MTHFR 677T Is a Strong Determinant of the Degree of Hearing Loss Among Polish Males with Postlingual Sensorineural Hearing Impairment

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Hearing impairment (HI) is the most common sensory handicap. Congenital HI often has a genetic basis, whereas the etiology of nonsyndromic postlingual HI (npHI) usually remains unidentified. Our purpose was to test whether the *MTHFR* C677T (rs1801133) polymorphism affecting folate metabolism is associated with the occurrence or severity of npHI. We studied rs1801133 genotypes in 647 npHI patients (age <40, sudden sensorineural loss excluded, HI characterized as mean of better ear hearing thresholds for 0.5–8 kHz) and 3273 adult controls from the background population. Genotype distribution among patients and controls was similar, but among male cases ($n=302$) we found a dose-dependent correlation of *MTHFR* 677T with the degree of HI (mean thresholds in dB: 38.8, 44.9, and 53.3, for CC, CT, and TT genotypes, respectively; $p=0.0013$, $p_{\text{cor.}}=0.017$). Among male patients rs1801133 TT significantly increased the risk of severe/profound HI (odds ratio=4.88, $p=0.001$). Among controls the known effect of *MTHFR* 677T on plasma total homocysteine was more pronounced in men than in women ($p<0.00004$ for genotype-sex interaction) suggesting that in Poland folate deficiency is more prevalent in males. In conclusion, we report a novel strong effect of *MTHFR* 677T among males with npHI. The functional significance of rs1801133 suggests that these patients may benefit from folate supplementation—an intervention which is simple, cheap, and devoid of side effects.

Introduction

HEARING IMPAIRMENT (HI) is the most common sensory handicap. Congenital HI has a genetic origin in ~50%, being frequently caused by mutations in *GJB2* (Pollak *et al.*, 2007, 2008), whereas the etiology of postlingual HI usually remains unidentified. Postlingual HI may be caused by environmental factors such as exposure to ototoxic drugs, noise, infections, including those localized to the ear, or meningitis (Smith and Mathers, 2006; Roeser *et al.*, 2007; Nadol, 2010). The well-identified group of risk factors of HI of adult onset includes systemic diseases such as diabetes mellitus, renal insufficiency, thyroid dysfunction, hyperlipidemia, and autoimmune disorders (Roeser *et al.*, 2007; Katz *et al.*, 2009; Nadol, 2010). Also, inner ear diseases such as cochlear otosclerosis, endolymphatic sac hydrops, or autoimmune diseases may begin in adulthood as a bilateral HI (Roeser *et al.*, 2007; Nadol, 2010). In some cases postlingual HI may have a genetic basis, being caused by mutations in *DIAPH1*, *WFS1*,

CCDC50, *TECTA*, *COL11A2*, *CCDC50*, *KCNQ4*, *GJB3*, *COCH*, or other yet unidentified genes (Smith *et al.*, 2010).

The *MTHFR* gene encodes 5-methylenetetrahydrofolate reductase catalyzing the conversion of 5,10-methylenetetrahydrofolate (methyleneTHF) to 5-methyltetrahydrofolate (methylTHF), which is essential for the transformation of potentially toxic homocysteine (Hcy) into methionine by 5-methyltetrahydrofolate-homocysteine methyltransferase. The *MTHFR* gene is polymorphic with the most frequently studied variation being a C/T substitution at nucleotide 677 (rs1801133) that converts alanine into valine at residue 222 and decreases enzyme activity to 30% and 65% of the wild-type for the TT and CT genotypes, respectively (Guenther *et al.*, 1999; Yamada *et al.*, 2001).

