Ana Lívia Silva Galbiatti

AVALIAÇÃO DE POLIMORFISMOS ENVOLVIDOS NO METABOLISMO DO FOLATO EM PACIENTES COM CÂNCER DE CABEÇA E PESCOÇO

São José do Rio Preto
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Dissertação apresentada à Faculdade de Medicina de São José do Rio Preto para obtenção do Título de Mestre no Curso de Pós-graduação em Ciências da Saúde, Eixo Temático: Medicina e Ciências Correlatas.

Orientadora: Profa. Dra. Eny Maria Goloni Bertollo

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SUMÁRIO

Dedicatória

Aos meus pais Luiz e Maria Rosa

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Epígrafe

"Parece-me que o preço mais alto possível para qualquer trabalho humano não é o que se recebe por ele, mas o que se torna através dele"
Brock Bell

"É melhor tentar e falhar, que preocupar-se e ver a vida passar. É melhor tentar ainda que em vão, que sentar-se, fazendo nada até o final "
Martin Luther King

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LISTA DE ABREVIATURAS E SÍMBOLOS

5,10-MTHF	5,10-metilenotetrahidrofolato (*5,10-methylenetetrahydrofolate*)
5-MTHF	5-metiltetrahidrofolato (5-*methyltetrahydrofolate*)
CBS	Cistationina β-sintase (*Cystathionine β-synthase*)
CH_3	Metil (*Methyl*)
CI 95%	Intervalo de confiança 95%
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico (*National Council for Scientific and Technological Development*)
CONEP	Comitê Nacional de Pesquisa (*National Research Commission*)
DHFR	Dihidrofolato redutase *(Dihydrofolate reductase)*
DNA	Ácido desoxirribonucléico *(Desoxirribonucleic acid)*
dTMP	Timidina monofosfato *(Deoxythymidine monophosphate)*
dUMP	Deoxiuridina monofosfato *(Deoxyuridine monophosphate)*
FAMERP	Faculdade de Medicina de São José do Rio Preto (*São José do Rio Preto Medical School*)
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo (*São Paulo State Research Foundation*)
FUNFARME	Fundação Faculdade Regional de Medicina de São José do Rio Preto
Hcy	Homocisteína (*Homocysteine*)
HNSCC	*Head and neck squamous cell carcinoma*
HPV	*Human Papiloma Virus*

HWE	*Hardy–Weinberg equilibrium*
INCA	Instituto Nacional do Câncer *(Brazilian National Cancer Institute)*
M	Metástase à distância
mRNA	Acido ribonucléico mensageiro (*Ribonucleic acid)*
MTHF	Metilenotetrahidrofolato *(methylenetetrahidrofolate)*
MTHFD1	Metilenotetrahidrofolato desidrogenase 1 (*Methylenetetrahydrofolate dehidrogenase 1*)
MTHFR	Metilenotetrahidrofolato redutase (*Methylenetetrahydrofolate reductase*
MTR	Metionina sintase (*Methionine synthase*)
MTRR	Metionina sintase redutase (*Methionine synthase reductase*)
N	Envolvimento de linfonodos
OR	*Odds ratio*
PCR	Reação em Cadeia da Polimerase *(Polymerase chain reaction)*
PCR-RFLP	Polimorfismo do Tamanho do Fragmento de Restrição *Polymerase chain reaction-restriction fragment length polymorphism*
RFC1	Carreador de folato reduzido 1 *(Reduced folate carrier 1)*
SAH	S- adenosilhomocisteína *(S-adenosylhomocysteine)*
SAM	S- adenosilmetionina *(S-adenosylmethionine)*
SHMT	Serina hidroximetiltransferase *(Serine hydroxymethyltransferase)*
SISNEP	Sistema Nacional de Informação sobre Ética em Pesquisa *(National Information System on Research Ethics)*

T	Tamanho de tumor
TNM	Classificação dos Tumores Malignos *(TNM classification)*
TYS	Timidilato sintase *(Thymidylate synthase)*
UICC	*International Union of Cancer Control*
UPGEM	Unidade de Pesquisa em Genética e Biologia Molecular (*Genetics and Molecular Biology Research Unit*)

RESUMO

Introdução: O carcinoma de cabeça e pescoço pode ser decorrente de alterações na metilação do DNA associadas ao metabolismo anormal do folato. Concentrações reduzidas desse nutriente podem diminuir a capacidade de reparo do DNA, resultando em alterações celulares malignas que modulam a função e expressão dos genes. Polimorfismos em genes que participam da via do folato têm sido investigados como fatores de risco para susceptibilidade ao carcinoma de cabeça e pescoço, entre eles, polimorfismos nos genes *MTR, RFC1, CBS* e *MTHFR*. **Objetivos:** Estabelecer a freqüência dos polimorfismos nos genes *MTR* (A2756G), *RFC1*(A80G), *CBS* (844 ins 68) e *MTHFR* (C677T e A1298C) em pacientes com carcinoma de cabeça e pescoço comparando-a com aquela observada em indivíduos sem história de neoplasia; avaliar a associação dos polimorfismos com os hábitos tabagista e etilista, gênero e idade e verificar associação entre os polimorfismos e parâmetros clínico-histopatológicos. **Casuística e Método:** Foram incluídos no estudo 854 indivíduos (322 pacientes com carcinoma de cabeça e pescoço e 531 indivíduos controles). Para análise molecular, o DNA genômico foi extraído a partir de leucócitos de sangue periférico e as técnicas de reação em cadeia da polimerase e digestão enzimática foram utilizadas para genotipagem dos indivíduos estudados. Os dados sócio-demográficos foram obtidos a partir do prontuário médico do paciente e entrevista realizada aos indivíduos controle. Para análise estatística foi utilizado os testes de qui-quadrado e regressão logística múltipla. **Resultados:** Em relação ao polimorfismo *MTR* A2756G, os resultados mostraram que os hábitos tabagista e etilista, idade acima de 42 anos, gênero masculino, genótipo 2756AG e alelo polimórfico 2756G podem aumentar o risco de carcinoma de

cabeça e pescoço (p<0,05). Houve alta freqüência do alelo *MTR* 2756G em pacientes do gênero masculino (p<0,05). A avaliação do polimorfismo *RFC1* A80G mostrou que gênero masculino, hábito tabagista e genótipos *RFC1* 80AG ou GG foram associados com risco aumentado da doença. O polimorfismo *CBS* 844ins68 não foi associado com o risco de carcinoma de cabeça e pescoço e houve alta freqüência dessa variante em pacientes que possuíam como sítio primário a cavidade oral. A análise para os polimorfismos no gene *MTHFR* (C677T e A1298C) mostrou que idade avançada, gênero masculino, hábitos tabagista e etilista, genótipos *MTHFR* 1298AC ou CC e genótipos combinados *MTHFR* 677CT/1298AC, 677TT/1298AC, 677CT/1298CC e 677TT/1298CC foram associados com aumento de risco para o carcinoma de cabeça e pescoço (p<0,05). Houve freqüência maior do que esperada do haplótipo *MTHFR* 677C-1298A e freqüência menor que esperada dos haplótipos 677T-1298C e 677C-1298C em ambos grupos (p<0,05). O polimorfismo *MTHFR* A1298C foi mais freqüente em pacientes que possuíam como sítio primário a cavidade oral. **Conclusões:** O carcinoma de cabeça e pescoço é mais frequente em homens, com idade acima de 42 anos, fumantes e etilistas. Os polimorfismos *MTR* A2756G, *RFC1* A80G, *MTHFR* A1298C e os polimorfismos combinados A1298C e C677T do gene *MTHFR* podem modular o risco para o carcinoma de cabeça e pescoço.

Palavras-chave: Câncer de cabeça e pescoço, metabolismo do folato, polimorfismo genético

ABSTRACT

Introduction: Head and neck cancer can be caused by alterations in DNA methylation associated associated with abnormal folate metabolism. Low concentrations of folate may reduce the capacity for DNA repair, resulting in malignant cell that alter the function and expression of genes. Polymorphisms in genes involved in folate pathway has been investigated as risk factors for susceptibility to head and neck carcinoma, among them polymorphisms in *MTR, RFC1, MTHFR* and *CBS* genes. **Objectives:** To establish the frequency of polymorphisms in the *MTR* (A2756G), *RFC1* (A80G), *CBS* (844 ins 68) and *MTHFR* (C677T and A1298C) genes in patients with head and neck carcinoma comparing to individuals with no history neoplasia; evaluate the association of polymorphisms with tobacco and alcohol consumption, gender and age and the association between polymorphisms and clinical-histopathological parameters. **Methods:** The study included 854 individuals (322 patients with head and neck cancer and 531 controls). For molecular analysis, genomic DNA was extracted from peripheral blood leukocytes and the techniques of polymerase chain reaction and restriction enzyme digestion were used for genotyping of subjects studied. The socio-demographic data were obtained from the patient's medical records and interview to control individuals. For statistical analysis used the chi-square and logistic regression. **Results:** For the *MTR* A2756G polymorphism, the results showed that tobacco and alcohol consumption, age over 42 years, male gender, genotype 2756AG and 2756G polymorphic allele may increase the risk of head and neck carcinoma ($p < 0.05$). There was a high frequency of the *MTR* 2756G allele in male patients ($p < 0.05$). The results showed that *RFC1* A80G polymorphism, male gender, tobacco habit and *RFC1* 80AG

or GG genotypes were associated with an increased risk of disease. Polymorphism *CBS* 844ins68 was not associated with risk of head and neck carcinoma and there was a high frequency of this variant in patients who had oral cavity as primary site. The analysis for the polymorphisms (C677T and A1298C) showed that advanced age, male gender, tobacco and alcohol consumption, *MTHFR* 1298AC and CC genotypes and combined genotypes *MTHFR* 677CT/1298AC, 677TT/1298AC, and 677CT/1298CC 677TT/1298CC were associated with an increased risk for head and neck carcinoma ($p < 0.05$). There was a higher frequency than expected of *MTHFR* 677C-1298AA haplotype and a lower frequency than expected of 677T- 1298C and 677C- 1298C observed in both groups ($p < 0.05$). The *MTHFR* A1298C was more frequent in patients who had primary site as the oral cavity. **Conclusions:** Head and neck carcinoma is more common in men, individuals with age over 42 years, smokers and drinkers. The polymorphisms *MTR* A2756G, *RFC1* A80G, *MTHFR* A1298C and A1298C and C677T polymorphisms combined *MTHFR* gene may modulate the risk for head and neck carcinoma.

Key words: Head and neck cancer, folate metabolism, genetic polymorphism

1. INTRODUÇÃO

1. INTRODUÇÃO

O carcinoma de cabeça e pescoço ocupa a quinta posição na lista das neoplasias mais frequentes, com uma incidência mundial estimada de 500.000 casos novos por ano. [1,2] O tipo histológico mais comum em tumores de cabeça e pescoço é o carcinoma de células escamosas (*HNSCC – head and neck squamous cell carcinoma*) e os sítios anatômicos que estão incluídos nesse grupo compreendem a cavidade oral, faringe e laringe, com ocorrência aproximada de 40%, 15% e 25% respectivamente. [2-5] No Brasil, a estimativa de câncer de cavidade oral para o ano de 2010 é de 14.120 casos novos, sendo 10.330 do gênero masculino e 790 para o gênero feminino. [6]

Esse tipo de tumor acomete em maior proporção indivíduos do gênero masculino e com idade avançada. [7,8]No entanto, a incidência de câncer de cabeça e pescoço tem aumentado em indivíduos com idade inferior a 45 anos e isso é atribuído ao aumento da prevalência da infecção pelo vírus HPV, que contribui para o desenvolvimento dessa neoplasia nos países em desenvolvimento. [9]

Os principais fatores de risco já estabelecidos para a doença são tabagismo e etilismo, que quando atuam em conjunto multiplicam o risco para câncer. [10] Em relação ao hábito alimentar, os resultados da literatura evidenciam que uma dieta rica em alimentos que possuem micronutrientes tais como vitaminas B, C e E, carotenóides, flavonóides entre outros, protege contra danos oxidantes do DNA, pois esses micronutrientes possuem propriedades antioxidantes e anticarcinogênicas. [11-14]

O folato (Vitamina B9) presente em frutas e vegetais, quando alterado no organismo, pode estar associado à etiologia do *HNSCC*, uma vez que para a regulação da síntese, metilação e reparo do DNA, é essencial que o folato, principal doador de metil para reações de metilação celular, esteja presente no organismo na quantidade

adequada.[15,16] Alterações nos níveis desse micronutriente podem ser ocasionadas por polimorfismos em genes que codificam enzimas envolvidas no metabolismo do folato e consequentemente podem dar início ao processo de carcinogênese .[8, 17-24]

Existem dois mecanismos pelos quais polimorfismos genéticos envolvidos no metabolismo do folato podem alterar os níveis de folato e contribuir para a carcinogênese: (1) hipometilação de DNA e subseqüente ativação dos proto-oncogenes [25,26] ; (2) erro de incorporação da uracila durante a síntese de DNA que leva à instabilidade genômica [25-27]aumentando assim o risco de adquirir o câncer. [26,28-32]

Na Figura 1 estão apresentadas as principais enzimas que participam do metabolismo do folato.

Polimorfismos genéticos e Câncer de cabeça e pescoço

O polimorfismo *MTHFR* C677T está associado à redução da atividade enzimática, limitando a conversão de 5,10 MTHF para 5-MTHF, forma de folato requerida para as reações de metilação do DNA [33]. Um estudo *in vitro* mostrou que o genótipo heterozigoto 677CT foi associado com redução de 40% da atividade enzimática, enquanto que o genótipo homozigoto polimórfico 677TT foi associado com redução de 70% da atividade enzimática. [34]

De acordo com dados da literatura, somente os estudos de Weistein *et al.,* 2002;[17] Kureshi *et al.*2004; [18] Neuman *et al.,* 20005; [19] Reljic *et al.,* 2006; [20] Vairaktaris *et al.,* 2006; [21] Suzuki *et al.,* 2007;[22] Solomon *et al.,* 2008; [23] Kruzsyna *et al.,* 2010 [24] avaliaram a associação desse polimorfismo em câncer de cabeça e pescoço.

Dentre esses estudos, somente Reljic *et al* (2006),[20] Vairaktaris *et al.*, 2006[21] e Solomon *et al* (2008)[23] confirmaram associação do polimorfismo *MTHFR* C677T no risco de câncer de cabeça e pescoço e os resultados foram contraditórios.

Reljic *et al* (2006)[20] avaliaram 81 pacientes com câncer de cabeça e pescoço e 102 indivíduos sem historia de câncer em uma população croata e encontrou que o genótipo 677TT diminui o risco desta doença. O estudo de Vairaktaris *et al* (2006)[21] em 110 indivíduos com câncer de cavidade oral e 102 indivíduos sem história de neoplasia realizado em alemães e gregos mostrou que o genótipo 677CT foi associado com aumento de risco desse tipo de câncer. Solomon *et al* (2008)[23] avaliaram 126 indivíduos etilistas (33 etilistas crônicos importantes, 56 etilistas moderados e 37 etilistas sociais) com câncer oral e mostrou que o genótipo 677TT foi associado com o grupo de indivíduos etilistas crônicos importantes e também com o grupo de indivíduos com hábito etilista moderado, em menor proporção.

