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RESEARCH ARTICLE

N-Acetyltransferase 2, glutathione S-transferase gene polymorphisms and susceptibility to hepatocellular carcinoma in an Algerian population

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ABSTRACT

1. This study was conducted to investigate the potential association of genetic polymorphisms of glutathione S-transferase M1/T1 (*GSTM1*, *GSTT1*), and N-acetyltransferase 2 (*NAT2*) genes and epidemiological parameters with the risk of HCC in the Algerian population.
2. A case-control study including 132 confirmed HCC patients and 141 cancer-free controls was performed. Genotyping analysis was performed using conventional multiplex PCR and PCR-RFLP. Statistical analysis was performed using the Chi-square test. Logistic regression analysis was used to estimate odds ratios and 95% confidence intervals (95% CI).
3. *GSTM1* null and *NAT2* slow acetylator genotypes confer an increased risk to HCC (OR = 1.88, 95% CI 1.16–3.05; OR = 2.30, 95% CI 1.26–4.18, respectively). This association was prevalent in smokers (OR = 2.00, 95% CI 1.05–3.8 and OR = 2.55, 95% CI 1.22–5.34, respectively). No significant association was observed for *GSTT1* null genotype in the contribution to HCC risk (OR = 0.76, 95% CI 0.46–1.27).
4. In conclusion, the *GSTM1* and *NAT2* gene polymorphisms are positively associated with the risk of HCC in older men and especially in smokers.

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Introduction

Hepatocellular carcinoma (HCC) is primary liver cancer that typically occurs in the setting of chronic liver disease, and predicted to be the fourth most common cause of cancer-related deaths worldwide (Kim and Viatour 2020). The global incidence of HCC is projected to increase to 1 million deaths annually by 2030 (Shek et al. 2021). The main risk factors for HCC are viral infections, such as hepatitis B virus (HBV) or hepatitis C virus (HCV), alcoholism, smoking and obesity-induced non-alcoholic fatty liver disease (NAFLD), all of which lead to chronic inflammation (Reghupaty et al. 2019). In addition, several epidemiological studies have indicated that genetic polymorphisms may play an important role in the pathogenesis of HCC (Ladero et al. 2006; Li et al. 2012; Liu et al. 2014).

In human populations, GST genes are highly polymorphic for alleles that can adversely affect enzyme function (Hayes et al. 2005). *GSTM1* and *GSTT1* codes for key xenobiotic-metabolising enzymes (XMEs) involved in the detoxification of xenobiotics or carcinogens such as polycyclic aromatic hydrocarbons and aromatic amines, which are abundantly present in cigarette smoke (Gelatti et al. 2005). Homozygous partial deletion of some GST genes (i.e. *GSTM1*-null and *GSTT1*-null) can lead to enzymatic activity deficiency and decreased detoxification capacity (Hayes et al. 2005), which impairs the ability to eliminate carcinogenic compounds metabolically, and increases the susceptibility to HCC (Dai Long et al. 2006; Asim et al. 2010).

The N-acetyltransferase 2 (*NAT2*) is a polymorphic gene, which is expressed predominantly in the liver and gut in a genotype-determined manner (Agúndez et al. 1996; Agúndez & García-Martín 2018). *NAT2* is involved in the activation and inactivation of several carcinogenic compounds such as arylamines and N-hydroxylated heterocyclic amines present in tobacco smoke through N- or O-acetylation (Smith et al. 1994; Agúndez et al. 1996). Several allelic forms lead to variable acetylation status that can be slow or fast acetylation of potentially toxic substances (Hirvonen 1999). Previous studies reported the involvement of *NAT2* in the development of hepatocellular carcinoma (Yu et al. 2000; Farker et al. 2003; Gelatti et al. 2005). However, the results are inconsistent.

The current study aims to ascertain whether *GSTM1*, *GSTT1*, and *NAT2* gene polymorphisms influence the susceptibility for HCC in the Algerian population in relation to smoking and alcohol consumption.

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Materials and methods

Study population

Blood samples were collected from 132 unrelated and untreated Algerian patients in whom a first incident of HCC occurred. The inclusion criterion was the presence of a suspicious hepatic mass confirmed by clinical histopathology. Control subjects (141) were unrelated healthy volunteer blood donors without any medication, had no history of prior malignant disease and showed no symptoms or pathological signs of liver disease. Controls were matched to patients according to age, sex, ethnicity, geographic origin, alcohol consumption and cigarette smoking. All subjects included in this study are only North Africans (from different provinces of eastern Algeria). The study was conducted according to the Declaration of Helsinki. Written informed consent was obtained from patients and controls prior to the start of the study.

