Polymorphisms in folate metabolism genes are associated with susceptibility to presbycusis

Article *in* Life Sciences · January 2018

DOI: 10.1016/j.lfs.2018.01.015

CITATIONS
2

READS
124

5 authors, including:

Santoshi Kumari Manche

15 PUBLICATIONS **26** CITATIONS

SEE PROFILE

Madhavi Jangala

Institute of Genetics & Hospital for Genetic Diseases

15 PUBLICATIONS **28** CITATIONS

SEE PROFILE

Life Sciences 196 (2018) 77–83

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Polymorphisms in folate metabolism genes are associated with susceptibility to presbycusis

Santoshi Kumari Manche[a,b], Madhavi Jangala[a,b], Dinesh Dudekula[a], Meganadh Koralla[a], Jyothy Akka[b,*]

[a] MAA Research Foundation, Somajiguda, Hyderabad, Telangana State, India
[b] Institute of Genetics and Hospital for Genetic Diseases, Osmania University, Hyderabad, Telangana State, India

ARTICLE INFO

Keywords:
Presbycusis
Susceptibility
Methylene tetrahydrofolate reductase
Methionine synthase
Thymidylate synthase

ABSTRACT

Aim: Presbycusis or age related hearing loss is caused by several extrinsic and intrinsic factors that damage the auditory system. Gene polymorphisms in folate metabolism were found to play an important role in the etiology of presbycusis. The present study aimed to investigate the role of 5,10-methylenetetrahydrofolate reductase (*MTHFR*), methionine synthase (*MTR*) and thymidylate synthase (*TYMS*) gene polymorphisms in the onset of presbycusis in a South Indian population.

Main methods: A total of 220 subjects confirmed with presbycusis along with 270 age and sex matched healthy controls visiting MAA ENT Hospitals, Hyderabad, India were enrolled for the study. Genotyping of *MTHFR* C677T (rs180133) and A1298C (rs1801131), *MTR* A2756G (rs1805087), *TSER* (rs1801136) and *TS*1494indel6 bp (rs16430) was carried out using PCR & PCR-RFLP methods.

Key findings: The 'TT' genotype of *MTHFR* C677T and '152 bp/152 bp' genotype of *TS*1494indel6 bp showed statistically significant risk for presbycusis while CC genotype of *MTHFR* A1298C, '2R/2R' genotype of *TSER at* 3′UTR and 6 bp ins/6 bp ins of *TYMS* at 5′UTR were found to be protective. The **T**-A-A haplotype combination of *MTHFR* C677T, *MTHFR* A1298C and *MTR* A2756G as well as 3R- 152 bp of *TYMS* at 5′UTR and 3′UTR were also found to contribute significant risk for the onset of presbycusis. Further, the combination of SNP loci *TSER*: *TS*1494indel6 bp exhibited moderate linkage in presbycusis.

Significance: The present pilot study identified the significant association of gene variants of *MTHFR* and *TYMS* with presbycusis. These findings aid in early diagnosis of hearing loss in the elderly population.

1. Introduction

Hearing loss is one of the major chronic health problems affecting 360 million people globally and the prevalence is higher in the aging population [1]. Approximately 24% of the subjects belonging to the age group of 65–74 years and about 40% of the subjects above 75 years are reported to be affected with hearing loss [2]. The loss of hair cells and other cellular elements in cochlea leads to auditory dysfunction in elderly causing presbycusis [3]. The dysfunction in cochlear transduction depends upon many extrinsic and intrinsic factors [4,5]. It is reported that deficiency of several metabolites and micronutrients like homocysteine, vitamin B12 and folate have an influence on the onset of hearing loss [6,7,8].

Folate, a vitamin of B group plays an important role in the synthesis and methylation of DNA and also contributes to sustain methionine levels in case of low methionine availability. Polymorphisms in genes encoding enzymes such as 5,10-methylenetetrahydrofolate reductase (*MTHFR*), methionine synthase (*MTR*), methionine synthase reductase (*MTRR*) and thymidylate synthase (*TYMS*) are reported to regulate dNTP levels [9,10]. The most associated SNPs with disease pathogenicity are *MTHFR* C677T (rs1801133); *MTHFR* A1298C (rs18011310) and *MTR* A2756G (rs1805087) (Fig. 1). These polymorphisms interfere with the synthesis of methyltetrahydrofolate and remethylation of homocysteine. 5,10-Methylenetetrahydrofolate reductase (*MTHFR*) is a key enzyme in folate metabolism that plays a central role in DNA methylation through the methionine-homocysteine cycle [10,11]. Methionine synthase (*MTR*) with vitamin B12 as a cofactor uses the methyl group from 5-*MTHF* to remethylate homocysteine and produce methionine and tetra hydrofolate (THF) [12,13]. The methionine thus formed is used for the production of S-adenosylmethionine that is required for DNA methylation. Thymidylate synthase, also a crucial enzyme of the folate metabolism along with 5,10-methylenetetrahydrofolate (5,10-

* Corresponding author at: Institute of Genetics and Hospital for Genetic Diseases, Osmania University, Hyderabad, Telangana State 500016, India.
E-mail address: jyothycell@rediffmail.com (J. Akka).

https://doi.org/10.1016/j.lfs.2018.01.015
Received 12 September 2017; Received in revised form 4 January 2018; Accepted 15 January 2018

