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PILOT CASE–CONTROL STUDY OF *MTHFR* C677T AND *MTRA*2756G POLYMORPHISMS AMONG ALCOHOL/ TOBACCO USERS IN EASTERN UTTAR PRADESH, INDIA (N = 200)

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Abstract–Folate-mediated one-carbon metabolism (OCM) supports nucleotide synthesis and methylation. Perturbations of OCM are linked to hyperhomocysteinemia and genomic instability, and functional polymorphisms in *MTHFR* (C677T) and *MTR* (A2756G) may modify susceptibility under exposures such as alcohol consumption and tobacco use. To estimate genotype/allele frequencies of *MTHFR* C677T and *MTR* A2756G and evaluate their association with alcohol/tobacco user status in a pilot cohort from Eastern Uttar Pradesh (UP), India. We constructed a pilot case–control subset (N = 200; 100 alcohol/tobacco users and 100 controls) from an institutional dataset with PCR–RFLP genotype calls. For *MTHFR* C677T, genotypes were coded as CC/CT/TT; for *MTR* A2756G, genotypes were coded as AA/AG/GG. We compared genotype distributions using a 2×3 chi-square test, assessed Hardy– Weinberg equilibrium (HWE) in controls, and estimated odds ratios (OR) under a dominant model (variant carriers vs. non-carriers) using Fisher's exact test and age/sex-adjusted logistic regression. Control genotypes satisfied HWE for both variants (*MTHFR*: p = 0.170; *MTR*: p = 0.140). *MTHFR* C677T showed no evidence of association with case status (genotype p = 0.538; dominant OR 1.16 [95% CI 0.62–2.16], p = 0.752). In contrast, *MTR* A2756G differed between groups (genotype p = 0.031), with a higher G-allele frequency in cases (21.5%) versus controls (12.0%). Under a dominant model (AG/GG vs. AA), *MTR* variant carriers had increased odds of being cases (unadjusted OR 2.31 [95% CI 1.23–4.32], p = 0.013; adjusted OR 2.34 [95% CI 1.18–4.61], p = 0.015). In this pilot Eastern UP cohort, *MTR* A2756G (but not *MTHFR* C677T) showed an association with alcohol/tobacco use. Larger, exposure-stratified analyses with balanced covariates and nutritional biomarkers (folate, vitamin B_{12}, homocysteine) are warranted to clarify gene–environment effects.

INTRODUCTION

Alcohol and tobacco exposures frequently co-occur and contribute substantially to preventable morbidity and mortality. Large prospective evidence indicates markedly elevated mortality risks among smokers compared with never-smokers (Carter et al., 2015). Epidemiologic analyses further show that alcohol use and tobacco use cluster within individuals and subgroups, motivating joint consideration of these exposures (Falk et al., 2006; Dawson, 2000). Identifying biological pathways that shape inter-individual susceptibility in exposure-prone settings is important for prevention and risk stratification.

A biologically plausible pathway connecting nutrition, environmental exposures, and genomic stability is folate-mediated one-carbon metabolism (OCM). OCM supplies one-carbon units required for (i) de novo nucleotide synthesis (thymidylate and purine synthesis) and (ii) methylation reactions via the methionine cycle that generates S-adenosylmethionine (SAM), the universal methyl donor. Disturbances in this pathway are often reflected in elevated homocysteine and reduced methylation capacity. Mechanistic and human evidence links folate deficiency to genomic uracil misincorporation, chromosome breakage, and broader DNA instability (Blount *et al*., 1997; Duthie, 1999).

Two enzymes central to OCM are methylenetetrahydrofolate reductase (*MTHFR*) and methionine synthase (*MTR*). *MTHFR* catalyzes conversion of 5, 10-methylenetetrahydrofolate to 5-

methyltetrahydrofolate, the methyl donor required for remethylation of homocysteine to methionine. The common *MTHFR* C677T polymorphism (Ala222Val) is associated with reduced enzyme activity and thermolability; biochemical and structural work supports decreased FAD binding and altered stability, with folate potentially exerting a stabilizing effect (Frosst *et al*., 1995; Rozen, 1997; Guenther *et al.,* 1999; Yadav *et al*., 2017). *MTR* is a vitamin B_{12}-dependent enzyme that remethylates homocysteine to methionine and supports SAM production; it is a modular protein with distinct binding regions, underscoring its central role in methyl-group transfer and methylation balance (Goulding *et al*., 1997).