Genotyping

Genotype analyses were carried out in genomic DNA obtained from 5 mL blood samples. A multiplex polymerase chain reaction (PCR) assay was used to determine the *GSTM1* and *GSTT1* genotypes. The Albumin gene was co-amplified as an internal control. The primers used were *GSTM1* (5′-GAACTCCCTGAAAGCTAAAGC and 3′-GTTGGGGCTCAAATAT ACGGTGG) and *GSTT1* (5′-TCCTTACTGGTCCTCACATCTC and 3′-CACCGGATCATGGCCAGCA), the albumin gene (5′-GCCCTCTGCTAACAAGTCCTAC and 3′-GCCCTAAAAAG-AAAA TCGCCAATC) (Arand et al. 1996). PCR conditions were 30 cycles with denaturing at 94 °C for 1 min, annealing at 64 °C for 1 min, and extension at 72 °C for 7 min. The amplificates were separated by ethidium bromide–stained 2% agarose gel electrophoresis and visualised under UV light (Figure 1).

Analysis of the most common *NAT2* alleles was done based on the method described by Bell et al. (1993). A combination of polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) was applied (Figure 2). Loss of restriction sites for the endonucleases enzymes, *KpnI*, *BamHI*, *TaqI* and *MspI*, was used to detect the *NAT2* alleles: *NAT2*5* (C481T), *NAT2*6* (G590A), *NAT2*7* (G857A) and *NAT2*14* (G191A) (Torkaman-Boutorabi et al. 2007).

Statistical analysis

A multiple logistic regression model was used to estimate odds ratios (ORs) together with their corresponding 95% confidence intervals (CIs) and the significance of the linear trends in HCC risk after adjusting for age, sex and smoking status. Statistical significance of differences in genotype frequencies between patients and controls was estimated by the Chi-square test. Differences considered significant for p values less than 0.05. The statistical power calculation was done based on the sample size, the Pearson Chi-Square test

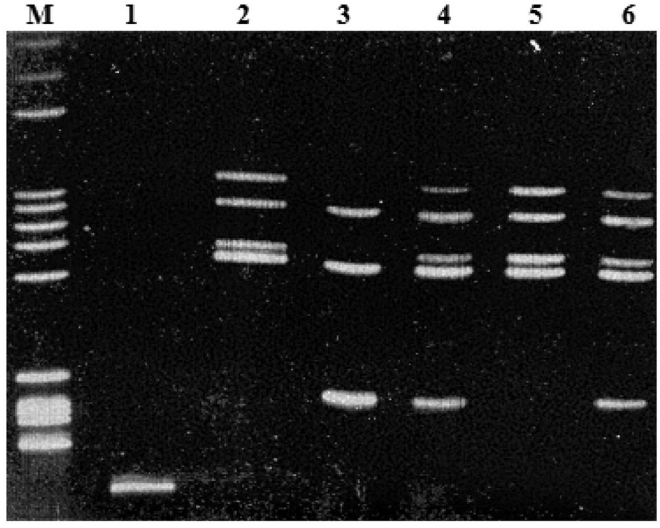

Figure 2. RFLP product of NAT2 G590A site digested by TaqI. M: molecular marker; Lane 1: Undigested DNA; lanes 2,5: NAT2*+/+; lane 3: NAT2*6/*6; lanes 4,6: NAT2*6/+.

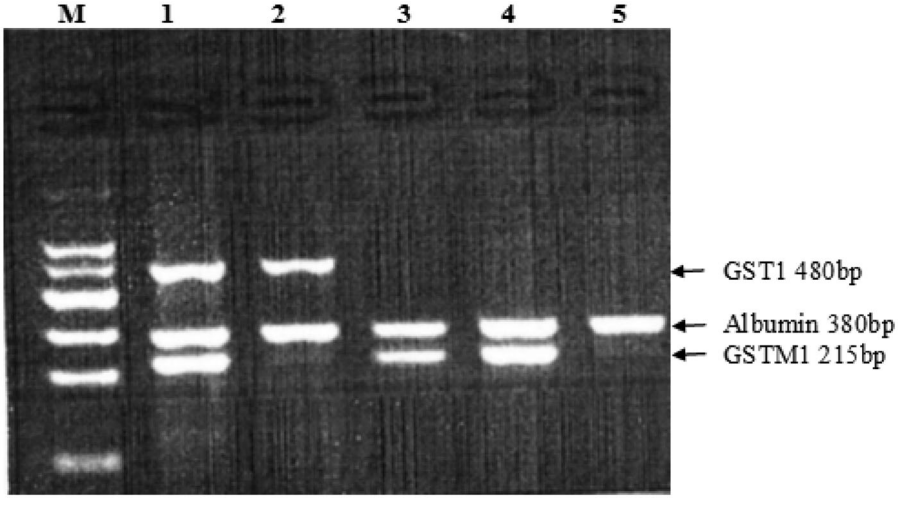

Figure 1. Representative gel photograph showing amplification of *GSTT1* (480 bp), *GSTM1* (215 bp) and the control Albumin (380 bp) gene products. Lane 1: is an individual with both *GSTT1* and *GSTM1* alleles present. Lane 2: is an individual with *GSTT1* present (480 bp) and *GSTM1* null alleles. Lanes 3, 4: are an individuals with *GSTT1* null and *GSTM1* present (215 bp) alleles. Lane 5: is an individual with null alleles for both *GSTT1* and *GSTM1* genes showing only one band at 380 bp (internal control). M: is a DNA molecular marker.

and the pooled standard deviation. IBM SPSS version 28 (free trial) was used for all statistical analyses.