The widely known result of decreased activity of the *MTHFR* enzyme is elevated plasma total homocysteine (ptHcy), which has been associated with increased risk of cardiovascular diseases (Klerk *et al.*, 2002; Wald *et al.*, 2002; Kim and Becker, 2003; Molloy, 2004; Holmes *et al.*, 2011). Impaired *MTHFR*

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metabolism also leads to hypomethylation of DNA and affects neurodevelopment. It is well known that folate-deficient diet during pregnancy causes developmental and neurological abnormalities (Obican *et al.*, 2010; Dunlap *et al.*, 2011). Interestingly, these effects may extend into later life, impairing mental development (del Rio *et al.*, 2009) and causing language delay (Roth *et al.*, 2011). Consistent with this, associations were found between folate metabolism and/or *MTHFR* C677T polymorphism and numerous psychiatric disorders (Bjelland *et al.*, 2003; Gilbody *et al.*, 2007; Ozbek *et al.*, 2008; Feng *et al.*, 2009; Goin-Kochel *et al.*, 2009; Peerbooms *et al.*, 2010).

The *MTHFR* T allele has been repeatedly shown to increase risk of sudden sensorineural hearing loss (SSNHL) possibly owing to its pro-coagulant action (Capaccio *et al.*, 2005, 2007; Yildiz *et al.*, 2008; Uchida *et al.*, 2010; Fusconi *et al.*, 2011). Interestingly, an inverse association between the same variant and age-related hearing loss (ARHL) was also reported with a suggested explanation invoking a protective effect on mitochondria (Durga *et al.*, 2006; Uchida *et al.*, 2011).

Despite the intriguing findings in SSNHL and ARHL, the role of *MTHFR* C677T variant has not been investigated in other forms of HI such as the relatively prevalent non-syndromic postlingual HI (npHI). The purpose of the present study was to evaluate whether *MTHFR* C677T polymorphism is associated with the occurrence or severity of npHI in a Polish population.

Materials and Methods

Subjects

We studied 635 consecutive patients (297 males and 338 females) with HI, who were treated at the Institute of Physiology and Pathology of Hearing, Warsaw, Poland. The selection criteria were as follows: postlingual bilateral sensorineural HI defined age at onset of HI ≥ 3 years, lack of 35delG mutation(s) in *GJB2*, and age at examination < 40 years. We excluded subjects with SSNHL and those with syndromic HI of known genetic origin. Clinical characterization of patients is shown in Table 1. As can be seen, males had a lower mean age at onset of HI than females (10.8 vs. 13.8 years, for males and females, respectively, $p = 0.0001$) and were younger at the time of examination (18.7 vs. 21.9, respectively, $p = 0.0023$). The mean degree of HI was similar for both sexes.

Hearing levels were determined by pure-tone audiometry at frequencies of 500 Hz, 1 kHz, 2 kHz, 4 kHz, and 8 kHz. For each individual frequency and in the calculation of mean HI the thresholds for the better ear were considered.

The bioethical commission at the Institute of Physiology and Pathology of Hearing approved the study and all subjects or their guardians gave signed consent.

The distribution of *MTHFR* genotypes in the general population of Poland and analysis of relationship between the *MTHFR* genotype and pTHcy were based on a sample of 3273 subjects from the WOBASZ cohort as recently described (Waskiewicz *et al.*, 2011). The WOBASZ project is the Polish National Multicenter Health Survey—a cross-sectional study on the prevalence and control of risk factors for cardiovascular disease. The WOBASZ study was conducted in 2003–2005 by the Institute of Cardiology in Warsaw and was based on a representative random sample of the Polish population aged 20–74 years with a response rate of 74.3% and 79.3% for men and women, respectively. The hearing status of WOBASZ subjects was not known (Broda and Rywik, 2005; Rywik *et al.*, 2005).

Genotyping

MTHFR C677T (rs1801133) genotyping was performed by real-time PCR using Assay on Demand reagents (Life Technologies) and an ABI Prism 7500 apparatus (Applied Biosystems). The distribution of the *MTHFR* genotypes did not show deviation from Hardy–Weinberg equilibrium (HWE, $p = 0.13$).