Outro polimorfismo do gene *MTHFR* também avaliado em câncer de cabeça e pescoço é o *MTHFR* A1298C, associado *in vitro* com diminuição da atividade enzimática, porém em menor proporção em relação ao polimorfismo *MTHFR* C677T. [35] A exata relevância biológica do polimorfismo *MTHFR* A1298C ainda não está clara e os resultados são inconsistentes. [36-38] Dados sobre o risco de câncer de cabeça e pescoço relacionados ao polimorfismo também foram contraditórios [15, 39]

O estudo de Suzuki *et al* (2007),[15] realizado no Japão, em 237 pacientes com câncer de cabeça e pescoço e 711 indivíduos sem história de neoplasia e o estudo de Kruzsyna *etal* (2010) [24] em 131 poloneses com câncer de laringe e 250 poloneses sem história de câncer não encontraram associação dessa variante com o risco de carcinoma de cabeça e pescoço. No entanto, o estudo de Neumann *et al* (2005)[39] realizado

no Texas em 537 pacientes com câncer de cabeça e pescoço e 545 indivíduos controle mostrou que indivíduos com genótipos 1298AC ou 1298CC apresentaram uma diminuição de 35% no risco de câncer de cabeça e pescoço.

Estudos têm demonstrado que o polimorfismo *MTR* A2756G aumenta o risco de câncer de cabeça e pescoço. Zhang *et al* (2005),[31] em um estudo caso-controle realizado no Texas avaliaram 721 pacientes com câncer de cabeça e pescoço e 1.234 indivíduos sem história de neoplasia e observaram que os genótipos *MTR* 2756AG ou GG aumentam o risco desta doença. Assim como em nosso estudo [8] com 236 pacientes brasileiros com câncer de cabeça e pescoço e 469 indivíduos controles e o estudo de Kruzsyna *et al* (2010) [24] nos quais o genótipo *MTR* 2756GG e o alelo *MTR* 2756G foram associados com risco aumentado de HNSCC.

O gene *CBS* apresenta-se polimórfico no nucleotídeo 844, com inserção de 68 pares de base (pb). Estudos mostram associação entre esse polimorfismo e redução nos níveis de Hcy na presença do fragmento inserido e, acredita-se que a inserção de 68 pb esteja associada com aumento da atividade da enzima CBS. [41] É possível que o aumento da atividade enzimática e, consequentemente, diminuição das concentrações de Hcy, comprometa a via de remetilação da Hcy para metionina, reduzindo a síntese de SAM e as reações de metilação celular. [40-42] No entanto, essa variante ainda não foi estudada em câncer de cabeça e pescoço.

O efeito do polimorfismo *MTHFD1* G1958A foi avaliado em câncer de cabeça e pescoço em apenas um estudo e não mostrou associação com risco da doença. [24] Os polimorfismos *RFC1* A80G, *BHMT* G742A, *SHMT* C1420T, *TC2* A67G e *TC2* C776G, também envolvidos no metabolismo do folato, ainda não foram estudados em

câncer de cabeça e pescoço, porém eles estão associados a outros tipos de câncer e poderiam modular o desenvolvimento de neoplasia de cabeça e pescoço. [43-46]

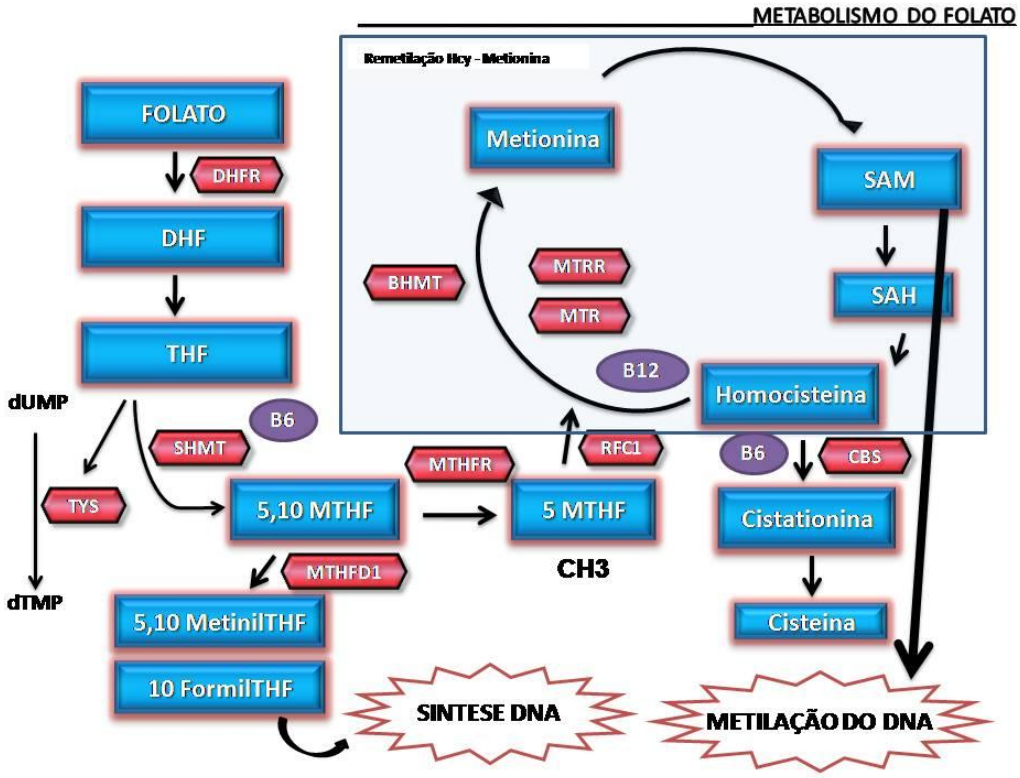

Figura 1. Esquema representando o metabolismo do folato com as principais enzimas envolvidas – DHF: Dihidrofolato; THF- Tetrahidrofolato; DHFR: Dihidrofolato redutase; SHMT: Serina hidroximetiltransferase; TYS: Timidilato sintase; MTHFD1: Metilenotetrahidrofolato desidrogenesase 1; MTHFR: Metileno tetrahidrofolato redutase; MTR: metionina sintase; MTRR: Metionina sintase redutase; BHMT: Betaína-homocisteína metiltransferase; CBS: Cistationina beta sintase; RFC1: Carreador de folato reduzido 1; SAM: S S-adenosilmetionina; SAH: S- adenosilhomocisteina; dUMP: Deoxiuridina monofosfato; dTMP: Timidina monofosfato.

OBJETIVOS

Considerando as evidências apresentadas, este estudo teve como objetivos:

1. Avaliar a associação dos polimorfismos *MTR* A2756G, *RFC1* A80G, *CBS* 844ins68, *MTHFR* 677T e *MTHFR* A1298C no risco de câncer de cabeça e pescoço, em um estudo caso-controle;

2. Avaliar a associação dos polimorfismos com os hábitos tabagista e etilista, gênero e idade (fatores de risco) no desenvolvimento do câncer de cabeça e pescoço;

3. Verificar associação entre os polimorfismos e sítios primários de ocorrência, extensão do tumor, comprometimento de linfonodos, e prognóstico da doença (tempo de recidiva e óbito).

2. ARTIGOS CIENTÍFICOS

2. ARTIGOS CIENTÍFICOS

Os resultados estão apresentados em forma de artigo. No total estão apresentados 04 artigos, dois artigos publicados, um aceito para publicação, e um a ser submetido

Artigo 1:

Título: 5-Methyltetrahydrofolate-homocysteine methyltransferase gene polymorphism (*MTR*) and risk of head and neck câncer.

Autores: Ana Lívia Silva Galbiatti, Mariangela Torreglosa Ruiz, Patricia Matos Biselli-Chicote, Luiz Sérgio Raposo, José Victor Maniglia, Érika Cristina Pavrino-Bertelli, Eny Maria Goloni-Bertollo.

Periódico: Brazilian Journal of Medical and Biological Research, 43: 445-450; 2010.

Artigo 2

Título: A80G polymorphism of reduced folate carrier 1 (*RFC1*) gene and head and neck squamous cell carcinoma etiology in Brazilian population.

Autores: Ana Lívia Silva Galbiatti, Mariangela Torreglosa Ruiz, Daniela Rezende Pinto, Luiz Sérgio Raposo, Jose´ Victor Maníglia, Erika Cristina Pavarino-Bertelli, Eny Maria Goloni-Bertollo.

Periódico: Molecular Biology Reports, Publicado online em 27/07/2010.

Artigo 3

Título: *CBS* 844ins68 polymorphism and head and neck squamous cell carcinoma risk: A case-control Analysis.

Autores: Ana Lívia Silva Galbiatti, Mariangela Torreglosa Ruiz, Luis Sérgio Raposo, José Victor Maníglia, Erika Cristina Pavarino-Bertelli, Eny Maria Goloni-Bertollo.

Periódico: Archives of Medical Science; 2010. Aceito para publicação.

Artigo 4

Título: Polymorphisms and haplotypes in methylenetetrahydrofolate reductase gene and risk of head and neck squamous cell carcinoma.

Autores: Ana Lívia Silva Galbiatti, Mariangela Torreglosa Ruiz, Juliana Olsen Rodrigues, Luiz Sérgio Raposo, José Victor Maníglia, Erika Cristina Pavarino-Bertelli, Eny Maria Goloni-Bertollo.

Periódico: Cancer Research and Clinical Oncology, a ser submetido para publicação.

ARTIGO CIENTÍFICO 1

Artigo 1:

Título: 5-Methyltetrahydrofolate-homocysteine methyltransferase gene polymorphism (*MTR*) and risk of head and neck câncer.

Autores: Ana Lívia Silva Galbiatti, Mariangela Torreglosa Ruiz, Patricia Matos Biselli-Chicote, Luiz Sérgio Raposo, José Victor Maniglia, Érika Cristina Pavrino-Bertelli, Eny Maria Goloni-Bertollo.

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5-Methyltetrahydrofolate-homocysteine methyltransferase gene polymorphism (MTR) and risk of head and neck cancer

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Abstract

The functional effect of the A>G transition at position 2756 on the *MTR* gene (5-methyltetrahydrofolate-homocysteine methyltransferase), involved in folate metabolism, may be a risk factor for head and neck squamous cell carcinoma (HNSCC). The frequency of *MTR* A2756G (rs1805087) polymorphism was compared between HNSCC patients and individuals without history of neoplasias. The association of this polymorphism with clinical histopathological parameters was evaluated. A total of 705 individuals were included in the study. The polymerase chain reaction-restriction fragment length polymorphism technique was used to genotype the polymorphism. For statistical analysis, the chi-square test (univariate analysis) was used for comparisons between groups and multiple logistic regression (multivariate analysis) was used for interactions between the polymorphism and risk factors and clinical histopathological parameters. Using univariate analysis, the results did not show significant differences in allelic or genotypic distributions. Multivariable analysis showed that tobacco and alcohol consumption ($P < 0.05$), AG genotype ($P = 0.019$) and G allele ($P = 0.028$) may be predictors of the disease and a higher frequency of the G polymorphic allele was detected in men with HNSCC compared to male controls ($P = 0.008$). The analysis of polymorphism regarding clinical histopathological parameters did not show any association with the primary site, aggressiveness, lymph node involvement or extension of the tumor. In conclusion, our data provide evidence that supports an association between the polymorphism and the risk of HNSCC.

Key words: Head and neck cancer; Polymorphism; Folate metabolism; *MTR* gene

Introduction

Head and neck squamous cell carcinomas (HNSCC) include malignant tumors of any site in the upper aero-digestive tract, occurring in 95% of cases and being the fifth most common cancer worldwide and the most common histological type (1). Smoking and alcohol consumption, viral infections, especially the papilloma virus subtypes 16 and 18, and vitamin and micronutrient deficiencies, including deficits of folate, vitamins A, C, and E, selenium and zinc, are risk factors for this disease (1-6).

Folate metabolism plays an important role in carcinogenesis because of its involvement in both DNA methylation and nucleotide synthesis. DNA methylation is the transfer of methyl groups (CH3) to the C5 position of cytosine residues located in cytosine-guanine dinucleotides, by reactions catalyzed by proteins called DNA methyltransferases (7). This epigenetic modification of the DNA has several functional roles including control of gene expression (8), maintenance of genomic stability (9) and stability of the chromatin structure (10).

The relationship between polymorphisms of genes involved in folate metabolism and the risk for cancer is related to their effect on DNA methylation and synthesis and, thus, on the maintenance of chromatin structure and chromosome stability (6).

The 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*) gene, located in chromosome 1q43 (10), encodes the methionine synthase enzyme, which has a role in folate metabolism, catalyzing the remethylation of

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homocysteine (Hcy) to methionine, a reaction essential to adequately maintain normal methionine and intracellular Hcy concentrations (11). An adenine to guanine transition at position 2756 (rs185087) of the *MTR* gene results in the substitution of the amino acid, aspartic acid, with glycine in codon 919 of the protein and is related to alterations in the folate metabolic pathway, thus possibly influencing the risk of cancer (12,13).

Research results on the influence of this polymorphism on the development of cancer are contradictory. Some studies have shown associations with colon (14), lung (15) and breast cancer (16), and relationships with tumor aggressiveness and variable responses to esophageal cancer treatment (17). Other studies did not confirm associations between the polymorphism and different types of cancer, including colorectal (18), lung (19), bladder (20), and stomach cancer (21).

Three studies investigated the *MTR* A2756G polymorphism with respect to head and neck cancer. Zhang et al. (22), when analyzing the *MTR* A2756G polymorphism in 721 cancer patients and 1234 control individuals, observed differences in the genotype frequencies between the two groups. They also showed that the 2756AG genotype was associated with a significantly increased risk of HNSCC among younger subjects, women and former drinkers, in particular tumors of the oral cavity. In another study, Suzuki et al. (23) analyzed 237 HNSCC patients and 711 controls in Japan but did not identify differences between the two groups. Kruszyna et al. (24) analyzed 131 men diagnosed with squamous cell carcinoma of the larynx and 250 randomly selected, unrelated healthy male blood donors and other healthy volunteers in the Polish population and suggested that the *MTR* 2756G allele may contribute to the risk of laryngeal cancer.

Based on the above evidence, the aim of this study was to compare the frequencies of the *MTR* A2756G polymorphism between head and neck cancer patients and individuals with no history of cancer, and to determine if there were an association of this polymorphism with clinical histopathological parameters.

Patients and Methods

A total of 705 individuals (236 patients and 469 controls) with a mean age of 52.5 ± 13.7 years were included in the study.

The study protocol was approved by the National Ethics Committee (SISNEP 0976.0.140.000-05). The case group consisted of 236 patients who were diagnosed with head and neck cancer at Hospital de Base, São José do Rio Preto, SP, Brazil. Informed consent was obtained from all individuals enrolled in the study. The diagnosis was made from pathological specimens after total excision or a biopsy. The inclusion criterion was squamous cell carcinoma tumor cell types and the exclusion criterion was patients previously treated for tumors. The primary anatomic sites were oral cavity (N = 92, 40%), pharynx (N = 59, 25%), and larynx (N = 76, 32.2%) and 9 (2.8%) patients with unknown primary site of the tumor.

All required information about clinical histopathological parameters was obtained from the patients' medical records. The average survival was 31.7 ± 27.1 months. The treatment options for the patients were surgery, radiotherapy and chemotherapy.

The tumors were classified according to the parameters of the International Union of Cancer Control (UICC), 2002, and the American Joint Committee for Cancer (AJCC), 2002, based on three criteria: extension of the tumor (T), presence of regional lymph node involvement (N), and presence of metastasis at a distance (M) (25). The clinical stage (TNM) was used to analyze aggressiveness, with tumors being grouped as non-aggressive (stages I and II) and aggressive (stages III and IV).

The control group consisted of 469 Brazilian blood donors without a diagnosis of cancer according to government guidelines for donated blood that is tested for 20 related diseases (http://www.hemonline.com.br/portarias/rdc153/indexframe.htm). The inclusion criterion was age of more than 40 years and the exclusion criterion was a family history of cancer. Each eligible subject was interviewed to obtain data on age, gender, smoking habit, use of alcohol, and family history of cancer. Patients and controls were followed up for 60 months.

