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ASSOCIAÇÃO ENTRE POLIMORFISMOS EM GENES ENVOLVIDOS NO METABOLISMO DO FOLATO E RISCO MATERNO PARA A SÍNDROME DE DOWN

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Associação entre polimorfismos em genes envolvidos no metabolismo do folato e risco materno para a síndrome de Down

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Sumário

Dedicatória..... i

Agradecimentos..... ii

Epígrafe..... iv

Lista de Figuras..... v

Lista de Tabelas..... vi

Lista de Abreviaturas e Símbolos..... vii

Resumo..... x

Abstract..... xii

1. Introdução..... 01

1.1. Objetivos..... 08

2. Artigos Científicos..... 09

Artigo 1. 19-base pair deletion polymorphism of the *Dihydrofolate reductase* (*DHFR*) gene: maternal risk for Down syndrome and folate metabolism..... 11

Artigo 2. *DNMT3B* -149C→T and -283T→C polymorphisms as a maternal risk factor for Down syndrome..... 29

3. Conclusões..... 47

4. Referências Bibliográficas..... 49

5. Apêndice..... 60

Synergistic effect of *DHFR* 19-bp deletion and *SHMT* C1420T polymorphisms on folate pathway metabolites concetrations in individuals with Down syndrome..... 62

6. Anexos..... 76

Anexo 1. Aprovação do Comitê de Ética em Pesquisa da FAMERP (CEP)..... 78

Anexo 2. Aprovação do Comitê Nacional de Ética (CONEP)..... 79

Anexo 3. Aprovação do Comitê de Ética em Pesquisa da FAMERP (CEP) para análise molecular dos polimorfismos dos genes *Dihidrofolato redutase* (*DHFR*) e *Serina hidroximetiltransferase* (*SHMT*)..... 80

Anexo 4. Aprovação do Comitê de Ética em Pesquisa da FAMERP (CEP) para análise molecular dos polimorfismos do gene *DNA metiltransferase 3B* (*DNMT3B*)..... 81

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Aos meus pais, que me ensinaram

o verdadeiro valor do estudo.

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Aos colegas de laboratório

Pela alegre convivência e sugestões essenciais no desenvolvimento desse trabalho.

Epígrafe

"Somente os desafios que não são seus

são intransponíveis"

Autor desconhecido

Lista de Figuras:

Introdução **Figura1.** Metabolismo do folato com as principais enzimas envolvidas..... 04

Lista de Tabelas

Artigo 1

Table 1. Allele and genotype frequencies of 19-bp deletion polymorphism in intron-1 of *Dihydrofolate reductase (DHFR)* gene in case and control groups……...…….. 27

Table 2. Distribution of serum folate and plasma homocysteine (Hcy) and methylmalonic acid (MMA) concentrations according to genotypes of the 19-bp deletion polymorphism in intron-1 of *Dihydrofolate reductase (DHFR)* gene in case and control groups...…… 28

Artigo 2

Table 1. Genotype distribution of *DNA methyltransferase 3B* (*DNMT3B*) -149C→T and *DNMT3B* -283T→C polymorphisms between the case and control groups..... 44

Table 2. Haplotype frequencies of *DNA methyltransferase 3B* (*DNMT3B*) -149C→T and *DNMT3B* -283T→C polymorphisms between the case and control groups…..... 45

Table 3. Distribution of serum folate and plasma homocysteine (Hcy) and methylmalonic acid (MMA) concentrations according to combined genotypes *DNA methyltransferase 3B* (*DNMT3B*) -149C→T/*DNMT3B* -283T→C..... 46

Apêndice

Table 1. Allele frequencies of 19-base pair (bp) deletion in intron-1 of *Dihydrofolate reductase* (*DHFR*) gene and *Serine hydroxymethyltransferase* (*SHMT*) C1420T polymorphisms in individuals with Down syndrome..... 74

Table 2. Distribution of serum folate and plasma homocysteine (Hcy) and methylmalonic acid (MMA) concentrations according to combined genotypes of the 19-base pair (bp) deletion in intron-1 of *Dihydrofolate reductase* (*DHFR*) gene and *Serine hydroxymethyltransferase* (*SHMT*) C1420T polymorphisms in individuals with Down syndrome..... 75

Lista de abreviaturas e símbolos

µmol/L	*Micromol / litre*
10-formilTHF	10-formiltetrahidrofolato
5,10-metinilTHF	5,10-metiniltetrahidrofolato
5,10-MTHF	5,10-metilenotetrahidrofolato (*5,10-methylenetetrahydrofolate*)
5-metilTHF	5-metiltetrahidrofolato (*5-methyltetrahydrofolate*)
5-MTHF	5-metilenotetrahidrofolato
A	Adenina (*Adenine*)
B_{12}	Vitamina B_{12}
BAP	Bolsa de Auxílio ao Pesquisador
bp	*Base pair*
C	Citosina (*Cytosine*)
Cbl	Cobalamina
CEP	Comitê de Ética em Pesquisa
CI	*Confidence interval*
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CpG	Dinucleotídeo citosina-fosfato-guanina
CβS	*Cystathionine beta-synthase*
D	*Allele with deletion*
del	*Allele with deletion*
DHF	Dihidrofolato (*Dihydrofolate*)
DHFR	Dihidrofolato redutase (*Dihydrofolate reductase*)
DNA	Ácido desoxirribonucléico (*Desoxirribonucleic acid*)

DNMT1	*DNA methyltransferase 1*
DNMT3A	*DNA methyltransferase 3A*
DNMT3B	DNA metiltransferase 3B (*DNA methyltransferase 3B*)
DNMTs	DNA metiltransferases (*DNA methyltransferases*)
DS	*Down syndrome*
dTMP	Deoxitimidina monofosfato
DTN	Defeitos de tubo neural
dUMP	Deoxiuridina monofosfato
Et al.	*Et alia*
FAMERP	Faculdade de Medicina de São José do Rio Preto
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
FUNFARME	Fundação Faculdade Regional de Medicina de São José do Rio Preto
G	Guanina (*Guanine*)
Hcy	Homocisteína (*Homocysteine*)
I	Wild allele
IC	Intervalo de confiança
ins	*Wild allele*
LD	*Linkage Disequilibrium*
MMA	Ácido metilmalônico (*Methylmalonic acid*)
mRNA	*Messenger ribonucleic acid*
MTHFR	Metilenotetrahidrofolato redutase (*Methylenetetrahydrofolate reductase*)
MTR	Metionina sintase (*Methionine synthase*)
ng/mL	*Nanogram / microlitre*

NTD	*Neural tube defects*
OR	*Odds ratio*
pb	Pares de base
PCR	Reação em Cadeia da Polimerase (*Polymerase chain reaction*)
q	Braço longo do centrômero ao telômero de um cromossomo
RFC1	Carregador de folato reduzido 1
SAH	S-adenosilhomocisteína (*S-adenosylmethionine*)
SAM	S-adenosilmetionina
SD	Síndrome de Down
SHMT	*Serine hydroxymethyltransferase*
T	Timina (*Thymine*)
TC2	Transcobalamina 2
THF	Tetrahidrofolato (*Tetrahydrofolate*)
UNICAMP	Universidade de Campinas
UPGEM	Unidade de Pesquisa em Genética e Biologia Molecular
USP	Universidade de São Paulo
$\chi 2$	*Chi-square*

RESUMO

Introdução: A síndrome de Down (SD) é a cromossomopatia humana mais frequente e, na maioria dos casos (cerca de 90%), é caracterizada pela trissomia livre do cromossomo 21, resultante de falhas na segregação cromossômica durante a meiose materna. Estudos sugerem que a ocorrência da SD independente da idade materna está relacionada à hipometilação do DNA centromérico como conseqüência do metabolismo anormal do folato e, polimorfismos genéticos envolvidos nesta via metabólica têm sido apontados como fatores de risco materno para a síndrome. Além dos polimorfismos genéticos, deficiências de micronutrientes, tais como folato e vitamina B_{12}, podem alterar os produtos resultantes da via metabólica do folato e resultar em hipometilação do DNA, instabilidade genômica e redução da capacidade de reparo do DNA. **Objetivos:** Avaliar a influência dos polimorfismos de deleção de 19 pares de base (pb) do gene *Dihidrofolato redutase* (*DHFR*), *DNA metiltransferase 3B* (*DNMT3B*) -149C→T e -283T→C como fatores de risco materno para a SD e investigar a associação entre esses polimorfismos e as concentrações de folato sérico, homocisteína (Hcy) e ácido metilmalônico (MMA) plasmáticos. **Casuística e Métodos:** Foram incluídas no estudo 105 mães de indivíduos com trissomia livre do cromossomo 21 e 185 mães de indivíduos sem a síndrome. O polimorfismo do gene *DHFR* foi avaliado por meio da Reação em Cadeia da Polimerase (PCR) por diferença de tamanho de fragmentos e os polimorfismos *DNMT3B* -149C→T e -283T→C foram analisados por PCR em tempo real. O folato sérico foi quantificado por quimioluminescência, e Hcy e MMA plasmáticos foram determinados por cromatografia líquida/espectrometria de massas sequencial. **Resultados:** Em relação ao polimorfismo de deleção do gene *DHFR*, não houve diferença entre os grupos em relação às frequências alélica e

genotípica (P = 0,44; P = 0,69, respectivamente) e as concentrações de folato, Hcy e MMA não mostraram diferença significativa entre os genótipos, entre grupos (P > 0,05). Os genótipos combinados *DNMT3B* -149TT/-283TC foram associados com o aumento do risco materno para a SD (OR = 4,61, IC 95% = 1,35 – 15,79; P = 0,02) e, concentração de folato aumentada foi observada em mães com os genótipos *DNMT3B* -149CT/-283CC quando comparados com os demais genótipos combinados (P = 0,03). **Conclusões:** O polimorfismo de deleção de 19 pb do gene *DHFR* não é um fator de risco materno para SD e não está relacionado com variações nas concentrações de folato sérico, Hcy e MMA plasmáticos. Por outro lado, os polimorfismos do gene *DNMT3B* aumentam o risco materno para a SD e modulam a concentração de folato na população estudada.

