Folate-related genes and the risk of tobacco-related cancers in Central Europe


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Background

Given the high risk of lung cancer that remains among former smokers, as well as the high risk of second cancers among those with a primary upper aero-digestive cancer, identification of chemopreventive agents for these cancers is clearly important (1). Numerous studies have reported that fruits and vegetables are protective against upper aero-digestive cancers (2), and folate has been suggested as one of the chemopreventive compounds possibly responsible for such a protective effect, although the evidence is not yet conclusive partially due to the high correlation among food items (3–7). Alcohol consumption on the other hand is an established risk factor for upper aero-digestive cancers (8), and has been shown to reduce the folate bioavailability (9). Folate deficiency may lead to DNA strand breaks, reduced DNA repair and aberrant DNA methylation (7,10,11). Sequence variants in genes encoding key enzymes in the folate metabolism, such as methylenetetrahydrofolate reductase (MTHFR) and methionine synthase (MTR), have been shown to be associated with altered folate levels in plasma or red blood cells (12–15).

MTHFR, a central enzyme in folate metabolism, converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the main circulating form of folate. Its substrate provides one-carbon units for DNA synthesis, and its metabolic product provides methyl group for the synthesis of methionine (11,16). MTR catalyzes the methylation of homocysteine to methionine, which results in S-adenosylmethionine, the universal methyl donor. The latter molecular is a key component for the process of DNA methylation (11,16) (see Figure 1).

Several sequence variants have been identified in MTHFR and MTR (16). The two variants of MTHFR that have been most studied are (i) a nucleotide change of C to T at position 677, which results in an amino acid substitution of alanine to valine at codon 222 (C677T, A222V, rs1801133) (17), and (ii) a nucleotide change of A to C at position 1298, which results in an amino acid change of glutamic acid to alanine at codon 429 (A1298C, E429A, rs1801131) (18). Various functional studies have been conducted for these two variants. In particular, the heterozygote and homozygous variant of C677T, the thermolabile variant, were shown to have 65 and 30% of the enzyme activity, respectively (19). Subjects who carried the homozygote of C677T were shown to have lower folate levels in red blood cells (12,20), and higher homocysteine levels in plasma (13). Furthermore, carriers of C677T C677T genotype who had low folate levels in plasma also displayed lower methylation levels (21). The A1298C variant carriers also exhibited lower enzyme activity but to a lesser extent (22), and the associations of this variant with plasma folate and homocysteine levels are unclear due to inconsistent results (14,23,24). Several studies have been conducted to investigate the association between these two variants and the risk of lung or upper aero-digestive cancers (26–34). However, most of them were relatively small in size, and reported either no association or increased risk of cancer for carriers of C677T variants, and contradictory results for carriers of the A1298C variant (26–34).

Regarding MTR, an A to G nucleotide change at position 2756, which results in an amino acid substitution of aspartic acid with glycine at codon 919 was identified in MTR (A2756G, D919G, rs1805087) (15). Despite limited studies aimed to investigate the functional significance of this variant, the evidence is not yet conclusive (15,35). Only few epidemiological studies have been conducted for MTR D919G and upper aero-digestive cancer risk, and the results have been contradictory (26,36,37).

We hypothesized that folate is an important factor in the etiology of lung and upper aero-digestive cancers, and that the variants of folate metabolism genes are associated with the risk of these cancers. Furthermore, any effect of gene variants may be modified by dietary folate intake and alcohol consumption. In order to test these hypotheses, we have conducted in Central Europe a large-scale genetic association study of lung and upper aero-digestive cancers for MTHFR C677T, A1298C and MTR D919G.

