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ORIGINAL ARTICLE

The Glutathion-S-Transferase and N-Acetyl Tranferase Gene Polymorphism in Tympanosclerosis

Kemal Gorur, Yusuf Vayisoglu, Cengiz Ozcan, Nilgun Guner, Hatice Yildirim Yaroglu, Lulufer Tamer

Mersin University, Faculty of Medicine, ENT Department (KG, YV, CO, NG)
Mersin University, Faculty of Medicine, Biochemistry Department (YY, LT)

Objective: The aim of this study is to evaluate the effect of Glutathion-S-Transferaz (GST) and N-Acetyl Transferase (NAT) gene polymorphism in the development of tympanosclerosis (TS).

Study Design: A prospective clinical study

Materials and Methods: The study population consisted of 95 (32 male, 68 female) healthy individuals and 95 (42 male, 53 female) patients with TS. 2ml of blood belonging to both groups were kept in EDTA-containing tubes. The polymorphisms of Glutathion-S-Transferaz T1 (GSTT1), Glutathion-S-Transferaz P1 (GSTP1), N-Acetyl Transferase 2*5A (NAT2*5A), N-Acetyl Transferase 2*6A (NAT2*6A) and N-Acetyl Transferase 2*7A/B (NAT2*7A/B) were assessed by real time PCR.

Results: The GST genotype profile did not correlate with the development of TS. The NAT2*5A mutant genotype was correlated to risk of developing TS but this increment was not statistically significant. (OR= 5.5, 95% CI 0.64-47.6, p: 0.1). The NAT2*6A mutant genotype presented increased risk (4.3 fold) on developing TS as compared to control subjects (OR=4.3 95% CI 1.15-16.1, p: 0.03). Following assigning the phenotypes into two groups as fast and slow acetylator, NAT2*6A slow acetylator was 4.46 fold high in patients with TS compared to control subjects (OR=4.46, 95% CI 1.20-16.5 p=0.02).

Conclusion: Slow acetylator antioxidant enzyme polymorphism contributes on the development of TS.

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Introduction

Tympanosclerosis (TS) is a common sequel of chronic or recurrent otitis media. It occurs as hyaline degeneration of the fibrous and elastic layers of the tympanic membrane and the lamina propria of the middle ear mucosa.[1,2]

It is generally asymptomatic or causes mild hearing loss. Moderate degree conductive hearing loss may occur if hyaline plaques involve great amount of the TM or bony annulus and ossicular chain.[3,4]

Because of high rate of serious complications, surgery of TS may not be suitable and easy. Therefore, effective and novel medical treatment models may be required. Development of a new effective medical treatment for TS depends on elucidating its pathogenesis. The exact etiology and pathogenesis of TS is not well known. Myringotomy, insertion of ventilation tubes, middle ear infections, [5] otitis media with effusion[6], trauma[7], various chemical agents[8], immunity[9], genetic and local metabolic changes are suggested as being among the etiologic factors of TS.[10,11] TS does not appear in all patients with these suggested cases. This difference may arise from personal genetic polymorphism like antioxidant system and wound healing capacity.

Several studies are performed to clarify the role of oxidative stress in the pathogenesis of TS. Many different chemical agents are used to decrease oxidative tissue trauma. It is shown that administration of antioxidants and reductants decrease or prevent formation of TS.[12-21]

Corresponding address:
Dr. Yusuf Vayisoglu
Mersin University, Faculty of Medicine, ENT Department
Phone: +90 3243374300-1160
E-mail: yvayisoglu@gmail.com

Development of TS in inter-individual variability may be related to personal antioxidant capacity. We aimed to assess genetic polymorphism of Glutathion-S-Transferaz (GST) and N-Acetyl Transferase (NAT) in patients with TS.