Palavras chave: Síndrome de Down, polimorfismo genético, fatores de risco, folato.

ABSTRACT

Introduction: Down syndrome is the most common genetic disorder and, in about 90% of the cases, is characterized by free trisomy of chromosome 21, caused by the failures of chromosomal segregation during maternal meiosis. Studies suggested that the occurrence of DS independent of maternal age is associated with DNA hypomethylation due to impairments in folate metabolism, and genetics polymorphisms involved in this metabolic pathway have been appointed as maternal risk factors for DS. Addition to the genetic polymorphisms, micronutrients deficiencies, as folate and B_{12} vitamin, can change the products of the folate pathway and result in DNA hypomethylation, genomic instability and reduced DNA repair capacity. **Objectives:** We evaluated the influence of the 19-base pairs (bp) deletion polymorphism of *Dihydrofolate reductase* (*DHFR*) gene, *DNA methyltransferase 3B* (*DNMT3B*) -149C→T and -283T→C on the maternal risk for DS and investigated the association between these polymorphism and variations in the concentrations of serum folate and plasma homocysteine (Hcy) and methylmalonic acid (MMA). **Methods:** 105 mothers of DS individuals with free trisomy 21 and 185 mothers of individuals without the syndrome were studied. Molecular analysis of the *DHFR* polymorphism was performed by the Polymerase Chain Reaction (PCR) by difference in the size of fragments and *DNMT3B* -149C→T and -283T→C were analyzed by real-time PCR. Folate was quantified by chemiluminescence and Hcy and MMA by liquid chromatography-tandem mass spectrometry. **Results:** The analysis of *DHFR* polymorphism showed no difference between the groups in relation to allele and genotype frequencies ($P = 0.44$; $P = 0.69$, respectively). In relation to gentoype, folate, Hcy and MMA concentrations did not differ between the groups ($P > 0.05$). The *DNMT3B* -149TT/-283TC combined

genotypes were associated with increased maternal risk for DS (OR = 4.61, CI 95% = 1.35 – 15.79; P = 0.02) and higher folate concentration was observed in mothers with *DNMT3B* -149CT/-283CC genotypes compared to other combined genotypes (P = 0.03). **Conclusions:** The 19-bp deletion polymorphism of *DHFR* gene is not a maternal risk factor for DS and is not related to variations in the concentrations of serum folate and plasma Hcy and MMA. On the other hand, the *DNMT3B* polymorphisms increase the maternal risk for DS and modulate the folate concentration in studied population.

Key words: Down syndrome, genetic polymorphism; risk factors, folate.

1. INTRODUÇÃO

1. INTRODUÇÃO

A síndrome de Down (SD) é uma doença genética conhecida há mais de um século, desde que John Langdon Haydon Down descreveu suas características fenotípicas. Trata-se da cromossomopatia humana mais frequente, com incidência de 1:660 nascidos vivos,[1] e, na maioria dos casos (cerca de 90%), é caracterizada pela trissomia livre do cromossomo 21, resultante de falhas na segregação cromossômica durante a meiose materna.[2-4]

Um dos mais importantes fatores de risco para a SD é a idade materna avançada.[2,5-7] Embora não esteja totalmente esclarecido, este risco pode estar associado com a perda da eficiência do processo meiótico, causado, por exemplo, por defeitos na coesão das cromátides irmãs ou por degradação de proteínas envolvidas na formação do fuso mitótico.[8-10] Entretanto, o nascimento de indivíduos com SD de mães jovens sugere a existência de outros fatores etiológicos.

Estudo de James et al.[11] propôs que a ocorrência da SD independente da idade materna está relacionada ao metabolismo anormal do folato. O metabolismo do folato é responsável, em uma de suas vias, pela síntese de S-adenosilmetionina (SAM), o maior doador intracelular de grupos metil para reações de metilação do DNA, além de estar envolvido na síntese de purinas e pirimidinas para a síntese e reparo do DNA (Figura 1). A deficiência de folato reduz a síntese de SAM, causando hipometilação do DNA que prejudica a formação da heterocromatina e o estabelecimento do cinetocoro, complexo DNA-proteína que garante a divisão precisa de cromossomos entre as células-filhas por meio da ligação do centrômero aos microtúbulos do fuso mitótico.[11,12,13] A formação do cinetocoro depende de padrões de metilação específicos e da ligação de proteínas sensíveis à metilação na cromatina centromérica e, alterações neste processo podem

resultar em não-disjunção cromossômica.[9,14] De fato, estudos mostram que a hipometilação do DNA genômico está associada à instabilidade genética e à ocorrência de aneuploidias. [12,15]

No metabolismo do folato, a enzima Dihidrofolato redutase (DHFR) é responsável pela conversão do ácido fólico sintético, presente em suplementos vitamínicos e alimentos fortificados, em dihidrofolato (DHF). Essa enzima também catalisa a conversão de DHF em tetrahidrofolato (THF), a forma metabolicamente ativa do folato no organismo humano.[16] Por sua vez, a enzima Metilenotetrahidrofolato redutase (MTHFR) catalisa a conversão do 5,10-metilenotetrahidrofolato (5,10-MTHF) para 5-metiltetrahidrofolato (5-MTHF), a principal forma circulante de folato, que atua como doador de grupos metil para a remetilação da homocisteína (Hcy) para metionina.[17,18] Esta reação de remetilação é catalisada pela enzima Metionina sintase (MTR), que requer a vitamina B_{12}, ou cobalamina (Cbl), como co-fator, e resulta na formação de SAM.[19,20]

As enzimas DNA metiltransferases (DNMTs) catalisam a transferência do grupo metil, resultante da transformação do SAM em S-adenosilhomocisteína (SAH), para citosinas localizadas, predominantemente, em dinucleotídeos citosina-fosfato-guanina (CpG).[21-23] A enzima DNA metiltransferase 3B (DNMT3B), pertencente à família das DNMTs, é essencial para a metilação de sítios anteriormente não metilados ou hemimetilados, processo denominado metilação *de novo*.[22,24,25]

