RFC-1 80G > A Polymorphism in Case-Mother/ Control-Mother Dyads Is Associated with Risk of Nephroblastoma and Neuroblastoma

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Aim: Embryonic tumors are associated with an interruption during normal organ development; they may be related to disturbances in the folate pathway involved in DNA synthesis, methylation, and repair. Prenatal supplementation with folic acid is associated with a decreased risk of neuroblastoma, brain tumors, retinoblastoma, and nephroblastoma. The aim of this study was to investigate the association between *MTHFR* rs1801133 (C677T) and *RFC-1* rs1051266 (G80A) genotypes with the risk of developing nephroblastoma and neuroblastoma. **Materials and Methods:** Case-mother/control-mother dyad study. Samples from Brazilian children with nephroblastoma ($n=80$), neuroblastoma ($n=66$), healthy controls ($n=453$), and their mothers (case $n=93$; control $n=75$) were analyzed. Genomic DNA was isolated from peripheral blood cells and/or buccal cells and genotyped to identify *MTHFR* C677T and *RFC-1* G80A polymorphisms. Differences in genotype distribution between patients and controls were tested by multiple logistic regression analysis. **Results:** Risk for nephroblastoma and neuroblastoma was two- to fourfold increased among children with *RFC-1* polymorphisms. An increased four- to eightfold risk for neuroblastoma and nephroblastoma was seen when the child and maternal genotypes were combined. **Conclusion:** Our results suggest that mother and child *RFC-1* G80A genotypes play a role on the risk of neuroblastoma and nephroblastoma since this polymorphism may impair the intracellular levels of folate, through carrying fewer folate molecules to the cell interior, and thus, the intracellular concentration is not enough to maintain regular DNA synthesis and methylation pathways.

Introduction

GENETIC SUSCEPTIBILITY, PRENATAL FACTORS, and neonatal exposure are probably involved in the development of embryonic tumors, which develop in very young children. Microscopically, they resemble structures seen in developing tissues of the embryo and fetus (Dehner, 1998). The risk of developing these cancers may be modified by polymorphic variants in genes (of the children or parents) involved in metabolism, growth, and development, as well as in DNA repair and the cell cycle (Blount *et al.*, 1997; Gemmati *et al.*, 2004).

Folate plays an important role in embryogenesis and early fetal development through its effects on DNA methylation and synthesis (Blount *et al.*, 1997). Folate insufficiency, due to low folate intake or defects in folate metabolism, may lead to DNA hypomethylation and genomic instability, which may play a role in carcinogenesis by altering gene expression

or increasing DNA damage. Prenatal folic acid supplementation has been shown to decrease several congenital anomalies and is recommended for women of childbearing potential and during early pregnancy to decrease the risk of neural tube defects (NTD) in several countries (Berry *et al.*, 1999). Multivitamin supplementation is thought to be associated with a reduced incidence of neuroblastoma, brain tumors, retinoblastoma, and nephroblastoma (Wilms' tumor) (Preston-Martin *et al.*, 1998; Goh *et al.*, 2007; Schuz *et al.*, 2007). Folic acid food fortification was associated with a decline in neuroblastoma in Canada and nephroblastoma incidence in the United States (French *et al.*, 2003; Grupp *et al.*, 2011; Linabery *et al.*, 2012).

Methylenetetrahydrofolate reductase (*MTHFR*) is one of the main regulatory enzymes of the folate metabolic pathway since it controls the balance between DNA methylation and synthesis through the irreversible conversion of

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5,10-methylenetetrahydrofolate required for DNA synthesis to 5-methyltetrahydrofolate, a methyl donor for conversion of homocysteine to S-adenosylmethionine. Reduced folate carrier-1 (RFC-1) is a well-characterized folate transporter in the cell membrane with a high affinity for physiological folate. Genes encoding these proteins harbor polymorphisms that may decrease the enzymatic activity and impair folate entrance into the cell and are associated with the development of several malignancies (Yin *et al.*, 2004; Kennedy *et al.*, 2012; Yu and Chen, 2012).