The variables analyzed were gender, exposure to risk factors (tobacco and alcohol consumption), primary site of occurrence, aggressiveness, extension of the tumor, and lymph node involvement. Individuals who had smoked more than 100 cigarettes in their lifetime and at the time of the interview continued to smoke either every day or at least on some days were considered to be smokers. Individuals who drank 4 doses of alcohol per week were considered to be alcohol consumers (26,27).

Genomic DNA was obtained from peripheral blood according to technique of Miller et al. (28). The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was used to determine genotypes of the *MTR* A2756G polymorphism (rs1805087). The primers used were: sense 5'-CCA GGG TGC GAC GTA TAC AG-3' and anti-sense 5'-GCC TTT TAC ACT CCT CAA AAC C-3'. Amplification was obtained with initial denaturation at 94°C for 4 min, followed by 30 cycles of 1 min of DNA denaturation at 94°C, 1-min primer annealing at 56°C, and 1-min extension at 72°C. A final extension of 10 min at 72°C was carried out. The product of 498 bp was submitted to digestion with the *Hae*III restriction enzyme for 2 h at 37°C according to manufacturer guidelines. Fragments of 390, 123, and 85 bp were generated when the G polymorphic allele was present and 413- and 85-bp fragments were observed with the A allele.

Statistical analysis

Groups were compared by the chi-square test (univariate analysis). Utilizing the BioEstat computer program, this test was also used to analyze Hardy-Weinberg equilibrium. Multiple logistic regression models were used to determine the interaction effect between the genetic polymorphisms and variables related to HNSCC. One model included gender (reference: female), smoking (reference: non-smokers) and drinking habits (reference: non-drinkers) using the Minitab for Windows computer program (Version 12.22). $P < 0.05$ was considered to be statistically significant. Results are shown as odds ratio (OR) and 95% confidence intervals (95%CI).

The clinical histopathological parameters were analyzed by multiple logistic regression. Tumors were classified as low T (T1, T2) and high T (T3, T4). The N classification was dichotomized into no lymph node involvement (N0) and involvement (N1, N2, N3). Tumors were divided into early stage (stages I and II) and advanced stage (stages III and IV) categories.

The Kaplan-Meier method was used to evaluate survival rates and time of disease recurrence. The log-rank test was used to assess differences related to the different genotypes.

Results

There were statistically significant differences between patients and controls regarding gender, alcohol consumption and smoking ($P < 0.05$). Five hundred and twenty-nine (75%) participants were men (203 patients and 326 controls) and 176 (25%) were women (33 patients and 143 controls). Of the cases, 77.97% consumed alcohol compared to 46.70% of the controls. Smoking also differed greatly between cases (89.41%) and controls (48.83%).

Hardy-Weinberg equilibrium showed that the genotypic distributions were in equilibrium for both groups (case: $X^2 = 0.028$; $P = 0.868$, and control: $X^2 = 2.868$; $P = 0.09$).

For the *MTR* 2756 polymorphisms, AA, AG, and GG genotype frequencies were 65.7, 31.0, and 3.3%, respectively, for the cases, and 72.5, 24.0, and 3.5%, respectively, for the controls. The variant *MTR* 2756A allele frequencies were 0.81 among the cases and 0.85 among the controls, while the *MTR* 2756G allele frequencies were 0.19 and 0.15 among cases and controls, respectively.

The frequency of the G polymorphic allele (individuals carrying at least one G allele - genotypes: AG and GG) was stratified by gender. There was a higher frequency of carriers of the G variant allele in men with head and neck cancer compared to the group of men with no history of cancer ($P = 0.008$). No difference between patients and controls ($P = 0.335$) was observed for the women.

As matching demographic data and risk factors between patients with cancer and control individuals was not possible, multivariable analysis was performed to adjust these variables. The following variables were included: gender, tobacco and alcohol consumption, and *MTR* A2756G polymorphism. Smoking ($P < 0.05$), alcohol consumption ($P < 0.05$), AG heterozygous genotype ($P = 0.019$), and the G polymorphic allele ($P = 0.028$) were predictors of the disease (Table 1).

Table 1. Distribution of demographic data, risk factors, genotypes, *MTR* 2756 alleles, and odds ratio (OR) for head and neck cancer.

Variables	N (%)	OR (95%CI)
Tobacco consumption		
Non-smokers	301 (37.3)	Reference
Smokers	504 (62.7)	4.49 (2.68-7.54)*
Alcohol consumption		
Alcohol non-consumers	338 (42)	Reference
Alcohol consumers	467 (58)	2.30 (1.46-3.63)*
Gender		
Female	263 (32.6)	Reference
Male	595 (67.4)	1.18 (0.69-2.01)
Age		
<42 years	217 (27)	Reference
42-51 years	185 (23)	4.72 (2.29-9.72)*
52-63 years	191 (23.7)	19.45 (9.55-39.59)*
>63 years	212 (26.3)	11.01 (5.33-22.72)*
MTR 2756 genotypes		
AA	523 (65)	Reference
AG	247 (30.6)	1.69 (1.09-2.62)*
GG	35 (4.4)	1.08 (0.37-3.13)
MTR 2756 alleles		
A	682 (76.4)	Reference
G	210 (23.6)	1.60 (1.05-2.44)*

MTR = 5-methyltetrahydrofolate-homocysteine methyltransferase. *P < 0.05 (muliple logistic regression).

Analysis of polymorphisms and clinical parameters did not detect any association with the primary tumor site ($P = 0.12$), tumor extension ($P = 0.38$), lymph node involvement ($P = 0.35$), or tumor stage ($P = 0.18$).

The Kaplan-Meier survival curves by genotype are presented in Figure 1A, and did not demonstrate any association between polymorphisms and overall survival ($P = 0.52$). Moreover, no association was observed for the time of recurrence of the disease ($P = 0.25$; Figure 1B).

Discussion

A review of published data showed that the most important factors predisposing factors to the development of HNSCC, present in 90% of cases, are alcohol and tobacco consumption (1) and our study confirms this association. Additionally, a significant association was confirmed between

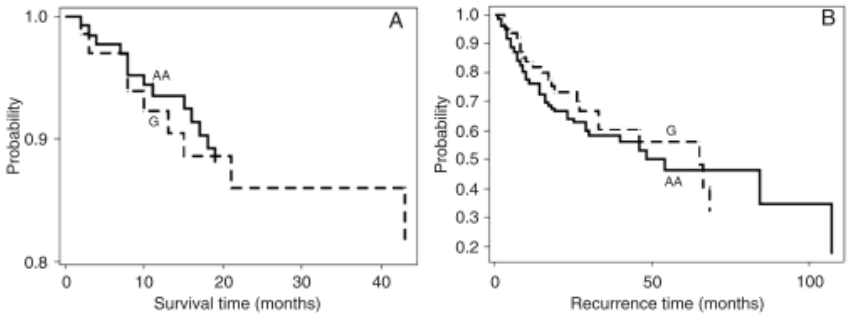

Figure 1. Kaplan-Meier curves for overall survival (P = 0.52) (A) and recurrence time (P = 0.25) (B) for patients according to *MTR* A2756G polymorphism. There was no statistical difference between the curves for subjects with the AA genotype and subjects with at least one mutant allele (G allele). *MTR* = 5-methyltetrahydrofolate-homocysteine methyltransferase.

gender and this disease, as previously reported by Argiris et al. (29), who showed that males are the most affected by this disease.

Using univariate analysis, the results of the current study did not demonstrate significant differences between groups with respect to allele and genotype distributions, similar to the data reported by Suzuki et al. (23). The results also show that men with head and neck cancer had a higher frequency of the G polymorphic allele than men with no history of cancer, thus supporting the results of Kruszyna et al. (24), who showed that the frequency of the G allele was 1.55 times higher in male patients with laryngeal cancer compared to male controls. Our findings did not confirm those of Zhang and et al. (22), who reported that the *MTR* 2756AG genotype is associated with an increased risk for this type of cancer, especially in younger individuals, women and former smokers.

Multivariable analysis performed after adjusting for age, gender and tobacco and alcohol consumption showed that the G polymorphic allele and the AG heterozygous genotype are associated with increased risk for the disease with OR of 1.60 and 1.69, respectively. Associations between *MTR* A2756G polymorphism and the development of some types of cancer have been shown in previous studies (30,31).

Only three studies have investigated this polymorphism regarding head and neck cancer (22-24). Zhang et al. (22) and Kruszyna et al. (24) reported differences in the *MTR* A2756G genotype frequencies between head and neck cancer patients and controls and between larynx cancer patients and controls, respectively, while Suzuki et al. (23) did not observe any association between the presence of the polymorphism and the risk for the disease. The latter investigators demonstrated an interaction of alcohol consumption with the 2756GG genotype in the risk for head and neck cancer.

Folate is an essential nutrient, which has important roles in the synthesis (genetics), repair, and methylation (epigenetics) of DNA. It is used to transport a methyl group, which is essential for the *de novo* synthesis of deoxynucleoside triphosphate and methionine.

The functional variant, *MTR* A2756G, encoding the *MTR* enzyme (OMIM 156570) uses methyl-tetrahydrofolate as a methyl donor for the remethylation of Hcy to form methionine. Methionine adenosyltransferase, using methionine and ATP, induces the formation of S-adenosylmethionine (32).

Folate deficiency associated with the *MTR* GG or *MTR* AG genotypes may increase cancerogenesis by reducing the formation of S-adenosylmethionine, leading to DNA hypomethylation and cancer and may also induce uracil misincorporation into DNA in place of thymine during DNA replication, which, along with DNA hypomethylation, results in impaired DNA repair, DNA strand breakage, and chromosome damage (33). The study of Paz and collaborators (34) showed that hypomethylation of genomic DNA may be associated with the *MTR* A2756G polymorphism.

Studies on other types of cancer also found a link between the *MTR* GG genotype and extensive genomic hypomethylation as well as decreased promoter hypermethylation of tumor suppressor genes (35,36).

In the present study, Hcy concentration was not measured but several studies have shown that the *MTR* A2756G polymorphism has also been associated with variations in Hcy concentrations. However, the quantitative impact of this polymorphism on cellular Hcy concentrations has not been determined. Studies have shown an association between the *MTR* GG genotype and lower plasma Hcy levels compared to the *MTR* AA genotype, showing that the *MTR* A2756G polymorphism contributes to enhanced methionine synthesis and an increase in intracellular S-adenosylmethionine concentration (13,37). However, Laraqui et al. (38) observed a correlation between the polymorphic allele (G) and moderately high Hcy levels.

Our study did not detect any associations of the *MTR* A2756G polymorphism with the site of occurrence, aggressiveness, tumor extension, or lymph node involvement. Zhang et al. (22) showed an increased frequency of the polymorphism related to tumors of the oral cavity, while Kruzsyna et al. (24) did not find any association of this polymorphism with laryngeal cancer. After an exhaustive literature review, no previous studies were found regarding the possible association between this polymorphism and clinical histopathological parameters or with the risk of cancer development.

Marchal et al. (39) reported that the presence of the *MTR* 2756 G allele is a factor of tumor aggressiveness in prostate cancer. No data were found in the literature assessing survival according to *MTR* A2756G polymorphism in patients with head and neck cancer.

In conclusion, although there is an association between *MTR* A2756G polymorphism and the risk for head and neck cancer and a higher frequency of carriers of the G allele variant in men with head and neck cancer compared to men without any history of cancer, studies of other enzymes involved in folate metabolism, their plasma concentrations and other derivatives could contribute to a better understanding of the factors involved in the etiology of head and neck cancer.

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ARTIGO CIENTÍFICO 2

Artigo 2

Título: A80G polymorphism of reduced folate carrier 1 (*RFC1*) gene and head and neck squamous cell carcinoma etiology in Brazilian population.

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A80G polymorphism of reduced folate carrier 1 (*RFC1*) gene and head and neck squamous cell carcinoma etiology in Brazilian population

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Abstract Reduced folate carrier is an essential folate transporter and the A80G polymorphism in reduced folate carrier 1 gene (rs1051266) has been shown to be associated with alterations in folate metabolism and consequently cancer development. We evaluated the association of this polymorphism with head and neck squamous cell carcinoma risk in a case–control study of 322 head and neck carcinoma patients and 531 individuals without cancer in a Brazilian population and association of this polymorphism with clinical histopathological parameters, and gender and lifestyle factors. The PCR-RFLP technique was used to genotype the polymorphism and multiple logistic regression was used for comparation between the groups and for interaction between the polymorphism and risk factors and clinical histopathological parameters. We observed association between the *RFC1* A80G polymorphism and the risk of this disease. Male gender, tobacco habit and *RFC1* AG or GG genotypes may be predictors of this disease ($P < 0.05$). The genotype, 80AG or GG was associated with for >50 years, male gender and non alcohol consumption ($P \leq 0.05$). The polymorphism did not show any association with the primary site, aggressiveness, lymph node involvement or extension of the tumor. In conclusion tobacco and male gender are associated with etiology of this disease and our data provide evidence that supports an association between the *RFC1* A80G polymorphism and head and neck squamous cell carcinoma risk, male gender, alcohol non consumption and age over 50 years. However, further studies of folate and plasma concentrations may contribute to the better understanding of the factors involved in the head and neck squamous cell carcinoma etiology.

Keywords Head and neck squamous cell carcinoma · Polymorphism · Folate metabolism · *RFC1* gene

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Introduction

Head and neck squamous cell carcinoma (HNSCC) includes malignant tumors site in the upper aero-digestive tract in oral cavity, larynx and pharynx. It is the fifth most common cancer worldwide with an estimation of 50,0000 new cases diagnosed annually [1–3]. In Brazil, the estimate of new cases of oral cavity for 2010 is 10,330 for men and 3,790 for women [4].

Tobacco and alcohol are the most common etiological factors. Human papilloma virus oral infection (HPV), genetic factors as family history and, polymorphisms in genes that codify enzymes involved on folate metabolism and polymorphisms in repair and cell cycle genes are also risk factors for this disease [3, 5–12].

Studies about genetic variants in enzymes involved in folate pathway suggest that the result of folate abnormal metabolism, a cofactor for the methylation and synthesis of

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nucleic acids, may be a cancer etiologic factor associated to DNA hypomethylation and consequently increase genome instability and mutation rates, DNA frequent breaks in cycle repair, thus potentially leading to an increase in cancer risk [5, 13–17].

The reduced folate carrier 1 gene (*RFC1*) (OMIM 600424) located on chromosome 21q22.3 is involved in folate intracellular transport. It participates in the process of folate absorption and on the transport of 5-MTHFR to the interior of a variety of cells, being an important determinant of folate intracellularly concentrations [18]. It is polymorphic in exon 2, with substitution of adenine for guanine at nucleotide 80 (A80G) (rs1051266) and although the exact functional relevance of this variant is unknown, RFC1 is one of the key enzymes in folate metabolism pathway, it is biologically plausible that this variant genotype may be involved in carcinogenesis by affecting plasma folate and homocysteine (Hcy) levels, which is associated with DNA methylation, DNA repair capacity and cancer susceptibility [18–22].

Studies have found association of this polymorphism and several types of cancer. Wang et al. [23] studied 633 patients with gastric cancer, 216 patients with oesophageal cancer and 673 cancer-free population controls and found significantly increased risk of both oesophageal cancer and gastric cancer associated with the *RFC1* 80AA homozygous, and these significant associations were more evident among women, older subjects, non-smokers and non-drinkers. The study of Di et al. [24] with 107 cases with cervical cancer and 107 controls with hysteromyoma (a benign tumour educing in a uterus muscular coat—myometriums) that were matched with age and risk factors showed that *RFC1* A80G polymorphism can increase the risk of cervical cancer. De Jonge et al. [25] that studied 245 pediatric acute lymphoblastic leukemia patients (cases) and 500 blood bank donors individuals (controls) concluded that *RFC1* AA genotype is associated with acute lymphoblastic leukemia increased risk.