Materials and Methods

The study population consisted of 95 (32 male, 68 female) healthy individuals and 95 (42 male, 53 female) patients with TS. The mean age was 37.7±12.1 in patients, and 40.3 ±17.5 in control subjects. TS diagnosed was confirmed with otoscopy and otomicroscopy. The patients and controls were from the same geographic region (southern Turkey) and of the same ethnic origin (Turkish-Caucasian). Control group chosen from the volunteers without having TS, Turkish individuals living in the southern region of Turkey and unrelated with the patient group, were accepted to this prospective study. After informed consent, 2 ml of blood belonging to both groups were kept in EDTA-containing tubes. Genetic laboratory specialist was blinded from the blood of each group.

DNA extraction and genotyping of GSTT1, GSTM1, GSTP1 and NAT2

Blood was collected into EDTA-containing tubes and DNA was extracted from the leucocytes by high pure template preparation kit (Roche diagnostics, GmbH, Mannheim, Germany).

The polymorphisms of GST were performed by real time PCR with LightCycler instrument using hybridization probes in combination with the LightCycler DNA Master Hybridization Probes Kit (Roche Diagnostics). The PCR conditions used were essentially those described by Ko et al.[22]

NAT2*5A, NAT2*6A and NAT2*7A/B polymorphism of NAT2 were employed an assay based on the LightCycler (LightCycler 480; Roche Diagnostics, Basel, Switzerland) technique to screen for the NAT2 SNPs, which determine NAT2 allelic variants. Template DNA amplification was performed with real-time PCR, and fluorescence resonance energy transfer (FRET) technology was applied to facilitate the online melting-curve analysis of oligonucleotide probes bound to the target SNPs. After LightCycler PCR, hybridization probes in combination with the LightCycler DNA Master Hybridization Probes Kit (Roche Diagnostics) were used. PCR primers and hybridization probes were synthesized by TIB Molbiol (Berlin, Germany). The PCR conditions used were essentially those described by Pistorius et al.[23]

The mutant alleles of NAT2 were accepted as a slow acetylation phenotype. The wild types were termed and heterozygous alleles were termed as fast acetylators.

Statistical analysis: Chi-square (χ^2) test was used to evaluate the distribution of the GST and NAT2 genotypes among the patients and control subjects. The association between GST, NAT2 genotypes and TS patients was estimated by computing odds ratios (OR) and 95% confidence intervals (CI) from logistic regression analyses. All statistical calculations were performed using the SPSS software package version (11.0 for Windows SPSS Inc., Chicago, IL). All tests were conducted at the P=0.05 level of significance.

Results

The frequencies of present and null GSTT1 genotypes were 85.3%, 14.7% in patients and 71%, 29% in controls, respectively. There was no significant difference between the patients and the control groups in the frequency of GSTT1 alleles. Subjects with the GSTP1 homozygous Val/Val genotype had a 1.14-fold increased risk of having TS when compared to control subjects (OR = 1.14; 95% CI, 0.59-5.24 p: 0.31), but this increased risk was not statistically significant (Table 1). The frequencies of wild, heterozygous and mutant NAT2*5A genotypes were 54.7%, 38.9% and 6.3% in cases and 48%, 51% and 1% in controls, respectively. The NAT2*5A mutant genotype was correlated to risk of developing TS but this increased risk was not statistically significant (OR= 5.5, 95% CI 0.64-47.6, p: 0.1). The NAT2*6A mutant genotype presented increased risk (4.3 fold) on developing TS as compared to control subjects (OR=4.3 95% CI 1.15-16.1, p: 0.03) (Table 1). NAT2*7A/B gene polymorphisms were not associated with the risk of developing TS.

Table 1. GST and NAT2 genotypes and the risk of developing TS.

