RFC - 1 Gene Polymorphism and the Risk of Down Syndrome in Romanian Population

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┌─ **ABSTRACT** ─

Background and aims: There is evidence that the polymorphisms of the genes involved in folate metabolism may be associated with higher risk of Down syndrome (DS) pregnancy. The aim of the present study was to investigate the effect of A80G polymorphism in reduced folate carrier 1 (RFC1) gene on the maternal risk for DS.

Methods: In our study, twenty-two DS mothers and forty-two mothers who had no children with DS were evaluated. Genomic DNA was isolated from whole peripheral blood collected on EDTA, using peqGOLD blood DNA mini kit (ATP Biotech) following the manufacturer's instructions.

Results: The results show that the frequencies of RFC1 alleles, as well as the frequencies of RFC1 A80G genotypes (GG, GA, AA, GA+AA) do not correlate with DS pregnancies, demonstrating no difference between the case and control groups.

Conclusions: In the present study, we did not find any statistically significant association between RFC-1 polymorphic genotype and history of DS pregnancies; thus, the relationship between RFC-1 polymorphism and DS appears to be only a supposition and the next step in our study is the catamnestic evaluation of our patients with DS babies for two years.

Keywords: Trisomy 21, Down syndrome, folate, RFC1 gene, RFC1 A80G polymorphism

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INTRODUCTION

Down syndrome (DS) or trisomy 21 is a genetic disease caused by abnormal chromosomal segregation (1). Free trisomy 21 is found in 95% of DS cases and is due to chromosome 21 nondisjunction, mostly occurring during maternal meiosis (2). A number of genetic and environmental factors have been suggested to play interactively a role in aneuploidization, among which dietary factors (3). Folate (pteroiloglutamic acid) has an important role in the process of genetic material distribution during cell division, because of its part in the cellular methylation reactions, which, in turn, epigenetically regulate segregation and other processes (4); a diagram illustrating folate metabolism is shown in figure 1.

After intestinal absorption, folate requires reduction and methylation into the liver to form 5-methyltetrahydrofolate (5-methylTHF), release into the blood and cellular uptake; then, it can be used for the synthesis of DNA and RNA precursors or for the conversion of homocysteine to methionine, which is then used for the synthesis of the main DNA methylating agent, S-adenosylmethionine (SAM) (5,6).

Reduced folate carrier protein (RFC1) is responsible for folate uptake from jejunum and its subsequent translocation across biological membranes in a variety of cells (7). The RFC1 gene is located on chromosome 21 and likely overexpressed in DS individuals. The A80G polymorphism of the RFC1 gene has been recently demonstrated to affect plasma folate and homocysteine levels, alone or in combination with the C677T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene. The first report of a RFC1 gene polymorphism described a highly frequent A80G single nucleotide polymorphism resulting in the replacement of an arginine residue by a histidine, with impact on folate status (8).

