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Impact of the genes UGT1A1, GSTT1, GSTM1, GSTA1, GSTP1 and NAT2 on acute alcohol-toxic hepatitis

Research Article

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Abstract: Alcohol metabolism causes cellular damage by changing the redox status of cells. In this study, we investigated the relationship between genetic markers in genes coding for enzymes involved in cellular redox stabilization and their potential role in the clinical outcome of acute alcohol-induced hepatitis. Study subjects comprised 60 patients with acute alcohol-induced hepatitis. The control group consisted of 122 healthy non-related individuals. Eight genetic markers of the genes *UGT1A1*, *GSTA1*, *GSTP1*, *NAT2*, *GSTT1* and *GSTM1* were genotyped. *GSTT1* null genotype was identified as a risk allele for alcohol-toxic hepatitis progression (OR 2.146, P=0.013). It was also found to correlate negatively with the level of prothrombin (β= –11.05, P=0.037) and positively with hyaluronic acid (β=170.4, P=0.014). *NAT2* gene alleles rs1799929 and rs1799930 showed opposing associations with the activity of the biochemical markers γ-glutamyltransferase and alkaline phosphatase; rs1799929 was negatively correlated with γ-glutamyltransferase (β=–261.3, P=0.018) and alkaline phosphatase (β= –270.5, P=0.032), whereas rs1799930 was positively correlated with γ-glutamyltransferase (β=325.8, P=0.011) and alkaline phosphatase (β=374.8, P=0.011). Enzymes of the glutathione *S*-transferase family and NAT2 enzyme play an important role in the detoxification process in the liver and demonstrate an impact on the clinical outcome of acute alcohol-induced hepatitis.

Keywords: *Hepatitis • Alcoholic • Oxidative stress • Pharmacogenetics*

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1. Introduction

Acute alcoholic hepatitis develops as a result of toxic additives in unrecorded low-quality alcohol or due to an individual's reduced metabolic ability to convert alcohol into non-toxic substances. The capability for alcohol degradation in humans is mostly genetically determined. Approximately 90% of alcohol is metabolized through the liver. Metabolism occurs predominantly in an oxidative manner, mainly involving the enzymes alcohol dehydrogenase, acetaldehyde dehydrogenase and the cytochrome P405 system enzyme CYP2E1.

The importance of acute alcoholic hepatitis lies in its significantly high morbidity and mortality, with a reported in-hospital mortality as high as 65% [1]. Furthermore, there is an alarmingly high number of people who have died because of alcohol overdose or misuse of unrecorded alcohol ('unrecorded' is an overview category for any kind of alcohol that is not taxed as beverage alcohol or registered in the jurisdiction where it is consumed) [2].

In Latvia, mortality from alcohol-induced liver diseases was 6.9 and 7.3 per 100,000 inhabitants in 2009 and 2011, respectively, but alcohol-induced intoxication mortality was 7.8 and 4.0 per 100,000

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inhabitants in 2008 and 2011, respectively (data from the Centre for Disease Prevention and Control). There is also a high rate of alcohol consumption in Latvia, with the average consumption for the period 2003–2005 being 9.5 litres per capita for recorded alcohol and 3.0 litres per capita for unrecorded alcohol (compared with the WHO European Region total alcohol consumption of 12.2 litres per capita (sum of recorded and unrecorded). The problem of unrecorded alcohol in Eastern Europe is particularly significant due to its ability to cause massive poisoning [2].

Oxidative alcohol metabolism generates reactive oxygen species that alter the redox state of cells, thereby causing damage. The redox status of cells is not only impacted by alcohol consumption and the activity of directly involved enzyme systems, but also by the activity of antioxidant systems within cells. There are many such systems which decrease the oxidative stress in cells, *e.g.* low-molecular weight antioxidants (*e.g.* bilirubin and glutathione) [3] and enzymes (*e.g.* arylamine *N*-acetyltransferases) [4].