Recently, Miranda *et al.* (2014) demonstrated that the RFC A80A genotype was significantly associated with the risk of developing neuroblastoma. *MTHFR* variants play a controversial role in the development of childhood acute leukemia (da Costa Ramos *et al.*, 2006; Zanrosso *et al.*, 2006; Metayer *et al.*, 2011). In a previous Brazilian series, *MTHFR* A1298C polymorphism was associated with an increased risk of acute lymphoblastic leukemia and acute myelogenous leukemia in nonwhite children (da Costa Ramos *et al.*, 2006; Zanrosso *et al.*, 2006). In embryonic solid childhood tumors, several data have suggested an association of *MTHFR* C677T with nephroblastoma, *MTHFR* A1298C with embryonic central nervous system tumors, and *MTR* (methionine synthase) A2756G with retinoblastoma (Sirachainan *et al.*, 2008; Ferrara *et al.*, 2009; De Lima *et al.*, 2010).

The present study aimed to evaluate the association between nephroblastoma and neuroblastoma with the polymorphisms of folate metabolism genes through case-mother/control-mother design.

Materials and Methods

Study population

Cases were identified from four Brazilian institutions in the southeast region of the country (São Paulo, Rio de Janeiro), between 2005 and 2012. One hundred and forty-six Brazilian children with embryonic tumors were enrolled, including 80 with nephroblastoma and 66 with neuroblastoma. There were 74 males and 74 females; patient age ranged from 5 days to 178 months. The control group consisted of 453 randomly selected healthy children, age ranged from 3 days to 168 months, obtained from multidisciplinary projects that have been running at the Hematology–Oncology Pediatric Program of Research Center-INCA. Eligibility consisted of children from the same region of the cases with no history of cancer and no congenital anomalies. The racial admixture in the trihybrid Brazilian population, with Amerindian, European, and African roots, produces special challenges to ethnic classifications in this population. For this study, race distribution was assessed according to the Instituto Brasileiro de Geografia e Estatística (www.ibge.gov.br/home), which relies on skin color self-definition. Then, we categorized individuals into two major groups: whites (cases $n=95$; controls $n=154$) and nonwhites (cases $n=51$; controls $n=181$). Skin color was unknown in 118 children among the control group and 1 child among the tumor group. Sixty-one mothers of children with nephroblastoma, 32 mothers of children with neuroblastoma and 75 of the control group were studied. No information of age and skin color was available. Peripheral blood samples or buccal cells were collected from cases and controls and their mothers. The study protocol was approved by the Ethics Committee of the

Brazilian National Institute of Cancer (136/09), and signed informed consent was obtained from all participants' parents.

Genetic analysis

Genomic DNA samples of children (cases and controls) were isolated from peripheral blood cells with the QIAamp DNA Blood Mini Kit (Qiagen®) and mothers' buccal samples (cases and controls) with Oragene-DNA (Genotek), both according to the manufacturer's instructions. Samples were genotyped through PCR-RFLP for two polymorphisms: *MTHFR* rs1801133 (C677T) and *RFC-1* rs1051266 (G80A) using previously described methods (Frosst *et al.*, 1995; Shaw *et al.*, 2002).

Statistical analysis

Allele frequencies were derived by gene counting. The differences between patients with embryonic tumors (nephroblastoma, neuroblastoma) and controls were assessed by the chi-square (χ^2) or Fisher exact test. The influence of alleles on the risk of developing nephroblastoma and neuroblastoma was evaluated by logistic regression to predict the odds ratios (OR) with 95% confidence intervals (95% CI). The Hardy–Weinberg equilibrium was tested for each SNP by comparing observed with expected frequencies using the χ^2 test. The software package SPSS version 13.0 was used for statistical analysis of the data, $p < 0.05$ was considered statistically significant.