Abbreviations: CI, confidence interval; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; OR, odds ratio.
Materials and methods

The study was conducted in 15 centers in six countries of Central and Eastern Europe including Czech Republic (Prague, Olomouc, Brno), Hungary (Borsod, Heves, Szabolcs, Szolnok, Budapest), Poland (Warsaw, Lodz), Romania (Bucharest), Russia (Moscow) and Slovakia (Banska Bystrica, Bratislava, Nitra). Each center followed an identical protocol and was responsible for recruiting a consecutive group of newly diagnosed cases of lung cancer and upper aero-digestive cancer, as well as a comparable group of population or hospital controls. All subjects were recruited between 1998 and 2003. Lung cancer cases were recruited in all centers, whereas upper aero-digestive cancer cases were recruited only in the centers in Russia, Czech Republic, Romania, Slovakia and Poland. The participation rates for both cases and controls were over 80% for both cases and controls in all centers. A total of 2250 lung cancer cases and 811 upper aero-digestive cancer cases (168 oral cavity, 113 pharynx, 326 larynx, 176 esophagus and 28 cases of cancers from overlapping oral/pharynx sites) were recruited and provided DNA samples. Cases were histologically confirmed, and upper aero-digestive cases were restricted to squamous cell carcinoma, the predominant histological type. Controls in all centers except Warsaw were chosen among patients admitted in the same hospital as the cases with conditions unrelated to tobacco including minor surgical conditions, benign disorders, common infections, eye conditions (except cataract or diabetic retinopathy), common orthopaedic diseases (except osteoporosis), etc. In Warsaw, population controls were selected by random sampling from the Polish Electronic List of Residents. Cases and controls were frequency matched by sex, age (±3 years), center and referral (or residence) area. This resulted in a total of 2899 controls, of which 2618 were available as controls for upper aero-digestive cancer cases. Both cases and controls underwent an identical interview based on the same questionnaire. Written consent for participation was obtained from all study subjects and ethical approval has been obtained for all study centers as well as at the International Agency for Research on Cancer, the coordinating center. Further details on the questionnaire, as well as case and control recruitment, have been reported elsewhere (38).

After DNA extraction from buffy coats, genotyping was performed by the 5’-nuclease assay (TaqMan, Applied Biosystem, Foster City, CA). Sequences of primers and probes for the MTHFR C677T, A1298C, MTR D919G variants were obtained from the TaqMan protocol on the SNP500 project (http://snp500cancer.nci.nih.gov/home.cfm). DNA from cases and controls was randomized on polymerase chain reaction plates and duplicate genotyping was performed for a random 10% of the samples for quality control. All genotyping was conducted centrally in the International Agency for Research on Cancer. Genotyping call rates were similar for cases and controls being 96.6% overall for the MTHFR C677T variant, 98.8% for the A1298C variant and 98.2% for the MTR D919G variant. Duplicate quality control genotypes showed 99.8, 99.7 and 99.9% concordance for the MTHFR C677T, A1298C, and MTR D919G sequence variant.

We conducted stratified analyses by histology for lung cancer to investigate the potential heterogeneity in the effect of each polymorphism by major histological subtypes (squamous cell carcinoma, adenocarcinoma and small cell carcinoma). We also evaluated the modulating effects of dietary folate intake, alcohol consumption and age of onset by stratifying and comparing the strata-specific estimates with test of heterogeneity. Folate intake was estimated from the food frequency questionnaire that included 23 food items, and specifically four items rich in folate contents (liver, spinach, cabbage and the combination of brussels sprouts with broccoli) (39). The questionnaire was repeated for two different periods: the year before interview and before political and market changes in 1989 (1992 in Russia); a weighted average (denoted as \( A_e \)) of the two intake frequencies was calculated on the basis of the age of the individual. The sum of \( A_e \) from the four food items was calculated as an indicator of the total folate intake.

Cumulative alcohol consumption was estimated based on the amount of alcohol beverages consumed per week at age 25, 40, 45 and 60, weighted by the alcohol content in different beverages (5% for beer, 12% for wine and 40% for spirits) (40) and multiplied by the estimated drinking duration. The unit of the cumulative alcohol consumption was grams of ethanol per week-years. The stratified analyses of folate intake and alcohol consumption were conducted based on categories derived from the control group.

We calculated odds ratios (ORs) and 95% confidence intervals (CIs) for the heterozygous and homozygous variant genotypes after adjusting for potential confounders including country of residence, age, sex and cumulative tobacco consumption using multivariate logistic regression. All the analyses were conducted with SAS software. Haplotypes frequencies were estimated by TagSNP software using the E-M algorithm (41). Linkage disequilibrium between variants was tested by the measures of \( D' \) and \( R^2 \). The false-positive reporting probability was estimated based on the method proposed by Wacholder et al. (42).