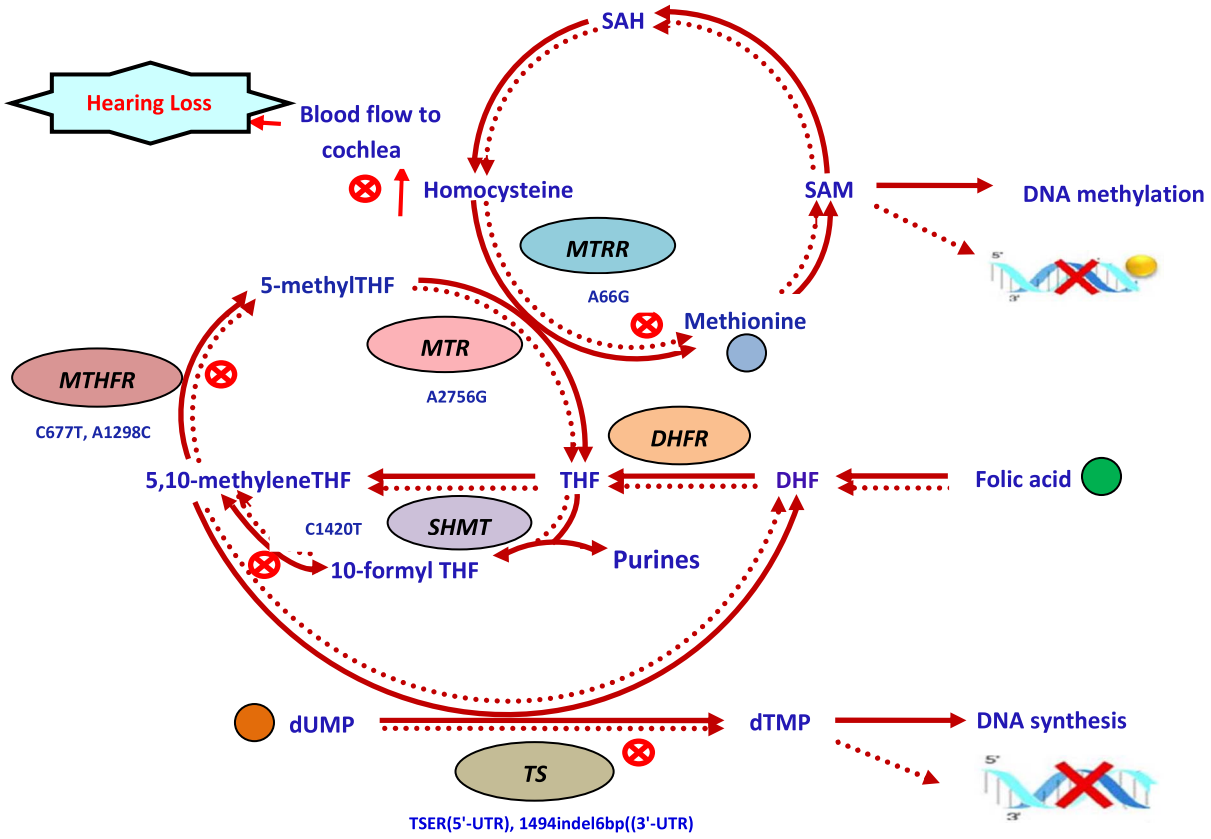

Fig. 1. Genes involved in folate metabolism.
(Source: Skibola et al., Blood 2004; 104: 2155–2162 (modified))

MTHF) produces thymidylate that plays a significant role in DNA synthesis and repair [14,15].

The *MTHFR* and *MTR* genes are located on chromosome 1p36.3 and *TYMS* gene is located on Chr 18p11.32. The two common *MTHFR* polymorphisms C677T in exon 4 and A1298C in exon 7 present in the catalytic domain of the protein result in decreased enzyme activity that has led to the onset of several diseases [12]. *MTR* A2756G polymorphism that results in substitution of aspartic acid for glycine decreases methionine synthase activity increases the cellular homocysteine level and causes hypomethylation of DNA [14]. The variations in *TYMS* gene promoter enhancer region (*TSER*: rs1801136) commonly constituted by a double repeat of 28 bp (2R, *TSER**2) and triple repeat (3R, *TSER**3) in 5′-untranslated enhancer region (5′-UTR) are involved in modulating *TYMS* mRNA expression and translational efficiency [16,17]. In addition, 3R allele with substitution of G > C (*TSER**3 G > C, rs2853542) at the 12th nucleotide in the E-box consensus element is implicated in decreased *TYMS* transcription [17]. Further, a 6 bp insertion/deletion in the 3′-UTR at 1494 position of *TYMS* (*TYMS*1494indel6bp: rs16430) has an influence on mRNA stability and protein expression [9,18].

Recent studies carried out in different populations have reported that the gene polymorphisms in folate metabolism played a key role in the etiopathogenesis of hearing loss [19]. In prebycusis, Uchida et al., (2011) reported an association of *MTHFR* and *MTR* gene polymorphisms in Japanese population and Durga et al., (2006) reported *MTHFR* 677 'TT' genotype to be associated in Netherlands population [20,21]. However, no studies have been reported on functional polymorphism of *MTHFR*, *MTR and TYMS* genes in relation with presbycusis in Indian population. Therefore, the present pilot study has been undertaken to evaluate the role of gene polymorphisms of folate metabolism pathway in the onset of presbycusis in a South Indian population.

2. Subjects and methods

2.1. Subjects

In the present case–control study, 220 cases confirmed with presbycusis at MAA ENT HOSPITALS, Hyderabad, Telangana State, over a period of three years from 2011 to 2014 and 270 age and sex matched healthy controls were recruited for the study. All patients underwent a detailed medical otoscopic examination that included tympanometry and pure tone audiometric test analysis for evaluating hearing loss at 0.5, 1, 2, 4 and 8 kHz frequencies. The patients whose age was equal or > 40 years and with no vestibular or any history of otological surgery, sensorineural form of hearing loss (bilateral as well as symmetrical) and co-morbidities such as diabetes, hypertension and hypothyroidism were included in the study. Subjects suffering from outer & middle ear diseases and noise induced hearing loss were excluded from the study. Informed written consent was taken from all participants, and the study was carried out with the Institutional Ethics committee (Institute of Genetics and Hospital for Genetic Diseases) approval. Blood samples were collected from all the study subjects in EDTA vials. Genomic DNA was isolated from whole blood samples by salting out method [22] and stored at −80 °C for molecular analysis.

2.2. Genotyping

The *MTHFR* C677T (rs1801133), A1298C (rs1801131), *MTR* A2756G (rs1805087), and *TYMS*1494indel6 bp (rs16430) polymorphisms were determined using the PCR-RFLP and *TSER* (rs1801136) by using amplified fragment length polymorphism (AFLP) assays. The details of the genes under study along with their respective primers, PCR reaction conditions, and restriction enzymes are presented in Table 1.

Table 1
Primers, PCR reaction conditions, PCR product, Restriction enzyme products for each type of gene polymorphism involved in folate metabolism.