Results

Sample characteristics

The frequency of the *MTHFR* rs1801133 (C677T) variant alleles was 27.6% and 27.3% and for *RFC-1* rs1051266 (G80A) was 58.4% and 57.1% in children with nephroblastoma and neuroblastoma, respectively. The frequencies of the *MTHFR* rs1801133 (C677T) variant allele were 28.2%, 27.5%, and 29.4% among the mothers of nephroblastoma, neuroblastoma, and controls, respectively. Also, the frequencies of *RFC-1* rs1051266 (G80A) variant allele were 47.2%, 57.6%, and 43.5% among the mothers of nephroblastoma, neuroblastoma and controls, respectively.

Genotype frequencies for *MTHFR* rs1801133 (C677T) and *RFC-1* rs1051266 (G80A) were in Hardy–Weinberg equilibrium in controls ($p=0.4934$; $p=0.9752$, respectively).

Association between case-mother/control-mother dyad polymorphism and risk of tumor

The risk for nephroblastoma was increased two- to fourfold in children with *RFC-1* GA (adjusted OR = 2.55; 95% CI 1.21, 5.35), AA (adjusted OR = 4.23; 95% CI 1.84, 9.71), and GA/AA (adjusted OR = 3.07; 95% CI 1.53, 6.16). The risk for neuroblastoma was increased two- to threefold in children with *RFC-1* GA (adjusted OR = 2.48; 95% CI 1.13, 5.44), AA (adjusted OR = 3.46; 95% CI 1.45, 8.24), and GA/AA (adjusted OR = 2.88; 95% CI 1.36, 6.07) (Table 1).

Maternal genotypes were associated with increased risk for neuroblastoma (*RFC-1* GA (OR = 3.09; 95% CI 1.02, 9.31) and *RFC-1* GA/AA (OR = 3.11; 95% CI 1.09, 8.90). Combined analysis of child and maternal polymorphisms increased significantly the risk for neuroblastoma (*RFC-1*

AA+AA and GA/AA+GA/AA) (Table 2). For nephroblastoma, looking at maternal and child *RFC-1* GA/AA separately, it is evident that the polymorphism was significant only for the child genotypes (OR = 2.56; 95% CI 1.33, 4.95), but not for the maternal. The combined effect of the child and maternal genotype increased the risk (OR = 4.52; 95% CI 1.18–17.25) (Table 3).

Discussion

Embryogenesis and carcinogenesis are complex multifactorial events arising from an array of genetic and environmental interactions. To the best of our knowledge, this is the first report evaluating the association of case-mother/control-mother dyads *MTHFR* rs1801133 (C677T) and *RFC-1* rs1051266 (G80A) polymorphisms with neuroblastoma and nephroblastoma.

In the present report, first, we analyzed the influence of each child and mother polymorphism separately on the risk of each tumor and found significant increased risk association conferred by child *RFC-1* A80G genotype. Next, we investigated the combination of child and mother genotype of *MTHFR* rs1801133 (C677T) and *RFC-1* rs1051266 (G80A) on the risk of developing tumors. The combination of mother and child genotype of *RFC-1* G80A showed an even higher risk of developing nephroblastoma and neuroblastoma. Our findings demonstrate a role for both maternal and child folate polymorphism genotypes on the risk of nephroblastoma and neuroblastoma and highlight the importance of the combined genotype analysis. We believe that the mother has a crucial role in the early-onset disease predisposition as she provides the prenatal environment and can influence her offspring's risk of disease not only as a genetic donor but also through the effects of her genes acting directly on the intrauterine milieu or indirectly through child maternal gene–gene interactions.

Zhao *et al.* (2001) shed light on the critical role that *RFC-1* plays in embryonic development. *RFC-1*-null mice embryos (genetically engineered by gene knockout) died *in utero* due to failure of hematopoietic organs. The embryonic lethality could be rescued by maternal folate supplementation. Folic acid is a very poor substrate for *RFC-1*, which has a much higher affinity for reduced folate that comes from natural resources. It is likely that appreciable quantities of folic acid may be delivered into cells by passive diffusion as well as other secondary transport routes. As *RFC-1* rs1051266 (G80A) polymorphism may reduce the intracellular folate concentration, based on the above data on *RFC-1*-null mice embryos, we can speculate that higher doses of folate might overcome this deficit.