However, the study of Eklof et al. [19] in 220 individuals with colorectal cancer and 414 healthy controls provided evidence that although *RFC1* 80A-allele may influence folate status, it is not likely to have a major independent role in the development of colorectal cancer. Kotsopoulos et al. [26] which studied 1,009 breast cancer patients and 907 healthy controls also did not found any association of this polymorphism and the risk of breast cancer.

This *RFC1* A80G polymorphism has not yet investigated in HNSCC risk and based on the present evidences and due to the lack of study, the aim of this study was to verify the main risk factors for HNSCC, to compare the frequency of the *RFC1* A80G polymorphism in HNSCC patients with individuals without history of cancer, and to investigate the association of this polymorphism with risk factors and clinical histopathological parameters.

Patients and methods

Study subjects

After approval by the National Ethics Committee (CONEP—No. 5566/2005) and obtaining of the Consent Term, peripheral blood leukocyte samples were collected from 322 patients with HNSCC and 531 individuals without cancer (controls) (SISNEP 0976.0.140.000-05).

The control group included Brazilian blood donors without diagnoses of cancer according to government guidelines for donated blood that tests for 20 related diseases (http://www.hemonline.com.br/portarias/rdc153/indexframe.htm). Individuals with family history of cancer were excluded and individuals with age greater than 40 years were included in the study. Each eligible subject was interviewed to obtain data on age, gender, tobacco habits, alcohol use and family history of cancer.

The cancer cases were treated at the Hospital de Base, São José do Rio Preto, São Paulo, Brazil. Diagnosis was made from pathological specimens either after total excision or biopsy. Patients with squamous cell carcinoma tumor cell types were included and patients previously treated for tumors were excluded. The primary anatomic sites were subdivided as oral cavity ($n = 129$–40%), pharynx ($n = 81$–25%), larynx ($n = 97$–30%) and 15 (05%) patients of unknown primary site of tumor. Demographic, lifestyle, clinical and clinical histopathological parameters were obtained from patient medical records. The average survival was 31.7 (±27.1) months. Fifth-seven (17.7%) patients presented tumor recurrence in a mean time of 20.7 months.

The tumors were staged according to TNM classification following three criteria: extension of the tumor (T), presence of regional lymph node involvement (N) and presence of metastasis at a distance (M) [27]. The clinical stage (TNM) was used to analyze aggressiveness being the tumors classified as non-aggressive (Stages I and II) and aggressive (Stages III and IV).

The variables analyzed were gender, exposure to risk factors (tobacco and alcohol consumption), primary site of occurrence, aggressiveness, extension of the tumor and lymph node involvement. Individuals who had smoked more than 100 cigarettes in their lifetime were considered smokers. Individuals who drank four doses of alcohol per week were considered alcohol consumers [28, 29].

Genotyping of RFC1 A80G

Genomic DNA was obtained from peripheral blood following a modified technique of Miller and collaborators [30]. Molecular analysis of the *RFC1* A80G polymorphism was performed by PCR-RFLP using primer sequences described by Födinger et al. [31] and *Hha*I enzyme was used to

recognize the polymorphic site. Amplification was obtained with initial denaturation at 94°C for 2 min, followed by 35 cycles of 1 min of DNA denaturation at 94°C, 1 min of primer annealing at 58°C and 1 min of extension at 72°C. A final extension of 10 min at 72°C was carried out. The product of 229 base pairs (bp) was submitted to digestion with *Hha*I enzyme, for 3 h at 37°C according to the manufacturer's guidelines. Fragments of 125, 68 and 37 bp were generated when the G polymorphic allele was present and 162 and 68 bp fragments were observed with the A allele. Heterozygous individuals presented 162, 125, 68 and 37 bp fragments.

Statistical analysis

Statistical analysis was performed using the Minitab software/Windows—Version 14.0 and Bioestat Program. The demographic and lifestyle data were analysed by multiple logistic regression. Chi-square test was conducted to examine whether the genotype frequency of the *RFC1* A80G was in Hardy–Weinberg equilibrium (HWE).

Multiple logistic regression models were used to determine the interaction effect between the genetic polymorphism and variables related to HNSCC carcinoma. The model included age (reference: <50 years; median), gender (reference: female), tobacco (reference: non-smokers), alcohol habits (reference: non-drinkers) and tobacco/alcohol together (reference: non-smokers and non-drinkers) using the Minitab for Windows computer program (Version 12.22). A P value ($P \leq 0.05$) was considered statistically significant. Results are shown as odds ratio (OR) and 95% confidence intervals (95% CI).

The clinical histopathological parameters were analyzed by multiple logistic regression. Tumor classification was divided into low T (T1, T2) and high T (T3, T4) classification categories. The N classification was dichotomized into no lymph node involvement (N0) and involvement (N1, N2, N3). Stage grouping was divided into early stage (Stages I and II) and advanced stage (Stages III and IV) categories.

Diseases-free was defined as the time from the date of diagnosis to the date of first local or distant recurrence or last contact. Overall survival analysis was defined as the time from the date of diagnosis to death if the patient died from HNSCC. Survival curves were plotted using Kaplan–Meier method and the differences between groups were calculated by the log-rank test.

Results

Gender and lifestyle factors

Statistical analysis for analyzed variables showed that male gender and tobacco consumption revealed significant statistically differences ($P < 0.05$) when comparing patients (86.9% male gender and 81.6% smokers) and controls (72.1% male gender and 40.4% smokers). Alcohol consumption did not show significant association with HNSCC, being that 69.2% of patients were alcohol consumers and 49.2% of controls were alcohol consumers (Table 1).

RFC1 genotype

The genotype distribution of the polymorphism studied showed deviation from Hardy–Weinberg equilibrium (HWE) for patients (χ^2: 7.15, $P = 0.007$) and controls (χ^2: 13.18, $P = 0.0003$).

The allele and genotype frequencies of the *RFC1* gene of HNSCC patients and of the control group are show in Table 2. According to multiple logistic regression test, there was no significant statistically difference in the allele distribution of the *RFC1* A80G polymorphism between controls and HNSCC patients. However differences in genotype frequencies between patients and controls were significant when was compared AA genotype with AG or GG genotypes (at least one polymorphic allele) (OR = 1.47; 95% CI = 1.02–2.12; $P = 0.04$) being that 71% patients and 65% control subjects had at least one polymorphic allele (AG or GG genotypes).

The interaction between the distribution of the *RFC1* genotype and exposure to risk factors for head and neck cancer are shown in Table 3 with significant statistically difference between the *RFC1* 80AG or *RFC1* 80GG genotypes (at least one polymorphic allele) and age >50 years (OR = 1.93; 95% CI = 1.22–3.03; $P = 0.005$), alcohol non-consumption (OR = 1.78; 95% CI = 0.99–3.21; $P = 0.05$) and male gender (OR = 1.75; 95% CI = 1.15–2.66; $P = 0.009$) when was used *RFC1* 80AA genotype (wild type genotype) as reference by multiple logistic regression. For analyses of alcohol and tobacco consumers our results did not found association between these combined factors and the *RFC1* A80G polymorphism (alcohol and tobacco habits: OR = 1.23; 95% CI = 0.74–2.03; $P = 0.428$).

Clinical histopathological parameters

The association between of clinical histopathological parameters of HNSCC patients and *RFC1* genotypes are shown in Table 4. Only patients with complete pathological data were considered for this analysis. The majority of patients who had the GG genotype or at least one polymorphic allele showed less advanced stage of tumor extension and lymph node involvement (72.8 and 68.5%, respectively). The site of tumor more prevalent was oral cavity (42.3%) with 28.9% of all patients with *RFC1* GG genotype or with at least one polymorphic allele. The

Table 1 Distribution in odds ratio (OR) of the gender and risk factors between head and neck squamous cell carcinoma patients and controls

Variables	Patients ($n = 323$) n (%)	Controls ($n = 531$) n (%)	OR (95% CI)	P value
Gender				
Female	43 (13.3)	147 (27.7)	1.00 (ref)	
Male	280 (86.7)	384 (72.3)	2.49 (1.72–3.62)	**<0.05**
Tobacco consumption				
Non-smokers	60 (18.6)	316 (59.5)	1.00 (ref)	
Smokers	263 (81.4)	215 (40.5)	4.07 (2.75–6.03)	**<0.05**
Alcohol consumption				
Alcohol non-consumers	100 (31)	269 (50.6)	1.00 (ref)	
Alcohol consumers	223 (69)	262 (49.4)	1.24 (0.84–1.83)	0.283

The tobacco habit was defined as the consumption of more than 100 cigarrettes in their life time. The drinking habit was defined as the consumption of an average of four drinks a week

Multiple logistic regression, $P \leq 0.05$ was considered significant

OR adjusted for gender, tobacco and alcohol habits and *RFC1* A80G polymorphism. There was difference statistically significant for age >50 years, male gender, and tobacco consumption

Table 2 Distribution of the *RFC1* A80G polymorphism between HNSCC patients and controls

RFC1 A80G polymorphism	Patients n (%)	Controls n (%)	OR (95% CI)	P value
Genotypes				
AA	93 (28.8)	184 (34.6)	1.00 (ref)	
AG	137 (42.5)	221 (41.6)	1.47 (1.02–2.12)	**<0.05**
GG	92 (28.7)	126 (23.8)		
Alleles frequencies				
A allele	0.51	0.55	1.00 (ref)	
G allele	0.49	0.45	1.31 (0.99–1.73)	0.06

RFC1 - Folate carrier 1, *AA*: wild type homozygous; *AG*: heterozygous or *GG*: variant genotype

Multiple logistic regression; $P \leq 0.05$ was considered significant

OR adjusted for age, gender, tobacco and alcohol habits. The genotypes was calculated for polymorphic homozygous individuals or carrying risk allele heterozygous vs. wild type homozygous. There was difference statistically significant for AG/GG genotypes

analysis of metastasis classification was not performed since all patients were classified as M0.

The Kaplan–Meier survival curves by genotype are presented in Fig. 1a, and did not demonstrate any association between polymorphisms and overall survival ($P = 0.26$). Moreover, no association was observed for the time of recurrence of the disease and genotype ($P = 0.50$; Fig. 1b).

Discussion

Our results indicate that tobacco is risk factor for HNSCC development. Stratification of cases and controls in tobacco consumers and tobacco non-consumers reveals a clear trend in risk increase, according to the literature data [10, 32]. Alcohol consumption did not show significant association with this disease.

Despite literature data show that the most important predisposing factors for in the development of this disease are alcohol and tobacco consumption [3], studies in animal models showed that alcohol consumption have no direct carcinogenic effect and it is not genotoxic [33]. Moreover, the study of Ragin et al. [34] showed that tobacco habit is a strong risk factor for head and neck cancer independent of alcohol consumption.

Additionally, our results show significant association between male gender and this disease. Studies show that male gender remains the most affected by this of tumor type, with higher proportions about the female gender [35, 36]. Nevertheless, while the HNSCC incidence is much higher in males, more and more females are HNSCC developing as

Table 3 Odds ratio of head and neck cancer related to *RFC1* genotypes by age, gender, tobacco habits and alcohol habits

Variables	AA genotype (cases/controls)	OR (95% CI)	AG or GG genotype (cases/controls)	OR (95% CI)[a]	*P* value
Age					
<50	21/120	1.00 (ref)	41/253	0.88 (0.48–1.60)	0.666
>50	73/184	1.00 (ref)	188/347	1.93 (1.22–3.03)	**<0.05**
Gender					
Female	17/51	1.00 (ref)	26/96	0.80 (0.37–1.74)	0.572
Male	77/133	1.00 (ref)	203/251	1.75 (1.15–2.66)	**<0.05**
Tobacco habits					
No	14/109	1.00 (ref)	46/207	1.67 (0.83–3.38)	0.152
Yes	75/80	1.00 (ref)	183/140	1.40 (0.90–2.16)	0.133
Alcohol habits					
No	26/93	1.00 (ref)	74/176	1.78 (0.99–3.21)	**0.05**
Yes	68/91		155/171	1.32 (0.82–2.13)	0.252
Alcohol and tobacco habits					
No	12/64	1.00 (ref)	35/123	1.54 (0.71–3.37)	0.277
Yes	66/46		144/87	1.23 (0.74–2.03)	0.428

RFC1 - Folate carrier 1, *AA*: wild type homozygous; *AG*: heterozygous or *GG*: variant genotype

Multiple logistic regression; $P \leq 0.05$ was considered significant

[a] OR adjusted for age, gender, tobacco and alcohol habits. *RFC1* AG or GG genotype compared with variables—Reference: *RFC1* AA. There were significant statistically difference for *RFC1* GG or AG genotypes (at least one polymorphic allele) and age >50 years, alcohol non-consumers and male gender

Table 4 Distribution of the clinical histopathological parameters and *RFC1* polymorphism

Variables	AA genotype	OR (95% CI)	AG or GG genotype	OR (95% CI)	*P* value
Site of tumor					
Oral cavity	41	1.00 (ref)	89	1.24 (0.76–2.02)	0.388
Pharynx	25	1.00 (ref)	55	1.16 (0.67–2.02)	0.592
Larynx	22	1.00 (ref)	75	0.54 (0.37–1.11)	0.102
Tumor extension					
T1/T2	44	1.00 (ref)	118	1.00 (ref)	
T3/T4	49	1.00 (ref)	105	0.80 (0.49–1.30)	0.364
N involvement					
No	63	1.00 (ref)	137	1.00 (ref)	
Yes	24	1.00 (ref)	46	1.35 (0.81–2.24)	0.241
Aggressiveness					
No	39	1.00 (ref)	89	1.00 (ref)	
Yes	25	1.00 (ref)	59	0.93 (0.60–1.46)	0.767

RFC1 - Folate carrier 1, *AA*: wild type homozygous; *AG*: heterozygous and *GG*: variant genotype

Multiple logistic regression; $P \leq 0.05$ was considered significant

The analysis was made to patients with complete data. *RFC1* AG or GG genotype compared with clinical histopathological parameters—Reference: *RFC1* AA. OR adjusted for age, gender, tobacco and alcohol habits. There was no difference statistically significant

women acquires a male habit of alcohol and tobacco consumption [37].

According to HWE analysis our study showed that *RFC1* gene is not in equilibrium. The deviation from the HWE equilibrium may result from the random selection of the studied individuals, the disease model adopted, and evolutionary factors which can influence changes in the genotype frequencies [38, 39]. On the other hand, this disequilibrium should be expected, considering that it reflects biologic and genetic characteristics in complex disease models [40].

To the better of our knowledge, this is the first molecular epidemiological study of *RFC1* A80G polymorphism in HNSCC and our results showed significant statistically

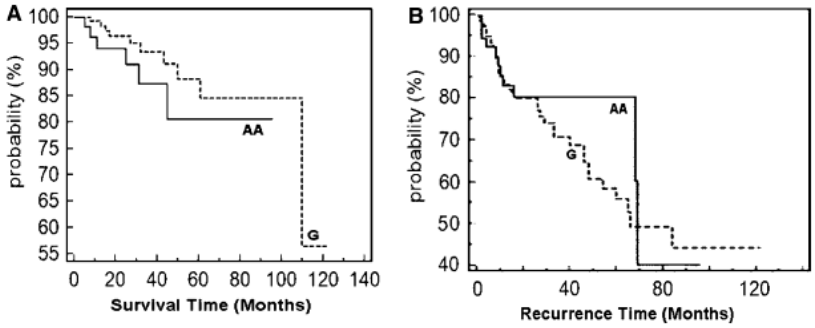

Fig. 1 Kaplan–Meier curves for the overall survival (P value = 0.26) (**a**) and recurrence time (P value = 0.50) (**b**) for patients according to the *RFC1* A80G polymorphism

differences between groups for genotype distributions, being that 71% of patients and 65% of control subjects had at least one polymorphic allele (AG or GG genotypes). The multivariate analysis adjusted for age, gender, tobacco and alcohol habits showed that individuals that had at least one polymorphic allele (*RFC1* 80AG heterozygous or *RFC1* 80GG polymorphic homozygous genotypes) are associated with increased risk for the disease with OR of 1.47 (95% CI = 1.02–2.12; $P < 0.05$). Studies of Wang et al. [23], Di et al. [24] and De Jonge et al. [25] also found association between this polymorphism and gastroesophageal cancer, cervical cancer and pediatric acute lymphoblastic leukemia respectively.