Gene NCBI rs No	Primers (5′-3′)	Sequence	PCR Reaction conditions	PCR product(bp)	Restriction enzyme And its products	References
MTHFR (C 677 T) (rs1801133)	Forward Reverse	5′-TGAAGGAGAAGGTGTCTGCGGGA-3′ 5′-AGGACGGTGCGGTGAGAGTG-3′	D- 94 °C /30 s, A-62 °C /30 s, E- 72 °C/30 s.; C-40	198	*Hinf I* TT genotype-175 and 23 bp CC genotype- 198 bp	[23]
MTHFR (A 1298 C) rs1801131	Forward Reverse	5′-AAGGAGGAGCTGCTGAAGATG-3′ 5′-CTTTGCCATGTCCACAGCATG-3′	D - 94 °C/30 s, A − 61 °C/30 s, E- 72 °C/30 s; C-35	237	*MboII* AA genotype- 182, 28,27 bp CC genotype- 210, 27 bp	[24]
MTR (A 2756 G) (rs 1,805,087)	Forward Reverse	5′-TGTTCCCAGCTGTTAGATGAAAATC-3′ 5′-GATCCAAAGCCTTTTACACTCCTC-3′	D- 95 °C/1 min, A-60 °C/1.5 min, E- 72 °C/ 1 min; C-35	211	*HaeIII* AA genotype- 211 bp GG genotype- 131,80 bp	[25,26]
TYMS 5′-UTR(AFLP) (rs1801136)	Forward Reverse	5′-GTGGCTCCTGCGTTTCCCCC-3′ 5′-GGCTCCGAGCCGGCCACAGGCATGGCGCGG-3′	D − 95 °C/ 30 s, A - 61 °C/30 s, E - 72 °C/45 s; C-35	327	3R3R- 243 bp 3R2R- 243, 215 bp 2R2R-215 bp	[27]
TYMS 3′-UTR(Ins6/del6) (rs16430)	Forward Reverse	5′-CAAATCTGAGGGAGCTGAGT-3′ 5′-CAGATAAGTGGCAGTACAGA-3′	D - 94 °C/5 min, A - 58 °C/1 min, E- 72 °C/40 s	152/158	*DraI* Ins6bp/Ins6bp–158 bp Ins6bp/del6 bp −158, 152 bp del6bp/del6bp–152 bp	[15]

a. D-Denaturation, A-Annealing, E-Elongation, C-Cycles, s-Seconds, min-minutes.

2.3. Statistical analysis

The data obtained was coded for statistical evaluations. Appropriate statistical analysis was performed using the Statistical Package for Social Sciences, PASW STATISTICS 18.0 software (SPSS Inc., Chicago, IL, USA). Continuous data is represented as means and standard deviations whereas categorical data as proportions. Statistical significance of the differences in frequency of genotypes was analyzed using the Fischer's exact test and binary logistic regression analysis. Hardy–Weinberg equilibrium was tested using goodness-of-fit. Linkage disequilibrium and haplotype frequencies were inferred using Haploview software (MIT/Harvard Broad Institute, Cambridge, USA).

3. Results

The demographic data revealed that the prevalence of presbycusis was found to be associated with advanced aging. The mean age of onset of presbycusis was 60.1 ± 10.38 years in the study subjects. The demographic, audiological and associated co-morbidities of the presbycusis subjects and controls are represented in Table 2. The genotype frequencies for all the polymorphisms of the genes involved in folate metabolism pathway under study were in agreement with the Hardy–Weinberg equilibrium. The genotypic and allelic frequencies between cases and controls for the *MTHFR* C677T, *TSER* and *TYMS*1494indel6 bp polymorphisms were statistically significant. However, no significant difference was observed for the *MTR* A2756G as well as *MTHFR* A1298C polymorphisms between cases and controls. The frequency distribution for the genes and alleles among study subjects is shown in Table 3.

3.1. Distribution of allelic and genotypic frequencies of gene polymorphisms under study

3.1.1. MTHFR C677T polymorphism

The frequencies of 'TT' genotype (7.3% vs 1.1%) and 'T' allele (23.2% vs 8.3%) were found to be significantly higher in presbycusis group (TT genotype: OR 9.07, 95% CI 2.59–31.71, p < .001; T allele: OR 3.32, 95% CI 2.28–4.84, p < .001) compared to controls respectively. With regard to inheritance models, the 'TT' genotype in recessive model (adjusted OR 2.75, 95% CI 1.77–4.29, p < .001) and 'CT' genotype in over-dominant model (adjusted OR 2.75, 95% CI 1.77–4.29, p < .001) were found to increase the risk for presbycusis (Table 3).

Table 2
Distribution of demographic, audiologic, associated risk factors and co-morbidities in the study subjects.

Parameters	Cases (%)	Controls (%)	P-value†
Gender			
Male	127(57.7)	160(59.3)	0.732
Female	93(42.3)	110(40.7)	
Age at visit(years)††	63.7 ± 9.98	62.9 ± 9.86	0.396
Age of onset(years)	60.2 ± 10.38	NA	NA
40–50	36(16.4)		0.970
50–60	80(36.4)		
> 60	104(47.2)		
Degree of hearing (Decibels-dB)	52.9 ± 8.51	12.1 ± 0.36	NA
< 40 dB	12(5.5)	0(0.0)	NA
> 40 dB	208(94.5)	0(0.0)	
Associated risk factors			
Tinnitus	42(19.1)	0(0.0)	NA
Vertigo	9(4.1)	0(0.0)	NA
Co-morbidities			
Diabetes	48(21.8)	18(6.7)	< 0.001
Hypertension	30(13.6)	33(12.2)	0.642
Hypothyroidism	3(1.4)	6(2.2)	0.481

a. † − $\chi 2$ test;††-Independent sample 't test.
b. NA-Not applicable.

3.1.2. MTHFR A1298C polymorphism

The frequencies of homozygotes 'CC' genotype (22.6% vs15.9%) and 'C' allele (46.1% vs 38.9%) were found to be predominant in controls indicating their protective role for presbycusis. (CC genotype: OR 0.56, 95% CI 0.34–0.94, p = .029; C allele: OR 0.74, 95% CI 0.58–0.96, p = .054). The inheritance models have also not showed any significant association of the genotypes with presbycusis (Table 3).

3.1.3. MTR A2756G polymorphism

The frequencies of 'GG' genotypic and 'G' allele was found to be high in controls when compared to presbycusis cases indicating no association. Inheritance models have also showed no significant association with presbycusis (Table 3).

3.1.4. TSER 2R/3R repeat polymorphism

The frequencies of '2R' allele (43.9% vs 23.9%), "3R2R" (48.5% vs 39.5%) and '2R2R' genotypes (19.6% vs 4.1%) were found to be predominant in controls when compared to presbycusis group ('2R' allele:

Table 3
Genotypic and Allelic frequencies of *MTHFR*, *MTR* and *TYMS* genes in the study subjects.

