JBUON 2014; 19(4): 1024-1028
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

The influence of methylenetetrahydrofolate reductase and thymidylate synthetase gene polymorphisms on lung adenocarcinoma occurrence

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Summary

Purpose: *Methylenetetrahydrofolate reductase (MTHFR) and thymidylate synthetase (TYMS) are suggested as risk factors for lung cancer. The purpose of this study was to analyze the association of MTHFR C677T polymorphism and variable number tandem repeat 2R/3R and single nucleotide polymorphism G→C in the 3R allele of the TYMS gene with lung adenocarcinoma.*

Methods: *A case-control study including lung adenocarcinoma patients and healthy subjects was performed. Restriction fragment length polymorphism analysis was used for genotyping. Descriptive analyses included genotype and allelic frequencies; the odds ratio and 95% confidence interval were calculated as an estimate of relative risk. Significance was set at p<0.05.*

Results: *A significant difference in CC vs TT+CT MTHFR genotype distribution was observed between patients and controls. There was no significant association between the TYMS polymorphisms and the risk of lung adenocarcinoma.*

Conclusions: *The MTHFR 677T allele is likely to have a protective effect against lung adenocarcinoma development.*

Key words: *gene polymorphism, lung adenocarcinoma, methylenetetrahydrofolate reductase, thymidylate synthetase*

Introduction

Lung cancer is one of the most frequent cancers and one of the leading causes of cancer-related deaths worldwide [1]. It is the most common neoplastic disease in Eastern and Central Europe [2], and in Serbia there are approximately 5200 newly diagnosed lung cancer cases each year, with a death rate of 4600 per year [3]. The majority of all lung cancers (85-90%) are categorized as non-small cell lung cancer (NSCLC), of which adenocarcinoma represents around 40% [4]. Although tobacco smoke is probably the predominant etiological risk factor for lung adenocarcinoma, it still develops in less than 11% of people who smoke throughout their life [5] and adenocarcinoma is also the most common type of lung cancer in non-smokers [4]. As the mechanisms driving the carcinogenesis of lung adenocarcinoma are still not well understood, many environmental and genetic risk factors are being explored [6]. Folate deficiency has been linked to alterations in DNA synthesis, repair and methylation, contributing to the overall genomic instability and increased cancer risk [5,7]. Polymorphisms of the folate metabolism enzymes *MTHFR* and *TYMS* could be risk factors in lung cancer and lead to poor prognosis and drug toxicity, as they are associated with altered levels of serum folate [8-10].

The most frequent *MTHFR* polymorphism is the cytosine to thymine transition at nucleotide 677 (C677T), positioned in exon 4 of the *MTHFR* gene. As a result of this single nucleotide polymorphism (SNP), an alanine to valine substitution in the N-terminal catalytic domain of the *MTHFR* protein occurs, which leads to the formation of a

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Received: 14/05/2014; Accepted: 01/06/2014

thermolabile variant of this enzyme. The 677 CT and TT genotypes have been shown to induce the production of enzyme variants with approximately 65% and 30% of the *MTHFR* wild-type enzyme activity *in vitro*, respectively [11]. The *TYMS* gene promoter has a variable number tandem repeat (VNTR) polymorphism in the enhancer region (28bp double or triple tandem repeat, 2R, 3R) which affects the *TYMS* protein expression. It has been shown that in 2R/2R homozygotes, and in carriers of a common SNP in the second repeat of the 3R allele (guanine to cytosine transition, G→C), there is lower intra-tumoral expression of the *TYMS* protein [12,13]. These observations led to a classification of patients into "low" (L) and "high" (H) *TYMS* expression groups [12].

In the present study, the C677T *MTHFR* gene polymorphism together with the 28 bp VNTR (2R/3R) and the SNP (G→C) in the 3R allele of the *TYMS* gene were evaluated as risk factors for lung adenocarcinoma.

Methods

The procedures in this study were performed in accordance with the ethical standards approved by the Ethical Committee of the Institute for Oncology and Radiology of Serbia and with the Helsinki Declaration of 1975, as revised in 2000.