Results

The frequency distribution of the demographic variables and putative risk factors is shown in Table I. The allele frequencies for MTHFR 677T, 1298C and MTR 919G among the control group are 30, 33 and 22%, respectively, with no meaningful differences by countries. The allele distribution of the two MTHFR variants fell within expected distributions of Hardy–Weinberg equilibrium among controls of lung and upper aero-digestive cancers, whereas the allele distribution of MTR D919G polymorphism among controls did not (\( P = 0.003 \)). When stratified the genotype frequencies of MTR D919G by country, the departure from Hardy–Weinberg equilibrium was observed in Hungary and Russia. Therefore, data of both cases and controls from these two countries were excluded from further analysis of MTR D919G.

![Folate pathway](https://example.com/folate_pathway.png)

**Fig. 1.** Folate pathway (modified from ref. 16). DHF, dihydrofolate; THF, tetrahydrofolate; SAM: S-adenosylmethionine; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate.
Education: low = basic/elementary levels; medium = secondary/middle school; high = university level or above.

The main effects of MTHFR and MTR variants are shown in Table II. The MTHFR C677T variant was associated with increased risk of lung cancer; the OR of lung cancer for homozygote variant was 1.37 (95% CI = 1.10–1.71), and that of upper aero-digestive cancers (OR of lung cancer = 1.29 (95% CI = 0.95–1.76). On the other hand, the MTHFR 1298C homozygous variant was associated with a reduced risk of lung cancer (OR = 0.80, 95% CI = 0.65–1.00). Stratified by age of onset, the risk estimates for MTHFR C677T/677T genotype were more prominent among subjects with onset of <50 for lung cancer with OR of 1.92 (95% CI = 1.12–3.29) (comparing with the subjects with older onset, P value for test of heterogeneity was 0.20). The three variants did not appear to confer differential cancer risk according to histology subtypes of lung cancer or topography of upper aero-digestive cancers. On the other hand, MTR D919G did not appear to be important for either lung or upper aero-digestive cancer risk.

The two MTHFR variants were in strong linkage disequilibrium (D' = −0.99, R2 = 0.21). The haplotype analysis suggested that 677TT/1298A was the primary haplotype to be associated with the risk of lung and upper aero-digestive cancers (OR of lung cancer = 1.16, 95% CI = 1.04–1.29, and OR of upper aero-digestive cancer = 1.14, 95% CI = 0.98–1.33 for carrying one copy of such haplotype) (Table III). The estimated false-positive reporting probability (42) showed that the positive finding of MTHFR C677T remained robust when the prior probability was at least 10% for lung cancer (false-positive reporting probability = 0.058).

When stratified by dietary intake of folate, the increased risk of lung cancer due to the MTHFR 677TT variant was more prominent among subjects with low intake of folate: the ORs of lung cancer for the carriers of 677TT/677TT genotype with the lowest quartile of folate intake frequency was 2.06 (95% CI = 1.35–3.15), and that for those whose folate intake in the lowest decile was 2.60 (95% CI = 1.39–4.88, P value for test of heterogeneity = 0.03) (Figure 2). An increased risk of upper aero-digestive cancer was also observed among subjects at the lowest decile of folate intake with OR of 4.14 (95% CI = 1.47–11.7, P value for test of heterogeneity = 0.02) for carriers of 677TT/677TT genotype; but no specific pattern in risk was observed for higher folate intake frequencies.

Figure 3 shows the results of stratified analysis by alcohol consumption. The OR of lung cancer among never drinkers for 677TT/677TT homozygous carriers was 2.39 (95% CI = 1.07–5.35) compared with 677T/677C carriers, and that of upper aero-digestive cancer was not informative given the small number of cases in this group.

