Association of Functional Polymorphisms in Matrix Metalloproteinase-9 and Glutathione S-Transferase T1 Genes with Temporomandibular Disorders

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Aims: To investigate the potential role of polymorphisms in matrix metalloproteinase-9 (MMP-9), glutathione S-transferase M1 (GSTM1) and T1 (GSTT1), and methylenetetrahydrofolate reductase (MTHFR) genes as risk factors for development of temporomandibular disorders (TMD) in a Serbian population. **Methods:** This case-control study included 282 subjects: 100 with TMD and 182 healthy controls. Genotyping was done by means of polymerase chain reaction (PCR)/restriction fragment length polymorphism (RFLP) for single nucleotide polymorphisms (SNPs) analysis (C-1562T MMP-9 and C677T MTHFR) or multiplex PCR and real-time PCR methods for deletion analysis (GSTM1, GSTT1) of DNA obtained from buccal swabs. The association of gene variants with TMD risk was determined by calculating odds ratios (OR) and their 95% confidence intervals (CI). **Results:** A statistically significant difference in genotype and allele frequencies was found between the TMD group and controls for the MMP-9 SNP. Heterozygotes (CT) were significantly more frequent in the TMD group than in the control group and carriers of the T allele had an approximately twofold increase of TMD risk (OR = 2.13, 95% CI = 1.24–3.67, P = .005). The null GSTT1 genotype as well as the combined non-null GSTM1/null GSTT1 were associated with lower risk of TMD (OR = 0.28, CI = 0.10–0.74, P = .004 and OR = 0.16, CI = 0.03–0.58, P < .001, respectively). GSTM1 alone and MTHFR polymorphisms did not show an association with TMD. **Conclusion:** The C-1562T SNP in the promoter region of the MMP-9 gene, the GSTT1 null, as well as the combined GSTM1 non-null and GSTT1 null genotypes are modulators of TMD risk in a Serbian population. *J Oral Facial Pain Headache 2015;29:279–285. doi: 10.11607/ofph.1343*

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Temporomandibular disorders (TMD) are defined as a subgroup of craniofacial conditions affecting the temporomandibular joint (TMJ), masticatory muscles, and associated head and neck musculoskeletal structures. The most common presenting symptom of these disorders is pain in the masticatory muscles and TMJ, and TMD are a major cause of chronic pain in the orofacial region.[1–3] These disorders may manifest an inability to open the mouth comfortably, limited joint movement, headaches, ear pain, occlusal changes, and TMJ clicking and/or crepitus sounds produced during mandibular function.[2,3]

Epidemiologic studies have shown that women experience a higher frequency of TMD than men, and also greater pain and muscle tenderness on palpation compared to male TMD patients.[3,4] The highest prevalence of TMD is found in women in their reproductive period (20 to 40 years of age); TMD are less common among children, adolescents, and the elderly.[5] There are multiple factors that have been proposed to cause TMD, including occlusal changes, trauma, infection, autoimmunity, and hormonal and psychological factors.[6–12]

In multifactorial or complex diseases, the genetic background is ubiquitously present but has a variable degree of impact, and its precise identification in a given disease usually represents a considerable scientific challenge.

Researchers and clinicians are becoming increasingly aware of the role that genetic factors may play in TMD. Expression studies relying on immunohistochemistry have shown that deregulation of several classes of genes is implicated in TMD. Leonardi et al have shown that alpha-smooth muscle actin (α-SMA) and heat shock protein 27 (HSP27) are overexpressed in samples of dysfunctional human TMJ discs.[13,14] More recently, the same group has demonstrated that the expression of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) may be correlated with TMJ disc degeneration and that matrix-metalloproteinases 7 and 9 (MMP-7 and MMP-9) are upregulated in TMJ discs with internal derangement.[15,16] Animal models have also illustrated the importance of a number of genes in TMD. Mutations in the collagen type II alpha 1 (Col2a1) gene caused TMJ osteoarthritis in mice, and ankylosis (ank) mutant mice developed fibrous ankylosis in the TMJ.[17,18] Nonetheless, gene mutations directly responsible for TMD in humans have not yet been described.