Recent evidence shows that almost 92% of the DS children are born from young mothers, suggesting that other risk factors than advanced maternal age must be involved. Within this context, some studies suggested and even demonstrated a possible link between DS and maternal polymorphisms in genes involved in folate metabolism (9,10). ❑
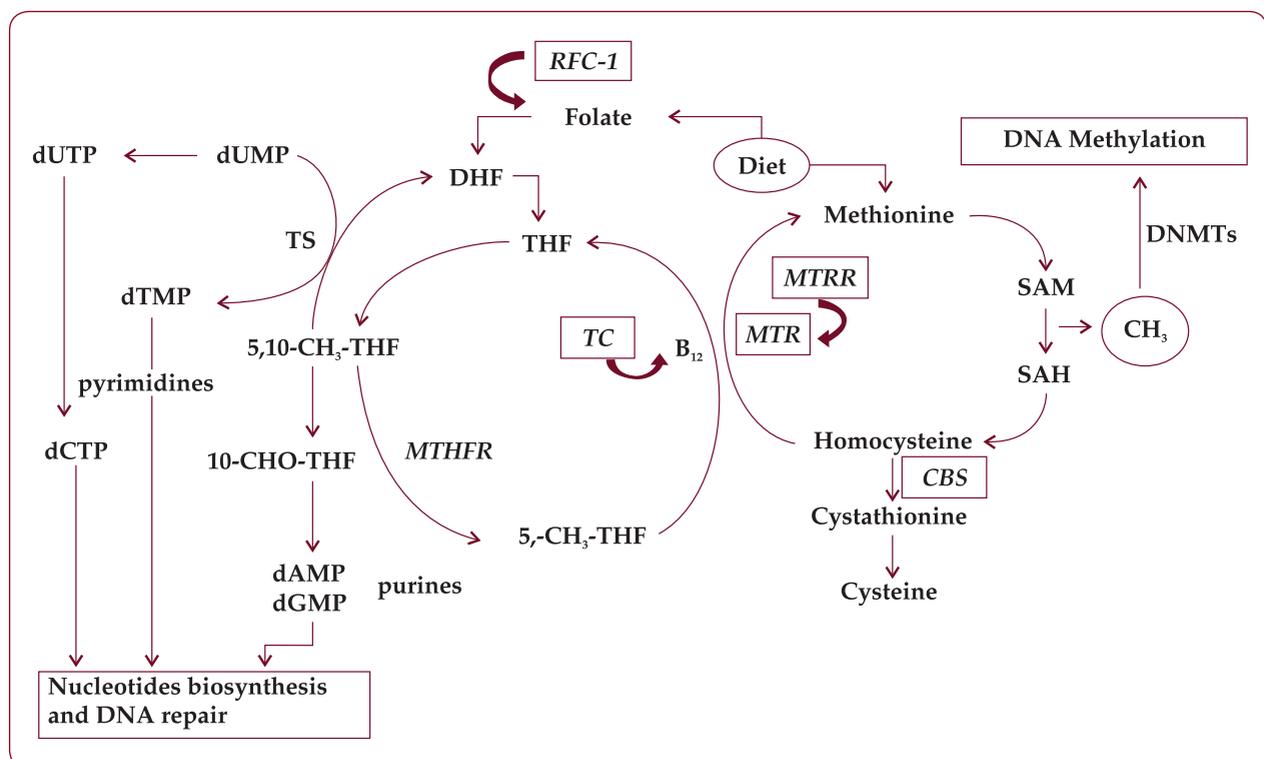

FIGURE 1. Folate metabolism and its interconnection with other essential metabolic pathways, such as biosynthesis of nucleotides, used next in the synthesis and repair of DNA and in cellular methylation reactions.

MATERIALS AND METHODS

Patients

The present study includes 72 women (ages 20-42 years old): 26 of them, that gave birth to DS children, citogenetically confirmed as regular trisomy 21, including 7 women with a history of spontaneous miscarriages, and 46 control mothers that gave birth only to healthy children, without any history of miscarriages or abnormal pregnancies. All women in our study reside in the same geographic area and have a similar social background. There was no periconceptional use of folic acid. Only 11.53% of the DS mothers had irregularly taken multivitamin preparations in the second or third trimester.

Sample specimens and DNA extraction

Peripheral blood samples (5 ml) were collected on EDTA from both DS and control mothers Genomic DNA was isolated from whole blood, using peqGOLD blood DNA mini kit (*ATP Biotech*) following the manufacturer's instructions.

Quantitative PCR

All genotype analyses were performed using PCR-RFLP (CfoI). The primers for amplification were: forward: 5'-AG TGT CAC CTT CGT CCC-3' and reverse 5'-TCC CGC GTG AAG TTC TTG-3'. PCR conditions were 44 cycles of 30 sec at 94°C, 30 sec at 52°C, and 45 sec at 72°C, preceded by an initial denaturation of 2 min at 94°C, and followed by a final extension of 7 min at 72°C. Three hours digestion with CfoI (Sigma) resulted in three fragments of 125, 68, and 37 bp, in the presence of the 80G allele, while the 80A allele produced two fragments of 162 and 68 bp.

Statistical analysis

Expected genotype frequencies were calculated from allele frequencies under the assumption of Hardy-Weinberg equilibrium. The differences in allele frequencies between mothers of children with DS and control mothers were determined using chi-square test .The interaction between *RCF1* genotypes was evaluated by calculating the odds ratios (OR) for mutant genotypes, as compared to wild types. 95% confidence intervals (95% CI) were calculated to estimate the risk of the different genotypes.