The study in gastroesophageal cancer [23] showed that *RFC1* 80AA was associated with a significantly increased risk for this disease with OR of 1.63 (95% CI = 1.30–2.04) similar to the findings of De Jonge et al. [25] that found increased risk for pediatric acute lymphoblastic leukemia 1.5 times (95% CI = 1.10–2.10) and 2.1 times (95% CI: 1.30–3.20) in A-allelic carriers and 80AA homozygous respectively while the study of Di et al. [24] showed that cervical cancer patients with polymorphic genotype (*RFC1* 80GG) had higher risk for cervical cancer with OR of 2.42 (95% CI: 1.01–5.81) when compared with individuals wild-type, according to our study.

The studies of Eklof et al. [19] and Kotsopoulos et al. [26] did not found association between *RFC1* A80AG polymorphism and colorectal cancer and breast cancer respectively, however Eklof et al. [19] showed that subjects with the *RFC1* 80AA genotype in combination with low plasma folate concentrations or *MTHFR* 677TT genotype had a reduced risk of colorectal cancer but it is not likely to have a major independent role in the development of colorectal cancer.

The polymorphism *RFC1* A80G also was associated with others diseases as congenital heart defects (CHD) and neonatal neural tube defects (NTD). The study of Pei et al. [41] analyzed 82 families with a child affected by cleft lip with or without cleft palate (CLP), 67 families with a child-affected by CHD, and 100 nonmalformed control families and the results suggest that the RFC1 G allele is probably an important candidate gene in folate transport and is associated with risk for CHD. In 2008, Shang et al. [42] analyzed the genotypic distributions and allele frequencies of *RFC1* A80G polymorphism from mothers with at least one previous child with NTDs showed that mothers with *RFC1* 80GG homozygous genotype presented an increased risk for neural tube defects. Biselli et al. [43] that studied 67 mothers of down-syndrome (DS) individuals with free trisomy 21 and 113 control mothers did not found association between the *RFC1* A80G polymorphism and the maternal risk of DS in the sample evaluated.

In our study, there was a significant interaction between the *RFC1* A80G polymorphism and age >50 years, male gender and alcohol non-consumption, suggesting that men with no-habit of alcohol consumption with genotypes AG or GG have increased risk of head and neck cancer compared with AA genotype. However, the study in esophageal and gastric cancer showed that *RFC1* 80AA genotype was more evident among women, older subjects, non-smokers and non-drinkers compared with AG or GG genotypes [23]. The study of Eklof et al. [19] in colorectal cancer showed no association between the polymorphism and the variables (Age, gender, tobacco and alcohol habits).

Tobacco and alcohol were analyzed together because these two factors when combined increase HNSCC risk [44], but we did not found association these factors together. In relation to the association between the polymophism and alchohol comsuption, further studies are necessary for the better understanding of the role of this polymorphism.

The Hcy and folate concentration was not measured in our study, but literature data shows differences between these concentrations and polymorphism *RFC1* A80G. One study showed that individuals with colorectal cancer carrying *RFC1* 80A allele had reduced plasma folate and elevated plasma total Hcy concentrations, but the result was statistically significant only for folate [19]. DeVos et al. [20] in a cohort study of 991 Puerto Rican individuals, confirmed that homozygous polymorphic (*RFC1* 80GG) have approximately 7% lower plasma total

homocysteine concentrations than AA and AG genotypes and plasma folate was unchanged. The study of Stanisławska-Sachadyn et al. [22] in men and women of Northern Ireland demonstrated that Northern Irish women with A allele have high folate concentrations and 80GG genotype is associated with relatively low folate concentrations in women.

In the clinical histopathological parameters analysis, our results did not show significant association of tumor extension, lymph node involvement, tumor aggressiveness and location of the primary site with the *RFC1* A80G polymorphism. During a review of the literature, it was not found publications about clinical histopathological parameters of this polymorphism and the risk cancer.

In our study, the primary site most prevalent was oral cavity (42.3%). These anatomical sites of primary tumors may vary according to the tobacco and alcohol consumption. Non-alcoholic and non-smokers usually have oral cavity primary site more prevalent and individuals who possess these two changes tend to have the larynx and pharynx as the most prevalent primary anatomical sites [8, 45] but in our study only 4.7% of HNSCC patients that had oral cavity as primary site were non-alcoholic and non-smokers.

The overall survival and disease recurrence were not associated with polymorphism. Despite the lack of studies of these features and *RFC1* A80G polymorphism, data show that the overall survival beyond to be low, did not increased significantly in recent years [46–48].

In conclusion, our results suggest that tobacco and male gender and age over 50 years are associated with HNSCC risk. The *RFC1* A80G polymorphism may be functionally relevant as evidenced by their associations with risk of HNSCC developing in Brazilian population. Moreover, alcohol non-consumers males with age over 50 years with *RFC1* 80AG or 80GG genotypes were associated with increased risk for this disease. Therefore, further studies about dietary folate intake and other potential exposure variables are needed to evaluate gene-environment or gene-nutrient interactions in larger researches in different populations.

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ARTIGO CIENTÍFICO 3

Artigo 3

Título: *CBS* 844ins68 polymorphism and head and neck squamous cell carcinoma risk: A case-control Analysis.

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CBS 844ins68 polymorphism and head and neck squamous cell carcinoma risk: A case-control Analysis

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Abstract

Introduction: Susceptibility to head and neck squamous cell carcinoma may be modified by functional polymorphisms in genes involved in folate pathway, such as cysthationine-beta-synthase (*CBS*). The *CBS* 844ins68 polymorphism lead to Hcy decreased concentrations, consequently low availability of S-adenosylmethionine (SAdoMet), the main methyl donor for methylation reactions. It is associated with DNA methylation changes and cancer development. This paper aims to investigate to investigate the association of *CBS* 844ins68 polymorphism with head and neck squamous cell carcinoma risk and with risk factors and clinical histopathological parameters of this disease.

Material and Methods. A case-control retrospective study was conducted in 322 head and neck squamous cell carcinoma patients and in 531 control subjects without diagnoses of cancer. The PCR technique was used to polymorphism genotyping. For statistical analysis, there were used the chi-square and Multiple logistic regression test.

Results. No significant diference in *CBS* 844ins68 genotype distribution was observed between groups. Age >50 years, male gender and tobacco consumption were predictors of the disease with increased risk of 7.89 (95%CI: 5.56-11.21), 2.49 (95%CI: 1,72-3,62), 6.44 (95%CI: 4,63-8,96) and 2.29 times (95%CI: 1,71-3,06) respectively. There were not association between the distribution of the *CBS* 844ins68 genotype and risk factors for this disease. For clinical histopathological parameters, *CBS* 884ins68 polymorphism presented high frequency in oral cavity ($p<0.05$).

Conclusion. We conclude that the *CBS* 844ins68 polymorphism is not associated with HNSCC risk and there is increased risk of this disease in men smokers with age over 50 years.

Key words: Genetic polymorphism, head and neck neoplasms, folate, metabolism, genes.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the fifth most common cancer worldwide and the most common neoplasm in the upper aerodigestive. In Brazil, expects 14.120 new cases of oral cancer for 2010, being 3.790 in women and 10.330 in men [1]. Despite advances in conventional therapies, including surgery, radiation and chemotherapy, the overall survival (OS) rate for HNSCC has not significantly improved in the past 3 decades [2].

The HNSCC anatomical region includes tumors of oral cavity, pharynx and larynx and the most common histological type is squamous cell carcinoma, present in 95% of cases.[2,3] Alcohol and tobacco use are commons factors for HNSCC, additionally evidence is accumulating for a role of folate in cancer. Studies to show the relationship between polymorphisms of genes involved in the folate metabolism and the HNSCC risk because to their influence on methylation and synthesis DNA [2, 4-11].

Methylation is responsible for gene expression control, structure chromatin stability and the maintenance of genomic stability. To regulate the synthesis, methylation and DNA repair is essential for folate, which is the methyl donor in reactions cellular methylation, is present in the body in adequate amounts, which does not occur in the presence of polymorphism in this pathway [5,12-14].

The *CBS* gene encodes the cystathionine beta synthase (*CBS*), involved in the

folate pathway, which is central enzyme in the transsulfuration pathway that irreversibly metabolizes homocysteine (removes homocysteine from the methionine) to cystathionine. It presents polymorphic in nucleotide 844 - exon 8 (*CBS*844ins68) with an insertion of 68 base pairs. Although the biologic impact of this polymorphism remains unclear, it seems to be associated with reduction of homocysteine levels and changes in DNA methylation because the low availability of S-adenosylmethionine (SAdoMet), the main methyl donor for methylation reactions and consequently may occur DNA hypomethylation and carcinogenesis [15-17].

Four authors groups studied the association about *CBS*844ins68 polymorphism and cancer etiology and these only one showed that *CBS*844ins68 variant allele may to be protective against colorectal cancer, but this association occurs together with other polymorphism of folate pathway [18]. However, the study of Pufulete *et al* (2003) [19] did not found association in colorectal cancer. Other studies also did not confirm association between the polymorphism and carcinomas of the upper gastrointestinal tract [20] and prostate cancer [21].

Concerning possible association between head and neck squamous cell carcinoma and *CBS* 844ins68 polymorphism have not been tested until now, we have conducted this case-control study in 853 individuals for investigate the association between *CBS* 844ins68 polymorphism and HNSCC etiology. Therefore, this study had the objectives to investigate the frequency of the *CBS* 844ins68 in head and neck squamous cell carcinoma patients and to compare with individuals with no history of cancer and to evaluate the association of the polymorphism with risk factors (tobacco and alcohol habits) and clinical histopathological parameters.

PATIENTS AND METHODS

Study Subjects

The study protocol was approved by the National Ethics Committee (CONEP - 5566/2005; SISNEP 0976.0.140.000-05).

The retrospective study population included eight hundred fifty-three (322 patients and 531 controls) with a mean age of 52.5 ± 13.7 individuals. The case group (86.7% men and 13.3% women) were treated at the Hospital de Base, São José do Rio Preto, São Paulo, Brazil – Public Institution. Diagnosis was made from pathological specimens either after total excision or biopsy. Patients with squamous cell carcinoma tumor cell types were included and patients previously treated for tumors were excluded.

The tumors were staged according to TNM classification following three criteria: extension of the tumor (T), presence of regional lymph node involvement (N) and presence of metastasis at a distance (M).[22] The clinical stage (TNM) was used to analyze aggressiveness with tumors being grouped as non-aggressive (Stage I and II) and aggressive (Stage III and IV). The average survival was 31.7 (± 27.1) months. Demographic data, lifestyle, clinical histopathological parameters were obtained from patient medical records.

The control group (72.3% men and 27.7% women) included was Brazilian blood donors without diagnoses of cancer according to government guidelines for donated blood that tests for 20 related diseases (http://www.hemonline.com.br/portarias/rdc153/indexframe.htm). Individuals with family history of cancer were excluded and individuals with age greater than 40 years

were included this study. Each eligible subject was interviewed to obtain data on age, gender, smoking habits, use of alcohol and family history of cancer.

The variables analyzed were gender, exposure to risk factors (tobacco and alcohol consumption), primary site of occurrence, aggressiveness, extension of the tumor and lymph node involvement. Individuals who had smoked more than 100 cigarettes in their lifetime were considered smokers. Individuals who drank four doses of alcohol per week were considered alcohol consumers [23,24].

Genotyping of CBS 844ins68

To determine the individual genotypes, genomic DNA was obtained from peripheral blood following technique of Miller and collaborators.[25] Molecular analysis of the *CBS 844ins68* polymorphism was performed by PCR technique (Polymerase chain reaction) by difference in size of amplification products, using primer sequences described by Dutta and collaborators (2005) [26].

Amplification was obtained with initial denaturation at 94ºC for 4 minutes, followed by 30 cycles of 1 minute of DNA denaturation at 94°C, 1 minute of primer annealing at 62ºC and 1 minute of extension at 72°C. A final extension of 5 minutes at 72°C was carried out. The PCR products were run onto 1.5% agarose gel, stained with ethidium bromide and visualized under UV illumination. The CBS gene thus included (I) or lacked (N) of 68 bp insertion at exon 8. The major allele (I) showed as a 239 bp product and normal allele showed as a 171 bp product. Fragments sizes were estimated by comparing with the 100 bp DNA size marker.

Statistical Analysis

Statistical analysis was performed using the Minitab software / Windows - Version 14.0 and Bioestat Program. Chi-square tests were conducted to examine whether the genotype frequency of the *CBS* 844ins68 was in Hardy-Weinberg equilibrium (HWE).

Differences in gender (reference: female), tobacco (reference: non-smokers) and alcohol habits (reference: non-drinkers) between the cases and controls were evaluated using Multiple logistic regression analysis. This model also was used to determine the interaction effect between the genetic polymorphism and variables related to head and neck squamous cell carcinoma.

The clinical histopathological parameters also were analyzed by binary logistic regression. Tumor classification was divided into low T (T1, T2) and high T (T3, T4) classification categories. The N classification was dichotomized into no lymph node involvement (N0) and involvement (N1, N2, N3). Stage grouping was divided into early stage (Stage I and II) and advanced stage (Stage III and IV) categories. A p-value < 0.05 was considered statistically significant. Results are shown as odds ratio (OR) and 95% confidence intervals (95% CI).

The Kaplan-Meier method was used to evaluate survival rates and time of disease recurrence. The Log-rank test was used to assess differences related to the different genotypes.

RESULTS

Demographic data and lifestyle factors

The case group with a mean age of 58.4 (9.9) years there were predominance of tobacco (80.7%) and alcohol (69.2%) consumers. The control group had a mean age of 47.4 (13.1) years, 40.4% tobacco consumers and 49.2% alcohol consumers.

As matching demographic data and risk factors between patients with cancer and control individuals was not possible, multivariable analysis was performed to adjust these variables. There were statistically significant differences between patients and controls for age >50 years (OR=7.89; 95%IC=5.56-11.21; $p<0.05$), male gender (OR=1.05; 95%CI=1.05-2.67; $p<0.05$) and tobacco habits (OR=4.09; 95%CI=2.77-6.03; $p<0.05$).

CBS genotype

The Hardy-Weinberg Equilibrium showed that the genotypic distributions were not expected both groups and are not in equilibrium (Case: $\chi 2$: 4.98, $p = 0.02$ and control: $\chi 2$: 8.05, $p = 0.004$).

The genotypic and allelic distributions of the *CBS* 844ins68 polymorphism were compared between groups and did not show statistically significant differences. Of 854 individuals studied, 702 (82.2%) being 443 controls and 259 patients did not have the polymorphism, 18 (2.1%) being 10 controls and 8 patients presented polymorphism *CBS* 844ins68 and 134 (15.7%) being 78 controls and 56 patients had the heterozygous genotype for this polymorphism (Table 1)

The potential interaction between the distribution of the *CBS* 844ins68 genotype and exposure to risk factors for head and neck squamous cell carcinoma are shown in Table 2, with no statistical difference.