It has been well documented that genetic variations or gene polymorphisms underlie differences in susceptibility to diseases. An increasing number of studies are dedicated to finding gene variants that might help in determining which individuals are more susceptible to TMD development or in predicting the severity of the disease process and disease symptoms.[19,20] In recent years different gene polymorphisms have been associated with risk of TMD or its clinical manifestations. In a large population-based prospective cohort study (Orofacial Pain Prospective Evaluation and Risk Assessment [OPPERA]) using the Pain Research Panel, an Affymetrix gene chip with 3,295 single nucleotide polymorphisms (SNPs) from over 300 genes, several polymorphisms have been shown to influence TMD risk, notably SNPs in the hydroxytryptamine receptor 2A (HTR2A) gene, Catechol-O-methyltransferase (COMT) gene, opioid receptor delta 1 (OPRD1) gene, etc, although without reaching statistical significance.[21] A more recent study within the OPPERA project has established an association between different polymorphisms in voltage-gated sodium channel, type I, alpha subunit (SCN1A), angiotensin I-converting enzyme 2 (ACE2), and prostaglandin-endoperoxide synthase 1 (PTGS1), etc, and clinical, psychological, and sensory TMD-related phenotypes.[22] Several studies dealing with single gene polymorphisms have reported as well on the importance of COMT in TMD; for instance, Michelotti and coworkers have shown that genetic polymorphisms rs165656 and rs4646310 play a role in TMD susceptibility, while Schwahn and coworkers have established that rs5993882 is related to TMD pain.[23,24] Serotonin transporter gene and estrogen receptor gene polymorphisms have also been related to TMD pain, and the human homolog of the murine progressive ankylosis (ANKH-OR) gene polymorphism has been associated with joint closed lock.[18,25,26]

Functional polymorphisms in genes controlling extracellular matrix degradation, oxidative metabolism, and folate pathways have also been analyzed, but the number of studies dealing with TMD susceptibility and the aforementioned gene polymorphisms are few and the results inconclusive.[27,28] Since the products of these genes have important metabolic roles and may ultimately contribute to TMD pathogenesis, they deserve further consideration. Matrix metalloproteinases (MMPs) are metal-dependent endopeptidases that degrade extracellular matrix components and play an important role in TMJ degeneration.[27] SNPs have been described in the promoter region of the MMP-9 gene (MMP-9 C-1562T, rs3918242) and shown to modify the basal and inducible gene-expression levels of MMP-9.[29]

GSTM1 and GSTT1 belong to a group of glutathione S transferases (GSTs) that have an important role in detoxification of electrophilic compounds, including products of oxidative stress, which is thought to be involved in TMD pathogenesis.[28] Polymorphisms in GST genes affect the activity of enzymes and consequently have functional effects on redox regulation.[30] Particular attention has been focused on complete gene deletion polymorphisms in mu (GSTM) and theta (GSTT) subfamilies, since they abolish enzymatic activity.[31] Approximately 50% of the European population lack the GSTM1 gene (so-called null genotype) due to inherited homozygous deletion of both alleles, and the incidence of GSTT1 homozygous deletion varies with ethnicity, from approximately 15% to 25% in Caucasians to over 60% in some Asian populations.[32,33] A functional enzyme is found in individuals with two or one copy of the gene (non-deletion alleles or heterozygous deletion). The null genotype (0 gene copy), on the other hand, will result in the complete absence of the enzyme.[32]

Folate plays a vital role in DNA synthesis, amino acid metabolism, and the generation of methyl groups.[34,35] Epigenetic DNA modification via methylation has several essential roles, such as controlling gene expression, stabilizing chromatin structure, and maintaining genomic stability, and thus can induce changes in the last growing period of development in humans and the appearance of TMD.[28] Methylenetetrahydrofolate reductase (MTHFR) is the key enzyme in folate metabolism, and a SNP in the coding region of the MTHFR gene C677T (rs1801133) produces an enzyme with decreased activity.[36] This means that, depending on the genotype, the balance in DNA methylation and DNA synthesis

may vary. In view of these findings with MMP-9, GST, and MTHFR genes, the aim of this study was to investigate the potential role of polymorphisms in MMP-9, GSTM1, GSTT1, and MTHFR genes as risk factors for TMD development in a Serbian population.

Materials and Methods

Study Group

A total of 282 systemically healthy individuals were recruited for the study between January 2011 and June 2013. The participants were anamnestically and clinically evaluated following the guidelines from the Research Diagnostic Criteria for TMD (RDC/TMD).[37] Of these participants, 100 (80 females and 20 males, mean age ± standard deviation (SD) 37.12 ± 14.58) were diagnosed with TMD and 182 (149 females and 33 males, aged 39.22 ± 13.64) without any TMD signs or symptoms. All participants were patients of the Clinic for Prosthodontics of the School of Dental Medicine, University of Belgrade, and all signed an informed consent. The inclusion criterion was at least one sign or symptom of TMD presence. The exclusion criteria were traumatic injury of the head and neck, mental disability, craniofacial anomalies, and neurologic disease. Participants were not related and originated from different parts of Serbia. All procedures were done in accordance with the Helsinki Declaration of 1975, and the study was approved by the Ethical Committee of the School of Dental Medicine, University of Belgrade.