Thus, the present study focused on investigating the relationship between markers in genes coding for enzymes involved in cellular redox stabilization and their potential role in the clinical outcome of acute alcohol-induced hepatitis. Specifically, we analysed polymorphisms in the following genes that contribute to the defence against reactive oxygen species: *GSTT1*, *GSTM1*, *GSTA1*, *GSTP1* (glutathione *S*-transferase-encoding genes involved in the metabolism of glutathione), *UGT1A1* (UDP glucuronosyltransferase) 1 family polypeptide A1-encoding gene involved in the metabolism of bilirubin) and *NAT2* (one of only two arylamine *N*-acetyltransferase-encoding genes in humans).

2. Experimental Procedures

2.1 Study subjects

Sixty patients were included in the present study. The gender distribution in the case group was as follows: 35 (58%) males and 25 (42%) females with mean age 46.5±10.0 years. Patients were hospitalized in the Latvian Centre of Infectious Diseases, Riga East University Hospital between 2006 and 2011 with a diagnosis of acute toxic hepatitis. Diagnosis was confirmed by anamnesis of an unrecorded alcohol usage or alcohol overuse, altered liver enzyme activity and the results of other clinical biochemistry tests. Nineteen patients (32%) had severe intoxication and died during hospitalization (nine male and ten female patients). Surviving patients were treated in the hospital for two weeks up to three months.

For the determination of genetic polymorphism frequencies in the overall population, a control group of 122 healthy non-related individuals was created. This group consisted of 71 male (58%) and 51 female (42%) individuals in the age group of 21–25 years (mean age 22.3±3.1 years) without any history of liver damage. The control group data were used for polymorphism frequency determination only and not for biochemical association analysis.

All study participants signed an informed consent form, issued according to the regulations of the Central Medical Ethics Committee of Latvia.

2.2 Selection of genetic markers

Genes involved in low-molecular weight antioxidant metabolism – *UGT1A1* (involved in bilirubin metabolism), *GSTM1*, *GSTT1*, *GSTA1* and *GSTP1* (involved in glutathione metabolism) – were chosen for study. The *N*-acetyltransferase enzymes are involved in the stabilization of the redox status of cells, thus one gene in this family, *NAT2*, which is predominantly expressed in the liver, small intestine and colon, was also chosen. Genetic markers included in our study were selected based on a review of the literature with regards to reported frequency differences in populations and the ability to influence gene product activity [5-10]. The genes and markers analysed in our study are summarized in Table 1.

2.3 Genotyping methods

Genomic DNA was isolated from peripheral blood by the standard chloroform/phenol purification method and ethanol precipitation with slight modification as described elsewhere [11]. Eight genetic markers – rs8175347 ($(TA)_n$ repeat in the *UGT1A1* gene promoter region, allele UGT1A1*28); rs3957356 (C69T polymorphism in the *GSTA1* gene); rs1695 (A333G polymorphism in the *GSTP1* gene); null genotype (*GSTT1*); null genotype (*GSTM1*); and three markers in the *NAT2* gene: rs1799929 (C481T), rs1799930 (G590A) and rs1799931 (G857A) – were analysed using multiplex PCR (polymerase chain reaction), RFLP (restriction fragment length polymorphism analysis) and fluorescent PCR, followed by sequencing on an automated sequencing instrument (Applied Biosystems, Carlsbad, CA, USA). Reactions were conducted under the standard conditions recommended by the manufacturer or following methods described elsewhere [12-17] (see Table 1 for details).