Clinical histopathological parameters and *CBS* polymorphism

Only patients with complete pathological data were considered for this analysis. There were significant associations of individuals with IN and II genotypes (at least one 68p insertion allele) with oral cavity (OR=1.93; 95%CI=1.10-3.40; $p<0.05$). An analysis of metastasis classification was not performed since all patients were classified as M0. Regarding the primary site of tumor, 40% (n=129) of patients had as primary site of tumor in the oral cavity, 25.1% (n=81) pharynx and 30.1% (n=97) larynx and 16 (05%) patients had unknown primary site of tumor the rest had unknown primary site (Table 3).

The Kaplan-Meier survival curves by genotype are presented in Figure 1 and there was association between polymorphism and survival time ($p = 0.02$) (Figure 1A) and did not demonstrate any association between polymorphism and time of recurrence of the disease (Figure 1B - $p= 0.52$).

DISCUSSION

The results show that the HNSCC is more common in smokers men with age over 50 years. Previous studies to show that male gender, alcohol and tobacco consumption are the most important predisposing factors for this disease [2,27-29].

However in our study alcohol consumption was not associated with HNSCC. A multicenter study confirmed that tobacco is a strong risk factor for HNSCC independent of alcohol consumption [30] and studies in animal models showed that alcohol not have direct carcinogenic effect and it is not genotoxic. However, this agent suppresses the removal of nitrosamines molecules of molecular weight low released by the tobacco in the liver through inhibition of multiple isoforms of cytochrome P450 superfamily. Thus, there is an increase of nitrosamines to the post-hepatic tissues and an increase in the formation of DNA adducts [31-33].

The male gender remains the most affected by this tumor type, with higher proportions about the female gender [27,29] as shown our findings. However more and more females are HNSCC developing as women adopt the male pattern of alcohol and tobacco consumption. [2]

According to Hardy-Weinberg equilibrium (HWE) analysis our study showed *CBS* gene is not in HWE. The departure from the HWE equilibrium may result from the random selection of the studied individuals, the disease model adopted, and evolutionary factors which can influence changes in the genotype frequencies [34,35]. On the other hand, this disequilibrium should be expected, considering that it reflects biologic and genetic characteristics in complex disease models [36].

In our case–control study *CBS* 68 bp insertion alelle (I) was not significant statistically with HNSCC risk (OR=1.20; 95%CI= 0.85-1.71; p=0.30) nor for heterozygous genotype (I/N) or polymorphic homozygous (I/I) with OR of 1.15 (95%CI= 0.74-1.79; p=53). We not found evidence that the *CBS* 844ins68

polymorphism may contribute to the individual risk for the development of head and

neck squamous cell carcinoma according with studies of Kimura *et al* (2000) [21] and Ott *et al* (2008) [20] in prostate cancer and the upper gastrointestinal tract respectively .

Ott *et al* (2008) [20] investigated the insertion of 68 bp in the CBS gene with susceptibility to carcinomas of the upper gastrointestinal tract. They studied 263 patients with esophageal cancer, 89 patients with barrett's esophagus-associated esophageal adenocarcinoma, 144 with cardiac carcinoma, 221 with gastric cancer and 257 healthy subjects and did not found association of these neoplasias, as in the case-control study of Kimura *et al* (2000) [21] in 132 patients with prostate cancer and 150 individuals without cancer and the study of Pufullete *et al* (2003) [19] that investigated thirty-five patients with adenoma, 28 with colorectal cancer patients and 76 controls.

Only study of Le Marchand *et al* (2002) [18] found association of *CBS* 844ins68 and cancer etiology. They investigated 727 colorectal cases of Japanese, caucasian, or Native Hawaiian and 727 controls without neoplasia matched on sex, age, and ethnicitys and showed that *CBS* 844ins68 variant allele may to be weakly protective against colorectal cancer, but this effect occurs only if this variant act together with the T allele (variant) of *MTHFR* C677T polymorphism. There was a suggestion that, in the presence of the *CBS* insertion polymorphism, the protective effect of the *MTHFR* 677T allele was stronger; however the risk estimates were highly variable due to the rarity of the *CBS* insert.

Our results for potential interaction between the distribution of the *CBS* 844ins68 genotype and exposure to risk factors for head and neck squamous cell carcinoma to showed any association significant statistically. Kimura *et al* (2000) [21] showed that the insertion allele was slightly more prevalent among females in homozygous or heterozygous form for control group and the polymorphic allele was

rarer in the older prostate cancer patient group but these differences were not statistically significant.

This polymorphism reside in key enzyme of one-carbon metabolism pathway and may result in aberrant DNA synthesis and this may lead uncontrolled growth but none study has significant results for *CBS* 844ins68 polymorphism and cancer risk[15-17]. For our knowledge, this is first study of *CBS* 844ins68 and HNSCC risk and despite a study to show association of the *CBS* polymorphism with etiology cancer, other studies to show association this polymorphism with other diseases as schizophrenia [37], neural tube defects [38], Alzheimer [39] and coronary artery disease [40].

The *CBS* 844ins68 polymorphism was first reported in a homocystinuric patient by Sebastio *et al* (1995) [41], it was initially thought to mandate the use of an insertion-associated premature stop codon in the CBS mRNA leading to the translation of a truncated inactive enzyme. Subsequently Tsai *et al* (1996) [15] showed that the 68 bp insertion generates an alternative splice site that permits the elimination of the entire inserted region, thereby allowing the formation of a normal mRNA transcript and a fully functional CBS enzyme. In 1998, De Stefano *et al* (1998) [42] reported that MTHFR 677TT homozygous who carry a CBS 844ins68 allele had lower homocysteine levels than noncarriers; however, folate levels were not presented in this report. More recently Dekou *et al* (2001) [43] also reported that the CBS 844ins68 allele appears to have a homocysteine lowering effect in *MTHFR* 677TT homozygous, but again no data were reported for the effect on folate levels.

In our study, it was possible to measure the homocysteine and folate concentration, but studies reported that individuals who have the insertion 68 bp present

had increased homocysteine and folate level compared with wild-type individuals [44,45].

For clinical histopathological parameters analyzed, our result suggests that the *CBS* –Insertion 68 bp allele was more frequent in patients with oral cavity as primary site. The tumor extension and lymph node involvement were not associated with the presence of polymorphism. This observation also not did found in prostate cancer [21].

The majority HNSCC patients showed less advanced stage classified as what T1/T2 and N0 (54,3% e 96%, respectively), is not in accordance with literature data, which shows a high frequency of head and neck squamous cell carcinoma in advanced stage (60% in the III e IV stage) [46]. However, in gastric and cardiac cancer category tumor size T1 and T2 was more prevalent, as in our study [20].

No data were found in the literature assessing survival according to the *CBS* 844ins68 polymorphism in patients with HNSCC. According to Esher *et al* (2008) [47], patients with HNSCC have a low survival rate among all cancers in spite of new surgical techniques, radiotherapy and concomitant chemotherapy, there not significant increase in survival rate [48-50].

CONCLUSION

Male gender and tobacco consumption are associated with HNSCC risk and there is no evidence of association between *CBS* polymorphism and head and neck carcinogenesis risk. Further studies in larger populations are required to better understanding this polymorphism.

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Table 1. Distribution of the *CBS* 844ins68 polymorphism between HNSCC patients and controls.

***CBS* 844ins68 polymorphism**	**Patients n (%)**	**Controls n (%)**	**OR(IC 95%)**	**P Value**
Genotypes				
NN (Non insertion)	258(80)	443 (83,4)	1.00 (ref)	
IN (Heterozygous)	56(17,5)	78(14,6)	1.15 (0.74 – 1.79)	0.53
II (Polymorphic)	08(2,5)	10(02)		
Alleles				
Non insertion (N) 68bp	315(83.1)	521(82.8)	1.00 (ref)	
Insertion 68 bp(I)	64(16.9)	88(17.2)	1.20 (0.85-1.71)	0.30

NN- 68 bp Non-insertion / **IN-** Heterozygous - *CBS* 844ins68 / **II-** *CBS* 844ins68 polymorphic.

Ajusted for age, gender, tobacco and alcohol habits. The genotypes was calculated for polymorphic homozygous individuals or carrying risk allele heterozygous vs wild-type homozygous.

P< 0.05 was considered significant. There was no difference statistically significant (Multiple logistic regression).

Table 2. Odds Ratio of head and neck cancer related to *CBS* genotypes by age, gender, tobacco and alcohol habits.

Variables	NN Genotype (Case/Controls)	OR(95%CI)	IN and II Genotype (Cases/Controls)	OR (95% CI)*	P value
Age					
<50	53/313	1.00 (ref)	10/60	1.19 (0.55- 2.57)	0.66
>50	205/130	1.00 (ref)	54/28	1.18 (0.68- 2.03)	0.55
Gender					
Female	34/125	1.00 (ref)	08/22	1.34 (0.49- 3.65)	0.56
Male	224/318	1.00 (ref)	56/66	1.12 (0.68- 1.83)	0.65
Tobacco Habits					
No	47/261	1.00 (ref)	12/55	1.14 (0.52- 2.50	0.73
Yes	211/182	1.00 (ref)	52/33	1.14 (0.66-1.95	0.64
Alcohol Habits					
No	81/221	1.00 (ref)	18/48	1.10 (0.55- 2.19)	0.78
Yes	177/222		46/40	1.21 (0.67- 2.17)	0.53
Tobacco and Alcohol Habits					
No	38/151	1.00 (ref)	08/36	0.79 (0.31-1.98)	0.61
Yes	168/112		42/21	1.04 (0.56-1.93)	0.90

CBS cystathionine ß-synthase: NN- 68 bp Non-insertion / **IN-** Heterozygous - *CBS* 844ins68 / **II-** *CBS* 844ins68 polymorphic genotype. *Ajusted for age, gender, tobacco and alcohol habits. *CBS* IN and II genotype compared with variables - Reference: *CBS* NN.

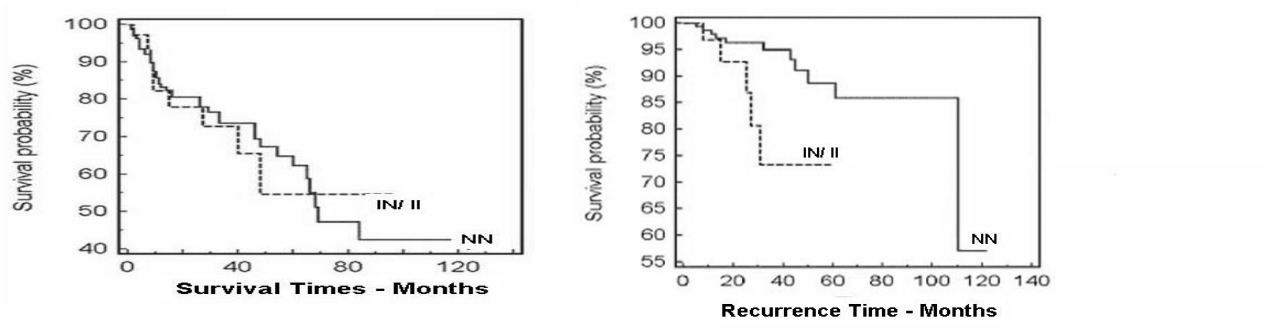
p<0.05 was considered significant. None of the differences between groups were statistically significant by multiple logistic regression.

Table 3. Distribution of the clinical histopathological parameters and *CBS* polymorphism*.

Variables	**NN Genotype**	**OR (95% CI)**	**IN/II Genotype**	**OR (95% CI)****	**P value**
Site of tumor					
Oral cavity	97	1.00 (ref)	33	193 (1.10-3.40)	**<0.05**
Pharynx	65	1.00 (ref)	15	0.92 (0.48- 1.76)	0.92
Larynx	85	1.00 (ref)	12	0.83 (0,50- 1,36)	0.45
Tumor extension					
T1/T2	106	1.00 (ref)	28	1.00 (ref)	
T3/T4	118	1.00 (ref)	29	0.48 (0.27- 0.86)	0.63
N involvement					
No	11	1.00 (ref)	01	1.00 (ref)	
Yes	213	1.00 (ref)	56	1.47 (0.37- 5.75)	0.58

CBS cystathionine ß-synthase: **NN-** 68 bp Non-insertion / **IN-** Heterozygous - *CBS* 844ins68 / **II-** *CBS* 844ins68 polymorphic genotype.

*The analysis was made to patients with complete data. *CBS* IN and II genotype compared with clinical histopathological parameters - Reference: *CBS* NN. p<0.05 was considered significant. There was difference statistically significant between oral cavity and *CBS* polymorphism. (Multiple logistic regression)



* NN- Non Insertion/ II – Polymorphic/ IN – Heterozygous)

Figure 1: Kaplan–Meier curves for the survival time (p=0.02) (A) and recurrence time (p=0.89) (B) for patients according to the *CBS 844ins68* polymorphism. There was statistical difference between the curve for subjects with at least one polymorphic allele (I or IN genotype) with survival time.

ARTIGO CIENTÍFICO 4

Artigo 4

Título: Polymorphisms and haplotypes in methylenetetrahydrofolate reductase gene and risk of head and neck squamous cell carcinoma.

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Polymorphisms and haplotypes in methylenetetrahydrofolate reductase gene and risk of head and neck squamous cell carcinoma.

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ABSTRACT

Purpose: Functional polymorphisms in genes encoding enzymes involved in folate metabolism might modulate head and neck carcinoma risk because folate participates in DNA methylation and synthesis. We therefore conducted a case-control study of 853 indiviuals (322 head and neck cancer cases and 531 non-cancer controls) to investigate associations among *MTHFR* C677T and *MTHFR* A1298C polymorphisms and head and neck squamous cell carcinoma risk. Interactions between these two polymorphisms and risk factors and clinical histopatological parameters were also evaluated. **Methods:** The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was used to genotype the polymorphisms and Chi-square test and multiple logistic regression were used for statistical analyses. **Results:** The variables age over 49 years, male gender, tobacco habits and alcohol consumption, *MTHFR* 1298 AC or CC genotypes, combined genotypes with two or more polymorphic alleles and 677T and 1298C polymorphic alleles were associated with increased risk for this disease ($p<0.05$). Furthermore, we found that 1298 AC or CC genotypes were associated with age over 49 years, tobacco and alcohol habits ($p<0.05$). Regarding clinical histopatological parameters, the A1298C polymorphism was more frequent in patients with oral cavity as primary site ($p<0.05$). **Conclusions:** *MTHFR* polymorphisms may contribute for increase risk for head and neck carcinoma and the variables age over 49 years, male gender, tobacco and alcohol habits were associated with *MTHFR* 1298AC or CC genotypes, confirming that individuals with these variables and *MTHFR* A1298C polymorphism has higher risk for this disease.

Key words: Head and neck squamous cell carcinoma, polymorphism, folate metabolism, MTHFR gene, haplotypes.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the fifth most common cancer worldwide (Marcu and Yeoh 2009) and in Brazil the estimative of new cases for oral cavity carcinoma for 2010 is 10.330 for men and 3.790 for women (INCA 2010). Alcohol and tobacco consumption are the predominant risk factors for HNSCC carcinogenesis. Alterations in folate pathway also may have play a role in the HNSCC risk (Suzuki *et al.* 2006; Sapkota *et al*. 2008; Garavelo *et al.* 2009; Marcu and Yeoh 2009).

Folate plays the fundamental role of providing methyl groups for de novo deoxynucleoside synthesis and for intracellular methylation reactions (James *et al.* 2003). Alterations in folate levels may cause DNA hypomethylation and thus to an increase cancer risk (Hoffman *et al.* 2005). However, although low folate has been shown as a risk factor for HNSCC (Almadori *et al.* 2005; Hsiung *et al*. 2007), the mechanism of this association has not been fully elucidated.