Statistical analysis

Pairwise tests for equality of means of hearing thresholds and age were performed by student *t*-test and Mann–Whitney test (a nonparametric test). Additive effects of the dosage of the *MTHFR* T allele on the hearing threshold were analyzed by Pearson *r* and Kendall tau tests (a nonparametric correlation test). The nonparametric tests were used to eliminate the influence of any departures from normal distribution on significance testing. Analysis of HWE (to verify typing accuracy), calculation of odds ratio (OR) assuming various genetic models of effect (dominant, codominant, and recessive), and assessment of the fit of the data to the respective models (p_{fit}) were performed using the Web-Assotest program (www.ekstroem.com/assotest/assotest.html), which is based on chi-square or logistic regression analysis (Hansen *et al.*, 2004). Whenever indicated as p_{cor} , the *p*-values were corrected for number of comparisons using the Bonferroni method. The correction factor was nine as we analyzed three models of genetic effects (dominant, additive, or recessive) in three cohorts (all patients, males, and females). Severe/profound HI (as opposed to mild/moderate) was defined using the previously proposed cutoff of > 70 dB at 0.5–2 kHz (Snoeckx *et al.*, 2005) calculated for the better ear. Confidence intervals (CI) were calculated as 95% CI. The effect size of *MTHFR* TT genotype on pTHcy in Polish males versus females from the WOBASZ cohort was analyzed by

TABLE 1. CLINICAL CHARACTERIZATION OF STUDIED PATIENTS

	All, n = 635	Males, n = 297	Females, n = 338	p-Value ^a
Mean hearing threshold HI (SD)	42.4 (22.8)	42.5 (24.0)	42.4 (21.7)	NS
Mean age at examination, years (SD)	20.4 (10.3)	18.7 (10.2)	21.9 (10.3)	0.0001
Mean age at onset of HI, years (SD)	12.0 (9.3)	10.8 (8.6)	13.0 (9.7)	0.0023

Mean age at onset of HI was defined as the age at which the patient or his/her parents first became aware of HI.

^aStudent's *t*-test.

HI, hearing impairment; NS, nonsignificant; SD, standard deviation.

TABLE 2. DISTRIBUTION OF *MTHFR* GENOTYPES AMONG HEARING IMPAIRMENT PATIENTS AND SUBJECTS FROM A BACKGROUND POLISH POPULATION (WOBASZ COHORT) (Waskiewicz *et al.*, 2011)

Cohort	n	MTHFR		
		CC n (%)	CT n (%)	TT n (%)
HI				
Males	302	154 (50.0)	122 (40.4)	26 (8.6)
Females	345	163 (47.3)	138 (40.0)	44 (12.8)
All	647	317 (49.0)	260 (40.2)	70 (10.8)
WOBASZ cohort				
Males	1561	730 (46.8)	673 (43.1)	158 (10.1)
Females	1712	842 (49.2)	709 (41.4)	161 (9.4)
All	3273	1572 (48.0)	1382 (42.2)	319 (9.7)

There were no statistically significant differences (all p -values >0.15) in any pairwise comparison between patients (males, females, and all) and population controls (males, females, and all).

including the appropriate interaction term in an ANOVA model of the effect of genotype and sex on log-transformed ptHcy. The log transformation of ptHcy was performed to achieve normal distribution of this variable. All calculations were performed with Statistica software package.

Our study had a power of 0.8 ($\alpha=0.05$) to detect a difference in prevalence of *MTHFR* T between cases and controls conferring allelic OR=1.2 (OR=1.3 in sex-stratified analyses). Regarding correlation between T-allele dosage and HI, we could detect an effect associated with $r=0.11$ with a power of 0.8 ($\alpha=0.05$; $r=0.16$ in sex-stratified analyses, power calculations performed with Statistica).

Results

MTHFR genotype distribution is similar among HI subjects and population controls

Distribution of the *MTHFR* genotypes among HI subjects and population controls is shown in Table 2. We did not find statistically significant differences in any pairwise comparison between patients (males, females, and all) and controls (males, females, and all) irrespective of the inheritance model tested (all p -values >0.15).

TABLE 3. BETTER EAR HEARING THRESHOLDS (MEAN OF ALL FREQUENCIES) AMONG HEARING IMPAIRMENT PATIENTS STRATIFIED BY THE *MTHFR* GENOTYPE

MTHFR	Better ear hearing thresholds (mean for all freq.)								
	All ^a			Males ^b			Females		
	Mean	SD	n	Mean	SD	n	Mean	SD	n
CC	41.4	22.3	313	38.8	20.9	151	44.0	23.4	162
CT	42.1	22.4	255	44.9	25.1	121	39.6	19.5	134
TT	48.3	25.5	67	53.3	31.8	25	45.4	20.7	42

^a $r=0.073$, $p=0.067$, Kendal Tau test: $p=0.06$.