Patients and controls

A case-control study was performed in a group of 55 chemonaive advanced lung adenocarcinoma patients (between 40-76 years of age, 21 males, 34 females) from various cancer centers in Serbia, and 53 healthy control subjects (between 23-63 years of age, 23 females, 30 males), all of Caucasian descent. All the patients were diagnosed with advanced primary lung adenocarcinoma (stage III or IV, grade 2 or 3, ECOG performance status 0 or 1) according to clinical and histological criteria. The healthy control group consisted of randomly chosen volunteers who work at the Institute and their friends and relatives, with no previous history of malignancies and no known folate metabolism deficiency. Formalin-fixed paraffin-embedded (FFPE) lung tissue blocks were obtained by biopsy/resection. Ethylenediaminetetraacetic acid (EDTA) peripheral blood was drawn by venipuncture and further used for leukocyte isolation.

DNA isolation

Genomic DNA was isolated from FFPE lung tissue blocks (patients) using the QIAamp® DNA FFPE Tissue isolation kit (Qiagen, UK), or from blood leukocytes (healthy controls) using BloodPrep Chemistry for ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems, CA, USA). Tissue blocks were not available from healthy controls and recent large-scale literature analysis provided information that the concordance between germline and somatic DNA in variants of pharmacogenetic genes is virtually 100% [14]. The concentration and purity of the isolated DNA samples was determined spectrophotometrically (Nanodrop, Shimadzu).

MTHFR and TYMS genotyping

To assure adequate genotyping, a previously established heterozygote sample was used as a method control and genotyping was carried out blind to case-control status. Restriction fragment length polymorphism analysis (PCR-RFLP) was used for *MTHFR* and *TYMS* genotyping as previously described [12,13,15].

Statistics

Descriptive analyses included gene and allelic frequencies. Logistic regression analysis was used to calculate the odds ratio (OR) and the 95% confidence interval (CI) as an estimate of relative risk. The Hardy-Weinberg equilibrium was tested using the x^2 test. Statistical significance was set at $p < 0.05$.

Results

A 198 bp PCR product containing the *MTHFR* C677T polymorphic site was successfully obtained from all patient and control samples (Figure 1a). After digestion of the PCR products, an undigested PCR product (198 bp) indicated the presence of a homozygous wild type genotype (CC), while heterozygotes (CT) produced three bands of 198, 175 and 23 bp, and homozygotes (TT) produced two fragments of 175 and 23 bp (Figure 1b). The RFLP analysis was successful for all patient and control samples.

The distribution of *MTHFR* C677T genotypes in patients and controls did not deviate from the Hardy-Weinberg equilibrium (x^2=0.09; p=0.76 and x^2=3.62; p=0.057). The allele frequencies of the *MTHFR* C677T polymorphic variants in patients vs controls showed that the frequency of the C allele was higher in patients (0.78) than in healthy controls (0.56). CC homozygosity at the 677 polymorphic site of the MTFHR gene was more prevalent in lung adenocarcinoma patients than in healthy control subjects (Figure 2). The *MTHFR* C allele was associated with lung adenocarcinoma in a recessive model, as a statistically significant difference for CC vs TT+CT genotype distribution was observed between lung adenocarcinoma patients and healthy controls (Table 1). In a dominant model, there was no statistically significant

Table 1. Association between *MTHFR* genotypes and lung adenocarcinoma risk

MTHFR genotype	*OR*	*95% CI*	*p-value*
CC vs TT+CT	4.98	2.17–11.41	0.0002
CT+CC vs TT	2.64	0.64–10.80	0.29

Table 2. Association between *TYMS* genotypes and lung adenocarcinoma risk

TYMS genotype	*OR*	*95% CI*	*p-value*
HH vs HL+LL	1.4	0.41–4.72	0.812
HH+HL vs LL	1.05	0.44–2.49	0.909