Alcohol and tobacco may modulate the phenotypic impact of OCM variants. Alcohol use can reduce folate availability and disrupt B-vitamin status, while tobacco exposure contributes oxidative stress and may correlate with nutritional depletion. Consistent with a gene–environment framework, prior work has reported that *MTR* A2756G can interact with alcohol and folate intake in determining downstream phenotypes in some settings (Yamaji *et al.,* 2009). Therefore, OCM variants remain plausible modifiers of exposure-associated biological vulnerability.

Population-specific information is also critical because allele frequencies vary across ancestries and regions. An updated meta-analysis reported substantial global heterogeneity in *MTHFR* C677T prevalence (Schneider *et al*., 1998).In Eastern UP specifically, an independent healthy-population study reported a T-allele frequency around 11% and TT genotype near 1% (1000 individuals), providing an important regional baseline for interpretation of local datasets (Yadav *et al*., 2017).

Eastern Uttar Pradesh (UP) comprises a large and diverse population with substantial prevalence of tobacco chewing/smoking and variable alcohol use. Establishing genotype frequencies and testing initial association signals in this population can inform larger, well-powered studies that incorporate exposure intensity and nutritional biomarkers.

In this paper, we report a pilot case-control analysis using an institutional dataset from Eastern UP. Our aims are to (i) estimate genotype and allele frequencies for *MTHFR* C677T and *MTR* A2756G in a balanced subset of 200 individuals and (ii) test whether these variants are associated with alcohol/tobacco user status. This pilot analysis is intended to generate initial evidence and guide subsequent full-cohort analyses incorporating exposure strata and biomarkers.

MATERIALS AND METHODS

Study design and pilot subset construction

We analyzed an institutional dataset comprising individuals recruited from Eastern UP with recorded demographics and genotype calls for *MTHFR* C677T and *MTR* A2756G. For this pilot paper, we constructed a case–control subset (N = 200):

% **Cases (**n = 100**):** individuals labelled as tobacco users, alcohol users, or combined alcohol+tobacco users.

% **Controls (**n = 100**):** individuals labelled as controls (non-users) or random population samples.

Participants with missing genotype entries for either locus were excluded. To reduce selection bias, the final n = 100 cases and n = 100 controls were selected by reproducible random sampling without replacement from eligible records (random seed documented in analysis scripts).

% **Ethics approval and informed consent**

The study was cleared by the **Institutional Ethics Committee** of Veer Bahadur Singh Purvanchal University, Jaunpur, Uttar Pradesh, India. Written informed consent was obtained from all participants prior to blood collection, and all procedures followed institutional and applicable ethical guidelines.

% **Sample collection, DNA extraction, and genotyping**

Peripheral blood (3 ml) was collected in EDTA vacutainers and genomic DNA was extracted using standard whole-blood protocols (Bartlett and White, 2003).

Genotyping was performed using PCR followed by restriction fragment length polymorphism (PCR–RFLP) digestion and agarose gel electrophoresis, with genotype calls recorded in the institutional database.

% ***MTHFR* C677T:** PCR–RFLP genotyping followed the widely used approach described by Frosst et al. and as implemented in regional studies (Frosst *et al.,* 1995; Yadav *et al.,* 2017). Briefly, PCR amplification was followed by *HinfI* digestion; the expected amplicon is 198 bp, with digestion producing 175 bp and 23 bp fragments for the T allele, while the C allele remains uncut (Yadav *et al.,* 2017).

***MTR* A2756G:** PCR–RFLP genotyping was performed using the institutional laboratory SOP with restriction digestion to distinguish alleles; genotype calls were recorded as AA/AG/GG in the database. (Primer sequences and PCR/digestion conditions are available from the institutional SOP and can be provided on request; primer sequences are omitted here to avoid transcription errors.)