2.4 Biochemical analysis

Biochemical analysis was performed in the laboratory of the Latvian Centre of Infectious Diseases, Riga East University Hospital using routine methods. The

Gene	Genetic variant	Enzyme activity compared to wild type allele	dbSNP nomenclature	Method of analysis	Method description
UGT1A1	UGT1A1*28	Lower activity (5)	rs8175347	Fluorescent PCR	Lin *et al.*, 2006 [12]
GSTA1	C69T	Lower activity (6)	rs3957356	RFLP (restrictase EamI)	Ping *et al.*, 2006 [13]
GSTP1	A333G	Lower activity (7)	rs1695	RFLP (restrictase Alw26I)	Harries *et al.*, 1997 [14]
GSTT1	Null genotype (gene deletion)	No activity (9)	–	Multiplex PCR	Kondo *et al.*, 2009 [15]
GSTM1	Null genotype (gene deletion)	No activity (9)	–	Multiplex PCR	Kondo *et al.*, 2009 [15]
NAT2	C481T (NAT2*5A[a])	Normal activity (25)	rs1799929	RFLP (restrictase Taq1)	Gelatti *et al.*, 2005 [17]
	G590A (NAT2*6[a])	Lower activity (10)	rs1799930	RFLP (restrictase KpnI)	Gelatti *et al.*, 2005 [17]
	G857A (NAT2*7[a])	Lower activity (10)	rs1799931	RFLP (restrictase BamHI)	Gelatti *et al.*, 2005 [17]

Table 1. Genetic markers selected for analysis in the present study.

[a]*SNPs for NAT2 alleles according to nomenclature louisville.edu/medschool/pharmacology/nat/Human.NAT2.pdf*

case group was examined for alanine transaminase, aspartate transaminase, hyaluronic acid, total bilirubin, direct bilirubin, prothrombin, γ-glutamyltransferase, alkaline phosphatase, cholesterol, reduced glutathione level, leukocyte and thrombocyte count, C-reactive protein and erythrocyte sedimentation rate. For control group biochemical analysis were not made.

2.5 Statistical analysis

Data analysis and quality control for the genotyping data were performed by PLINK software (http://pngu.mgh.harvard.edu/purcell/plink/) [18] with the following criteria: minor allele frequency (MAF) <5%, call rate <98% and Hardy-Weinberg equilibrium test (HWE) (P≤0.05). HWE were not applied for null genotypes in the genes *GSTT1* and *GSTM1,* because the method used for analysis did not allowed us to detect heterozygous deletion. The case and control groups were compared by the χ^2 (chi-square) test, significance threshold applied when P<0.05. For case-control analysis, Bonferroni correction was applied for multiple testing. Comparison of mean biochemical marker values between the case and control groups was performed by the ANOVA test (SPSS software v.16.0).

Genetic marker association analysis with biochemical markers in patient group was conducted using linear regression with additive model. Effect of genetic marker was evaluated as the regression coefficient (β) and data were accepted as statistically significant if P<0.05– values were adjusted by gender, age and clinical outcome (lethal or non-lethal) that were used as covariates.

3. Results

Data analysis and quality control for the genotyping data rs1799931 were excluded from further analysis because of the criterion MAF < 5% (MAF_{cases} = 0.0092 and $MAF_{controls}$ = 0.0098). There was no evidence of deviation from HWE for markers in the genes *GSTA1, GSTP1, UGT1A1* and *NAT2.*

The mean age in the patient group was 46.5 ± 10.0 years. Mean age was not significantly different between lethal and non-lethal cases (43.9 ± 10.9 and 47.6 ± 9.5 years, respectively; P=0.183, CI 95% 9.27–1.81) and between genders (47.6 ± 10.1 and 45.0 ± 10.0 years for males and females, respectively; P=0.318, CI 95% 7.89–1.61).

Genetic marker analysis in the toxic hepatitis patient group and healthy control group did not reveal any significant associations except for the *GSTT1* null genotype (MAF_{cases} = 0.2 and $MAF_{controls}$ = 0.104, P=0.013, OR 2.146, CI 95% 1.160–3.971), which lost significance after Bonferroni correction for multiple testing. The non-significant results of the other markers are not shown.