**Discussion**

Based on the largest study of lung and upper aero-digestive cancers to date, we identified a moderate effect of MTHFR C677T variant on the risk of lung cancer, which is consistent with results of the previous functional studies (12,13,17,19–21). Moreover, the effect of C677T variant on aero-digestive cancers appeared to be modified by folate intake. The protective effect of MTHFR A1298C on lung cancer risk is likely to be driven by the linkage disequilibrium with C677T, which is confirmed by the haplotype analysis. A previous functional study also showed that DNA hypomethylation is mainly associated with 677TT when both polymorphisms were evaluated simultaneously (24).

The association between MTHFR C677T homozygous variants and risk of lung cancer agrees with our prior hypothesis, given that carriers of C677T homozygous variant have been shown to have lower enzyme activity, lower folate levels and subsequently aberrant gene methylation levels. Interestingly, a more prominent effect was observed among subjects with a very low intake of folate for both lung and upper aero-digestive cancers, also in agreement with our prior hypothesis.

Low folate intake could have an impact on the folate metabolism due to reduced availability of carbon donors. In vitro studies suggested that the C677T variant was associated with impaired stability and reduced activity under low folate status (17,43). In addition, it has been shown that, when plasma folate levels are low, DNA methylation levels are decreased in carriers of 677TT/677TT homozygotes, since MTHFR 677TT variant might have a higher folate requirement (11,21). Our observation is compatible with the model hypothesized by Friso et al. (11) that in circumstances of folate deficiency, less 5-methylTHF is available for the remethylation of homocysteine to methionine in carriers with 677TT/677TT genotype than in other subjects, resulting in lower methylation status. Several epidemiological studies have also reported a similar effect modification of C677T by folate intake (44,45), although the others have not (34).

It has been suggested that whether the C677T variant will act as beneficial or deleterious depends on the subjects’ folate status or the preferred downstream pathway after the conversion of 5,10-methyltetrahydrofolate to 5-methyltetrahydrofolate. Specifically, the 677T allele, which has a reduced enzyme activity, is considered deleterious since it was shown to result in DNA hypomethylation (possibly via a reduced level of S-adenosylmethionine) (46), which may lead to genome instability and altered expression. On the other hand, the 677T allele can also exert a protective effect since it may lead to increased levels of 5,10-methyltetrahydrofolate, which is essential for nucleotide biosynthesis. This may partially explain the inconsistent results in the previous studies, which did not always take folate intake or alcohol consumption into account. In addition, one would expect that MTHFR C677T might not have an effect on the risk of aero-digestive cancers in the population with high prevalence of folate supplement intake, and this might at least partially explain the null results from the previous studies conducted in USA, where common food type such as bread are fortified with folate (29,30).
Since alcohol was shown to decrease the bioavailability of folate (9), we hypothesized a more important role of sequence variations in folate-related genes among heavy drinkers. However, we did not observe such an effect modification; instead, the effect of MTHFR 677T/677T genotype on lung cancer risk appeared to be more prominent among never and light drinkers. Few studies have investigated the potential effect modification of alcohol consumption on folate-related sequence variants but the results were inconsistent. In particular, a study on head and neck cancers from USA reported no interaction between MTHFR variants and alcohol drinking (29), whereas a study from Japan reported an interaction between MTHFR C677T and heavy alcohol drinking of esophageal cancer, although the sample size was relatively small (26). Given the small number of never and light drinkers in our study, chance finding might be a likely explanation of the fluctuating pattern observed.

Although high folate intake has been associated with a reduced risk of most cancer sites studied, the effect of MTHFR C677T variant appears to be different for different cancer sites. It is worthwhile to point out that the effect of MTHFR C677T observed in our study is in the opposite direction observed for colorectal cancer (16). On the other hand, the effect modification of MTHFR 677T/677T genotype by folate intake appeared to be consistent across cancer sites: subjects with the 677T/677T genotype who had lower folate intake levels had less of a protective effect for colorectal cancer and higher risk for lung and upper aerodigestive cancer, as opposed to subjects who had higher folate intake levels. The detailed mechanism of how the MTHFR C677T variant may lead to different effects for different cancer sites is unclear. Nevertheless, MTHFR plays a central role in balancing DNA synthesis (which involves 5,10-methylenetetrahydrofolate) and DNA methylation (which involves 5-methyltetrahydrofolate).