The complex interaction between genetic variants in the folate pathway and nutrient intake during pregnancy warrants further exploration. The preventive effect of folic acid supplementation is not questionable, but its protective mechanism is still poorly understood. The demand for folate during pregnancy has increased because it is required for growth and development of the fetus. It is well known that folic acid supplementation or fortification has reduced NTD and other congenital malformations (Berry *et al.*, 1999; Mezzomo *et al.*, 2007; Orioli *et al.*, 2011). Introduction of folic acid fortification of flour and grains in Canada produced a 30% reduction in the risk of nephroblastoma (Grupp *et al.*, 2011). Regarding neuroblastoma, the number of cases decreased from 1.57 cases per 10,000 births to 0.62 cases per 10,000 births ($p < 0.0001$)

TABLE 1. *MTHFR* C677T AND *RFC-1* GENOTYPE DISTRIBUTIONS AND POLYMORPHISM SUSCEPTIBILITY RISKS IN PATIENTS WITH NEPHROBLASTOMA AND NEUROBLASTOMA, CRUDE AND ADJUSTED BY SKIN COLOR

Genotype	Controls			Nephroblastoma			Neuroblastoma		
	n (%)	n (%)	n (%)	Crude OR (95% CI)	p	Adjusted ^a OR (95% CI)	Crude OR (95% CI)	p	Adjusted ^a OR (95% CI)
<i>MTHFR</i> (C677T)									
rs1801133									
CC	137 (56.6)	43 (53.7)	36 (54.5)	1 (ref)		1 (ref)	1 (ref)		1 (ref)
CT	87 (36.0)	30 (37.5)	24 (36.4)	1.09 (0.64–1.88)	0.73	1.09 (0.62–1.92)	1.05 (0.59–1.88)	0.87	1.05 (0.57–1.92)
TT	18 (7.4)	7 (8.8)	6 (9.1)	1.24 (0.48–3.16)	0.65	1.92 (0.65–5.61)	1.27 (0.47–3.43)	0.64	1.52 (0.48–4.79)
CT/TT ^b	105 (43.4)	37 (46.3)	30 (45.5)	1.12 (0.68–1.86)	0.65	1.18 (0.69–2.03)	1.09 (0.63–1.88)	0.76	1.12 (0.63–2.00)
<i>RFC-1</i> (G80A)									
rs1051266									
GG	76 (34.2)	13 (17.0)	11 (17.2)	1 (ref)		1 (ref)	1 (ref)		1 (ref)
GA	103 (46.4)	38 (49.3)	33 (51.6)	2.16 (1.07–4.33)	0.03	2.55 (1.21–5.35)	2.21 (1.05–4.66)	0.04	2.48 (1.13–5.44)
AA	43 (19.4)	26 (33.7)	20 (31.2)	3.53 (1.65–7.59)	<0.01	4.23 (1.84–9.71)	3.21 (1.41–7.33)	<0.01	3.46 (1.45–8.24)
GA/AA ^b	146 (65.8)	64 (83.0)	53 (82.8)	2.56 (1.33–4.95)	<0.01	3.07 (1.53–6.16)	2.51 (1.24–5.08)	0.01	2.88 (1.36–6.07)

^aOR adjusted by skin color.

^bCT/TT, GA/AA indicates at least one variant allele.

CI, confidence interval; *MTHFR*, methylenetetrahydrofolate reductase; OR, odds ratio; *RFC-1*, reduced folate carrier-1.