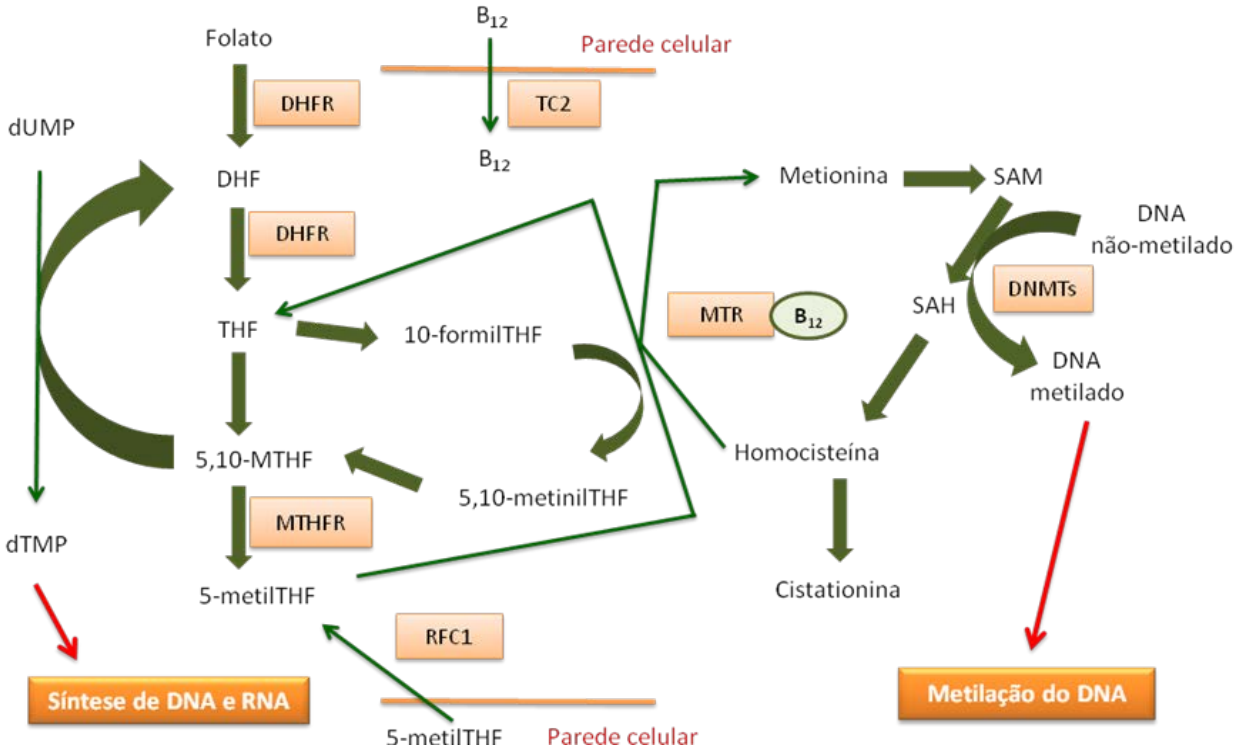

Figura 1. Metabolismo do folato com as principais enzimas envolvidas. TC2 = Transcobalamina 2, DHFR = Dihidrofolato redutase, MTHFR = Metilenotetrahidrofolato redutase, RFC1 = Carregador de folato reduzido 1, MTR = Metionina sintase, DNMTs = DNA metiltransferases, DHF = Dihidrofolato, THF = Tetrahidrofolato, 5,10-MTHF = 5,10-metilenotetrahidrofolato, 5-MTHF = 5-metiltetrahidrofolato, 5,10-metinilTHF = 5,10-metiniltetrahidrofolato, 10-formilTHF = 10-formiltetrahidrofolato, B_{12} = Vitamina B_{12}, SAM = S-adenosilmetionina, SAH = S-adenosilhomocisteína, dUMP = Deoxiuridina monofosfato, dTMP = Deoxitimidina monofosfato.

Proteínas transportadoras de folato reduzido (proteína carregadora de folato reduzido 1, gene *RFC1*) e Cbl (proteína transcobalamina II, gene *TC2*), são também importantes para o metabolismo do folato. A proteína RFC1 localiza-se na membrana das células da mucosa intestinal e participa do processo de absorção do ácido fólico, realizando o transporte do 5-MTHF para o interior de uma variedade de células, constituindo um importante determinante das concentrações de folato disponíveis dentro das células.[26] Por sua vez, a proteína TC2 é sintetizada no endotélio vascular da vilosidade intestinal e liga-se à Cbl livre no fluido intersticial. A proteína TC2 ligada à Cbl (complexo TC2-Cbl) passa, então, a microcirculação da vilosidade intestinal e por meio da veia porta alcança a circulação sistêmica.[27]

Polimorfismos em genes envolvidos no metabolismo do folato parecem modular as concentrações de metabólitos dessa via.[28-30] e estudos apontam para o papel de alguns destes polimorfismos na modulação do risco materno para a SD.[11,31-36] O polimorfismo *MTHFR* C677T está associado à redução da atividade da enzima MTHFR[37,38] e estudos mostram que esse polimorfismo está relacionado ao aumento das concentrações de Hcy e redução das concentrações de folato.[11,29,32,38-41] Vários estudos evidenciaram a influência do polimorfismo *MTHFR* C677T no aumento do risco materno para a SD, sendo este o polimorfismo mais investigado como fator de risco materno para a SD.[11,32,34-36,42-46]

A substituição A→G no nucleotídeo 2756 do gene *MTR* também foi associada ao risco materno para SD,[31,32,35,47,48] embora sua influência nas concentrações de Hcy permaneça contraditória. Enquanto alguns estudos associaram a presença do alelo selvagem *MTR* 2756A a concentrações elevadas de Hcy,[30,49-51] outros observaram

relação com o alelo polimórfico *MTR* 2756G.[33,52] Além disso, estudo de Chen et al.[49] associou esse polimorfismo ao aumento das concentrações de folato.

O gene *RFC1* apresenta-se polimórfico no nucleotídeo 80 (A→G), e a avaliação do impacto deste polimorfismo nas propriedades funcionais da proteína RFC1 resultante da variante polimórfica demonstrou que existem somente mínimas diferenças na sua afinidade por substratos e/ou eficiência de transporte em relação à enzima do tipo selvagem.[53] Recente estudo mostrou que o polimorfismo *RFC1* A80G está associado à diminuição das concentrações de folato.[30] Em relação à influência do polimorfismo *RFC1* A80G no risco materno para a SD, Chango et al.[47] e Fintelman-Rodrigues et al.[48] não observaram contribuição desta variante como fator de risco na população francesa, enquanto Biselli et al.,[34] Brandalize et al.[35] e Coppedè et al.[42] sugerem um papel para este polimorfismo, juntamente com outros polimorfismos da via metabólica do folato no risco para a SD.

O polimorfismo *TC2* C776G pode influenciar a quantidade de Cbl disponível. Seetharam and Li[54] observaram que a concentração do complexo TC2-Cbl foi significantemente mais alta na presença do polimorfismo *TC2* C776G em homozigose (GG). Além disso, concentrações médias de ácido metilmalônico (MMA), um indicador do status de Cbl,[28,55] foram significantemente mais baixas na presença dos genótipos *TC2* 776GG e CG em relação ao genótipo CC.[56] Estudo de Fintelman-Rodrigues et al.[48] mostrou uma maior frequencia de genótipos combinados *TC2* 776CC / *MTHFR* 677TT e *TC2* 776CC / *MTR* 2756AG em mães de indivíduos sem SD.

É possível que outros polimorfismos em genes que participam do metabolismo do folato possam modular o risco materno para SD. O gene *DHFR*, localizado no cromossomo 5q11.2, possui um polimorfismo de deleção de 19 pares de base (pb)

localizado no íntron 1 e há evidências da presença de um elemento inibitório de transcrição na sequência de 19 pb, que ao ser deletado pode resultar em expressão aumentada do gene *DHFR*.[57-59] Estudos mostram que esse polimorfismo está associado à redução da concentração de Hcy e aumento da concentração de folato.[16,60] Embora não haja estudo que avalie a influência desse polimorfismo no risco materno para SD, esse polimorfismo foi associado à modulação do risco materno para defeitos de tubo neural (DTN), doenças genéticas influenciadas pelos mesmos determinantes genéticos do metabolismo do folato.[61]

Em relação ao gene *DNMT3B*, estudos em SD e DTN são ausentes. Esse gene, localizado no cromossomo 20q11.2, contém um polimorfismo de transição C→T na região promotora, localizado a -149 pb do sítio de início da transcrição que foi associado ao aumento de 30% na atividade gênica.[62,63] Outra variante, *DNMT3B* -283T→C, mostrou uma redução de 50% da atividade gênica em estudo *in vitro*.[64] Estudos evidenciam que os polimorfismos no gene *DNMT3B* podem influenciar a atividade da enzima DNMT3B na metilação do DNA, aumentando a suscetibilidade ao câncer.[65-67] Além disso, expressão elevada de DNMT3B foi associada à hipermetilação das ilhas CpG em câncer colorretal.[68]

A relação entre a presença de polimorfismos genéticos envolvidos no metabolismo do folato e o risco materno para a SD deve-se provavelmente à influência dos mesmos nas reações de metilação e, assim, na manutenção da estrutura da cromatina e na segregação cromossômica.[69] Considerando a importância do gene *DNMT3B* nas reações de metilação do DNA, a investigação de suas variantes no risco materno para a SD torna-se relevante.

1.1. OBJETIVOS

1. Avaliar a influência dos polimorfismos *DHFR* del 19 pb, *DNMT3B* -149C→T e *DNMT3B* -283T→C como fatores de risco materno para a SD;

2. Investigar a associação entre os polimorfismos *DHFR* del 19 pb, *DNMT3B* -149C→T e *DNMT3B* -283T→C e as concentrações de folato sérico, Hcy e MMA plasmáticos.

2. ARTIGOS CIENTÍFICOS

2. ARTIGOS CIENTÍFICOS

Os resultados referentes a esta dissertação estão apresentados em forma de artigos. São apresentados 02 artigos: um publicado e um a ser submetido para publicação.

Artigo 1

Título: 19-base pair deletion polymorphism of *Dihydrofolate reductase* (*DHFR*) gene: maternal risk for Down syndrome and folate metabolism.

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Periódico: *São Paulo Medical Journal*, 2010 Jul;128(4):215-218.

Artigo 2

Título: *DNMT3B* -149C→T and -283T→C polymorphisms as a maternal risk factor for Down syndrome.