Statistical analysis

We summarised age (mean ± SD) and sex distribution by group. Genotype counts and allele frequencies were computed separately for cases and controls. Hardy–Weinberg equilibrium (HWE) in controls was assessed using a chi-square test with 1 degree of freedom.

Association with alcohol/tobacco user status was tested using:

% A 2 × 3 chi-square test comparing genotype distributions between cases and controls.

% A dominant genetic model: for *MTHFR* (CT/TT vs. CC) and for *MTR* (AG/GG vs. AA), reported as odds ratio (OR) with 95% confidence interval (CI). Unadjusted *p*-values were obtained via Fisher's exact test, and adjusted ORs were obtained from logistic regression including age and sex.

All tests were two-sided with $\acute{a} = 0.05$.

RESULTS

Participant characteristics

Table 1 summarizes baseline characteristics. Cases were older on average (46.78±14.83 years) than controls (41.55±13.41 years). The case group had a higher proportion of males (81%) than the control group (51%).

Table 1. Participant characteristics in the pilot cohort.

Group	*n*	Age (years; mean ± SD)	Male
Cases	100	46.78 ± 14.83	81 (81.0%)
Controls	100	41.55 ± 13.41	51 (51.0%)

Genotype and allele frequencies

Genotype distributions and allele frequencies are shown in Table 2. In controls, both variants were consistent with HWE (*MTHFR*: $p = 0.170$; *MTR*: $p = 0.140$). *MTHFR* C677T showed similar genotype proportions across groups (genotype test $p = 0.538$), and the T-allele frequency was comparable in cases (15.5%) and controls (15.0%). In contrast, *MTR* A2756G differed between cases and controls (genotype test $p = 0.031$), with a higher G-allele frequency in cases (21.5%) than controls (12.0%).

Association under a dominant model

Table 4 reports dominant-model association estimates. *MTHFR* variant-carrier status was not associated with case status (unadjusted OR 1.16, $p = 0.752$; adjusted OR 1.37, $p = 0.355$). In contrast, *MTR* variant carriers (AG/GG) had increased odds of being alcohol/tobacco users (un- adjusted OR 2.31 [95% CI 1.23–4.32], $p = 0.013$). The association remained after adjustment for age and sex (adjusted OR 2.34 [95% CI 1.18–4.61], $p = 0.015$).

DISCUSSION

This pilot case–control analysis from Eastern UP suggests differential behavior of two OCM variants with respect to alcohol/tobacco user status. We observed no association between *MTHFR* C677T and case status, while *MTR* A2756G showed a statistically detectable enrichment of the G allele among cases.

Interpretation of the *MTHFR* C677T null finding

The lack of association for *MTHFR* C677T is consistent with the near-identical genotype distributions and T-allele frequencies observed across cases and controls in this pilot subset. Mechanistically, the Ala222Val substitution can reduce *MTHFR* activity and stability, partly through altered FAD binding (Frosst *et al*., 1995; Rozen, 1997; Guenther *et al*., 1999; Yadav *et al*., 2017). However, the phenotypic impact of this variant is known to depend on nutritional context (folate/B-vitamin status) and possibly exposure intensity. In our dataset, we did not measure folate, vitamin B_{12}, or homocysteine, and we used a broad case definition (any alcohol/tobacco use) rather than graded exposure, which could dilute gene-environment effects.

A second consideration is regional allele frequency context. A large independent study of healthy individuals from Eastern UP reported a T-allele frequency around 11% and TT genotype near 1% (Yadav *et al*., 2017). Our controls show T-allele frequency of 15% and TT of 2%, which is directionally similar but somewhat higher; given the modest sample size ($n = 100$ controls), sampling

Table 2. *MTHFR* C677T genotype and allele frequencies ($N = 200$). Genotype p from 2 × 3 chi-square.