^b $r=0.182$, $p=0.002$, $p_{\text{cor.}}=0.018$. Kendal Tau test: $p=0.0013$, $p_{\text{cor.}}=0.017$; Bonferroni correction factor=9.

MTHFR genotype is associated with higher hearing thresholds in males

Mean hearing thresholds for the better ear among HI patients stratified by *MTHFR* genotype and sex are shown in Table 3. Among males, we found a correlation between the number of *MTHFR* T alleles and the hearing threshold with the mean thresholds (dB) being 38.8, 44.9, and 53.3, for CC, CT, and TT genotypes, respectively ($r=0.182$, $p=0.002$, $p_{\text{cor.}}=0.018$; Kendal Tau test: $p=0.0013$, $p_{\text{cor.}}=0.017$). Among males, the difference in hearing thresholds between the CC and TT genotypes was 14.5 dB with CI from 28.0 to 1.1 dB ($p<0.04$, by both t -test and Mann-Whitney test).

There was no correlation between mean hearing thresholds and the *MTHFR* genotype among females, regardless of the model considered, whereas in the whole cohort there was a trend for correlation in the same direction as that found among males ($r=0.073$, $p=0.067$; Kendal Tau test: $p=0.06$, Table 3).

Hearing thresholds at individual frequencies among patients stratified by sex and *MTHFR* genotype are shown in Figure 1.

FIG. 1. Hearing thresholds at individual frequencies among patients stratified by sex and the *MTHFR* genotype. r , Pearson correlation coefficient, p - p -value for Pearson correlation test, p_{Ken} , p -value for Kendall tau correlation test. Vertical bars show 95% confidence intervals.

The graph for males shows curves that are nonoverlapping and consistent with the highest degree of HI among *MTHFR* TT homozygotes, intermediate among CT heterozygotes, and the lowest among those with CC genotypes (Fig. 1). For all frequency, correlations between the dose of the T allele and the degree of HI were found using both parametric and nonparametric tests, but the most pronounced effects were observed at lower frequencies, that is, 0.5–2 kHz. The mean hearing thresholds at 0.5–2 kHz were higher by 17.7 dB among TT versus CC carriers ($p=0.007$, nonoverlapping CI in Fig. 1); whereas at 4–8 kHz, the difference was not statistically significant ($p=0.09$, overlapping CI in Fig. 1).

Strong association between severe/profound HI and MTHFR genotype in males

We also analyzed distribution of the *MTHFR* genotypes among subjects stratified according to clinical relevance of HI defined as severe/profound HI versus mild/moderate HI (Table 4). We found that among males under an additive model, a single copy of the T allele conferred risk for severe/profound HI associated with $OR=2.20$ ($p=0.003$). An even stronger association was found under a recessive model ($OR=4.88$, $p=0.001$). Formal comparison of the two models showed that although the recessive model had a better fit, the additive model could not be rejected ($p_{fit}=0.4$ vs. $p_{fit}=0.16$, for the recessive and additive model, respectively, Table 4). Severe/profound HI was not associated with *MTHFR* genotype among females or in the total cohort (Table 4).

In the general population of Poland the effect of MTHFR genotype on pHcy is stronger in males than in females

In a recent study based on 3273 subjects from the WOBASZ cohort it was shown that the Polish adult population is characterized by a higher mean pHcy among males than females and that this difference is particularly pronounced among carriers of the *MTHFR* TT genotype (Waskiewicz *et al.*, 2011). In the WOBASZ data set, the difference in age-adjusted geometric mean of pHcy (μM) between *MTHFR* TT

versus CT/CC genotypes was $13.12-10.18=2.94$ (22.4%) for males and $9.69-8.76=0.93$ (9.6%) for females. The stronger effect of *MTHFR* TT genotype on pHcy in Polish males versus females was further evidenced by a highly statistically significant interaction between sex and genotype observed when log-transformed pHcy was analyzed by ANOVA ($p<0.00004$).