Inheritance model		Subjects(%)	Controls(%)	OR (95 CI)†	p-value†	Adjusted OR (95% CI)††
MTHFR C677T rs1801133 (677C > T, Ala222Val)						
Co-dominant	Genotypes					
	CC	134(60.9)	228(84.4)	1.00(Reference)		1.00(Reference)
	CT	70(31.8)	39(14.4)	3.05(1.96–4.77)	< 0.001	3.04(1.95.34–4.75)*
	TT	16(7.3)	3(1.1)	9.08(2.60–31.72)	0.001	9.07(2.59–31.71)*
Dominant	CC vs CT + TT	86(39.1)	42(15.6)	3.48(2.28–5.34)	< 0.001	3.47(2.29–5.35)
Recessive	CC + CT vs TT	16(7.3)	3(1.1)	6.98(2.01–24.28)	< 0.001	6.98(2.00–24.27)*
Over-dominant	CC + TT vs CT	70 (33.4)	39(14.4)	2.76(1.78–4.30)**	< 0.001	2.75(1.77–4.29)*
	Alleles					
	C	338(76.8)	495(91.7)	1.00(Reference)	< 0.001	
	T	102 (23.2)	45(8.3)	3.32(2.28–4.84)		
MTHFR A1298C rs1801131 (1298A > C, Glu429Ala)						
	Genotypes					
Co-dominant	AA	84(38.2)	82(30.4)	1.00(Reference)		1.00(Reference)
	AC	101(45.9)	127(47)	0.78(0.52–1.16)	0.216	0.77(0.51–1.14)
	CC	35(15.9)	61(22.6)	0.56(0.34–0.94)	0.027	0.56(0.33–0.94)*
Dominant	AA vs AC + CC	136(61.8)	188(69.6)	0.71(0.47–1.03)	0.069	0.71(0.49–1.03)
Recessive	AA + AC vs CC	35(15.9)	61(22.6)	0.65(0.41–1.03)	0.064	0.65(0.41–1.03)
Over-dominant	AA + CC vs AC	101(45.9)	127(47)	0.96(0.67–1.37)	0.803	0.95(0.66–1.36)
	Alleles					
	A	269(61.1)	291(53.9)	1.00(Reference)	0.054	
	C	171(38.9)	249(46.1)	0.74(0.58–0.96)		
MTR A2756G rs1805087 (2756A > G, Asp919Gly)						
	Genotypes					
Co-dominant	AA	116(52.7)	144(53.3)	1.00(Reference)		1.00(Reference)
	AG	92(41.8)	105(38.9)	1.09(0.75–1.58)	0.658	1.09(0.75–1.58)
	GG	12(5.5)	21(7.8)	0.71(0.34–1.50)		0.73(0.34–1.56)
Dominant	AA vs AG + GG	104(47.3)	126(46.7)	1.03(0.72–1.46)	0.894	1.02(0.72–1.46)
Recessive	AA + AG vs GG	12(5.5)	21(7.8)	0.68(0.33–1.42)	0.307	0.69(0.33–1.44)
Over-dominant	AA + G/G vs AG	92(41.8)	105(38.9)	1.13(0.79–1.62)	0.511	1.13(0.78–1.62)
	Alleles					
	A	324(73.6)	393(72.7)	1.00(Reference)	0.763	
	G	116(26.4)	147(27.3)	0.96(0.72–1.27)		
TSER rs1801136(*TS* 5′-UTR)						
	Genotypes					
Co-dominant	3R3R	124(56.4)	86(31.9)	1.00(Reference)		1.00(Reference)
	3R2R	87(39.5)	131(48.5)	0.46(0.31–0.68)	< 0.001	0.46(0.31–0.68)*
	2R2R	9(4.1)	53(19.6)	0.12(0.06–0.25)	< 0.001	0.12(0.05–0.25)*
Dominant	3R3R vs 3R3R + 2R2R	96(43.6)	184(68.2)	0.36(0.25–0.52)	< 0.001	0.36(0.25–0.52)
Recessive	3R3R + 3R2R vs 2R2R	9(4.1)	53(19.6)	0.18(0.08–0.36)	< 0.001	0.17(0.08–0.36)
Over-dominant	3R3R + 2R2R vs 3R2R	87(39.5)	131(48.5)	0.69(0.48–0.99)	0.047	0.70(0.49–1.00)*
	Alleles					
	3R	335(76.1)	303(56.1)	1.00(Reference)	< 0.001	
	2R	105(23.9)	237(43.9)	0.40(0.30–0.53)		
*TYMS*1494indel6 bp rs16430 (*TYMS* 3′-UTR)						
	Genotypes					
Co-dominant	6 bp ins/6 bp ins	50(22.7)	85(31.5)	1.00(Reference)		1.00(Reference)
	6 bp ins/ 6 bp del	107(48.6)	141(52.2)	1.29(0.84–1.98)	0.246	1.29(0.84–1.99)
	6 bp del/6 bp del	63(28.6)	44(16.3)	2.43(1.45–4.09)	0.001	2.45(1.45–4.14)**
Dominant	6 bp ins/6 bp ins vs	170(77.3)	185(68.5)	0.64(0.43–0.96)	0.031	0.64(0.42–0.96)
	6 bp ins/ 6 bp del +6 bp del/6 bp del					
Recessive	6 bp ins/6 bp ins +6 bp ins/6 bp del vs 6 bp del/6 bp del	63(28.6)	44(16.3)	2.06(1.33–3.19)	0.035	2.07(1.33–3.22)
Over-dominant	6 bp ins/6 bp ins + 6 bp ins/6 bp del vs 6 bp ins/6 bp del	107(48.6)	141(52.2)	0.87(0.61–1.24)	0.43	0.87(0.61–1.24)
	Alleles					
	6 bp ins	207(47.1)	311(57.6)	1.00(Reference)	< 0.001	
	6 bp del	233(52.9)	229(42.4)	1.53(1.19–1.97)		

a. †-Binary logistic regression analysis; ††-Multinomial logistic regression analysis.
b. OR-Odds ratio; CI-Confidence interval.
c. Adjusted OR for gender and age.
d. Significance of *p-value = .01, **p-value ≤ .001 after Bonferroni correction.