DNA Extraction

Genomic DNA was isolated from the buccal swabs by DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's recommendations. Isolated DNA was stored in sterile double-distilled water at +4°C until further analysis.

Genotyping

MMP-9 C-1562T (rs3918242) polymorphism. The sequence surrounding the SNP position in the MMP-9 gene promoter was amplified using specific primers 5'-GCCTGGCACATAGTAGGCCC-3' (forward) and 5'-CTTCCTAGCCAGCCGGCATC-3' (reverse).[38] Polymerase chain reaction (PCR) was carried out in a total volume of 50 µL, containing 500 ng genomic DNA; 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 1 µM of each primer; 200 µM each of dATP, dCTP, dGTP, and dTTP; and 2.5 U Taq DNA polymerase (Amersham Pharmacia Biotech AB). The solution was incubated for 3 minutes at 95°C, followed by 35 cycles of 1 minute at 95°C, 45 seconds at 65°C, and 45 seconds at 72°C, with a final extension of 72°C for 7 minutes.

Each PCR product was digested with three units of SphI (MBI, Fermentas) overnight and the fragments separated on an 8% polyacrilamide gel stained with ethidium bromide. After digestion, wild type homozygotes (CC) showed one band of 435 bp, mutated homozygotes (TT) had two bands (247 and 188 bp), and heterozygous (CT) had three bands (435, 247, and 188 bp).[38]

GSTM1 and GSTT1 polymorphisms. For simultaneous detection of GSTM1 and GSTT1 genotypes, a multiplex PCR and real-time PCR were performed with primers described by Voso and coworkers.[39] As an internal amplification control, primers for β-globin gene were used to exclude false-negative results.[40]

The PCR mixture for multiplex PCR (total volume 50 µL) contained 2X Multiplex PCR Master Mix (2X-concentrated solution containing HotStart Taq DNA polymerase, reaction buffer, $MgCl_2$, and dNTP [Qiagen]), 0.5 µM of each primer (Metabion), and 0.2 µg of genomic DNA. The amplification products from this reaction were separated on 3% agarose gel, stained with ethidium bromide (0.5 µL/mL), and visualized under ultraviolet light for determination of genotypes. Subjects with null genotypes (M1–/– and T1–/–) did not show amplification of corresponding fragments of 215 bp and 480 bp, respectively. Amplified β-globin gene fragment (110 bp) was observed in every PCR reaction as an indicator of successful reaction.[40]

To avoid false-negative results, genotyping for 50 samples with lower DNA concentrations (29 TMD subjects and 21 controls) was also done by real-time PCR and melting curve analysis. Reaction mixes for real-time PCR were prepared according to the manufacturer's recommendations (for total volume 25 µL): 2X Maxim SYBR Green/ROX qPCR Master Mix (Fermentas Life Sciences), 0.35 µM of each primer (Metabion), < 50 ng template DNA, and nuclease-free water to 25 µL. There was no discrepancy between multiplex PCR and real-time PCR results.

MTHFR C677T (rs1801133) polymorphism. MTHFR gene polymorphism was determined by PCR-restriction fragment length polymorphism (PCR-RFLP). A 198 base-pair fragment, surrounding the 677 position, was amplified using the following primers: 5'-TGAAGGAGAAGGTGTCTGCGGGA-3' and 5'-AGGACGGTGCGGTGAGAGTG-3'.[36] The amplified fragment was digested with the enzyme HinfI (MBI, Fermentas), resulting in products of 198 base pairs for the wild type allele C, and 175 and 23 base pairs for the variant allele T.

Genotypes for the analyzed SNPs (MTHFR C677T and MMP-9 C-1562T) were confirmed by randomly re-genotyping 10% of samples. There were no discrepancies between genotypes determined in duplicate.

Table 1 MMP-9 Genotype Distribution and Allele Frequencies in TMD Patient and Control Groups

Genotype	Patients (100) n (%)	Controls (182) n (%)	OR	95% CI	*P*
CC	64 (64)	144 (79)	1.00	Reference	
CT	33 (33)	36 (20)	2.06	1.18–3.60	.008*
TT	3 (3)	2 (1)	3.37	0.55–20.69	
CT + TT	36 (36)	38 (21)	2.13	1.24–3.67	.18
C	0.80	0.89	1.00	Reference	.005*
T	0.20	0.11	2.02	0.91–4.48	.058

*Significant.
OR = odds ratio; 95% CI = 95% confidence interval; Reference = genotype or allele indicator.