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ARTIGO CIENTÍFICO 1

Original article

19-base pair deletion polymorphism of the dihydrofolate reductase (DHFR) gene: maternal risk of Down syndrome and folate metabolism

Polimorfismo de deleção de 19 pares de bases do gene dihidrofolato redutase (DHFR): *risco materno para síndrome de Down e metabolismo do folato*

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ABSTRACT

CONTEXT AND OBJECTIVES: Polymorphisms in genes involved in folate metabolism may modulate the maternal risk of Down syndrome (DS). This study evaluated the influence of a 19-base pair (bp) deletion polymorphism in intron-1 of the *Dihydrofolate reductase* (*DHFR*) gene on the maternal risk of DS, and investigated the association between this polymorphism and variations in the concentrations of serum folate and plasma homocysteine (Hcy) and plasma methylmalonic acid (MMA).

DESIGN AND SETTING: Analytical cross-sectional study carried out at Faculdade de Medicina de São José do Rio Preto (Famerp).

METHODS: 105 mothers of individuals with free trisomy of chromosome 21, and 184 control mothers were evaluated. Molecular analysis on the polymorphism was performed using the polymerase chain reaction (PCR) through differences in the sizes of fragments. Folate was quantified by means of chemiluminescence, and Hcy and MMA by means of liquid chromatography and sequential mass spectrometry.

RESULTS: There was no difference between the groups in relation to allele and genotype frequencies ($P = 0.44$; $P = 0.69$, respectively). The folate, Hcy and MMA concentrations did not differ significantly between the groups, in relation to genotypes ($P > 0.05$).

CONCLUSIONS: The 19-bp deletion polymorphism of *DHFR* gene was not a maternal risk factor for DS and was not related to variations in the concentrations of serum folate and plasma Hcy and MMA in the study population.

KEY WORDS: Down syndrome, genetic polymorphism, folic acid, genetic nondisjunction, risk factors.

RESUMO

CONTEXTO E OBJETIVOS: Polimorfismos em genes do metabolismo do folato podem modular o risco materno para síndrome de Down (SD). Este estudo avaliou a influência do polimorfismo de deleção de 19 pares de base (pb) no íntron 1 do gene *Dihidrofolato redutase* (*DHFR*) no risco materno para SD e investigou a associação entre esse polimorfismo e variações nas concentrações de folato sérico, homocisteína (Hcy) e ácido metilmalônico (MMA) plasmáticos.

TIPO DE ESTUDO E LOCAL: Estudo transversal analítico realizado na Faculdade de Medicina de São José do Rio Preto (Famerp).

MÉTODOS: 105 mães de indivíduos com trissomia livre do cromossomo 21 e 184 mães controles foram avaliadas. A análise molecular do polimorfismo foi realizada pela reação em cadeia da polimerase (PCR) por diferença de tamanho dos fragmentos. O folato foi quantificado por quimioluminescência, e Hcy e MMA foram determinados por cromatografia líquida/espectrometria de massas sequencial.

RESULTADOS: Não houve diferença entre os grupos em relação às frequências alélica e genotípica ($P = 0,44$; $P = 0,69$, respectivamente). As concentrações de folato, Hcy e MMA não mostraram diferença significativa entre os genótipos, entre grupos ($P > 0,05$).

CONCLUSÕES: O polimorfismo de deleção de 19 pb do gene *DHFR* não é um fator de risco materno para SD e não está relacionado com variações nas concentrações de folato sérico, Hcy e MMA plasmáticos na população estudada.

PALAVRAS-CHAVE: Síndrome de Down, polimorfismo genético, não-disjunção genética, ácido fólico, fatores de risco.

INTRODUCTION

Down syndrome (DS) is a genetic disease characterized, in most cases, by free trisomy of chromosome 21 caused by non-disjunction in maternal meiosis.[1,2] James et al.[3] were the first to observe an increased risk of chromosome non-disjunction due to abnormal folate metabolism, and this is responsible for abnormalities in the pattern of deoxyribonucleic acid (DNA) methylation.

Folate plays an essential role in several complex metabolic pathways, including those leading to DNA synthesis or conversion of homocysteine (Hcy) to methionine, which is then used to form the main DNA methylating agent: S-adenosyl methionine (SAM).[4] Studies have shown that polymorphisms in genes encoding enzymes involved in this metabolic pathway, and higher concentrations of Hcy and lower folate concentrations, modulate the maternal risk factor for DS.[5-8]

The *Dihydrofolate reductase* (*DHFR*) gene encodes an enzyme that catalyzes the conversion of dihydrofolate (DHF) into tetrahydrofolate (THF). It is also needed for the intracellular conversion of synthetic folic acid (which is consumed in supplements and fortified foods) to DHF and THF, which are the forms that participate in folate and Hcy metabolism.[9] Johnson et al.[10] described a 19-base pair (bp) deletion polymorphism in

intron-1 of the *DHFR* gene and hypothesized that this polymorphism could be functional because the deletion removes a possible transcription factor binding site that affects gene regulation.

A study on the mothers of individuals with spina bifida showed that the messenger ribonucleic acid (mRNA) expression of the *DHFR* gene was 50% higher in the del/del genotype than in the ins/ins genotype.[11] Moreover, in a study on women with breast cancer, Xu et al.[12] also observed mRNA concentrations that were 2.4 and 4.8 times higher in individuals with the ins/del and del/del genotypes, respectively, in comparison with individuals with the ins/ins genotype.

This polymorphism has been correlated with modulation of the metabolites involved in the folate pathway. Gellekink et al.[13] reported that the del/del genotype was associated with lower plasma Hcy concentrations, but did not find any association between this genotype and concentrations of serum and erythrocyte folate. Another study did not find any effect on Hcy concentration, but found increased plasma and erythrocyte folate levels in del/del individuals.[9]

To the best of our knowledge, no published studies have evaluated the association of 19-bp deletion polymorphism in intron-1 of the *DHFR* gene with the maternal risk of DS.

OBJECTIVES

This study aimed to evaluate the influence of the 19-bp deletion polymorphism in intron-1 of the *DHFR* gene on the maternal risk of DS and to investigate the association between this polymorphism and variations in the concentrations of serum

folate and plasma Hcy and methylmalonic acid (MMA), an indicator of vitamin B_{12} status.

METHODS

This analytical cross-sectional study was carried out at Faculdade de Medicina de São José do Rio Preto (Famerp) and was composed of a case group formed by 105 mothers of DS children with karyotypically confirmed free trisomy 21 and a control group consisting of 184 mothers with healthy offspring and no experience of miscarriages. Informed consent was obtained from all these volunteers.

The mothers' median ages at delivery (maternal age) and when the blood samples were obtained (age at presentation) were 30.4 years (range 12.9-46.3 years) and 43.1 years (range 22.5-69.3 years) in the case group, respectively, and 26.4 years (range 15.4-40.7 years) and 36.1 years (13.2-68.8 years) in the control group. The maternal age was calculated as the age of the mother at the birth of the DS child for the case group, and the age at the birth of the last child for the control group.

Fasting blood samples were collected for molecular and biochemical analysis (serum folate and plasma Hcy and MMA). DNA extraction was performed as previously described by Miller et al.[14] and the 19-bp deletion polymorphism in *DHFR* gene was analyzed by means of the polymerase chain reaction (PCR) using the difference in the size of fragments to determinate the genotypes, using primer sequences described by Dulucq et al.[15] The PCR-amplified fragments were analyzed by means of electrophoresis in 6% polyacrylamide gel.

Folate was quantified by means of chemiluminescence (Immulite Kit, DPC Medlab, Brazil) and liquid chromatography-tandem mass spectrometry was used to determine the plasma Hcy and MMA concentrations, as previously described.[16-18]

The existence of Hardy-Weinberg equilibrium was tested using the chi-square test, and genotype frequencies among the case and control mothers were compared by means of the likelihood ratio test and logistic regression. Mood's median test was used to investigate the association between the polymorphism in the *DHFR* gene and variations in the serum folate and plasma Hcy and MMA concentrations. P values $\leq$ 0.05 were taken to be statistically significant.

RESULTS

Table 1 presents the allele and genotype frequencies of 19-bp deletion polymorphism in the *DHFR* gene in the case and control groups. The allele frequencies were in Hardy-Weinberg equilibrium in both groups, and there were no differences in allele and genotype frequencies between the DS and control mothers. There were no significant differences in the distributions of serum folate and plasma Hcy and MMA concentrations between the genotypes of the case and control groups ($P > 0.05$) (**Table 2**).