OR 0.40, 95% CI 0.30–0.53, p < .001; '3R2R' genotype: adjusted OR 0.46, 95% CI 0.31–0.68, p < .001; '2R2R' genotype (adjusted OR 0.12, 95% CI 0.05–0.25, p < .001 respectively). '2R2R' genotype in recessive model (adjusted OR 0.17, 95% CI 0.08–0.36, p < .001), and '3R2R' genotype in over-dominant model (adjusted OR 0.70, 95% CI 0.49–1.00, p = .047), were also high in controls. Thus, indicating the protectiveness of '2R' allele in presbycusis subjects (Table 3).

3.1.5. *TYMS1494indel6 bp gene polymorphism*

The frequencies of '6 bp del/6 bp del' homozygous variant genotype (8.5% vs 16.3%, adjusted OR 2.43, 95% CI 1.45–4.09, p < .001 and '6 bp del ' allele (52.9% vs 42.4%, adjusted OR 1.53, 95% CI 1.19–1.97, p < .001) were found to be predominant in presbycusis group compared to controls. Among all the inheritance models, 'the frequency of 6 bp del/6 bp del' genotype in the recessive model, when compared with '6 bp ins/6 bp ins + 6 bp ins/6 bp del' genotype was found to be a high in presbycusis when compared to controls (adjusted OR 2.07, 95%

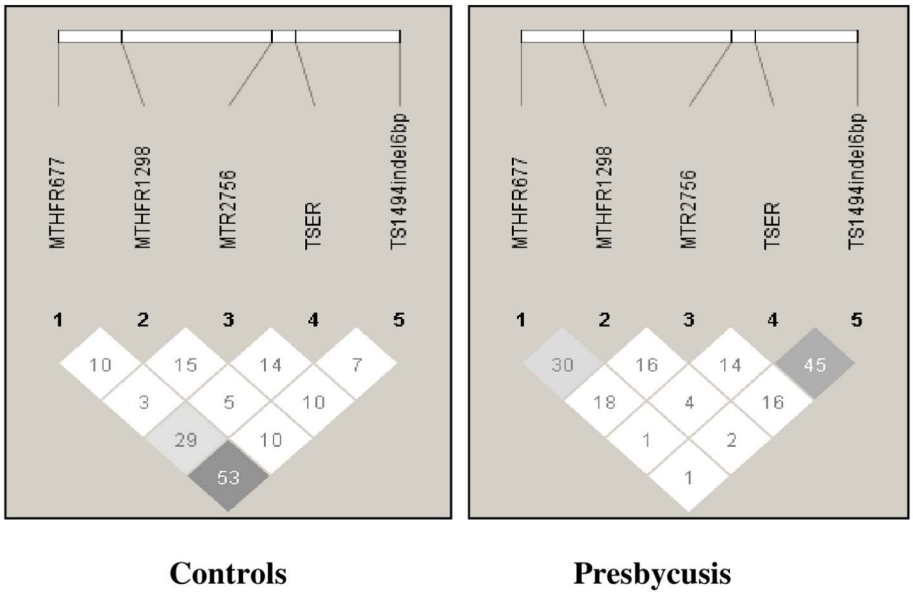

Fig. 2. Linkage disequilibrium plot of the folate genes in the study subjects.

CI 1.33–3.22, p < .001) confirming the risk of '6 bp del' allele to presbycusis (Table 3).

3.1.6. Linkage disequilibrium

Linkage disequilibrium occurs when genotypes at the two loci are dependent on each other while logarithm of the odds (LOD) is the probability of the inheritance of two gene loci as a unit indicating genetic linkage. In the present study, pair-wise linkage disequilibrium (LD) estimates obtained separately for the five gene polymorphisms of *MTHFR* C667T & A1298C, *MTR* A2756G, *TSER* and *TYMS*1494indel6 bp in cases and controls are depicted in the LD plot (Fig. 2). In case of presbycusis subjects only two of the SNP marker combinations (*TSER*: *TYMS*1494indel6 bp and *MTHFR* C677T: *MTHFR* A1298C) exhibited moderate association between the SNPs when compared to controls (D′ = 0.536, LOD = 0.84, r^2 = 0.019) (Table 4).

3.1.7. Haplotype analysis

Haplotype analysis provides valuable information about the influence of SNP combinations in the manifestation of the disease. Hence, haplotypes were constructed and analyzed for the five polymorphisms for their association with presbycusis (Table 4). The T-A-A haplotype combination of *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G conferred 4.77 fold increased risk for presbycusis. The allelic combination of 3R-152 bp at T*SER 5′ UTR* and *TYMS*1494indel6 bp also contributed risk for presbycusis (Table 5).

Table 4
Pair-wise linkage disequilibrium estimates in the study subjects.

Combination	D′	LOD	r^2
Controls			
MTHFR A1298C:*MTHFR* C677T	0.103	0.06	0.001
MTHFR A1298C:*MTR* A2756G	0.039	0.03	0.000
MTHFR A1298C:*TSER*	0.293	0.48	0.006
MTHFR A1298C:*TYMS*1494indel6bp	0.536	0.84	0.019
MTHFR C677T: *MTR* A2756G	0.155	0.5	0.008
MTHFR C677T: *TSER*	0.051	0.12	0.002
MTHFR C677T: *TYMS*1494indel6bp	0.103	0.33	0.007
MTR A2756G: *TSER*	0.143	0.58	0.010
MTR A2756G: *TYMS*1494indel6bp	0.109	0.4	0.006
TSER: *TYMS*1494indel6bp	0.075	0.26	0.005
Cases			
MTHFR A1298C:*MTHFR* C677T	0.307	1.29	0.013
MTHFR A1298C:*MTR* A2756G	0.184	1.63	0.020
MTHFR A1298C:*TSER*	0.016	0.02	0.000
MTHFR A1298C:*TYMS*1494indel6bp	0.019	0.01	0.000
MTHFR C677T: *MTR* A2756G	0.16	0.6	0.014
MTHFR C677T: *TSER*	0.041	0.04	0.001
MTHFR C677T: *TYMS*1494indel6bp	0.027	0.002	0.001
MTR A2756G: *TSER*	0.144	0.82	0.180
MTR A2756G: *TYMS*1494indel6bp	0.185	0.42	0.009
TSER: *TYMS*1494indel6bp	0.451	3.52	0.072

4. Discussion

Presbycusis is the most common auditory dysfunction associated with sensorineural hearing loss (SNHL) of higher frequencies that lead to gradual progression later in life causing social isolation, depression, diminished cognitive function, and poor quality of life. Presbycusis is influenced by multiple factors such as demographic, socioeconomic, noise exposure, ototoxins (e.g., aminoglycosides, chemotherapeutic agents, heavy metals etc.,), infections, smoking, co-morbidities, pathological conditions and senescence [28,29]. The progression and severity of auditory dysfunction in elderly were reported to show high inter-individual variability which is influenced by deficiency of

Table 5
Frequencies of the *MTHFR*, *MTR* and *TYMS* haplotypes and their associated risk in study subjects.