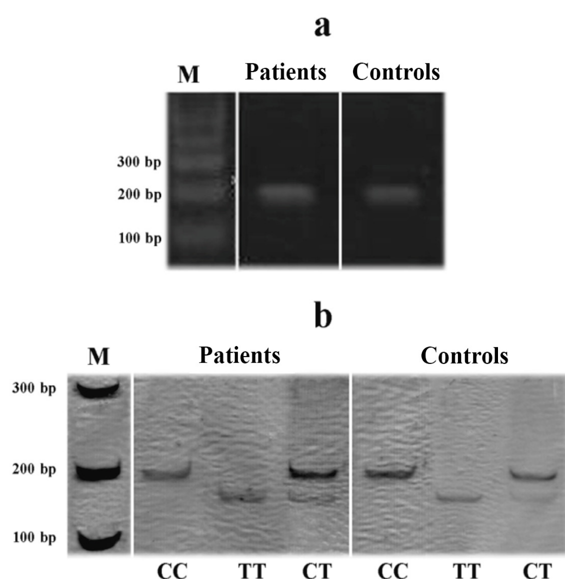

Figure 1. PCR (**a**) and RFLP (**b**) analysis of *MTHFR* C677T polymorphic variants. M – DNA bp marker.

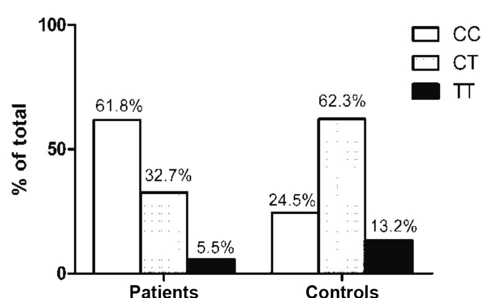

Figure 2. Genotype distribution of *MTHFR* C677T polymorphic variants.

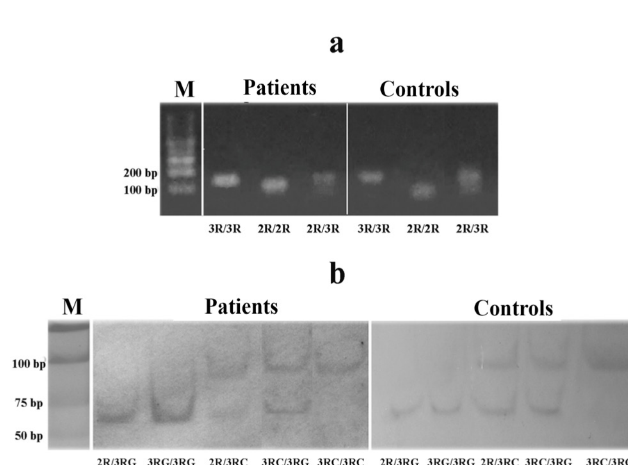

Figure 3. PCR (**a**) and RFLP (**b**) analysis of *TYMS* polymorphic variants. M – DNA bp marker.

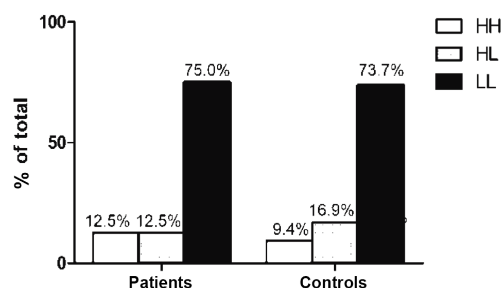

Figure 4. Genotype distribution of *TYMS* polymorphic variants.

difference in CT+CC vs TT genotype distribution.

Bands of 108 bp or 136 bp containing the *TYMS* VNTR polymorphic site were successfully obtained from all patient and control samples (Figure 3a). The 2R/2R variant produced one band of 108 bp, the 3R/3R variant produced one band of 136 bp, and the 2R/3R variant produced two bands of 108 and 136 bp. The 2R/3R and 3R/3R PCR products were further digested to determine the presence of the (G→C) SNP in the 3R allele. After digestion, the 2R allele produced three fragments of 66, 32 and 10 bp, the 3RG allele produced four fragments of 66, 32, 28 and 10 bp, while the 3RC allele produced three fragments of 94, 32 and 10 bp (Figure 3b). The RFLP analysis was successful for all patient and control samples. Depending on the presence of high (H, 3RG) or low (L, 2R and 3RC) expression alleles, *TYMS* functional groups were further subclassified into HH, HL and LL groups. No apparent difference in the frequency of the H allele between patients (0.19) and healthy controls (0.18) was observed. The frequencies of the *TYMS* polymorphic variants in patients vs controls are presented in Figure 4, and the analysis of the association between *TYMS* genotypes and lung adenocarcinoma risk is presented in Table 2. The *TYMS* H allele was not associated with lung adenocarcinoma either in a recessive (HH vs HL+LL) or in a dominant (HH+HL vs LL) model (p=0.812 and p=0.909, respectively).