DISCUSSION

Independent of maternal age, DS has been associated with other etiological factors. Studies have shown that cell folate deficiencies result in aberrant DNA methylation, point mutations, chromosome breakage, defective chromosome recombination and aneuploidy of chromosome 21.[19-21] Folate metabolism plays an

important role in the synthesis of nucleotides and of SAM, which is the main donor of methyl groups for DNA, protein and phospholipid methylation reactions.[22] Many genes are involved in these metabolic pathways and, in 1999, James et al.[3] hypothesized that abnormalities in DNA methylation are a potential causative mechanism of meiotic non-disjunction. Their report stimulated considerable investigation into the possible role of folate metabolism in relation to the risk of having a DS child, and several studies have found this association.[5-8,23,24]

One important gene that is involved in this metabolism is *DHFR*, which encodes the enzyme responsible for reducing folic acid in THF.[9] A common polymorphism in this gene, 19-bp deletion polymorphism in intron-1, has been correlated with modulation of the maternal risk of neural tube defects (NTDs),[10,11] and of the concentrations of the metabolites involved in the folate pathway.[9,13,25]

Originally, Johnson et al.[10] observed that the del/del genotype was related to increased risk of having offspring with spina bifida. On the other hand, another study has suggested that the del/del genotype has a protective effect and decreases the maternal risk of spina bifida,[11] while yet another study reported no effect.[26] Thus, the contribution of the 19-bp deletion polymorphism in intron-1 of the *DHFR* gene towards the risk of NTDs remains a matter of controversy.

Considering the high frequency of DS cases in families with higher risk of NTDs, and vice versa,[27] and the fact that both diseases are influenced by the same genetic determinants of folate metabolism,[28] it is possible that the 19-bp deletion polymorphism in intron-1 of the *DHFR* gene modulates the maternal risk of DS. However, in the present study, no association between this polymorphism and the maternal risk of DS was observed. In addition, no association was observed between the

19-bp deletion polymorphism of the *DHFR* gene and the folate, Hcy and MMA concentrations in the present study, although the presence of the polymorphic allele has been associated with increased concentrations of serum and erythrocyte folate, and reduction in the concentration of plasma Hcy in previous studies.[9,13,25] The variations between studies may have been caused by nutritional and ethnic differences between the populations studied, as well as differences in sample sizes and other genetic factors.

One of the limitations of our study is that serum folate was quantified instead of erythrocyte folate. The erythrocyte folate content represents the time average of the folate concentrations occurring at the genesis of each red cell and is therefore less susceptible to rapid changes in diet.[29,30] One study observed a significant difference in erythrocyte folate quantification between case and control mothers, which was not observed when quantifying serum folate.[31] However, quantification of erythrocyte folate is more complex to perform[29.30] and, for this reason, many studies have measured serum folate.[5,32-34] In addition, a recent study has indicated that serum folate assays provide information that is equivalent to erythrocyte folate measurements for attempting to determine folate deficiency.[30]

CONCLUSIONS

In this study, no evidence for an association between the 19-bp deletion polymorphism in intron-1 of the *DHFR* gene and the maternal risk of DS was observed. Moreover, this polymorphism was not related to variations in the concentrations of serum folate and plasma Hcy and MMA in the study population.

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Table 1. Allele and genotype frequencies of 19-bp deletion polymorphism in intron-1 of *Dihydrofolate reductase (DHFR)* gene in the case and control groups of mothers.

DHFR	**Case mothers**	**Control mothers**	**P**
Allele frequencies			
Ins	0.50	0.46	0.44
Del	0.50	0.54	
Genotype frequencies			
ins/ins	0.26	0.23	
ins/del	0.48	0.46	0.69
del/del	0.26	0.31	

Table 2. Distribution of serum folate and plasma homocysteine (Hcy) and plasma methylmalonic acid (MMA) concentrations according to genotypes of the 19-bp deletion polymorphism in intron-1 of *Dihydrofolate reductase (DHFR)* gene, in the case and control groups of mothers.

Concentration	**ins/ins**	**ins/del**	**del/del**	**P**
Case group				
Folate (ng/mL)	14.10	11.85	12.30	0.57
Hcy (µmol/L)	6.21	7.25	5.34	0.20
MMA (µmol/L)	0.18	0.17	0.17	0.41
Control group				
Folate (ng/mL)	15.60	14.10	14.60	0.74
Hcy (µmol/L)	8.85	8.15	8.19	0.29
MMA (µmol/L)	0.15	0.14	0.14	0.98

ARTIGO CIENTÍFICO 2

DNMT3B -149C→T and -283T→C polymorphisms as a maternal risk factor for Down syndrome

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Abstract

Down syndrome (DS) results from failures in chromosomal segregation during maternal meiosis in about 90% of the cases. Many mothers of DS individuals are young and studies show that polymorphisms in genes involved in folate metabolism might modulate maternal risk for DS. We investigated the influence of *DNA methyltransferase 3B* (*DNMT3B*) -149C→T and *DNMT3B* -283T→C polymorphisms as a maternal risk factor for DS and the association between these polymorphisms and the concentrations of folate, homocysteine (Hcy) and methylmalonic acid (MMA) in Brazilian population. 105 mothers of DS individuals with free trisomy 21 and 185 control mothers were studied. Molecular analysis of the *DNMT3B* -149C→T and *DNMT3B* -283T→C polymorphisms was performed by real-time polymerase chain reaction allelic discrimination. Serum folate was quantified by chemiluminescence and Hcy and MMA plasma by liquid chromatography-tandem mass spectrometry. The *DNMT3B* -149TT/-283TC combined genotypes were associated with increased maternal risk for DS with OR = 4.61 (CI 95% = 1.35 – 15.79; P = 0.02) and higher folate concentration was observed in mothers with *DNMT3B* -149CT/-283CC genotypes compared to other combined genotypes (P = 0.03). These results show that *DNMT3B* polymorphisms increase the maternal risk for DS and modulate the folate concentration in studied population.

Keywords: Down syndrome, genetic polymorphism, folate, risk factor.

1. Introduction

Down syndrome (DS) is characterized by free trisomy of chromosome 21, resulting from failures in chromosomal segregation during maternal meiosis in about 90% of the cases [1-3] and the molecular mechanisms responsible for meiotic nondisjunction are still poorly understood. The advanced maternal age at conception is considered the major risk factor for trisomy 21 [1,4-6], however, many mothers of DS individuals are young, suggesting the existence of other etiological factors.

James et al. [7] suggested that impaired folate metabolism due to polymorphisms in genes involved in this pathway is a risk factor for DS in young mothers. Folate is essential for the production of S-adenosylmethionine (SAM), the primary methyl donor for DNA methylation, which regulates gene expression and prevents chromosomal fragility in specific regions, such as the centromere [8-11]. Folate deficiency reduces SAM synthesis causing DNA hypomethylation [12-15]. The pericentromeric hypomethylation could impair the heterochromatin formation and kinetochore establishment, resulting in chromosomal nondisjunction [7]. This can happen because the stable centromeric DNA chromatin depends on the epigenetic inheritance of specific centromeric methylation patterns and on the binding of specific methyl-sensitive proteins in order to maintain the higher-order DNA architecture necessary for kinetochore assembly [16,17].

DNA methylation is mediated by DNA methyltransferases (DNMTs) enzymes, specifically DNMT1, DNMT3A and DNMT3B. Although DNMTs act cooperatively in order to achieve the establishment and maintenance of a genomic methylation pattern, DNMT1 is responsible for maintaining pre-existing methylation patterns due to its ability to preferentially methylate hemimethylated DNA during DNA replication.

DNMT3A and DNMT3B act as *de novo* methyltransferases, which methylate unmethylated and hemimethylated DNA with equal efficiencies after replication [11,18,19]. DNMT3B is specialized for the methylation of CpG dinucleotides within repeated sequences of the pericentromeric regions of chromosomes. Mutations within the *DNMT3B* gene can be associated with centromere instability [20,21].

Studies have suggest that some polymorphisms in genes involved in the folate metabolism modulate the maternal risk for DS [7,22-27]. *DNMT3B* gene, located on chromosome 20q11.2, contains a C→T transition polymorphism at -149 base pair (bp) from the transcription start site, which confers 30% increase in promoter activity [28,29]. Other variant, a T to C transition at position -283 from the exon 1A transcription start site has been shown to decrease the promoter activity in 50% [30].

The relationship between the presence of genetic polymorphisms involved in folate metabolism and maternal risk for DS is probably due to the influence of the same in the DNA methylation reactions and thus in maintaining the structure of chromatin and chromosome segregation [31]. Considering the importance of the *DNMT3B* gene in DNA methylation reactions investigation of polymorphisms in this gene as maternal risk factor for DS becomes relevant.

In this study, we investigated the influence of *DNMT3B* -149C→T and *DNMT3B* -283T→C polymorphisms on maternal risk for DS and evaluated the association between these polymorphisms and the concentrations of serum folate and plasma Hcy and MMA in a Brazilian population.

2. Subjects and methods

2.1. Subjects

This study was composed of a case group formed by 105 mothers of DS children with karyotypically confirmed free trisomy 21 and a control group consisted of 185 mothers with healthy offspring and no experience of miscarriages. Mothers with DS offspring presenting translocation or mosaicism were not included in the case group. Informed consent was obtained from all volunteers, according to the research protocols approved by the institutional Ethics Committees.