Haplotypes	Cases(n = 440 alleles)	Controls(n = 540 alleles)	P-value	OR(95% CI)†
TSER-*TYMS*1494indel6bp				
3R-158 bp	133(30.2)	184(34.1)	–	1.00(Reference)
3R-152 bp	202(45.9)	119(22.0)	< 0.001	2.35(1.71–3.23)
2R-158 bp	74(16.8)	127(23.5)	0.245	0.81(0.56–1.16)
2R-152 bp	31(7.1)	110(20.4)	< 0.001	0.39(0.25–0.62)
MTHFR C677T- *MTHFR* A1298C- *MTR* A2756G				
C-A-A	154(35.0)	187(34.6)		1.00(Reference)
C-A-G	45(10.2)	75(10.2)	0.146	0.73(0.48–1.12)
C-C-A	97(22.0)	176(64.5)	0.016	0.67(0.48–0.93)
C-C-G	42(9.5)	57(10.6)	0.630	0.89(0.57–1.41)
T-A-A	55(12.5)	14(2.6)	< 0.001	4.77(2.56–8.91)
T-A-G	15(3.4)	15(2.8)	0.610	1.21(0.58–2.57)
T-C-A	18(4.1)	16(3.0)	0.387	1.37(0.67–2.77)
T-C-G	14(3.2)	0(0.0)	NA	NA

a. †-Binary logistic regression analysis.
b. OR-Odds Ratio; CI-Confidence interval.
NA- Not applicable at point b.

micronutrients that affect the vascular blood flow in cochlea [30]. The *MTHFR* C677T and A1298C, *MTR* A2756G, *TSER* and *TYMS*1494indel6 bp gene polymorphisms of folate metabolism have been implicated in the etiopathogenesis of various diseases including hearing disorders [31]. Hence, the present study has attempted to evaluate the association of folate gene polymorphisms in the etiopathogenesis of presbycusis in a South Indian population.

The prevalence of presbycusis in the present study was more in men than in women which is in accordance with many of the earlier studies [32,33,34]. This is mainly attributed to the environmental and occupational exposure as well as the presence of associated co-morbidities and risk factors. Presbycusis tends to manifest in the fifth decade of life and will gradually progress over the years with symptoms of tinnitus and vertigo [35,36,37]. It was noticed that the prevalence of presbycusis in the study population increased with advancing age. The pathophysiology of adult onset hearing loss is not only influenced by the cumulative effect of genetic and physiologic factors but also due to environmental factors such as nutrition.

Several epidemiological studies have shown an association between certain nutritional deficiencies such as homocysteine and folate with development of sensorineural hearing loss (SNHL). Folate deficiency in mouse models has also indicated a significant impact on cochlear homocycteine levels thereby leading to progression of hearing loss [19,38,39]. Experiments on SNHL prone C57BL/6 J mice exhibited increased hearing thresholds after being fed on a folate deficient diet for 2 months that caused decreased serum concentrations of folate and increased tHcy levels leading to the onset of SNHL [39]. Cappaccio et al., (2005) have reported that *MTHFR* gene polymorphisms act as crucial risk factor for the onset of sensorineural hearing loss [40]. *MTHFR* C677T polymorphism converts alanine into valine at residue 222 which decreases 5,10-methylenetetrahydrofolate reductase enzyme activity to 30% in heterozygotes and 65% in variant genotypes and affects vascular function [24,41,42]. Uchida et al., (2011) also reported *MTHFR* 677 'TT' genotype to be the risk for hearing impairment in the Japanese elderly population [20]. Similarly, the present study also identified the frequency of 'T' allele and 'TT' genotype to be predominant and increased risk for presbycusis in South Indian population. The polymorphism of *MTHFR* at 1298 of the regulatory domain in the present study showed higher frequency of 'AA' genotype compared to homozygous 'CC' genotype in Presbycusis. However, the *MTR* polymorphism at A2756G did not show any significant difference between cases and controls.

The *TYMS* gene polymorphism at promoter-enhancer region (*TSER*) of 5′-UTR is involved in transcriptional autoregulation mechanism. The variation in '2R2R' repeat sequence was reported to have decreased expression of thymidylate synthase compared to '3R3R' repeat sequence [43,44]. In the present study, the heterozygous '3R2R' and homozygous '2R2R' genotypes of *TYMS* showed significant protective role in the onset of presbycusis. The 3′ UTR *TYMS* 6 bp ins/del-type polymorphism has been reported to have an influence on the stability of *TYMS* transcript [45,46]. In the present study, we found that subjects bearing homozygous 6 bp deletion genotype at nucleotide 1494 in *TYMS*-3′ UTR had nearly 1.53 fold increased risk for presbycusis.

Linkage disequilibrium analysis has revealed a moderate LD effect with the SNP loci combination of *TYMS*1494indel6bp: *TSER* (D′ = 0.45) and *MTHFR* C677T: *MTHFR* A1298C (D′ = 0.30) in presbycusis. Further, haplotype analysis has revealed that combination of T-A-A haplotype of *MTHFR* C677T, *MTHFR* A1298C, *MTR* A2756G to be associated with increased risk for the onset of presbycusis. Also, 3R-152 bp combination of *TSER* at 5′UTR and *TYMS* at 3′UTR increased the risk for presbycusis.

5. Conclusion

The present pilot study identified the association of gene variants of folate-metabolism (*MTHFR*, *MTR* and *TYMS*) with presbycusis in South Indian population. Also, the present study is the first to report the association of thymidylate synthase with presbycusis. The above genetic variants seem to be promising for early diagnosis of hearing loss in the elderly population to improve the quality of life. Further, studies on the epistatic interactions of folate genes in age related hearing loss are warranted to elucidate their possible underlying molecular mechanisms.

Acknowledgements

We would like to thank MAA Research Foundation for the funding and Ms. B. Sunita G Kumar, CMD, MAA ENT Hospitals for her support and cooperation in carrying out the study. We also thank J.V. Ramakrishna and P. Padmavathi for their techinical assistance in carrying out the work.

Conflicts of interest

There are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2018.01.015.