Analyses were performed using the software SPSS. P was considered statistically different if 0.05 or lower. ❑

RESULTS

The status of *RCF1* A80G polymorphism was addressed by PCR amplification of genomic DNA using the primers described in *Materials and Methods* section, followed by restriction digestion with an appropriate endonuclease. The results of the mutational analysis are shown for few representative cases in the figure 2, illustrating homozygous 80AA genotype (cases 13 and 58), homozygous 80GG genotype (cases 31 and 20), and heterozygous 80 GA genotype (and cases 18 and 4).
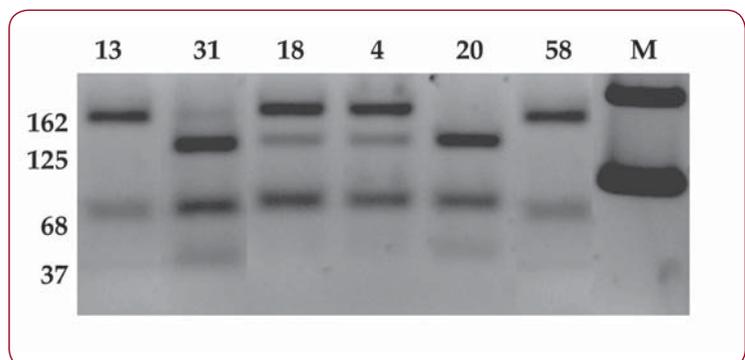

FIGURE 2. PCR-RFLP (*CfoI*) mutational analysis of *RFC1* gene. Lane 1 – case 13; 2 – case 31; 3 – case 18; 4 – case 4; 5 – case 20; 6 – case 58; M – pGEM 100 bp molecular weight marker (Promega).

Following mutational analysis, the frequencies of the polymorphic alleles of *RFC1* were calculated (Table I), and, next, the genotype frequencies (Table II, Fig. 3).

The results suggested that there was no significant difference in heterozygous genotype frequencies between the two groups. Moreover, while AA homozygous genotype frequency was higher among control mothers than among DS mothers (10.87% versus 3.85%), the overall combination of GA heterozygous and AA homozygous *RFC1* variant genotypes did not show significant difference between the case and control groups (OR 0.59 [0.21–1.70] P 0.33).

RFC1 A80G polymorphism and maternal age at conception

Twenty-six case and forty-six control mothers were stratified in three groups by age at conception, i.e., maternal age ≤26 years, 27–

Allele	DS mothers (%)	Control mothers (%)	χ²	P
G	34 (65.4)	52 (56.5)	1.085	0.297
A	18 (34.6)	40 (43.5)		
Total	52	92		

TABLE 1. *RCF1* allele frequencies in mothers of DS children and control mothers

Genotype	DS mothers (%)	Control mothers (%)	Odds ratio	95% CI	P
GG	9 (34.62)	11 (23.91)	1	Referinta	
GA	16 (61.54)	30 (65.22)	0.65	0.22-1.89	0.43
AA	1 (3.85)	5 (10.87)	0.24	0.02-2.49	0.21
GA or AA	17 (65.38)	35 (76.08)	0.59	0.21- 1.70	0.33

TABLE 2. *RCF1* 80A/G genotype frequencies in mothers of DS children and in control mothers
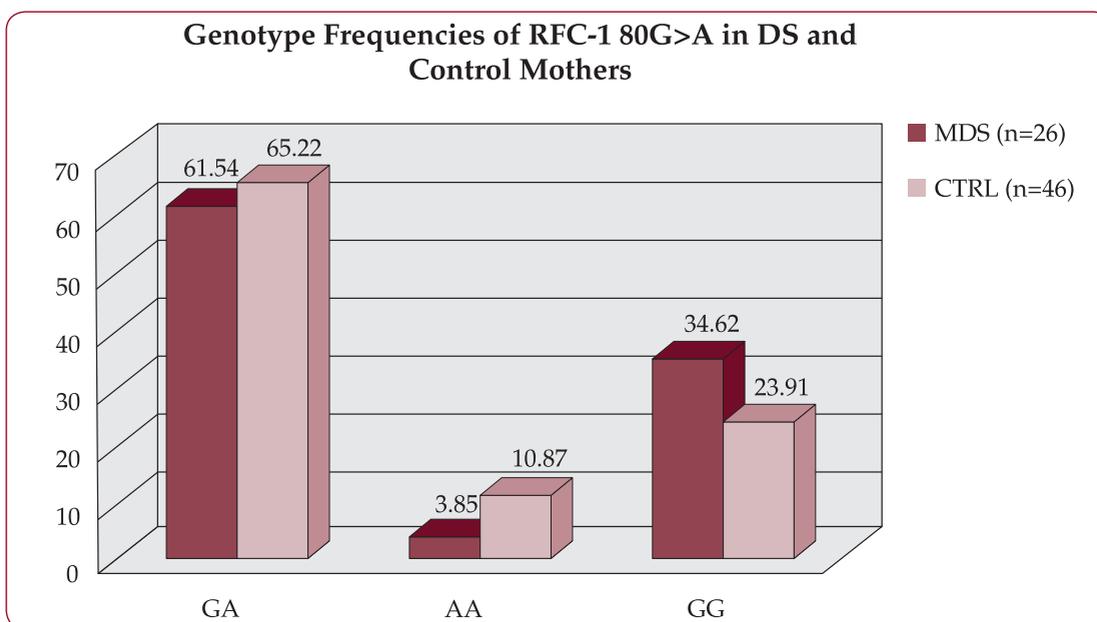