Group	CC	CT	TT	T-allele freq.
Controls	72 (72.0%)	26 (26.0%)	2 (2.0%)	15.0%
Cases	72 (72.0%)	25 (25.0%)	3 (3.0%)	15.5%

$p = 0.538$

Table 3. *MTR* A2756G genotype and allele frequencies ($N = 200$). Genotype $p$from 2 × 3 chi- square.

Group	AA	AG	GG	G-allele freq.
Controls	77 (77.0%)	22 (22.0%)	1 (1.0%)	12.0%
Cases	65 (65.0%)	27 (27.0%)	8 (8.0%)	21.5%

$p = 0.031$

Table 4. Association of variant-carrier status with alcohol/tobacco use under a dominant model. Unadjusted p-values are from Fisher's exact test; adjusted estimates are from logistic regression including age and sex.

Variant	Unadjusted OR (95% CI)	p	Adjusted OR (95% CI)	p
MTHFR C677T (CT/TT vs CC)	1.16 (0.62–2.16)	0.752	1.37 (0.71–2.66)	0.355
MTR A2756G (AG/GG vs AA)	2.31 (1.23–4.32)	0.013	2.34 (1.18–4.61)	0.015

variability and cohort composition could explain this difference. More generally, meta-analytic evidence indicates substantial geographic heterogeneity in *MTHFR* C677T prevalence (Schneider *et al.*, 1998; Wilcken *et al*., 2003; Yadav *et al*., 2017), reinforcing the importance of local baselines when interpreting pilot samples.

Interpretation of the *MTR* A2756G association signal

In contrast to *MTHFR*, *MTR* A2756G showed higher G-allele frequency among cases and an approximately 2.3-fold increase in odds of being a user among AG/GG carriers, persisting after adjustment for age and sex. *MTR* encodes methionine synthase, a key vitamin B_{12}-dependent enzyme that supports homocysteine remethylation and SAM generation (Goulding *et al*., 1997). Variation affecting remethylation capacity could plausibly alter methylation balance and homocysteine under nutritional stress.

Importantly, prior work suggests that *MTR* A2756G effects may be modified by environmental context, including alcohol intake and folate status (Yamaji et al., 2009). Our pilot analysis is consistent with such a gene-environment framework but does not establish interaction formally because exposure intensity, diet, and biomarkers were unavailable. It is also possible that the observed association reflects residual confounding by unmeasured factors correlated with alcohol/tobacco use (e.g., diet quality, socioeconomic factors), highlighting the need for richer covariate capture in the full-cohort study.

Strengths, limitations, and next steps

This pilot has several strengths: balanced case-control sample size, internal consistency of genotyping, HWE satisfaction in controls, and concordant unadjusted/adjusted estimates for *MTR*. However, limitations are important. First, covariate imbalance (notably sex) may bias crude comparisons; while we adjusted for age/sex, additional confounders were not available. Second, broad exposure classification may mask dose-response or subgroup effects (tobacco-only vs alcohol-only vs dual use). Third, small counts for rare homozygotes (TT for *MTHFR*, GG for *MTR*) limit precision. Finally, population stratification cannot be excluded in an institutional convenience sample.

These limitations directly motivate the planned full-cohort analysis. Key next steps include: (i) exposure-stratified analyses and intensity/duration modeling, (ii) inclusion of nutritional biomarkers (folate, vitamin B_{12}, homocysteine) and dietary assessments to test biologically grounded mediation/moderation hypotheses, (iii) expanded genetic coverage (e.g., *MTRR*, *CBS*, *SHMT*) and haplotype-based modeling, and (iv) careful matching or propensity approaches to reduce imbalance.

CONCLUSION

In this pilot Eastern UP case–control analysis (N = 200), we observed no evidence that *MTHFR* C677T is associated with alcohol/tobacco user status. In contrast, *MTR* A2756G showed a detectable association: variant carriers (AG/GG) had approximately 2.3-fold higher odds of being classified as alcohol/tobacco users, and cases exhibited a higher G-allele frequency than controls. Larger, exposure-stratified studies incorporating nutritional biomarkers are warranted to confirm and mechanistically interpret this preliminary *MTR* signal.

Conflict of Interest–None

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