### Table II. Effect of MTHFR C677T, A1298C and MTR D919G variants overall and stratified by age of onset

<table>
<thead>
<tr>
<th>MTHFR C677T</th>
<th>Lung cancer</th>
<th>Upper-aero-digestive cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>1009</td>
<td>1397</td>
</tr>
<tr>
<td>C/T</td>
<td>929</td>
<td>1147</td>
</tr>
<tr>
<td>T/T</td>
<td>231</td>
<td>259</td>
</tr>
<tr>
<td>Onset &lt; 50 year old</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>131</td>
<td>234</td>
</tr>
<tr>
<td>C/T</td>
<td>144</td>
<td>202</td>
</tr>
<tr>
<td>T/T</td>
<td>39</td>
<td>47</td>
</tr>
<tr>
<td>Onset ≥ 50 year old</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C/C</td>
<td>878</td>
<td>1163</td>
</tr>
<tr>
<td>C/T</td>
<td>785</td>
<td>945</td>
</tr>
<tr>
<td>T/T</td>
<td>192</td>
<td>212</td>
</tr>
<tr>
<td>OR1</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>95% CI</td>
<td>Reference</td>
<td></td>
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</table>

### Table III. Haplotype and combined analysis for folate metabolic genetic variants

<table>
<thead>
<tr>
<th>MTHFR haplotype</th>
<th>Lung cancer</th>
<th>Upper-aero-digestive cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>C677T-A1298C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>677C-1298A</td>
<td>777</td>
<td>1036</td>
</tr>
<tr>
<td>677C-1298C</td>
<td>680</td>
<td>918</td>
</tr>
<tr>
<td>677T-1298A</td>
<td>689</td>
<td>923</td>
</tr>
<tr>
<td>677T-1298C</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

OR adjusted for age, sex, country and tobacco pack-years.
This polymorphism may reduce the likelihood of uracil misincorporation into DNA and thereby may diminish the appearance of mutations that could lead to a protective effect for colorectal cancer or lower DNA methylation that could lead to higher risks of lung and upper aero-digestive cancer. This hypothesis may also at least partially explain the high allele frequency remained in the human population for a variant that may be deleterious.

There are several limitations in our study apart from those inherent in the hospital-based design. Firstly, our dietary questionnaire was relatively simple and did not provide information regarding quantities or usual portions of different food items. Therefore, we were not able to estimate folate intake in a precise fashion, and validity of the folate intake indicator has not been cross-validated with the folate levels in blood. Nevertheless, any misclassification or measurement error of folate intake may at least partially explain the high allele frequency remained in the human population for a variant that may be deleterious.
Folate intake cannot explain the current findings and may suggest the possibilities of a greater risk of aero-digestive cancers among those with low folate intake in the absence of misclassification. Secondly, we were not able to take other one-carbon nutrients (such as riboflavin, vitamin B6 and vitamin B12) into account based on the simple dietary questionnaire, which limited our ability of understanding the one-carbon pathway comprehensively. In particular, previous studies have shown that the association between MTHFR genotype and homocysteine concentration might be modified by riboflavin level (47). Thirdly, our study is focused on the variants with well-described functional consequences. However, these variants only accounts for a fraction of the total genetic variation in these genes. It is therefore possible that there are additional functional variants that we did not consider in the present analysis. Furthermore, there are other genes involved in the folate metabolism. However, other sequence variants in folate-related genes were not as well characterized and were therefore not considered in the present analysis.

In conclusion, if our findings are true, they provide support of the protective effect of folate in aero-digestive tract cancer, the evidence of which is unlikely to be confounded by other dietary or lifestyle factors. To further verify the role of folate in tobacco-related cancers, replication in other large studies is required. Subsequent studies ideally will be based on prospective cohorts, which include detailed questionnaire data on dietary folate intake, serological biomarkers of folate levels and other genetic variants in this pathway. Such studies may help to elucidate the potential chemopreventive role of folate and open the way for future chemoprevention studies of tobacco-related cancers.

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Conflict of Interest Statement: None declared.

References


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