FIGURE 3. *RCF1* 80A/G genotype frequencies in mothers of DS children and in control mothers

33 years and ≥34 years, respectively, where the first group corresponded to the first quartile, the second group to the second and third quartiles and the third group to the fourth quartile. The allele frequencies were calculated for the 3 groups, the results indicating non-association between either of the RFC1 alleles and any maternal age at conception, regarding the risk of a DS pregnancy (Table III). ❑

DISCUSSION

Down syndrome is the most commonly identified chromosome abnormality in humans. Birth prevalence increases with maternal age from 0.6 in 1,000 live births at 20 years up to 11 in 1,000 live births at 40 years. [11]. Although maternal age is the major risk factor for trisomy 21, most children with Down syndrome are born to young mothers (less than 35 years) (12). During the last years, research has been carried out to elucidate the mechanisms and physiological conditions that are favorable to the appearance of DS fetuses. Studies performed on cell cultures have clearly demonstrated that folate deficiency from the media is able to induce chromosome 21 aneuploidy (13). The mechanism underlying the meiotic nondisjunction is poorly understood and is thought to have a multifactorial aetiology, being influenced by both genetic and acquired factors (9,14).

Steps were taken to investigate the genes involved in the folate metabolism and their polymorphisms. Regarding *RFC1,* one study performed in Southern Italy suggests that the

Age (quartiles)	RFC1 (alleles)	Cases N=26	Controls N=46	Odds ratio	95% CI	P
≤ 26 years	G	3	16	0.53	0.05-5.86	0.6
	A	1	10			
27-33 years	G	16	30	0.75	0.27-2.08	0.58
	A	8	20			
≥ 34 years	G	15	6	0.36	0.1-1.33	0.12
	A	9	10			

TABLE 3. *RCF1* allele, maternal age at conception, and DS risk in case versus control mothers

80G allele might increase DS risk in mothers aging more than 34 years at conception (15). Further, *RFC1* GG genotype was associated with the risk of neural tube defects (NTD) (16). There is additional evidence that some mothers of infants with DS have abnormal folate and methyl metabolism, similar to mothers of infants with NTD (17); even more, sometimes DS and NTD occur in the same family, suggesting that, at least in a proportion of cases, DS and NTD could have a common etiological pathway (18).

However, the majority of the studies performed so far agree that the RFC1 A80G polymorphism is not an independent risk factor for having a DS child (19-22). While overall *in vitro* and *in vivo* studies indicate that an impaired folate/homocysteine metabolism can result in chromosome 21 nondisjunction, ultimately, the birth of a DS child seems to be the result of the interplay of several factors of genetic, epigenetic, environmental, and stochastic origin, making it difficult to discriminate the single contribution of each of them. Further studies are also required to address the possible contribution of both the paternal diet and the maternal grandmother dietary habits to chromosome 21 nondisjunction events. ❑

CONCLUSION

To our knowledge, this is the first study that has analyzed the *RFC1* A80G polymorphism as a maternal risk factor for meiotic nondisjunction of chromosomes 21, causing DS, in a cohort of Romanian mothers of DS children, in comparison with control mothers.

No evidence for an association between *RFC1* A80G polymorphism and the maternal risk of bearing a DS child was observed in this study. Thus, further research including other polymorphisms involved in folate metabolism could provide a better understanding of the role of genetic variants in the etiology of the chromosomal nondisjunction those results in DS. ❑

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