2.2. Molecular and biochemical analysis

Fasting blood samples were collected for molecular and biochemical analysis (serum folate and plasma Hcy and MMA). DNA extraction was performed as previously described by Miller et al. [32] and the polymorphisms in *DNMT3B* gene were analyzed by real-time polymerase chain reaction (PCR) allelic discrimination. *DNMT3B* -149C→T was detected using *Taqman SNP Genotyping Assays* (C__25620192_20) and *DNMT3B* -283T→C was genotyped using TaqMan® probes and primer sequences described by Jung et al. [33].

Folate was quantified by chemiluminescence (*Immulite Kit, DPC Medlab, Brazil*) and liquid chromatography-tandem mass spectrometry was used to determine the plasma Hcy and MMA concentrations, as previously described [34-36].

2.3. Statistical analysis

Hardy-Weinberg equilibrium was tested by the chi-square test using the *BioEstat program* (version 5.0), and genotype frequencies in case and control mothers were compared by the likelihood ratio and logistic regression tests. The haplotypes frequencies of *DNMT3B* gene were inferred by the *Haploview program* (version 4.0).

Folate and Hcy data presented normal distribution after logarithmic transformation and were analyzed as mean values in the logarithmic scale. MMA data

did not show normal distribution, even after Log-transformation, and was analyzed as median values in the natural scale. Association between *DNMT3B* polymorphisms and serum folate and plasma Hcy concentration was investigated by T-test and by Mood´s median test for the MMA data.

The computer-assisted statistical analyses were carried out using the *Minitab for Windows program* (*Release 14*). P values ≤ 0.05 were taken to be statistically significant.

3. Results

We were able to amplify 84 case mothers and 177 control mothers for *DNMT3B* -149C→T polymorphisms and 104 case mothers and 185 control mothers for *DNMT3B* -283T→C polymorphism. Allele frequencies were in Hardy-Weinberg equilibrium and no difference between DS mothers and control groups allele and genotype frequencies was observed. The genotype distribution of the polymorphisms is presented in Table 1.

The results of haplotype analysis showed that variants at positions -149 and -283 of the *DNMT3B* gene are strongly linked (LOD = 43.55; D' = 0.77). However, there was no difference in the haplotype frequencies between the groups (Table 2).

The influence of *DNMT3B* -149C→T and *DNMT3B* -283T→C polymorphisms on maternal risk for DS and the association between these polymorphisms and folate, Hcy and MMA concentrations were evaluated, both alone and in combination with the other polymorphism. The logistic regression analysis showed that *DNMT3B* -149C→T and *DNMT3B* -283T→C polymorphisms were not associated with the risk of offspring with DS, on the other hand, *DNMT3B* -149TT/-283TC combined genotypes were

associated with increased maternal risk for DS with OR = 4.61 (CI 95% = 1.35 – 15.79; P = 0.02).

The analysis of combined genotypes showed that *DNMT3B* -149CT/-283CC were associated with higher folate concentration compared to the other combined genotypes (P = 0.03) in the studied population (case and control mothers). The distribution of the Hcy, folate and MMA concentrations according to the combined genotypes are presented in Table 3.

4. Discussion

Folate is an essential nutrient required for DNA synthesis and epigenetic processes [37]. Deficiency in cellular folate results in aberrant DNA methylation, point mutations, chromosome breakage, defective chromosome recombination and aneuploidy [12-15]. Considering that impairments in folate metabolism due to genetic polymorphisms in genes coding metabolic enzymes can increase the risk for having an infant with DS [7,22-27], this study evaluated the influence of *DNMT3B* -149C→T and *DNMT3B* -283T→C polymorphisms on maternal risk for DS and on the concentrations of folate pathway metabolites.

Our results show that *DNMT3B* -149TT/-283TC combined genotypes increase the risk of having a child with DS. DNMT3B enzyme is responsible for methylation of cytosine to 5-methylcytosine after replication [38]. It is known that *DNMT3B* -149C→T and *DNMT3B* -283T→C polymorphisms affect enzyme activity, resulting in changes in DNA methylation [28-30]. Thus, the presence of *DNMT3B* -149C→T and *DNMT3B* -283T→C polymorphisms might cause DNA hypomethylation, and consequent instability of centromeric DNA chromatin and chromosomal nondisjunction. Regarding

haplotype analysis, although there was evidence of Linkage Disequilibrium (LD) between the *DNMT3B* -149C→T and -283T→C polymorphisms, no haplotype frequency was association with maternal risk for DS.

High folate concentration was observed in mothers with *DNMT3B* -149CT/-283CC combined genotypes in relation to the others mothers. This association is unknown, although Lee et al. [30] showed that *DNMT3B* -283C polymorphic allele reduces the enzymatic activity. It is possible that decreased enzymatic activity increases the amount of methyl groups. In a state of excess methyl groups, the resulting increase in SAM acts as an allosteric inhibitor of the enzyme Methylenetetrahydrofolate reductase (MTHFR) decreasing its activity and the subsequent production of 5-methyltetrahydrofolate (5-methylTHF) [39,40]. Thus, the lower 5-methylTHF concentration might results in accumulation of folate in mothers with *DNMT3B* -149CT/-283CC combined genotypes. However, *DNMT3B* -149C→T polymorphism was associated with increased enzymatic activity [28,29]. Therefore, further studies are needed to clarify the association of the *DNMT3B* -149CT/-283CC combined genotypes with increased folate concentration.

In conclusion, the results of the present study indicate that *DNMT3B* polymorphisms increase the maternal risk for DS and modulate folate concentrations in the studied population.

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Table 1. Genotype distribution of *DNA methyltransferase 3B* (*DNMT3B*) -149C→T and *DNMT3B* -283T→C polymorphisms between the case and control groups.

Genotypes	Case mothers n (%)	Control mothers n (%)	P*
***DNMT3B* -149C→T**			
CC	16 (19.0)	43 (24.3)	
CT	44 (52.4)	91 (51.4)	0.57
TT	24 (28.6)	43 (24.3)	
***DNMT3B* -283T→C**			
TT	23 (22.1)	53 (28.7)	
TC	59 (56.7)	87 (47.0)	0.30
CC	22 (21.2)	45 (24.3)	

***** Likelihood ratio test

Table 2. Haplotype frequencies of *DNA methyltransferase 3B* (*DNMT3B*) -149C→T and *DNMT3B* -283T→C polymorphisms in the case and control groups.

Haplotypes	Frequencies	χ^2	P
***DNMT3B* -149/-283**			
TT	0.46	0.33	0.57
CC	0.41	0.68	0.41
CT	0.07	0.17	0.68
TC	0.06	0.098	0.32

Table 3. Distribution of serum folate and plasma homocysteine (Hcy) and methylmalonic acid (MMA) concentrations according to combined genotypes *DNA methyltransferase 3B* (*DNMT3B*) -149C→T/*DNMT3B* -283T→C.

Genotypes *DNMT3B* -149 / -283	**Folate (ng/mL)**	**Hcy (µmol/L)**	**MMA (µmol/L)**
CC/TT[a]	2.42	1.94	0.27
CT/TT	2.66 ± 0.41	2.12 ± 0.60	0.15 (0.07-0.35)
CC/TC	2.58 ± 0.34	2.07 ± 0.64	0.18 (0.11-0.28)
CT/TC	2.69 ± 0.43	1.99 ± 0.45	0.15 (0.11-0.25)
TT/TC	2.60 ± 0.33	1.96 ± 0.48	0.16 (0.08-0.67)
CT/CC	3.03 ± 0.50*	1.88 ± 0.72	0.16 (0.09-1.41)
CC/CC	2.62 ± 0.46	1.89 ± 0.47	0.16 (0.06-0.72)
TT/TT	2.66 ± 0.44	2.03 ± 0.39	0.15 (0.05-0.53)
TT/CC[a]	2.75	2.14	0.23

Folate and Hcy data are reported as means ± SD; MMA data are reported as median and range.

[a]Genotypes observed in one individual.

*Statistically significant.

3. CONCLUSÕES

3. CONCLUSÕES

1. O polimorfismo de deleção de 19 pb do gene *DHFR* não é um fator de risco materno para SD e não está relacionado com variações nas concentrações de folato sérico, Hcy e MMA plasmáticos na população estudada.

2. Os genótipos combinados *DNMT3B* -149TT/-283TC são fatores de risco materno para a SD e os genótipos *DNMT3B* -149CT/-283CC estão associados ao aumento da concentração de folato sérico na população estudada.

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5. APÊNDICE

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Os resultados apresentados nesse artigo foram obtidos durante o mestrado e o artigo submetido para publicação.

Título: Synergistic effect of *DHFR* 19-bp deletion and *SHMT* C1420T polymorphisms on folate pathway metabolites concentrations in individuals with Down syndrome.