Polymorphisms in key enzymes in the folate metabolism pathway, such as methylenetetrahydrofolate reductase *(MTHFR*), were suggested to be associated with folate levels and DNA methylation and consequentely HNSCC development (Neumman *et al.*, 2005; Reljic *et al*., 2006; Vairaktaris *et al.*, 2006; Solomon *et al*., 2008). *MTHFR* catalyzes the conversion of 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-

MTHF, the primary circulating form of folate, thus playing a central role in balancing DNA synthesis (which involves 5,10-MTHF), and DNA methylation (which involves 5-MTHF). Two common polymorphisms associated with lower enzyme activity were described: C677T in exon 4 (rs1801133) and A1298C in exon 7 (rs1801131) (Weisberg *et al.,* 1998; Weisberg *et al*., 2002).

Studies on *MTHFR* C677T polymorphism showed that variant genotype (TT) is associated with decreased risk of development of esophageal cancer (Yang *et al.* 2005), lung cancer (Liu *et al.* 2009) and colorectal cancer (Fernández-Peralta *et al.* 2010, Levine et al. 2010), and whereas other authors found association with increased risk for cancer types different (Larsson *et al*. 2006; Wang *et al.* 2007; Lin *et al.* 2007; Hiyama *et al.* 2007, Li *et al*. 2008, Qin *et al*. 2008; Jin *et al.* 2009; Lagevin *et al.* 2009; Boccia *et al.* 2009, Cai *et al.* 2009, Qi *et al.* 2010, Naghibalhossaini *et al.* 2010), including HNSCC (Neumman *et al.* 2005; Vairaktaris *et al.* 2006; Reljic *et al*. 2007; Solomon *et al.* 2008). However other three studies in HNSCC not found association with *MTHFR* C677T polymorphism on risk this disease (Suzuki *et al.* 2007; Boccia *et al.* 2009; Kruszyna *et al.* 2010).

The *MTHFR* A1298C polymorphism was associated with increased risk of esophageal squamous cell carcinoma (Song *et al.* 2001), pancreatic cancer (Matsubayashi *et al.* 2005) and colorectal cancer (Levine *et al.* 2010). In contrast, this polymorphism was associated with decreased risk of HNSCC (Neumann *et al.* 2005). Other studies either not observe any significant association between *MTHFR* A1298C polymorphism and the risk of several cancer types (Miao *et al.,* 2002; Shen *et al.*,2005; Kim *et al.,* 2005, Li *et al*., 2005, Wang *et al.,* 2005, Larson *et al.,* 2006, Boccia *et al.,* 2009, Liu *et al.,* 2009, Cai *et al.,* 2009, Fernández-Peralta *et al.,* 2010;

Qi *et al.*, 2010), including HNSCC (Kruszyna *et al.*, 2010., Boccia *et al.*, 2009., Suzuki *et al.*, 2007).

In order to provide additional information on association between genetic polymorphisms of genes that encode enzymes envolved in folate metabolism and risk of HNSCC we investigated the association of the MTHFR C677T and A1298C polymorphisms and their haplotypes with HNSCC risk. Moreover, we determined if there is an association of these polymorphisms with variables such as age, gender, tobacco and alcohol habits related to HNSCC risk and clinical histopathological parameters.

PATIENTS AND METHODS

Study Subjects

The study comprised a series of 853 individuals being 322 patients with head and neck cancer (case group - 280 males; 42 females) and control samples from 531 healthy people that have never been diagnosed with head and neck tumor or other tumors (control group - 384 males; 147 females). The study protocol was approved by the National Ethics Committee (CONEP-5566/2005; SISNEP 0976.0.140.000-05).

The patients of case group were enrolled at Hospital de Base, São José do Rio Preto, São Paulo, Brazil and diagnosis was made from pathological specimens either after total excision or biopsy. Patients with squamous cell carcinoma tumor cell types were included and patients previously treated for this tumor were excluded this study. The primary anatomic sites were subdivided as oral cavity (n= 129 - 40%), pharynx (n = 81 - 25%) and larynx (n = 97 – 30%). Fifteen patients (05%) had unknown tumor primary site.

The tumors were staged according to TNM classification following three criteria: extension of the tumor (T), presence of regional lymph node involvement (N) and presence of metastasis at a distance (M) (Sobin and Wittelind 2000). Tumor classification was divided into low T (T1, T2) and high T (T3, T4) classification categories. The N classification was dichotomized into no lymph node involvement (N0) and involvement (N1, N2, N3). The clinical stage (TNM) was used to analyze aggressiveness with tumors being grouped as non-aggressive (Stage I and II) and aggressive (Stage III and IV). The survival time was defined as the time from the date of diagnosis to death if the patient died from HNSCC.

The control group included Brazilian blood donors without cancer diagnoses of according to government guidelines for donated blood that tests for 20 related diseases (http://www.hemonline.com.br/portarias/rdc153/indexframe.htm). Individuals with family history of cancer were excluded and individuals with age greater than 40 years were included this study. Each eligible subject was interviewed to obtain data on demographic and lifestyle factors.

Genomic DNA was obtained from peripheral blood following the technique of Miller and collaborators (1988). The genotypes for the C677T (rs1801133) and A1298C (rs1801131) polymorphism of MTHFR gene were determined by polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis using Hinf I and Mbo II enzymes for C677T and A1298C respectively . The primers sequences were sense 5'- TGA AGG AGA AGG TGT CTG GGG GA – 3’ and anti-sense 5' - AGG ACG GTG CGG TGA GAG TG – 3’ for the C677T polymorphism and sense 5'- CAA GGA GGA GCT GCT GAA GA- 3 and anti-sense 5’ – CCA CTC CAG CAT CAT CAC TCA CT – 3’ for the A1298C (Frosst et al. 1995; Yi et al. 2002).

The statistical significance was performed using the Minitab software program / Windows - Version 14.0. Chi-square test was conducted to examine whether the genotypes frequency of *MTHFR* genes were in Hardy-Weinberg equilibrium (HWE) and multiple logistic regression was used to comparison between the groups, to evaluate the interaction effect between the genetic polymorphisms and variables related to HNSCC and to examine the association between these polymorphisms and clinical histopathological. The variables analyzed were age, gender, exposure to risk factors (tobacco and alcohol consumption), primary site of occurrence, aggressiveness, extension of the tumor, and lymph node involvement. Individuals who had smoked more than 100 cigarettes in their lifetime were considered to be smokers and individuals who drank 4 doses of alcohol per week were considered to be alcohol consumers (Kjaerhein *et al.* 1998; Ahrendt *et al.* 2000).

For combined genotypes analysis, the *MTHFR* 677CC/1298AA genotypes were used as reference. The MTHFR 677CT/1298AA or 677CC/1298AC genotypes (One wild-type homozygote and other heterozygote) were considered as risk 1 and; 677CT/1298AC genotype (both heterozygote genotypes) was definite as risk 2 and MTHFR 677TT/1298AC or 677CT/1298CC or 677TT/1298CC (One polymorphic homozygote and other heterozygote or both polymorphic genotypes) were classified as risk 3 or 4.

A p-value < 0.05 was considered statistically significant. Results are shown as odds ratio (OR) and 95% confidence intervals (95% CI). *MTHFR* haplotypes were inferred using the Haploview 4.2 statistical program, which creates population frequency estimates of the haplotypes.

RESULTS

The analysis for comparison between groups showed that age over 49 years (Median; OR: 23.63 CI95%: 12.27 -45.50, p<0.05), male gender (OR: 1.63 CI95%: 1.01- 2.64, P<0.05), tobacco habit (OR: 4.24 CI95%: 2.83- 6.34, p<0.05) and alcohol consumption (OR:1.22 CI95%:0.82- 1.83, p<0.05) were more frequent in case group than in the control group

The *MTHFR* genotype distribution for the C667T polymorphism was in Hardy-Weinberg equilibrium (HWE) (X2 = 0.111, p=0.739 and X2 = 1.357, p=0.243 for patients and controls respectively) but not for the A1298C polymorphism (X2 = 14.229, p=0.0002 and X2 = 11.753, p= 0.0006 for patients and controls respectively). The distributions of the *MTHFR* alleles, genotypes and combined genotypes (*MTHFR* C677T and A1298C) between the cases and controls are shown in Table 1. We found that patients with either *MTHFR* 1298AC or CC genotypes displayed a 1.96-fold increased risk of HNSCC compared with the 1298AA genotype (OR: 1.96; 95% CI = 1.37 - 2.81, p<0.05). There was no statistically significant difference in the genotype frequencies of the *MTHFR* C677T polymorphism between controls and HNSCC patients. The *MTHFR* 677T and *MTHFR* 1298C variant allele frequencies were 0.36 and 0.33, respectively, among the cases and 0.33 and 0.24 among the controls, and these differences were statistically significant for the 677T allele (OR= 2.05; 95%CI=1.66 – 2.53; p <0.05 and for 1298C allele (OR= 1.48; 95% CI=1.15, p= 0.001).

We combined the genotypes with zero risk allele and used this combined group as the reference group, the results showed that risk estimates of HNSCC increased as the number of risk alleles increased: (MTHFR 677CT/1298AC genotypes, risk 2 OR:

1.80; 95%CI= 1.11 – 2.89; p=0.01 and 677TT/1298AC or 677CT/1298CC or 677TT/1298CC genotypes, risk 3 and 4 OR: 4.29; 95%CI= 2.02 - 9.10; p=0.00)

The haplotype analysis showed a higher frequency of the C-A haplotype (677C-1298A) observed in both groups (Case group: 0.346, Control group: 0.451; X2= 18.19, p<0.05). The other haplotypes frequencies were 0.295 and 0.302 for T-A (677T-1298A; X2= 0.085, p=0.7705), 0.286 and 0.210 for haplotype C-C (677C-1298C; X2= 12.889, p=0.0003) and 0.073 and 0.038 for haplotype T-C (677T-1298C; X2=10.196, p=0.0014) in patients and controls respectively.

The potential interaction between the distribution of the *MTHFR* genotypes and variables related to HNSCC are shown in Table 2. The multiple logistic regression analysis showed that *MTHFR* C677T polymorphism is not associated with age, gender, tobacco and alcohol habits, but for *MTHFR* A1298C polymorphism, we found that 1298AC or 1298CC genotypes were associated with a significantly increased risk of HNSCC among subjects with age over 49 years (OR 2,42; 95% CI, 1.21 – 2.73) smokers (OR, 2.00 95% CI, 1,34 - 3,00) and etilists (OR, 1.69; 95% CI, 1.09 – 2.62).

For combined genotypes, we found that 677CT/1298CC or 677TT/1298CC genotypes (3 and 4 alleles risk) were associated with age over 49 years (OR: 7.15; 95%CI:1.47-34.75, p=0.013), male gender (OR: 4.62; 95%CI: 1.85-11.63; p=0.001) and tobacco (OR: 4.66; 95%CI:1.73-12.57; p=0.002) and alcohol habits (OR: 4.37; 95%CI:1.47- 12.96; p=0.008).

The clinical histopathological parameters analysis confirmed that *MTHFR* 1298AC or CC genotypes were more frequent in patients that had oral cavity as primary site (Table 3). The C677T polymorphism did not show any association with clinical

histopathological parameters. The Kaplan-Meier survival curves by genotypes are presented in Figure 1, and did not demonstrate any association between polymorphisms and recurrence time of the disease (Figure A: C677T, p= 0.06 and Figure B: A1298C, p= 0.96). Moreover, no association was observed for the survival time and polymorphisms (Figure C: C677T,p = 0.76 and Figure D: A1298C, p= 0.78).

DISCUSSION

The results of the current study indicate that age > 49 years, male gender, tobacco habits and alcohol consumption are associated with HNSCC increased risk. According Marron *et al* (2010) the cessation of alcohol consumption and tobacco habits, calculed from age at reference date (interview) and age at which the individual stopped drinking and smoking, were associated with a reduction in the risk of this disease, which concludes that these two factors to increase HNSCC risk. Moreover, this cancer type occurs most often in older men, as to show our results (Sapkota *et al.* 2008, Marron *et al.* 2010, Galbiatti *et al*. 2010).

The HWE analysis revealed significant deviation of Hardy-Weinberg equilibrium for *MTHFR* A1298C gene in both groups, according with results of Neumann *et al* (2005). This disequilibrium should be expected, considering that could reflect biologic and genetic characteristics in complex disease model (Wittke-Thompson *et al.* 2005).

In present study we sought to determine whether *MTHFR* C677T and A1298C genetic variants are associated with HNSCC risk. Our observations suggested that individuals that carrying at least a variant allele for *MTHFR* A1298C (1298AC or 1298CC genotypes) may contribute to the increased risk of this disease in Brazilian

individuals. In contrast, Neumman *et al* in 2005 in a hospital-based case-control study of 537 non-Hispanic white individuals with HNSCC and 545 cancer-free controls confirmed that *MTHFR* 1298AC heterozygote had a decreased significantly risk for HNSCC, being that individuals that carrying at least a variant allele for *MTHFR* A1298C (1298AC or 1298CC genotypes) were associated with a 35% reduction risk. Levine *et al* in 2010 also found that 1298 CC variant genotype may be associated with a decrease colorectal cancer risk, which are inconsistent with our findings.

On the other hand, the case-control study of Song *et al* (2001) in 240 esophageal squamous cell carcinoma (ESCC) cases and 360 controls from China northern showed that 1298CC genotype was associated with an elevated risk of ESCC compared with the 1298AA genotype as in our study in HNSCC. However these authors found that the 1298AC genotype had no effect on the ESCC risk, but in our study this genotype also was associated with HNSCC risk.

Other studies did not found any association with HNSCC risk (Suzuki *et al.* 2007, Kruszyna *et al.* 2010, Boccia *et al.* 2009). Suzuki *et al* (2007) examined the *MTHFR* A1298C polymorphism distribution in 237 HNSCC cases and 711 age- and sex-matched non-cancer from Japan, Kruszyna *et al* (2010) studied patients with larynx cancer (n = 131) and controls (n = 250) from Poznan-Poland and Boccia *et al* (2009) reported a meta-analisys of four studies, 1.439 cases and 3.941 controls (Europeans, Japanese, Italians and Americans individuals) and did not found association this variant on HNSCC risk.

For C677T polymorphism, our results did not show association of 677CT or 677TT genotypes and HNSCC risk, according studies of Suzuki *et al* (2007), Boccia *et al* (2009) and Kruzyna *et al* (2010). However, we found that *MTHFR* 677T allele

variant was associated with increased risk for HNSCC. Our findings did not confirm those of Neuman *et al* (2005) Vairaktaris *et al* (2006) and Reljic *et al* (2007).

Neuman *et al* (2005) reported that the *MTHFR* 677CT heterozygote appeared to have a higher risk for this cancer type and 677T variant allele frequency was identical between cases and controls. Vairaktaris *et al* (2006) that analyzed 110 patients with oral squamous cell carcinoma and 120 healthy controls of comparable ethnicity also found that 677T variant allele displayed no statistical difference, but revealed an association of *MTHFR* 677CT or TT genotypes with an increased risk for oral cancer while Reljic *et al* (2007) that studied 81 HNSCC patients and 102 healthy controls found that 677CT genotype has a possible protective role, but 677T allele did not show significant statistically difference.

In our study, the combined analysis suggested that *MTHFR* C677T and A1298C polymorphisms togheter (risk of 2,3 or 4 polymorphic alleles) can modify the risk for HNSCC. The study of Neuman *et al* in 2005 that associated three polymorphisms (MTHFR A1298C, C677T and G1793A) showed that the the adjusted risk estimates of SCCHN increased as the number of risk alleles increased up to 2, as our study. To the best of our knowledge, only our study and the study of Neumann *et al* (2005) has simultaneously investigated the associations between MTHFR polymorphisms and HNSCC risk.