TABLE 2. *MTHFR* C677T and *RFC-1* POLYMORPHISM SUSCEPTIBILITY RISKS IN PATIENTS WITH NEUROBLASTOMA, THEIR MOTHERS, AND THE COMBINATION OF GENOTYPES

Neuroblastoma													
Child					Mother					Combined ^a			
Polymorphism	Genotypes	n (%)	OR (95% CI)	p	Controls, n (%)	Case, n (%)	OR (95% CI)	p	Controls, n	Case, n	OR (95% CI)	p	
<i>MTHFR</i> (C677T)													
rs1801133	CC	36 (54.5)	1 (ref)		37 (49.3)	17 (55.0)	1 (ref)		4	14	1 (ref)		
	CT	24 (36.4)	1.05 (0.59–1.88)	0.87	32 (42.7)	11 (35.0)	0.75 (0.31–1.83)	0.52	—	—	—		
	CT + CT	—	—	—	—	—	—	—	4	6	0.43 (0.08–2.31)	0.32	
	TT	6 (9.1)	1.27 (0.47–3.43)	0.64	6 (8.0)	3 (10.0)	1.09 (0.24–4.88)	0.91	—	—	—		
	TT + TT	—	—	—	—	—	—	—	0	2	—		
	CT/TT ^b	30 (45.5)	1.09 (0.63–1.88)	0.76	38 (50.7)	14 (45.0)	0.80 (0.35–1.86)	0.61	—	—	—		
	CT/TT + CT/TT	—	—	—	—	—	—	—	6	10	0.48 (0.11–2.14)	0.33	
<i>RFC-1</i> (G80A)													
rs1051266	GG	11 (17.2)	1 (ref)		30 (35.7)	5 (15.2)	1 (ref)		7	3	1 (ref)		
	GG + GA	—	—	—	—	—	—	—	4	1	0.58 (0.04–7.66)	0.68	
	GA + GG	—	—	—	—	—	—	—	3	2	1.56 (0.16–14.65)	0.70	
	GA	33 (51.6)	2.21 (1.05–4.66)	0.04	35 (41.7)	18 (54.5)	3.09 (1.02–9.31)	0.05	—	—	—		
	GA + GA	—	—	—	—	—	—	—	5	10	4.67 (0.83–26.24)	0.08	
	AA	20 (31.2)	3.21 (1.41–7.33)	<0.01	19 (22.6)	10 (30.3)	3.16 (0.93–10.67)	0.06	—	—	—		
	GA + AA	—	—	—	—	—	—	—	1	3	7.00 (0.50–97.75)	0.15	
	AA + GA	—	—	—	—	—	—	—	0	5	0	0	
	AA + AA	—	—	—	—	—	—	—	2	7	8.17 (1.03–64.94)	0.05	
GA/AA ^b	53 (82.8)	2.51 (1.24–5.08)	0.01	54 (64.3)	28 (84.8)	3.11 (1.09–8.90)	0.03	—	—	—			
GA/AA + GA/AA	—	—	—	—	—	—	—	8	25	7.29 (1.52–35.03)	0.01		

^aCombination of child–mother genotypes.

^bCT/TT, GA/AA indicates at least one variant allele.

TABLE 3. *MTHFR* C677T AND *RFC-1* POLYMORPHISM SUSCEPTIBILITY RISKS IN PATIENTS WITH NEPHROBLASTOMA, THEIR MOTHERS, AND THE COMBINATION OF GENOTYPES