Table 2 GSTM1 and GSTT1 Genotype Distribution in TMD Patient and Control Groups

Genotype	Patients (100) n (%)	Controls (182) n (%)	OR	95% CI	*P*
GSTM1					
Non-null (+/+, +/–)	57 (57)	120 (65.9)	1.00	Reference	
Null (–/–)	43 (43)	62 (34.1)	1.46	0.88–2.41	.09
GSTT1					
Non-null (+/+, +/–)	95 (95)	153 (84.1)	1.00	Reference	
Null (–/–)	5 (5)	29 (15.9)	0.28	0.10–0.74	.004*

*Significant.
OR = odds ratio; 95% CI = 95% confidence interval; Reference = genotype or allele indicator.

Table 3 Logistic Regression Analysis of Combined GSTM1/GSTT1 Genotypes in TMD Patient and Control Groups

GSTM1/ GSTT1 genotypes	Patients (100) n (%)	Controls (182) n (%)	OR	95% CI	*P*
M1 non-null/ T1 non-null (+/+)	55 (55)	94 (51.7)	1.00	Reference	
M1 non-null/ T1 null (+/–)	2 (2)	26 (14.3)	0.16	0.03–0.58	< .001*
M1 null/ T1 non-null (–/+)	40 (40)	59 (32.4)	1.16	0.69–1.95	.34
M1 null/ T1 null (–/–)	3 (3)	3 (1.6)	1.71	0.33–8.76	.40

*Significant.
OR = odds ratio; 95% CI = 95% confidence interval; Reference = genotype indicator.

Table 4 MTHFR Genotype Distribution and Allele Frequencies in TMD Patient and Control Groups

Genotype	Patients (100) n (%)	Controls (182) n (%)	OR	95% CI	*P*
CC	41 (41)	72 (40)	1.00	Reference	
CT	44 (44)	85 (46)	0.91	0.54–1.54	.41
TT	15 (15)	25 (14)	1.01	0.50–2.22	.52
CT + TT	59 (59)	110 (60)	0.94	0.57–1.55	.46
C	.63	.63	1.00	Reference	
T	.37	.37	1.00	0.56–1.78	.56

OR = odds ratio; 95% CI = 95% confidence interval; Reference = genotype or allele indicator.

Statistical Analyses

Chi-square test and Fisher exact test were used to determine possible differences in the genotype and allele frequencies. The association of gene variants with risk of disease was examined by use of unconditional logistic regression analysis to calculate odds ratios (OR) and their 95% confidence intervals (CI). *P* values less than .05 were considered statistically significant. The variant was used as a categorical variable in these analyses. The expected frequency of variants in controls was analyzed by the Hardy-Weinberg equilibrium test.[41] Calculations were performed using SPSS 10.0 statistical software (SPSS Inc).

Results

A significant difference in genotype and allele frequencies was found between the TMD group and controls for the C-1562T SNP (chi-square 7.926, *df* = 2, *P* = .019). The percentage of heterozygotes (CT) was considerably higher in TMD subjects (33.0%) than in control subjects (17.0%). Carriers of the variant allele T had a twofold increase of susceptibility for TMD compared to wild type homozygotes (CC) (OR = 2.13, 95% CI = 1.24–3.67, *P* = .005). The observed genotype and allele frequency distribution and risk estimates are given in Table 1.

A significant difference in GSTT1 null genotype percentage between TMD and control subjects was also observed (5.0% vs 15.9%, chi-square 7.277, *df* = 1, *P* = .007), and logistic regression analysis showed a lower risk for TMD in individuals with the GSTT1 null genotype (OR = 0.28, 95% CI = 0.10–0.74, *P* = .004). No association was found between GSTM1 null genotype and TMD (Table 2).

GSTM1/GSTT1 combined genotype percentages were significantly different between TMD and control subjects (chi-square 11.559, *df* = 3, *P* = .009). There was an association between GSTM1+/GSTT1– and TMD. Carriers of the +/– combination had a substantial decrease of the risk for TMD (OR = 0.16, 95% CI = 0.03–0.58, *P* < .001) (Table 3).

MTHFR C677T SNP did not show any statistical difference between TMD and control subjects (chi-square 0.209, *df* = 2, *P* = .901). Logistic regression analysis did not show association between the variant allele and TMD (OR = 0.94, 95% CI = 0.57–1.55, *P* = .46) (Table 4).

Discussion

Molecular mechanisms underlying TMD development are numerous and can be grouped into different categories, all genetically controlled.[1] The relevance of DNA polymorphisms as TMD risk factors and TMD phenotype predictor is still under debate, and more studies dedicated to common polymorphisms are needed.