For the four possible haplotypes based on the combinations of the distributions of the two known genotypes, we found consistent associations between the variant haplotypes genotypes and the risk of HNSCC. Although haplotypes C- C and T-C were present in our study population, their frequency was lower than the expected allele combination frequency calculated by the Haploview program, confirming negative

selection of these haplotypes. Linkage disequilibrium between *MTHFR* C677T and A1298C genotypes has been reported (Stegmann *et al.* 1999; Chen *et al.* 2002; Shi *et al.* 2003; Ito *et al.* 2003), including the study of Neuman *et al* (2005) in HNSCC that confirmed evidence linkage disequilibrium among *MTHFR* C677T and A1298C with AG1793A polymorphism providing an increased risk of this disease. However, a study of 102 women diagnosed with invasive cervical cancer did not observe any linkage disequilibrium between the C677T and A1298C polymorphisms, but this study may have been biased by the small sample size. (Gehard *et al.* 2003).

In our study, there was an interaction significant between the *MTHFR* A1298C polymorphism and the risk factors age over 49 years, male gender, alcohol and tobacco habits, suggesting that these variables and *MTHFR* 1298AC or 1298CC genotypes have increased HNSCC risk. No association was observed for the *MTHFR* C677T regarding risk factors. The analyses for combined genotypes showed increased risk between 677CT-1298CC and 677TT-1298CC genotypes for male gender, age over 49 years, tobacco and alcohol habits. Suzuki *et al* (2007) showed risk reduction with alcohol comsuption between individuals with the *MTHFR* 677TT genotype and did not found any association for the *MTHFR* A1298C and risk factors, which inconsistent with our study (Suzuki *et al.* 2007). The significant interaction for combined genotypes on HNSCC risk factors has not been reported previously.

Analysis of significance of genotypes distribution between clinical histopathological parameters revealed association of *MTHFR* 1298AC or CC genotypes with oral cavity. There are no studies that evaluated the association of the oral cavity and this polymorphism. Kruszyna *et al* (2010) assessed the association of this polymorphism with larynx cancer and did not confirm association.

Our study did not confirm the impact of *MTHFR* polymorphisms on overall survival and no data was found in the literature assessing survival according to *MTHFR* polymorphisms in patients with HNSCC. However, a study in gastric cancer showed that patients with who had folate intake over 260 micrograms/day and *MTHFR* 677 TT polymorphism were associated with better survival (Shitara *et al.* 2010). Moreover, Zhang *et al* (2007) in colorectal cancer (CRC) reported a influence of MTHFR 1298AA genotype and greater overall survival in CRC women patients and Etienne-Grimaldi in 2010 also in colorectal cancer confirmed that both *MTHFR* 677CT/ 677TT and 1298AC/1288CC were linked to better clinical response.

In conclusion, our case-control study provide evidences that *MTHFR* A1298C polymorphism , *MTHFR* combined genotypes (at least 2 risk alleles) significantly contribute to increased risk for HNSCC and *MTHFR* A1298C polymorphism were associated with age over 49 years, male gender, tobacco and alcohol habits, confirming that individuals with these variables and *MTHFR* 1298AC or CC genotypes has higher risk for this disease.

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Table 1. Distribution of the *MTHFR* C677T and A1298C genotypes, combined genotypes and allele frequencies among head and neck squamous cell carcinoma patients and controls.

***MTHFR* polymorphism**	**Patients n=322** n (%)	**Controls n=531** n (%)	**OR(IC 95%)**	**P Value**
C677T Genotypes				
CC	130 (40.3)	226 (42.6)	1.00 (Ref)	1.00
CT	147 (45.6)	250 (47.0)	0.91 (0.64 - 1.31)	0.61
TT	45 (5.3)	55 (4.4)		
T-allele frequency	0.36	0.33	2.05 (1.66 – 2.53)	**<0.05**
A1298C Genotypes				
AA	148 (46.0)	316 (59.5)	1.00 (Ref)	1.00
AC	117 (36.4)	168 (31.7)	1.96 (1.37 - 2.81)	**<0.05**
CC	57 (17.6)	47 (8.8)		
C- allele frequency	0.35	0.24	1.48 (1.15 – 1.90)	**<0.05**
Combined genotypes				
(0)677CC/1298AA	49 (15.2)	109 (20.5)	1.00 (Ref)	1.00
677CT/1298AA or	67 (20.8)	157 (29.5)		
(1)677CC/1298AC	47 (14.5)	86 (16.1)	1.15 (0.71 – 1.84)	0.57
****(2)***677CT/1298AC or	58 (18.0)	79 (14.8)	1.80 (1.11 – 2.89)	**<0.05**
677CC/1298CC or	36 (11.1)	31 (5.9)		
677TT/1298AA	34 (10.5)	49 (9.3)		
****(3/4)***677TT/1298AC or	10 (3.0)	04 (0.8)	4.29 (2.02 – 9.10)	**<0.05**
677CT/1298CC or	19 (6.0)	14 (2.7)		
677TT/1298CC.	02 (0.7)	02 (0.4)		

OR data were adjusted for age, gender, tobacco status and alcohol habits. $p < 0.05$ was considered significant in multiple logistic regression model.

*Risk 2: Both heterozygote genotypes; *Risk 3 or 4: One polymorphic homozygote and other heterozygote or both polymorphic genotypes

Table 2: Distribution in Odds ratio (OR) of *MTHFR* genotypes and variables related with head and neck carcinoma (gender, age, tobacco, and alcohol habits).

Variables	***1- MTHFR* C677T genotypes (Patients/Controls)**		**OR(IC 95%)**	***2- MTHFR* A1298C genotypes (Patients/Controls)**		**OR(IC 95%)**
	CC (Ref)	**CC and CT**		**AA** (Ref)	**AC and CC**	
Age						
<49	29/163	36/210	0,47 (0.13 – 1.67)	28/223	38/150	2.50 (0.70 – 8.94)
≥49	101/63	156/95	1,09 (0,76 - 2,16)	124/93	132/65	**2.42 (1.21 – 2.73)***
Gender						
Female	18/64	25/85	0,98 (0,45 - 2,13)	19/86	24/63	1,67 (0,77 - 3,65)
Male	112/162	168/220	0,90 (0,62 - 1,30)	129/229	151/153	**1,96 (1,36 - 2,82)***
Tobacco Habits						
No	29/140	31/176	1,26 (0,68 - 2,32)	28/188	32/128	1,69 (0,93 - 3,09)
Yes	108/86	155/129	0,80 (0,53 - 1,20)	120/128	143/87	**2,00 (1,34 - 3,00)***
Alcohol Habits						
No	40/122	60/147	1,08 (0,65 - 1,81)	42/156	58/113	2,20 (0,92 - 5,26)
Yes	90/104	133/158	0,81 (0,52 - 1,27)	105/160	118/102	**1,69 (1,09 - 2,62)***

OR data were adjusted for age, gender, tobacco and alcohol habits. $p < 0.05$ was considered significant in multiple logistic regression model.

* $p < 0.05$ (multiple logistic regression)

Table 3. Distribution of the clinical histopathological parameters and *MTHFR* polymorphisms.

Variables	*1- MTHFR* C677T genotypes		OR(IC 95%)	*2- MTHFR* A1298C genotypes		OR(IC 95%)
	CC (Ref)	**CC and CT**		**AA** (Ref)	**AC and CC**	
Site of tumor						
Oral cavity	49	81	1.34 (0.84 - 2.14)	49	81	**1.81 (1.17 – 2.82)***
Pharynx	27	53	1.55 (0.90 – 2.65)	43	37	0.69 (0.42 – 1.14)
Larynx	44	53	0.77 (0.47 – 1.26)	48	49	0.79 (0.50 – 1.25)
Tumor extension						
T1/T2	72	102	1.00 (Ref)	81	93	1.00 (Ref)
T3/T4	70	104	0.82 (0.49 – 1.35)	79	95	0.82 (0.49 – 1.35)
N involvement						
No	91	139	1.00 (Ref)	107	123	1.00 (Ref)
Yes	39	53	0.80 (0.48 – 1.34)	25	28	0.99 (0.59 – 1.64)
Aggressiveness						
No	48	69	1.00 (Ref)	55	62	1.00 (Ref)
Yes	82	123	0.91 (0.56 – 1.50)	93	112	0.87 (0.53 – 1.43)

OR data were adjusted for age, gender, tobacco, and alcohol habits. $p < 0.05$ was considered significant in multiple logistic regression model. *$p < 0.05$

The analysis was made to patients with complete data. *MTHFR* genotypes compared with clinical histopathological parameters - Reference: *MTHFR* 677CC and *MTHFR* 1298AA. There was difference statistically significant for oral cavity and *MTHFR* 1298 AC/CC genotypes.

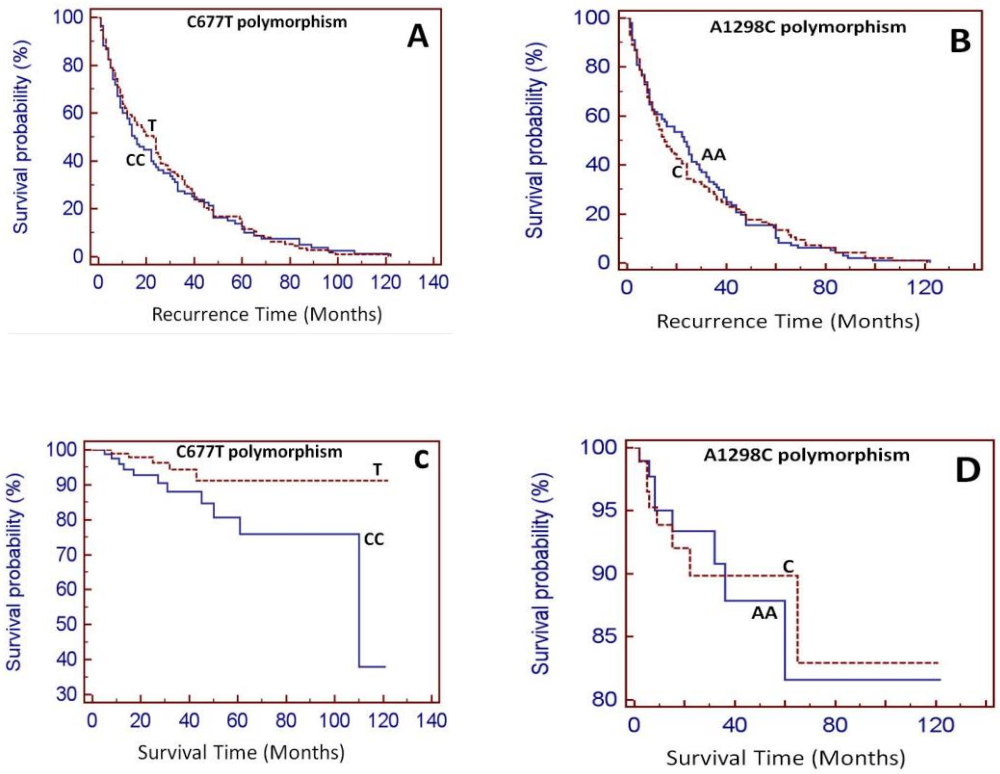

Figure 1. Kaplan-Meier curves for recurrence time for C677T (p= 0.76, A) and A1298C (p=0.77, B) and survival time for C677T (p= 0.06, C) and A1298C (p=0.96, D) for patients. There was no statistical difference between the curves for subjects with the selvage genotype and subjects with at least one mutant allele.

3. CONCLUSÕES

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1. Os genótipos *MTR* 2756AG, *RFC1* 80AG ou GG, *MTHFR* 1298AC ou CC e genótipos combinados *MTHFR* 677CT/1298AC, 677TT/1298AC, 677CT/1298CC , 677TT/1298CC estão associados com aumento no risco de câncer de cabeça e pescoço.

2. O genótipo *MTR* 2756 AG associado com gênero masculino; os genótipos *MTHFR* 1298AC ou CC e genótipos combinados *MTHFR* 677CT/1298AC, 677TT/1298AC, 677CT/1298CC e 677TT/1298CC associados com os hábitos tabagista e etilista, idade acima de 49 anos e gênero masculino e os genótipos *RFC1* 80AG ou GG associados com não consumo de álcool, idade acima de 50 anos e gênero masculino aumentam o risco de câncer de cabeça e pescoço.

3. Há evidências de associação entre os polimorfismos *CBS* 844ins68 e *MTHFR* A1298C e o sítio primário de ocorrência cavidade oral. O polimorfismo *CBS* 844ins68 está associado com menor tempo de sobrevida dos pacientes.

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5. ANEXOS

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)

COMITÊ DE ÉTICA EM PESQUISA

O Comitê de Ética em Pesquisa da Faculdade de Medicina de São José do Rio Preto tomou ciência e aprovou a ampliação da metodologia datada de 11 de novembro de 2005, referente ao protocolo n.º *5566/2005* sob a responsabilidade de Maurício José Cabral Ruback, com o título "Câncer de cabeça e pescoço: um levantamento epidemiológico do Hospital de Base/FAMERP de SãoJosé do Rio Preto".

São José do Rio Preto, 19 de dezembro de 2005.

Prof. Dr. José Paulo Cipullo
Vice- Coordenador do CEP/FAMERP

Av. Brigadeiro Faria Lima 5416 - 15090-000 São José do Rio Preto SP - Brasil
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FACULDADE DE MEDICINA DE SÃO JOSÉ DO RIO PRETO
Autarquia Estadual - Lei n.° 8899 de 27/09/94
(Reconhecida pelo Decreto Federal n.° 74.179 de 14/06/74)

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 autorizou a **dispensa do Termo de Consentimento Livre e Esclarecido conforme solicitação datado de 03/07/2008**, referente ao protocolo n.° **5566/2005** sob a responsabilidade de **Maurício José Cabral Ruback**, com o título "Câncer de cabeça e pescoço: um levantamento epidemiológico do Hospital de Base/FAMERP de São José do Rio Preto".

São José do Rio Preto, 08 de julho de 2008.

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

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Errata enviada a revista ***Brazilian Journal of Medical and Biological Research****.*

Artigo: 5-Methyltetrahydrofolate-homocysteine methyltransferase gene polymorphism (*MTR*) and risk of head and neck cancer*. 43: 445-450; 2010*

Table 1: Distribution of demographic data, risk factors, genotypes, *MTR* 2756 alleles and odds ratio (OR) for head and neck cancer

Variables	**n (%)**	**OR (95% CI)**
Tobacco consumption		
Non-smokers	265 (37.6)	Reference
Smokers	440 (62.4)	4.49 (2.68 – 7.54)*
Alcohol consumption		
Alcohol non-consumers	301 (42.7)	Reference
Alcohol consumers	404 (57.3)	2.30 (1.46 – 3.63) *
Gender		
Female	175 (24.9)	Reference
Male	530 (75.1)	1.18 (0.69 – 2.01)
Age		
<42	117 (16.6)	Reference
42-51	185 (26.2)	4.72 (2.29 – 9.72) *
51-63	191 (27.0)	19.45 (9.55 – 39.59)*
>63	212 (30.2)	11.01 (5.33 – 22.72)*
MTR 2756 Genotypes		
AA	448 (63.5)	Reference
AG	211 (29.3)	1.69 (1.09 – 2.62) *
GG	46 (7.2)	1.08 (0.37 – 3.13)
MTR 2756 Alleles		
A	659 (71.9)	Reference
G	257 (28.1)	1.60 (1.05 – 2.44)*

* $p < 0.005$