Nephroblastoma													
Child					Mother					Combined ^a			
Polymorphism	Genotypes	n (%)	OR (95% CI)	p	Controls, n (%)	Case, n (%)	OR (95% CI)	p	Controls, n	Case, n	OR (95% CI)	p	
<i>MTHFR</i> (C677T)													
rs1801133	CC	43 (53.7)	1 (ref)		37 (49.3)	24 (50.0)	1 (ref)		4	18	1 (ref)		
	CT	30 (37.5)	1.09 (0.64–1.88)	0.73	32 (42.7)	21 (43.7)	1.01 (0.48–2.15)	0.98	—	—	—	—	
	CT+CT	—	—	—	—	—	—	—	4	11	0.61 (0.13–2.95)	0.54	
	TT	7 (8.8)	1.24 (0.48–3.16)	0.65	6 (8.0)	3 (6.3)	0.77 (0.18–3.38)	0.73	—	—	—	—	
	TT+TT	—	—	—	—	—	—	—	0	0	0	0	
	CT/TT ^b	37 (46.3)	1.12 (0.68–1.86)	0.65	38 (50.7)	24 (50.0)	0.97 (0.47–2.01)	0.94	—	—	—	—	
	CT/TT+CT/TT	—	—	—	—	—	—	—	6	18	0.67 (0.16–2.77)	0.58	
<i>RFC-1</i> (G80A)													
rs1051266	GG	13 (17.0)	1 (ref)		30 (35.7)	15 (28.3)	1 (ref)		7	6	1 (ref)		
	GG+GA	—	—	—	—	—	—	—	4	5	1.46 (0.26–8.05)	0.66	
	GA+GG	—	—	—	—	—	—	—	3	9	3.50 (0.64–19.19)	0.15	
	GA	38 (49.3)	2.16 (1.07–4.33)	0.03	35 (41.7)	26 (49.0)	1.49 (0.67–3.31)	0.33	—	—	—	—	
	GA+GA	—	—	—	—	—	—	—	5	11	2.57 (0.56–11.72)	0.22	
	AA	26 (33.7)	3.53 (1.65–7.59)	<0.01	19 (22.6)	12 (22.7)	1.26 (0.49–3.27)	0.63	—	—	—	—	
	GA+AA	—	—	—	—	—	—	—	1	4	4.67 (0.40–53.95)	0.22	
	AA+GA	—	—	—	—	—	—	—	0	9	0	0	
	AA+AA	—	—	—	—	—	—	—	2	7	4.08 (0.60–27.65)	0.15	
GA/AA ^b	64 (83.0)	2.56 (1.33–4.95)	<0.01	54 (64.3)	38 (71.7)	1.41 (0.67–2.97)	0.37	—	—	—	—		
GA/AA+GA/AA	—	—	—	—	—	—	—	8	31	4.52 (1.18–17.25)	0.03		

^aCombination of child–mother genotypes.

^bCT/TT, GA/AA indicates at least one variant allele.

(French *et al.*, 2003). Rates of nephroblastoma were significantly lower after mandatory US folic acid fortification, with stronger effects detected in infants (Linabery *et al.*, 2012). Schuz *et al.* (2007) observed similar results with the use of vitamins, folate, and/or iron supplements during pregnancy in Germany. In Brazil, fortification of foods has been mandatory since 2004 (www.anvisa.gov.br/alimentos/farinha.htm). Supplementation with folic acid is also recommended during pregnancy in Brazil, but data on compliance are scarce. On the other hand, some countries do not mandate folic acid fortification, in part, because of concerns about the potential for enhancing cancer development (Wright *et al.*, 2007). The main strength of this study was our ability to investigate maternal genotypes together with children's genotypes in case and control samples. Maternal effects can arise because the genetic variants carried by the mother influence the prenatal environment in which the fetus develops, thereby increasing disease risk (Vermeulen *et al.*, 2009). The major limitation is the lack of information about folate levels and supplementation. Based on literature data, we assume that polymorphisms in these genes influence enzyme activity and receptor function (Kim, 1999; Kim *et al.*, 2011).

Further studies assessing folate levels and gene-environment interactions in childhood embryonic tumor development are still necessary.

In summary, our results suggest that individual carriers of *RFC* variant genotype present a higher risk for the development of nephroblastoma and neuroblastoma. It is likely they have an impaired absorption of folic acid and this may affect the folate metabolism pathway causing genomic instability and promoting cancer development.

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Patient Consent

Parents provided written informed consent to study participation.

Ethics Approval

The Ethics and Scientific Committees of Instituto Nacional de Câncer approved the study (CEP #136/09). All Brazilian collaborating institutions also approved the study.

Authors' Contributions

B.D.C., G.M.V., F.V., and M.S.P.O. contributed to the study design, data collection, data interpretation, and manuscript writing. L.C.T. participated in statistical analysis and data interpretation. R.M.A. contributed to all laboratory work and the results are part of her master's degree. The Brazilian Embryonic Tumor Group participated in the study design and data collection. All authors contributed equally to the revising and approval of the final version of the article.

Author Disclosure Statement

No competing financial interests exist.

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