	Cases (n=95)	Controls (n=100)			
Variable	**n (%)**	**n (%)**	**OR ‡**	**95% CI**	**p**
GSTT1*					
Present	81 (85.3)	71 (71.0)	1 (reference)	—	
Null	14 (14.7)	29 (29.0)	0.44	0.20-0.92	0.03
GSTP1					
Ile/Ile	47 (49.5)	48 (48.0)	1 (reference)	—	
Ile/Val	38 (40.0)	43 (43.0)	0.91	0.49-1.63	0.73
Val/Val	10 (10.5)	9 (9.00)	1.14	0.42-3.04	0.80
NAT2*5A					
Wild+	52 (54.7)	48 (48.0)	1 (reference)	—	
Heterozygous	37 (38.9)	51 (51.0)	0.87	0.41-1.81	0.71
Mutant	6 (6.3)	1 (1.0)	5.50	0.64-47.6	0.10
NAT2*6A					
Wild+	63 (66.3)	74 (74.0)	1 (reference)	—	
Heterozygous	21 (22.1)	23 (23.0)	1.09	0.37-3.20	0.84
Mutant	11 (11.6)	3 (3.0)	4.3	1.15-16.1	0.03
NAT2*7A/B					
Wild+	60 (37.5)	70 (70.0)	1 (reference)	—	
Heterozygous	35 (36.8)	30 (30.0)	1.0	0.31-2.69	0.87

n: *number of sample, ‡ ORs (odds ratio); CI (confidence interval) from binary logistic regression.*
** Carriers of at least one intact allele are used as reference, + Wild genotypes are used as reference.*
p: *values of significance with difference of each group*

To investigate whether profiles of GST genotypes were associated with the risk of TS or not, we examined the risk of TS associated with combinations of genotypes. The reference group consisted of individuals with all two putative low-risk genotypes, i.e., the presence of GSTT1 genotypes and the homozygous Ile/Ile genotype for GSTP1. Individuals heterozygous and homozygous for the Ile105val allele combined for this analysis. There was no association between GST genotype profile and the development of TS (Table 2). The phenotypes were classified as fast and slow acetylator. The NAT2*6A slow acetylator had a 4.46 fold increased risk developing TS compared to control subjects (OR=4.46, 95% CI 1.20-16.5 p:0.02) There was no association between NAT2*5A, NAT2*7A/B genotypes and the development of TS. (Table 3).

Table 2. Association between GST genotype profile and the development of TS.

GSTT1	GSTP1	Cases n (%)	Control n (%)	‡OR (95% CI)	p
Present	Ile/Ile	42 (42.1)	34 (34.0)	1 (reference)	
Present	Ile/Val or Val/Val	39 (43.2)	37 (37.0)	0.90 (0.45-1.61)	0.6
Null	Ile/Ile	7 (6.3)	13 (13.0)	1 (reference)	
Null	Ile/Val or Val/Val	7 (8.4)	16 (16.0)	0.81(0.22-2.91)	0.7

n: *number of sample, ‡ ORs (odds ratio); CI (confidence interval) from binary logistic regression. p: values of significance with difference of each group*

Table 3. The distribution of the mutations NAT2*5A, NAT2*6A, NAT2*7A/B as phenotypes in-groups.

Acetylator type	Cases n, (%)	Control n, (%)	‡OR (95% CI)	p
NAT2*5A Fast	89 (93.7)	98 (44.0)	1 (reference)	
Slow	6 (6.3)	2 (56.0)	3.65 (0.71-18.6)	0.11
NAT2*6A Fast	84 (88.4)	97 (65.0)	1 (reference)	
Slow	11 (11.6)	3 (35.0)	4.46 (1.20-16.5)	0.02
NAT2*7A/B Fast	95 (95)	100 (100)	—	—

n: *number of sample, ‡ ORs (odds ratio);* ***CI*** *(confidence interval) from conditional logistic regression.* ***p:*** *values of significance with difference of each group*

Discussion

In the current study, we analyzed the gene polymorphism incidence of antioxidant enzymes (GST, NAT) in the patients with TS. The NAT2*5A and NAT2*6A gene polymorphism was observed significantly higher in patients with TS compared to control groups.