As *MTHFR* and *TYMS* enzymes interact in the folate metabolic pathway by competing for the pool of 5,10-methylene THF, the combined effect of *MTHFR* and *TYMS* polymorphisms was analyzed. The risk of lung adenocarcinoma was not modified by an interaction between these polymorphic variants, but a nonsignificant difference in LL vs HL+HH *TYMS* genotype distribution between patients and controls was found in the CT+TT *MTHFR* subgroup (x^2=0.011, p=0.918, OR=1.07, 95% CI=0.31–3.67).

Discussion

This study showed an increased frequency of the C allele in Serbian lung adenocarcinoma patients compared to healthy controls. Moreover, a statistically significant correlation between the homozygous CC genotype and the risk of lung adenocarcinoma was determined. Therefore, the *MTHFR* 677T allele is likely to have a protective effect in lung adenocarcinoma development in the Serbian population. This is in accordance with previous findings of an inverse association of the *MTHFR* 677T allele with other types of malignancies, such as colorectal cancer [16], prostate cancer [17], acute lymphoblastic leukemia [18], as well as lung cancer in females [19]. However, other types of malignancies, such as chronic myeloid leukemia [15], gliomas [20] and breast cancer [21], showed either no association of the *MTHFR* genotypes with elevated cancer risk, or the *MTHFR* 677T allele was a low-penetrant risk factor. As the *MTHFR* enzyme resides at the crossroads of DNA synthesis and methylation, two opposing theories concerning its activity as a cancer risk factor are present in the literature. According to the hypomethylation theory, the *MTHFR* 677T allele should increase the risk of cancer development, as it leads to the production of a thermolabile *MTHFR* enzyme and hence reduces DNA methylation in the cell. DNA hypomethylation, particularly within promoter regions, might affect the methyl-mediated silencing of oncogenes and lead to proto-oncogene expression and cancer [22]. On the other hand, T allele carriers with lower *MTHFR* enzyme activity have a larger pool of 5,10-methylene THF and methyl groups for the conversion of dTMP from dUMP. High dUMP levels might lead to mis-incorporation of uracil into DNA and increase the frequency of DNA double-strand breaks. Therefore, it is possible that the T allele has a protective effect against cancer through a more reliable DNA synthesis and repair which overcome the inadequate DNA methylation [23]. Opposing results of the effect of *MTHFR* genotypes on the risk of various cancers might be explained by the different biology of their development, as carcinogenesis in one tissue might be more driven by hypomethylation effects, while in another tissue protection from erroneous DNA synthesis and repair might be more important. This effect has even been observed in different types of tumors of the same localization [24]. There have been reports that the DNA of cancerous lung tissue is generally hypomethylated in comparison to non-cancerous tissue in patients who have the *MTHFR* 677TT genotype [25]. The protective effect of the 677T allele observed in this study might signify that for the specific carcinogenesis of this subtype of lung cancer a more accurate DNA synthesis and repair can overcome the negative effects caused by DNA hypomethylation.

Although some studies have shown that the *TYMS* expression level might be a lung cancer risk factor in some countries [26], more studies reported that it has no significant effect on lung carcinoma occurence [9,26,27]. Local specific lifestyles and dietary habits in populations might explain these differences, as well as polymorphisms in other genes encoding for folate metabolism enzymes. We showed that *TYMS* polymorphisms had no significant influence on the susceptibility to lung adenocarcinoma in Serbia. The risk of lung adenocarcinoma was also not modified when both *MTHFR* and *TYMS* polymorphic variants were analyzed together. Finding a potential correlation between the polymorphisms of folate metabolizing enzymes and lung adenocarcinoma could be useful in elucidating the role of these enzymes in lung carcinogenesis.

Conclusions

A significant corelation between the 677CC *MTHFR* genotype and lung adenocarcinoma occurence was found. The *MTHFR* 677T allele is likely to have a protective effect against lung adenocarcinoma development.

Acknowledgements

This study was supported by a grant from the Ministry of Education and Science of the Republic of Serbia (Grant number III41026).

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