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Synergistic effect of *DHFR* 19-bp deletion and *SHMT* C1420T polymorphisms on folate pathway metabolites concentrations in individuals with Down syndrome

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Abstract

Down syndrome (DS) results from the presence and expression of three copies of the genes located on chromosome 21. Studies have shown that, in addition to over-expression of *Cystathionine β-synthase* (*CβS*) gene, polymorphisms in genes involved in folate/homocysteine (Hcy) metabolism may also influence the concentrations of metabolites of this pathway. Thus, this study evaluated the influence of the *Dihydrofolate reductase* (*DHFR*) 19-base pair (bp) deletion and *Serine hydroxymethyltransferase* (*SHMT*) C1420T polymorphisms on serum folate and plasma Hcy and methylmalonic acid (MMA) concentrations in eighty-two individuals with DS. Genotyping of *DHFR* 19-bp deletion and *SHMT* C1420T polymorphisms were performed by polymerase chain reaction (PCR) by difference in the size of fragments and real-time PCR allelic discrimination, respectively. Serum folate was quantified by chemiluminescence and Hcy and MMA plasma by liquid chromatography-tandem mass spectrometry. The polymorphisms of *DHFR* and *SHMT* genes alone showed no association with folate, Hcy and MMA concentrations. However, the analysis of combined genotypes showed that individuals with *DHFR* II / *SHMT* TT combined genotypes presented higher concentrations of Hcy ($P < 0.001$) in relation to the others genotypes. Moreover, *DHFR* DD / *SHMT* TT combined genotypes presented higher concentrations of folate ($P < 0.001$) and the *DHFR* II / *SHMT* CT genotypes presented lower concentrations of folate ($P = 0.01$). There was no association between MMA concentrations and genotypes. These results demonstrate a synergistic effect of polymorphisms of *DHFR* and *SHMT* genes on the modulation of folate/Hcy pathway in individuals with DS.

Key words: Down syndrome, genetic polymorphism, homocysteine, folate.

Introduction

Down syndrome (DS) results from the presence and expression of three copies of the genes located on chromosome 21 [1,2]. *Cystathionine β-synthase* (*CβS*) gene, located on chromosome 21, is responsible for the condensation of homocysteine (Hcy) and serine to cystathionine and is over-expressed in individuals with DS [2]. Increased concentrations of CβS enzyme results in lower concentrations of Hcy, methionine, S-adenosylhomocysteine, and S-adenosylmethionine [3-5], substrates of folate metabolism.

Studies have shown that, in addition to over-expression of *CβS* gene, polymorphisms in genes involved in folate/Hcy metabolism may also influence metabolites' concentrations of this pathway [6-9]. A 19-base pair (bp) deletion polymorphism in intron-1 of the *Dihydrofolate reductase* (*DHFR*) gene has been identified [10] and Kalmbach et al. [11] demonstrated that this is a functional polymorphism. Study shows that 19-bp deletion polymorphism is associated with increased expression of *DHFR* gene, responsible for the conversion of dihydrofolate (DHF) in tetrahydrofolate (THF) [12], and changes the folate/Hcy metabolic pathway [11,13,14].

Other polymorphism, C1420T, which results in the substitution of the amino acid leucine by phenylalanine, was identified in *Serine hydroxymethyltransferase* (*SHMT*) gene [15]. This gene encodes the enzyme that catalyzes the reversible conversion of serine and THF to glycine and 5,10-methyleneTHF [16] and the Fu et al.

[17] showed that the *SHMT* C1420T polymorphism may compromise the formation of the SHMT enzyme.

Both, *DHFR* 19-bp deletion and *SHMT* C1420T polymorphisms involved in folate/Hcy metabolism, have been associated with variations in the concentrations of Hcy and folate in several populations [11,13-15,18,19]. Thus, the aim of the present study was to evaluate the influence of *DHFR* 19-bp deletion and *SHMT* C1420T polymorphisms on serum folate and plasma Hcy and methylmalonic acid (MMA) concentrations in individuals with DS.

Subjects and methods

This study was composed by eighty-two individuals with full trisomy 21 confirmed by karyotype recruited at the General Genetics Outpatient Service of Hospital de Base, São José do Rio Preto, SP, Brazil. The study protocol was approved by the Research Ethics Committee of Sao Jose do Rio Preto Medical School (CEP-FAMERP), in Sao Paulo state, and informed consent was obtained for all family.

Fasting blood samples were collected for molecular and biochemical analysis (serum folate and plasma Hcy and MMA). DNA extraction was performed as previously described by Miller et al. [20] and polymorphisms in *DHFR* and *SHMT* genes were analyzed by polymerase chain reaction (PCR) using difference in the size of fragments and real-time PCR allelic discrimination, respectively. The 19-bp deletion polymorphism in *DHFR* gene was detected using primer sequences described by Dulucq et al. [21] and *SHMT* C1420T was detected using TaqMan® probes and primer sequences described by Skibola et al. [22]. Serum folate was quantified by

chemiluminescence and liquid chromatography-tandem mass spectrometry was used to determine plasma Hcy and MMA concentrations, as previously described [23-25].

In this study, the allele with 19-bp deletion in *DHFR* gene was denominated D and the allele without the deletion was named I.

Statistical analysis

Hardy-Weinberg equilibrium was tested by chi-square test, using the BioEstat program (version 5.0). Folate and Hcy data presented normal distribution after logarithmic transformation and were analyzed as mean values in the logarithmic scale. MMA data was not normal, even after Log-transformation, and was analyzed median values in the natural scale. To evaluate the association of *DHFR* 19-bp deletion and *SHMT* C1420T polymorphisms with biochemical parameters, the t-test was used for Hcy and folate data and the Mood´s median test was used for MMA data. The computer-assisted statistical analyses were carried out using the Minitab for Windows program (Release 14). Values of $P \leq 0.05$ were considered significant.

Results

Table 1 presents allele frequencies of *DHFR* 19-bp deletion and *SHMT* C1420T polymorphisms in individuals with DS. The allelic distribution of the *DHFR* 19-bp deletion and *SHMT* C1420T polymorphisms were in Hardy-Weinberg equilibrium ($\chi 2 = 2.079$; $p = 0.15$; $\chi 2 = 0.004$; $p = 0.95$, respectively).

We evaluated the influence of *DHFR* 19-bp deletion and *SHMT* C1420T polymorphisms on folate, Hcy and MMA concentrations in individuals with DS, both alone and in combination with other. The analysis showed no association between genotypes and folate, Hcy and MMA concentrations. However, when we proceed the

analysis of combined genotypes, the results showed that individuals with *DHFR* II / *SHMT* TT genotypes presented higher concentrations of Hcy ($P < 0.001$) in relation to the others combined genotypes. In another way, individuals with *DHFR* DD / *SHMT* TT combined genotypes presented higher concentrations of folate ($P < 0.001$) and the *DHFR* II / *SHMT* CT genotypes presented lower concentrations of folate ($P = 0.01$) in relation to the others combined genotypes. The distribution of MMA concentrations showed no significant difference between the genotypes. The distribution of the Hcy, folate and MMA concentrations according to the combined genotypes are presented in Table 2.

Discussion

The overexpression of genes results in biochemical alterations that affect the multiple interacting metabolic pathways culminating in cellular dysfunction and contributing to the pathogenesis of DS [3]. The presence of three copies of the *CßS* gene, located on chromosome 21, and of the polymorphisms *Methylenetetrahydrofolate reductase* (*MTHFR*) C677T and *Methionine synthase* (*MTR*) A2756G, involved in the folate/Hcy metabolic pathway, have been associated with variations on the concentrations of metabolites of this pathway [3-9].

Guéant et al. [7] observed that individuals with DS that present *MTHFR* 677T allele and elevated Hcy concentration had low intelligence quotient and Licastro et al. [8] found that the *MTHFR* 677TT genotype increases the concentrations of Hcy in these individuals. However, Fillon-Emery et al. [6] found no difference in Hcy concentrations according to the *MTHFR* C677T genotype in adults with DS. In another

study, the heterozygous genotype *MTR* 2756AG was associated with increased in plasma Hcy concentrations in individuals with DS [9].

To the best of our knowledge, there is no published study that has evaluated the influence of the *DHFR* 19-bp deletion and *SHMT* C1420T polymorphisms on the concentrations of metabolites of folate/Hcy pathway in individuals with DS. In the present study, the *DHFR* 19-bp deletion and *SHMT* C1420T polymorphisms alone were not associated with the folate, Hcy and MMA concentrations. However, considering that some polymorphisms may interact to produce a synergistic effect, the contribution of the combined genotypes to folate, Hcy and MMA concentrations was evaluate and the results show an association between the *DHFR* II / *SHMT* TT combined genotypes and higher Hcy concentration. Moreover, *DHFR* II / *SHMT* CT combined genotypes were associated with lower folate concentrations and *DHFR* DD / *SHMT* TT genotypes were associated with higher concentration of folate.

DHFR is an important folate-metabolizing enzyme responsible for reduction of folic acid into THF [14]. A common polymorphism in this gene, a 19-bp deletion polymorphism in intron-1, was associated with alterations on the concentration of metabolites involved in the folate/Hcy pathway [11,17,18]. Gellekink et al. [13] reported that the *DHFR* DD genotype is associated with lower plasma Hcy concentration in Caucasian individuals, but no association between this genotype and serum and erythrocyte folate concentrations was observed. Other study found no effect of this polymorphism on Hcy concentration in healthy adults, but the DD genotype was associated with increased serum and erythrocyte folate concentrations relative to the II genotype in women [14]. Kalmbach et al. [11] also observed no association between genotypes and plasma Hcy or plasma total folate in young adults, however *DHFR* DD

genotype was associated with lower erythrocyte folate concentration compared to *DHFR* ID and II genotypes.