For further analysis, the patient group was divided according to survival status. The results showed a significant association for rs1799930 only ($MAF_{lethal\ cases}$ = 0.395 and $MAF_{non\text{-}lethal\ cases}$ = 0.218, P=0.045, OR 2.340, CI 95% 1.006–5.441). The non-significant results for the other markers are not shown.

We found statistically significant correlations between biochemical markers characteristic of liver damage and polymorphisms in genes *GSTM1*, *GSTT1* and *NAT2* (Table 2).

The strongest association with total bilirubin level was for the *GSTM1* null genotype (β = 67.23, P=0.014). The association with the direct fraction of bilirubin was not as strong as with total bilirubin (β = 53.89, P = 0.023). This association was mainly influenced by age.

GSTT1 null genotype showed a negative correlation with prothrombin (β = –11.05, P=0.037) and a positive

Biochemical parameter	Genetic marker	ζϕ	p value
Bilirubin	*GSTM1* null genotype	67.23	0.014
	G590A (*NAT2*)	86.17	0.045
Direct bilirubin	*GSTM1* null genotype	53.89	0.023
γ-glutamyltransferase		(65.29)[a]	(0.0098)[a]
	C481T (*NAT2*)	–261.3	0.018
	G590A (*NAT2*)	325.8	0.011
Alkaline phosphatase		(378.2)[b]	(0.004)[b]
	C481T (*NAT2*)	–270.5 (–288.4)[a]	0.032 (0.024)[a]
	G590A (*NAT2*)	374.8	0.011
Cholesterol	C481T (*NAT2*)	–2.254	0.018
Prothrombin	*GSTT1* null genotype	–11.05	0.037
Hyaluronic acid	*GSTT1* null genotype	170.4	0.014

Table 2. Biochemical changes in acute alcohol-toxic hepatitis patients and their association with genetic markers.

β - *regression coefficient*
[a]β *(regression coefficient) adjusted and P value adjusted if age used as covariate*
[b]β *(regression coefficient) adjusted and P value adjusted if clinical outcome (lethal or non-lethal) used as covariate*

correlation with one of the inflammation markers, hyaluronic acid (β =170.4, P=0.014).

Further biochemical associations were detected with the *NAT2* gene alleles rs1799929 and rs1799930. rs1799929 showed a negative correlation with γ-glutamyltransferase, (β = –261.3, P=0.018), alkaline phosphatase (β = –270.5, P=0.032) and cholesterol (β = –2.254, P=0.018). Additionally, allelic association revealed the rs1799929 marker as more common in non-lethal cases; however, this was not statistically significant (OR 0.852, P=0.687).

The rs1799930 allele showed a positive correlation with bilirubin (β =86.17, P=0.045), γ-glutamyltransferase (β=325.8, P=0.011) and alkaline phosphatase (β=374.8, P= 0.011). As mentioned above, this allele was also associated with lethality of acute alcoholic hepatitis. Using covariate analysis, the strongest impact on γ-glutamyltransferase association was determined for clinical outcome ($P_{\text{adjusted for clinical outcome}}$ = 0.004), because γ-glutamyltransferase is one of the main biochemical markers of liver damage.

One of the risk factors for alcoholic liver disease is female gender [19]. Considering this, biochemical markers were compared between genders. In females, hyaluronic acid, prothrombin, cholesterol and erythrocyte count showed more obvious deviation from normal values (P<0.05; data not shown). Comparing biochemical markers between groups of lethal and non-lethal outcome, the lethal outcome group showed significantly abnormal values for prothrombin, leukocyte count and C-reactive protein (P <0.05; data not shown).