Reactive oxygen species (ROS) are kept in strict balance by antioxidant system, consisting of enzymatic and non-enzymatic mechanism in physiological conditions. These systems protect the tissues from the harmful effect of oxidative stress. The enzymatic antioxidant system is under the genetic control system and it is important in maintaining homeostasis of the body. GST and NAT are the most important enzymes that have important roles in the activation and detoxification of several endogenously formed or exogenously derived electrophiles and their metabolites. GST consists of four main classes of isoenzymes including α, μ, Л, θ. The GST genes are polymorphic and include a non-functional null allele.[24,25] N-acetylation (detoxification) and O-acetylation (activation) are catalyzed by two NAT isoenzymes (NAT1 and NAT2).[26]

It has been shown that low NAT-acetylation activity could increase the risk of developing age dependent cataract, suggesting that NAT detoxification function might be important for lens cells' homeostasis. NAT activity depends on genetic polymorphisms and environmental factors. Human arylamine NATs are known to exist as two isoenzymes, NAT1 and NAT2, with different, though overlapping, substrate specificities.[27] Aromatic amines and hydrazine (N-acetylation) and N-hydroxy-aromatic and heterocyclic amines (O-acetylation) are both examples of acceptor substrates. In general, they are deactivated (N-acetylation) or activated (O-acetylation) by NAT1 and/or NAT2. NAT1 and NAT2 also catalyze the intramolecular N, O-acetyltransfer of N-hydroxy- N-acetyl-aromatic amine. [28-30] The NAT2 gene contains a number of sites that are polymorphic, and these allelic variants have been reported to be codominant.[31] The individuals with the alleles encode the fast (F) or slow (S) enzyme variants may be phenotypically fast (FF), intermedia/heterozygotes (FS), or slow (SS), depending on the presence of these two alleles. Slow acetylator status has been associated with conditions such as urinary bladder cancer,[32] epilepsy,[33] Gilbert syndrome,[34] endometrious,[35] renal cell carcinoma,[36] esophagal carcinoma,[37] allergic and atopic disease,[38] and Behçet disease.[39] On the other hand, fast aceylator status has been associated with conditions such as type 1 diabetes mellitus,[40] lung cancer,[41] benign breast cancer,[42] laryngeal cancer,[43] and phenylketonuria. [44]

TS is a degenerative healing process in the connective tissue layer. TS is formed from a non-infective process and it does not develop in the presence of primarily infected ear. Surgical procedures may also cause local damage and create an aseptic inflammation in the middle ear and TM. It occurs following a middle ear or TM inflammation as an end stage sequel.

Oxidative stress is accepted as one of the main factors in the etiology and pathogenesis of TS. Over production of ROS and/or decreasing of antioxidant system capacity can cause tissue injury. It is suggested that myringotomy may cause an increment of oxygen concentration in the middle ear cavity and it is thought

to be a crucial factor for TS development.[45] The concentration of oxygen is 5-10 % in the middle ear. Myringotomy and VT insertion creates a relative hyperoxic condition.[46]

Hyperoxia and inflammation can induce production of ROS, and potentially initiate irreversible tissue damage. Following the irreversible tissue injury, hyaline degeneration and calcification can occur in the middle ear. Wound healing, epithelial regrowth and collagen synthesis are very important factors in the repair of traumatized tympanic membrane. Connective tissue layer is mainly composed of collagen and it is primarily damaged in the TS.[2,17] Collagen synthesis is regulated by several factors and controlled by genetically following tissue damage. Fibronectine is an important factor in this regulation and it is important in the development and severity of TS.[47]

The cells have several mechanisms to prevent or reduce oxidative tissue damage. GST and NAT are two of the enzymes which have an important role in both activation and detoxification of endogenously formed or exogenously derived electrophiles and their metabolites. There are three NAT genes in humans, one a pseudogene and two functional genes.48 Enzymatic antioxidant system has rapid and slow acetylator regarding their reaction velocity. In the present study, a significant relation was detected between development of TS and slow acetylator enzymes polymorphism. Because of TS is a chronic, end stage sequel, this relation may be important.

Conclusion

In this stage, we conclude that NAT2 slow acetylation may be a contributory factor in the development of TS.

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