SHMT enzyme plays a pivotal role in folate/Hcy metabolism by carrying out the reversible conversion of serine and glycine with THF and 5,10-methyleneTHF [16]. Heil et al. [15] identified the *SHMT* C1420T polymorphism and reported that individuals with neural tube defects and *SHMT* CC genotype had decreased erythrocyte and plasma folate concentrations and increased Hcy concentration. In study involving men with cardiovascular disease, the *SHMT* TT genotype was associated with lower Hcy concentration [19]; yet another study found no significant association between *SHMT* C1420T and plasma folate and Hcy concentrations in colorectal cancer [18].

In conclusion, these results demonstrate a synergistic effect of *DHFR* and *SHMT* genes polymorphisms on the modulation of the concentrations of folate/Hcy pathway metabolites in individuals with DS.

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Table 1. Allele frequencies of 19-base pair (bp) deletion in intron-1 of *Dihydrofolate reductase* (*DHFR*) gene and *Serine hydroxymethyltransferase* (*SHMT*) C1420T polymorphisms in individuals with Down syndrome.

	Allele frequencies	P-value*
DHFR		
I	0.52	0.15
D	0.48	
SHMT		
C	0.68	0.95
T	0.32	

* Chi-square test.

Table 2. Distribution of serum folate and plasma homocysteine (Hcy) and methylmalonic acid (MMA) concentrations according to combined genotypes of the 19-base pair (bp) deletion in intron-1 of *Dihydrofolate reductase* (*DHFR*) gene and *Serine hydroxymethyltransferase* (*SHMT*) C1420T polymorphisms in individuals with Down syndrome.

Genotypes *DHFR* / *SHMT*	Folate (ng/mL)	Hcy (µmol/L)	MMA (µmol/L)
ID / CT	24.12 ± 16.87	5.52 ± 2.63	0.32 (0.14-4.26)
ID / TT	18.00 ± 11.69	6.63 ± 3.78	0.26 (0.13-0.68)
ID / CC	18.18 ± 8.89	6.74 ± 4.28	0.23 (0.10-3.21)
II / CT	11.88 ± 5.20*	5.06 ± 2.37	0.22 (0.15-1.56)
II / TT	13.50 ± 1.70	8.14 ± 0.25*	2.50 (0.23-4.77)
II / CC	24.95 ± 11.74	4.36 ± 1.54	0.25 (0.09-0.32)
DD / CT	16.02 ± 4.93	5.33 ± 2.99	0.23 (0.14-1.21)
DD / TT	44.25 ± 1.77*	2.48 ± 1.72	0.12 (0.11-0.13)
DD / CC	19.46 ± 12.70	6.95 ± 4.45	0.30 (0.12-2.26)

Folate and Hcy data are reported as means ± SD; MMA data are reported as median and range.

* Statistically significant.

6. ANEXOS

6. ANEXOS

De acordo com Normas Regulamentares de Pesquisa em Seres Humanos, Resolução 196/96 do Ministério da Saúde, esse projeto foi aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Medicina de São José do Rio Preto/SP, CEP–FAMERP (Anexo 1) e pela Comissão Nacional em Pesquisa de Brasília/DF – CONEP (Anexo 2). Para a realização da análise molecular dos polimorfismos dos genes *Dihidrofolato redutase* (*DHFR*) e *Serina hidroximetiltransferase* (*SHMT*), foi aprovada pelo CEP-FAMERP uma extensão na data de 19 de maio de 2008 (Anexo 3) e outra na data de 29 de maio de 2010 para a genotipagem dos polimorfismos do gene *DNA metiltransferase 3B* (*DNMT3B*) (Anexo 4).

Esclareço também que foram utilizadas amostras de material biológico provenientes do banco de material biológico aprovado pela CONEP (Anexo 2) e regularizado junto ao CEP-FAMERP.

FACULDADE DE MEDICINA DE SÃO JOSÉ DO RIO PRETO
AUTARQUIA ESTADUAL - LEI Nº 8899 ,de 27/09/94
(Reconhecida pelo Decreto Federal nº 74.179, de 14/06/74)

Parecer n.º 165/2004

COMITÊ DE ÉTICA EM PESQUISA

O Protocolo n.º 3340/2004 sob a responsabilidade de Érika Cristina Pavarino Bertelli com o título "Avaliação Genético-Clínica e Molecular em Síndrome d Down" está de acordo com a Resolução CNS 196/96 e foi **aprovado** por esse CEP.

Lembramos ao senhor(a) pesquisador(a) que, no cumprimento da Resolução 251/97, o Comitê de Ética em Pesquisa (CEP) deverá receber relatórios semestrais sobre o andamento do Estudo, bem como a qualquer tempo e a critério do pesquisador nos casos de relevância, além do envio dos relatos de eventos adversos, para conhecimento deste Comitê. Salientamos ainda, a necessidade de relatório completo ao final do Estudo.

São José do Rio Preto, 12 de julho de 2004.

Prof.ª Dr.ª Patrícia Maluf Cury
Coordenadora do CEP/FAMERP

Av. Brigadeiro Faria Lima, 5416 - Cep 15.090-000 Fone: (017) 210 - 5700
Fax : 227-6201- São José do Rio Preto - São Paulo - Brasil

MINISTÉRIO DA SAÚDE
Conselho Nacional de Saúde
Comissão Nacional de Ética em Pesquisa - CONEP

PARECER Nº2400/2004

Registro CONEP: 10618 (Este nº deve ser citado nas correspondências referentes a este projeto)

Registro CEP: 3340/04 **Processo nº 25000.106488/2004-41**
Projeto de Pesquisa: *"Avaliação genético clínica e molecular em Síndrome de Down."*
Pesquisador Responsável: Dra. Érika Cristina Pavarino Bertelli
Instituição: Faculdade de Medicina de São José do Rio Preto - FAMERP
Área Temática Especial: Genética Humana

Ao se proceder à análise das respostas ao parecer CONEP nº 2001/2004, relativo ao projeto em questão, considerou-se que:

1) tendo em vista a afirmação da pesquisadora responsável que será estabelecido um banco de material biológico, solicita-se que seja feito um banco de dados junto ao CEP da instituição, informando: quem será o responsável pelo banco, condições de armazenamento, segurança do banco, como será o acesso pelos pesquisadores a esse banco, de que forma será garantida a confidencialidade dos indivíduos que doarem o material para a formação desse banco;
2) as informações enviadas atendem aos aspectos fundamentais da Res. CNS 196/96 sobre diretrizes e normas regulamentadoras de pesquisas envolvendo seres humanos;
3) o projeto foi aprovado pelo Comitê de Ética em Pesquisa – CEP da instituição supracitada.

Diante do exposto, a Comissão Nacional de Ética em Pesquisa - CONEP, de acordo com as atribuições definidas na Resolução CNS 196/96, manifesta-se pela aprovação do projeto de pesquisa proposto com a recomendação 1. acima citada, devendo esta ser acompanhada pelo CEP, para posterior início da pesquisa.

Situação: Projeto aprovado com recomendação

Brasília, 29 de Novembro de 2004

WILLIAM SAAD HOSSNE
Coordenador da CONEP/CNS/MS

FACULDADE DE MEDICINA DE SÃO JOSÉ DO RIO PRETO
Autarquia Estadual - Lei n.° 8899 de 27/09/94
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COMITÊ DE ÉTICA EM PESQUISA

O Comitê de Ética em Pesquisa em Seres Humanos da Faculdade de Medicina de São José do Rio Preto tomou ciência e aprovou a **prorrogação para agosto de 2009 para extensão da metodologia e tomou ciência do relatório parcial**; referente ao projeto n.° **3340/2004** sob a responsabilidade de **Érika Cristina Pavarino Bertelli** com o título "Avaliação Genético-Clínica e Molecular em Síndrome de Down".

São José do Rio Preto, 19 de maio de 2008.

Prof. Dr. Antonio Carlos Pires
Coordenador do CEP/FAMERP

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FACULDADE DE MEDICINA DE SÃO JOSÉ DO RIO PRETO
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COMITÊ DE ÉTICA EM PESQUISA

O Comitê de Ética em Pesquisa em Seres Humanos da Faculdade de Medicina de São José do Rio Preto tomou ciência e aprovou a **extensão da metodologia e tomou ciência do relatório parcia**l; referente ao projeto n.° **3340/2004** sob a responsabilidade de **Érika Cristina Pavarino Bertelli** com o título "Avaliação Genético-Clínica e Molecular em Síndrome de Down".

São José do Rio Preto, 29 de maio de 2010.

Prof. Dr. Antonio Carlos Pires
Coordenador do CEP/FAMERP

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