living individuals with a confirmed diagnosis of TE. The eNOS polymorphisms are neither necessary nor sufficient for the occurrence of embryopathy (TE). The presence of the allele with lower production of NO in affected individuals supports the hypothesis of teratogenesis via antiangiogenic mechanism, since NO plays important role in promoting angiogenesis. However, thalidomide victims were identified without this genotype demonstrating the complex feature of this syndrome and that others molecular mechanisms are also involved in the occurrence of embryopathy. Therefore, other molecular targets linked to this and other mechanisms, including oxidative stress and CRBN binding are part of this context, and must be experimentally investigated in model organisms, helping to understand the origin of malformations, phenotypic variability, and interspecific differences observed in TE. Anyhow, the identification of these genotypes, which do not favor the availability of eNOS in individuals with TE, can generate insights for the recognition of other molecular mechanisms of teratogenesis and thus assist in developing safer analogues of thalidomide.

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