Discussion

We found that *MTHFR* C677T genotype distribution among patients with postlingual sensorineural HI was similar to the general population, whereas there was a dose-dependent correlation between the T variant and the degree of HI. The effect was found among males but not females and resulted in a relatively large mean difference of 17.7 dB between TT and CC *MTHFR* carriers when measured at 0.5–2 kHz. Further, the TT genotype conferred high risk for severe/profound HI ($OR=4.88$, $p=0.001$) among males. In parallel to the sex-specific correlation among HI patients, we found that in the general population of Poland the known effect of *MTHFR* 677T on pHcy was also distinctly more pronounced in males than in females.

Similar distribution of *MTHFR* C677T genotypes among patients and subjects from the background population (WOBASZ cohort) suggests that the T allele alone is not a risk factor for developing postlingual HI before age 40. However, the correlation between the dose of the T allele and hearing thresholds indicates that the *MTHFR* genotype modifies the effect of yet unknown primary factor(s) causing HI. This effect is novel since the *MTHFR* C677T has not been previously analyzed in HI patients apart from distinct subsets with SSNHL (Rudack *et al.*, 2004; Gross *et al.*, 2006; Capaccio *et al.*, 2007; Yildiz *et al.*, 2008; Uchida *et al.*, 2010; Fusconi *et al.*, 2011) or ARHL (Durga *et al.*, 2006; Uchida *et al.*, 2011) who were both excluded from our cohort.

The direction of association found by us (i.e., unfavorable effect of the T allele) is similar as previously found in a range of cardiovascular diseases (Klerk *et al.*, 2002; Wald *et al.*, 2002; Kim and Becker, 2003; Molloy, 2004; Holmes *et al.*, 2011) and in SSNHL (Capaccio *et al.*, 2005, 2007; Gross *et al.*, 2006; Yildiz *et al.*, 2008; Uchida *et al.*, 2010; Fusconi *et al.*, 2011). Interestingly, an association in the opposite direction (i.e., protection conferred by *MTHFR* T) was reported in ARHI (Durga *et al.*, 2006; Uchida *et al.*, 2011). These divergent effects of *MTHFR* genotypes on various kinds of HI are intriguing and call for more studies in well-defined cohorts of patients.

Although association does not imply causation, it is tempting to suggest that worse hearing among males with *MTHFR* 677T is linked with the known functional defect of *MTHFR* 222Val combined with folate deficiency. *MTHFR* 222Val (encoded by the 677T allele) is characterized by easy flavin loss that lowers the enzyme activity. Importantly, this defect can be corrected by increased concentration of folate derivatives (Guenther *et al.*, 1999; Yamada *et al.*, 2001). In keeping with this, the effect of *MTHFR* TT on pHcy is weak/absent in populations with high supply of folate, for example, due to food fortification (Brattstrom *et al.*, 1998; De Bree *et al.*, 2003; Yang *et al.*, 2008; Papoutsakis *et al.*, 2010; Holmes *et al.*, 2011). Further, folate status modulates the association between *MTHFR* T and cardiovascular phenotypes such as coronary heart disease (Klerk *et al.*, 2002; Wald *et al.*,

TABLE 4. DISTRIBUTION OF THE *MTHFR* GENOTYPES ACCORDING TO OCCURRENCE OF SEVERE/PROFOUND OR MILD/MODERATE HEARING IMPAIRMENT (0.5–2 kHz, MEAN OF BETTER EAR)

HI	N	MTHFR		
		CC n (%)	CT n (%)	TT n (%)
All				
Mild/moderate	578	285 (49.3)	235 (40.7)	58 (10)
Severe/profound	69	32 (46.4)	25 (36.2)	12 (17.4)
Males				
Mild/moderate	266	141 (53)	108 (40.6)	17 (6.4)
Severe/profound ^a	36	13 (36.1)	14 (38.9)	9 (25)
Females				
Mild/moderate	312	144 (46.2)	127 (40.7)	41 (13.1)
Severe/profound	33	19 (57.6)	11 (33.3)	3 (9.1)

^aAdditive model: $OR=2.20$, $CI:1.32-3.67$, $p=0.003$, $p_{fit}=0.16$, recessive model: $OR=4.88$, $CI:1.98-12.01$, $p=0.001$, $p_{fit}=0.4$.