MMPs are zinc-dependent endopeptidases capable of degrading almost all components of extracellular matrix, including interstitial and basement membrane collagens, fibronectin, laminin, and proteoglycan core proteins.[42] They are involved in connective tissue remodeling and degradation, and they play an important role in TMJ degeneration.[43] This study is the first to establish an association between the promoter polymorphism C-1562T in the MMP-9 gene and increased risk of TMD. Since the T allele has a higher transcriptional activity, it is quite plausible that a higher expression of MMP-9 may influence TMJ degeneration. Indeed, a recent study using an immunohistochemical approach has shown overexpression of MMP-9 in TMJ discs with internal derangement, thus corroborating the direct genotype-phenotype link.[16] Interestingly, in accordance with the findings of the present study, Sun et al have shown that subjects with the CT/TT genotypes have a higher risk of developing degenerative lumbar disc disease, in comparison to those with the CC genotype.[44] Conversely, Planello et al have not found an association between increased risk of TMJ degeneration and polymorphisms in MMP-9, or in MMP-3, but have established that MMP-1 polymorphism is a risk factor for TMJ degeneration in Italian subjects.[27] A Turkish group studying MMP-1 and MMP-3 gene polymorphisms and TMD could not establish any association.[43]

GSTs are highly expressed enzymes with a complex transcriptional and posttranscriptional regulation. The level of expression of GSTs is a crucial factor in determining the sensitivity of cells to a broad spectrum of toxic chemicals. The role of GSTM1 and GSTT1 in diseases related to enhanced oxidative stress and oxidative damage has been well documented; these diseases include, among others, diabetes mellitus, Alzheimer disease, and cancer.[45–48] Several studies have demonstrated the influence of free radicals, oxidative stress, and antioxidants serum concentration on pain disorders related to TMD, such as fibromyalgia, a rheumatic disease characterized by widespread musculoskeletal pain, stiffness, and tenderpoints.[49,50] The release of free radicals resulting in oxidative stress and imbalance in redox biomarkers is also found in trauma, mechanical stress, disc derangements, and degenerative TMJ changes.[51] Therefore, the lack of enzymatic activity due to complete gene deletion could understandably influence TMD risk. Indeed in the present study, logistic regression analysis showed that carriers of the null genotype have a greater risk of developing TMD (although this did not reach statistical significance; $P = .09$). A larger group of patients would perhaps confirm the established trend. Only one study has been previously carried out on the role of the GSTM1 null variant in TMD, and it established that this variant was a susceptibility factor for TMD.[28] Surprisingly, the present study showed that GSTT1 null allele exerts a protective role and that individuals with homozygous deletion have a lower risk of developing TMD. The protective role of GST null genotypes has previously been described in acute myocardial infarction (GSTT1 null) and diabetes mellitus type 1 (GSTM1 null).[52,53] Carriers of the GSTM1 non-null and GSTT1 null combination also have a substantial decrease of the risk for TMD.

Folate metabolism can influence the final form of growing tissue owing not only to its involvement in nucleic acid synthesis but also to its known function in regulating DNA and protein methylation.[34,35,54] Nutritional deficiencies such as low levels of vitamins B1, B6, and B12 and/or folic acid can induce myofascial dysfunction and pain, and these deficiencies are relatively common in cases of TMD mechanical stress.[55] Functional polymorphisms in genes that control folate metabolism could accordingly be modifiers of TMD susceptibility. In the present study, there was no association between MTHFR gene C677T polymorphism and TMD. Namely, MTHFR C677T SNP did not show a statistical difference between TMD cases and controls, which is in agreement with the results of Aneiros-Guerrero et al.[28] Interestingly, these authors found a significant association between TMD and polymorphisms in four other genes also involved in folate metabolism regulation. Although the C677T SNP did not appear to be a TMD risk factor, different MTHFR genotypes should be analyzed in relation to specific clinical symptoms (mandibular deviation, pain, limited jaw opening, etc) and some association could potentially emerge. The same consideration applies to other polymorphisms in the present study as well.

Conclusions

It is reasonable to assume that individuals are not equally susceptible to TMD. Different genetic polymorphisms were investigated in order to uncover how unique genetic makeup contributes to the development of TMD, and the results suggest that a SNP in MMP-9 and a deletion polymorphism in GST genes modulate the risk of developing this condition in a

Serbian population. For a consistent conclusion to be drawn about these polymorphisms as molecular markers of TMD, more data from genetic studies on populations of different geographic and ethnic origins are still needed. Also, future studies should correlate these polymorphisms to clinical characteristics in order to estimate whether they may predict TMD phenotype severity.

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