References

[1] B.O. Olusanya, K.J. Neumann, J.E. Saunders, The global burden of disabling hearing impairment: a call to action, Bull. World Health Organ. 92 (2014) 367–373, http://dx.doi.org/10.2471/BLT.13.128728.
[2] J. Jerger, R. Chmiel, N. Wilson, R. Luchi, Hearing impairment in older adults: new concepts, J. Am. Geriatr. Soc. 43 (1995) 928–935.
[3] P. Perez, J. Bao, Why do hair cells and spiral ganglion neurons in the cochlea die during aging? Aging. Dis 2 (2011) 231–241.
[4] H.F. Schuknecht, Presbycusis, Laryngoscope 65 (1955) 402–419.
[5] K.K. Ohlemiller, Mechanisms and genes in human strial presbycusis from animal models, Brain Res. 1277 (2009) 70–83, http://dx.doi.org/10.1016/j.brainres.2009.02.079.
[6] L.J. Brant, S. Gordon-Salant, J.D. Pearson, L.L. Klein, C.H. Morrell, E.J. Metter, et al., Risk factors related to age-associated hearing loss in the speech frequencies, J. Am. Acad. Audiol. 7 (1996) 152–160.
[7] S. Péneau, C. Jeandel, P. Déjardin, V.A. Andreeva, S. Hercberg, P. Galan, et al., Intake of specific nutrients and foods and hearing level measured 13 years later, Br. J. Nutr. 109 (2013) 2079–2088, http://dx.doi.org/10.1017/S0007114512004291.
[8] T. Yamasoba, F.R. Lin, S. Someya, A. Kashio, T. Sakamoto, K. Kondo, Current concepts in age-related hearing loss: epidemiology and mechanistic pathways, Hear. Res. 303 (2013) 30–38, http://dx.doi.org/10.1016/j.heares.2013.01.021.
[9] V. Adleff, E. Hitre, I. Köves, Z. Orosz, A. Hajnal, J. Kralovánszky, Heterozygote deficiency in thymidylate synthase enhancer region polymorphism genotype distribution in Hungarian colorectal cancer patients, Int. J. Cancer 108 (2004) 852–856.
[10] Q. Shi, Z. Zhang, A.S. Neumann, G. Li, M.R. Spitz, Q. Wei, Case-control analysis of thymidylate synthase polymorphisms and risk of lung cancer, Carcinogenesis 26 (2005) 649–656.
[11] I.T. de Arruda, D.C. Persuhn, N.F. de Oliveira, The MTHFR C677T polymorphism and global DNA methylation in oral epithelial cells, Genet. Mol. Biol. 36 (2013) 490–493.
[12] S. Gilbody, S. Lewis, T. Lightfoot, Methylenetetrahydrofolate reductase (MTHFR) genetic polymorphisms and psychiatric disorders: a HuGE review, Am. J. Epidemiol. 165 (2007) 1–13.
[13] M. van den Donk, M.H. Visker, J.L. Harryvan, F.J. Kok, E. Kampman, Dietary intake of B-vitamins, polymorphisms in thymidylate synthase and serine hydroxymethyltransferase 1, and colorectal adenoma risk: a Dutch case-control study, Cancer Lett. 250 (2007) 146–153 PMID: 17113224.
[14] P. Capaccio, F. Ottaviani, V. Cuccarini, U. Ambrosetti, E. Fagnani, A. Bottero, S. Cenzuales, B.M. Cesana, L. Pignataro, Methylenetetrahydrofolate reductase gene mutations as risk factors for sudden hearing loss, Am. J. Otolaryngol. 26 (2005) 383–387.
[15] C.M. Ulrich, J. Bigler, C.M. Velicer, E.A. Greene, F.M. Farin, J.D. Potter, Searching expressed sequence tag databases: discovery and confirmation of a common polymorphism in the thymidylate synthase gene, Cancer Epidemiol. Biomark. Prev. 9 (2000) 1381–1385.
[16] K. Kawakami, D. Salonga, J.M. Park, K.D. Danenberg, H. Uetake, J. Brabender, et al., Different lengths of a polymorphic repeat sequence in the thymidylate synthase gene affect translational efficiency but not its gene expression, Clin. Cancer Res. 7 (2001) 4096–4101.
[17] F. Thomas, J.M. Hoskins, A. Dvorak, B.R. Tan, H.L. McLeod, Detection of the G > C