4. Discussion

Alcohol metabolism is a well-studied process which is known to change the redox state of cells, thus leading to cellular oxidative stress [20]. In the present study, genetic polymorphisms were found to be in association with reactive oxygen species deactivation and, thereby, involved in alcohol metabolism. As previously suggested by Stewart *et al.* [19], our study also demonstrates female gender to be one of the risk factors in alcohol-toxic hepatitis (significantly higher hyaluronic acid and lower prothrombin, cholesterol). A limitation of our study was the small group of patients, and control group that was not age matched, which may explain why we did not detect previously described associations. However, significant associations were found with the analysed alleles of *GSTT1*, *GSTM1* and *NAT2* genes.

Glutathione *S*-transferases are enzymes that exert a critical role in cellular protection against reactive oxygen species. Although the *GSTA1* gene is the most abundantly expressed glutathione *S*-transferase in liver [6], there are very few publications on its association with liver damage. We were unable to confirm its association with any of the biochemical markers analysed here. This may be a consequence of this enzyme not having a specific substrate and its deficiency being compensated by other isoenzymes. Another glutathione *S*-transferase-coding gene, *GSTP1*, has shown conflicting results as a risk factor for alcoholic liver disease development in a recent meta-analysis conducted by Marcos *et al.* [21]. As with *GSTA1*, we did not find any associations with

the biochemical markers analysed here, which may be a consequence of the small number of cases studied.

The most studied glutathione *S*-transferases are the *GSTT1* and *GSTM1* null genotypes, which are commonly used as population markers. The meta-analysis conducted by Marcos *et al.* [21] showed no difference between alcoholics with alcoholic liver disease for *GSTT1* and *GSTM1*; however, the *GSTM1* null genotype was determined to be a risk factor in alcoholics to develop alcoholic liver disease (OR 1.43, CI 95% 1.14–1.78). Our data reveal *GSTM1* as a risk factor for hyperbilirubinaemia. Despite the findings of a genome-wide association analysis [22], *UGT1A1* is considered to be the main genetic risk factor for hyperbilirubinaemia [7]. We were unable to detect such an association. This may be due to either the small number of patients or the fact that because alcohol induces *UGT1A1* transcription [23] and probably mutations in this gene abolish its significant role in determining bilirubin levels. We found that the *GSTT1* null genotype had the greatest impact on inflammation (positive correlation with hyaluronic acid). Marcos and co-workers' meta-analysis [21] presented evidence for a small and statistically non-significant *GSTT1* overall risk of developing alcoholic liver disease (OR 1.11, CI 95% 0.91–1.36). Therefore, although glutathione *S*-transferases play a role in detoxification processes, our data suggest it is not a significant role.

The most interesting results in our patient group emanated from the *NAT2* genotype analysis. We studied three polymorphisms: rs1799929 (silent polymorphism, normal acetylation) [24,25], rs1799930 (slow acetylation) and rs1799931 (slow acetylation) [10].

Our data imply that rs1799930 is strongly associated with lethality in patients with acute alcohol-toxic hepatitis. As the encoded enzyme is not directly involved in alcohol metabolism [26], there are only a few reports describing *NAT2* allelic association with alcohol metabolism and alcohol-induced liver diseases. In our patient group, the rs1799930 allele was significantly associated with bilirubin, γ-glutamyltransferase and alkaline phosphatase – markers for liver damage. This finding supports the assumption that slow acetylation is a risk factor for alcohol-induced liver disease, because rs1799930 is mostly characterized as a slow acetylator [10]. This notion conflicts with the findings of Agúndez *et al.* [26]. Clarification of the situation requires an enlarged patient group and improved *NAT2* gene function illustration through phenotyping and a more detailed haplotype analysis. rs1799929 alone has no impact on enzyme activity [24,25], but it is included in the *NAT2*5* haplotype that is accepted as a slow acetylator. In our study, these two genetic markers showed different associations with biochemical markers (γ-glutamyltransferase and alkaline phosphatase), perhaps indicating different acetylation activities. In general, slow acetylation in alcohol-induced hepatitis could be a risk factor, whereas fast acetylation could be a protective factor. Further studies assessing the rate of acetylation and analysing haplotypes are required [27].

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