CI, confidence interval; OR, odds ratio.

2002), intima-media thickness (Liu *et al.*, 2007), or stroke (Holmes *et al.*, 2011).

Thus, it could be speculated that the male-specific association between *MTHFR* 677T and HI is caused by more prevalent/pronounced folate deficiency among males than females in our cohort. Although we did not test folate concentration among patients, an indirect argument for this hypothesis comes from higher pHcy in males versus females found in the WOBASZ cohort—a large sample representative of the general Polish population (Waskiewicz *et al.*, 2011). Importantly, in the WOBASZ we also showed that pHcy elevating effect of *MTHFR* genotype is stronger in males than females. These observations are consistent with recently reported lower response of erythrocyte folate to folic acid supplementation in males than females, indicating that males are generally more prone to folate deficiency (Winkels *et al.*, 2008). The most frequent causes of folate deficiency, which could explain its high apparent prevalence among Polish men, are poor diet and/or alcohol abuse (Hamid *et al.*, 2009).

An alternative explanation of male-specific association between the degree of HI and the *MTHFR* genotype would be to postulate an interaction with recessive X-linked loci, whose defects, in theory, could be responsible for a portion of HI among males in our cohort (<http://hereditaryhearingloss.org>). Such an interpretation would fit the observation that male and female patients also differed in age at HI onset and at the time of examination. These differences may stem from chance or an unknown referral bias, whereas theoretically they could also be caused by cases of recessive X-linked HI, provided that they are characterized by relatively early onset of HI.

If the negative effect of *MTHFR* T on HI is indeed dependent on folate deficiency our findings would indicate that folate supplementation—an intervention which is simple, cheap, and devoid of side effects—could be beneficial for young subjects with postlingual HI. Interestingly, notwithstanding the counterintuitive findings in genetic studies (Durga *et al.*, 2006; Uchida *et al.*, 2011) folate supplementation has shown a benefit in ARHL (Durga *et al.*, 2007; Shargorodsky *et al.*, 2010).

Although subjects with *MTHFR* CT genotype have intermediate enzyme activity compared to CC and TT homozygotes, the increase of pHcy mediated by *MTHFR* T, which is the best-known phenotypic effect of this variant, appears to be a recessive trait. This may seem at odds with correlations between HI and *MTHFR* T in our cohort, which were found assuming an additive effect (Table 3 and Fig. 1). One explanation of this discrepancy could be the low power of our dataset to precisely establish the model of inheritance. Notably, the recessive effect was actually favored by the analysis after dichotomization into mild/moderate versus severe/profound HI. Further, inspection of Figure 1 also shows a greater difference between *MTHFR* TT versus CT and *MTHFR* CT versus CC genotypes, again precluding a definite conclusion regarding the effect mode.

Another possibility is that the mode of effect of *MTHFR* T need not be the same for pHcy and HI. In line with this, it was recently suggested that effects of *MTHFR* variants on SSNHL or atherosclerosis are not mediated by Hcy but by 5-methyltetrahydrofolate, possibly acting through modulation of nitric oxide bioavailability and superoxide generation in blood vessels (Antoniades *et al.*, 2009; Uchida *et al.*, 2010).

Interestingly, inspection of the results of Antoniades *et al.* shows that effects of *MTHFR* genotype on these markers are generally consistent with an additive or dominant, but not a recessive model (Antoniades *et al.*, 2009).

In conclusion, we found that the *MTHFR* 677T allele is associated with distinctly higher hearing thresholds among young (<40 years old) Polish males with postlingual non-syndromic HI of unknown cause. The known functional significance of *MTHFR* variation suggests that folate supplementation may be beneficial for these patients.

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Disclosure Statement

The authors declare no conflict of interest.

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