SNP and rare mutations in the 28-bp repeat of TYMS using gel-based capillary electrophoresis, Pharmacogenomics 11 (2010) 1751–1756, http://dx.doi.org/10.2217/pgs.10.170.
[18] M.V. Mandola, J. Stoehlmacher, W. Zhang, S. Groshen, M.C. Yu, S. Iqbal, et al., A 6 bp polymorphism in the thymidylate synthase gene causes message instability and is associated with decreased intratumoral TS mRNA levels, Pharmacogenetics 14 (2004) 319–327.
[19] T. Partearroyo, N. Vallecillo, M.A. Pajares, G. Varela-Moreiras, I. Varela-Nieto, Cochlear homocysteine metabolism at the crossroad of nutrition and sensorineural hearing loss, Front. Mol. Neurosci. 10 (2017) 107, , http://dx.doi.org/10.3389/fnmol.2017.00107.
[20] Y. Uchida, S. Sugiura, F. Ando, T. Nakashima, H. Shimokata, Hearing impairment risk and interaction of folate metabolism related gene polymorphisms in an aging study, BMC, Med. Genet. 12 (2011) 35, http://dx.doi.org/10.1186/1471-2350-12-35.
[21] J. Durga, L.J. Anteunis, E.G. Schouten, M.L. Bots, F.J. Kok, P. Verhoef, Association of folate with hearing is dependent on the 5,10-methylenetetrahdyrofolate reductase 677C→T mutation, Neurobiol. Aging 27 (2006) 482–489.
[22] D.K. Lahiri, J.I. Nurnberger Jr., A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies, Nucleic Acids Res. 19 (1991) 5444.
[23] P. Frosst, H.J. Blom, R. Milos, P. Goyette, C.A. Sheppard, R.G. Matthews, et al., A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase, Nat. Genet. 10 (1995) 111–113.
[24] C.F. Skibola, M.T. Smith, E. Kane, E. Roman, S. Rollinson, R.A. Cartwright, G. Morgan, Polymorphisms in the methylenetetrahydrofolate reductase gene are associated with susceptibility to acute leukemia in adults, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 12810–12815, http://dx.doi.org/10.1073/pnas.96.22.12810.
[25] D.L. Harmon, D.C. Shields, J.V. Woodside, D. McMaster, J.W. Yarnell, I.S. Young, et al., Methionine synthase D919G polymorphism is a significant but modest determinant of circulating homocysteine concentrations, Genet. Epidemiol. 17 (1999) 298–309, http://dx.doi.org/10.1002/(SICI)1098-2272(199911)17:4<298::AID-GEPI5>3.0.CO;2-V.
[26] H. Morita, H. Kurihara, T. Sugiyama, C. Hamada, Y. Kurihara, T. Shindo, et al., Polymorphism of the methionine synthase gene: association with homocysteine metabolism and late-onset vascular diseases in the Japanese population, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 298–302, http://dx.doi.org/10.1161/01.atv.19.2.298.
[27] N. Horie, H. Aiba, K. Oguro, H. Hojo, K. Takeishi, Functional analysis and DNA polymorphism of the tandemly repeated sequences in the 5′-terminal regulatory region of the human gene for thymidylate synthase, Cell Struct. Funct. 20 (1995) 191–197.
[28] C.M. McMahon, A. Kifley, E. Rochtchina, P. Newall, P. Mitchell, The contribution of family history to hearing loss in an older population, Ear Hear. 29 (2008) 578–584, http://dx.doi.org/10.1097/AUD.0b013e31817349d6.
[29] K.J. Cruickshanks, D.M. Nondahl, T.S. Tweed, T.L. Wiley, B.E. Klein, R. Klein, et al., Education, occupation, noise exposure history and the 10-yr cumulative incidence of hearing impairment in older adults, Hear. Res. 264 (2010) 3–9, http://dx.doi.org/10.1016/j.heares.2009.10.008.
[30] Y. Ghodke, A. Chopra, P. Shintre, A. Puranik, K. Joshi, B. Patwardhan, Profiling single nucleotide polymorphisms (SNPs) across intracellular folate metabolic pathway in healthy Indians, Indian J. Med. Res. 133 (2011) 274–279.
[31] G.A. Gates, J.H. Mills, Presbycusis, Lancet 366 (2005) 1111–1120.
[32] C.S. Sousa, Castro Júnior Nd, E.J. Larsson, T.H. Ching, Risk factors for presbycusis in a socio-economic middle-class sample, Braz. J. Otorhinolaryngol. 75 (2009) 530–536.
[33] L.A. Raynor, J.S. Pankow, K.J. Cruickshanks, C.R. Schubert, M.B. Miller, R. Klein, et al., Familial aggregation of olfactory impairment and odor identification in older adults, Laryngoscope 120 (2010) 1614–1618, http://dx.doi.org/10.1002/lary.20989.
[34] R. Jönsson, U. Rosenhall, Hearing in advanced age. A study of presbyacusis in 85-, 88- and 90-year-old people, Audiology 37 (1998) 207–218.
[35] D.H. Wilson, P.G. Walsh, L. Sanchez, A.C. Davis, A.W. Taylor, G. Tucker, et al., The epidemiology of hearing impairment in an Australian adult population, Int. J. Epidemiol. 28 (1999) 247–252.
[36] D. Megighian, M. Savastano, L. Salvador, A. Frigo, M. Bolzan, Audiometric and epidemiological analysis of elderly in the Veneto region, Gerontology 46 (2000) 199–204.
[37] B.D. Guenther, C.A. Sheppard, P. Tran, R. Rozen, R.G. Matthews, M.L. Ludwig, The structure and properties of methylenetetrahydrofolate reductase from Escherichia coli suggest how folate ameliorates human hyperhomocysteinemia, Nat. Struct. Biol. 6 (1999) 359–365.
[38] R. Martínez-Vega, S. Murillo-Cuesta, T. Partearroyo, G. Varela-Moreiras, I. Varela-Nieto, M.A. Pajares, Long-term dietary folate deficiency accelerates progressive hearing loss on CBA/Ca mice, Front. Aging Neurosci. 8 (2016) 209.
[39] R. Martínez-Vega, F. Garrido, T. Partearroyo, R. Cediel, S.H. Zeisel, C. Martínez-Álvarez, G. Varela-Moreiras, I. Varela-Nieto, M.A. Pajares, Folic acid deficiency induces premature hearing loss through mechanisms involving cochlear oxidative stress and impairment of homocysteine metabolism, FASEB J. 29 (2015) 418–432, http://dx.doi.org/10.1096/fj.14-259283.
[40] P. Capaccio, F. Ottaviani, V. Cuccarini, U. Ambrosetti, E. Fagnani, A. Bottero, S. Cenzuales, B.M. Cesana, L. Pignataro, Sudden hearing loss and MTHFR 677C > T/1298A > C gene polymorphisms, Genet. Med. 7 (2005) 206–208.
[41] K. Yamada, Z. Chen, R. Rozen, R.G. Matthews, Effects of common polymorphisms on the properties of recombinant human methylenetetrahydrofolate reductase, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 14853–14858.
[42] A. Pollak, M. Mueller-Malesinska, U. Lechowicz, A. Skorka, L. Korniszewski, A. Sobczyk-Kopciol, et al., MTHFR 677T is a strong determinant of the degree of hearing loss among Polish males with postlingual sensorineural hearing impairment, DNA Cell Biol. 31 (2012) 1267–1273, http://dx.doi.org/10.1089/dna.2012.1607.
[43] B.N. Ames, DNA damage from micronutrient deficiencies is likely to be a major cause of cancer, Mutat. Res. 475 (2001) 7–20.
[44] J. Murthy, G. Venkatesh Babu, L.V. Bhaskar, TYMS gene 5′- and 3′-untranslated region polymorphisms and risk of non-syndromic cleft lip and palate in an Indian population, J. Biomed. Res. 29 (2015) 337–339, http://dx.doi.org/10.7555/JBR.29.20140140.
[45] E. Chu, D.M. Koeller, J.L. Casey, J.C. Drake, B.A. Chabner, P.C. Elwood, et al., Autoregulation of human thymidylate synthase messenger RNA translation by thymidylate synthase, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 8977–8981.
[46] C.F. Skibola, M.S. Forrest, F. Coppedé, L. Agana, A. Hubbard, M.T. Smith, P.M. Bracci, E.A. Holly, Polymorphisms and haplotypes in folate-metabolizing genes and risk of non-Hodgkin lymphoma, Blood 104 (2004) 2155–2162.