Results

The demographic characteristics of the recruited cases and controls are given in Table 1. The study population included 132 patients with HCC (110 men and 22 women) and 141 controls (111 men and 30 women) with an average age of 63.10 ± 9.53 and 61.60 ± 10.88 years for HCC cases and controls respectively.

The distribution of subjects according to the aetiological variables such as smoking and alcohol consumption shows that the patient population has more smokers than the control population with 51.06% for the controls and 65.91% for the HCC cases. A significant association was found between this factor and the risk of developing hepatocellular cancer (OR = 1.85, 95% CI 1.14–3.02, $p = 0.013$). Considering alcohol consumption, 19.86% of controls were alcohol users compared to 21.97% of patients. No statistically significant difference was observed between cases (63.37%) and controls (55.37%) regarding alcohol consumption ($p > 0.05$).

The distribution of *GSTM1*, *GSTT1*, and NAT2 polymorphisms with the corresponding ORs and 95% CIs are given in Table 2. *GSTM1* null genotype was observed in 63.6% of HCC cases against 48.23% in controls. This difference was significant ($p = 0.011$). The adjusted OR showed an association between the *GSTM1* genotype and the risk of developing HCC (OR = 1.88, 95% CI 1.16–3.05, $p < 0.05$). However, no relationship between *GSTT1* null genotype and HCC risk was observed (OR = 0.76, 95% CI 0.46–1.27, $p > 0.05$).

For the NAT2 gene, the slow acetylator phenotype represented by *NAT2*5, NAT2*6, NAT2*7*, and *NAT2*14* alleles in the homozygous or double heterozygous state is significantly more present in patients (84.85%) than in controls (70.92%). Adjusted OR showed an increased risk of developing HCC in subjects with the slow acetylator genotype for *NAT2* (OR = 2.30, 95% CI 1.26–4.18, $p < 0.01$).

The HCC odds ratios adjusted for tobacco consumption are presented in Table 3. An increased risk of HCC was observed in smokers with the *GSTM1* null genotype (OR = 2, 95% CI 1.05–3.8) and those with slow acetylator genotype for *NAT2* (OR = 2.55, 95% CI 1.22–5.34) compared to non-smokers.

The results of the gene-gene interaction analysis are listed in Table 4. The GSTM1 null/slow acetylator combination is strongly associated with the risk of HCC (OR = 2.27, 95% CI 1.29–4.01, $p < 0.005$). The others *GSTM1* null/*GSTT1* null and *GSTT1* null/slow acetylator combinations did not show any significant association with the risk of developing HCC.

Discussion

Xenobiotic-metabolising enzymes (XMEs) constitute an important line of defence against a variety of carcinogens. Genetic polymorphism of xenobiotic-metabolising genes

Table 1. Epidemiological characteristics of the studied population.

Variables	HCC cases $n = 132$ (%)	Controls $n = 141$ (%)	OR (95%CI)[a]	p Value
Gender				
Male	110 (83.33)	111 (78.72)	1.0	
Female	22 (16.67)	30 (21.28)	0.74 (0.40–1.36)	0.333
Age (years)				
Mean ± SD	63.1 ± 9.53	61.6 ± 10.88		
<40	03 (2.27)	07 (4.96)	1.0	
40–60	52 (39.39)	46 (32.62)	2.64 (0.64–10.80)	0.167
>60	77 (58.33)	88 (62.41)	2.04 (0.51–8.17)	0.306
Smoking Habit				
Non-smokers	45 (34.09)	69 (48.94)	1.0	
Smokers	87 (65.91)	72 (51.06)	1.85 (1.14-3.02)	0.013*
Alcohol intake				
Negative	103 (78.03)	113 (80.14)	1.0	
Positive	29 (21.97)	28 (19.86)	1.14 (0.63-2.04)	0.669

[a]OR adjusted for age, gender, smoking and alcohol intake; *$p < 0.05$ *vs* control. HCC: hepatocellular carcinoma; OR: odds ratios; CI: confidence intervals; SD: stander deviation.

Table 2. Distribution of *GSTM1/T1* and *NAT2* genotypes among controls and HCC cases.

Genotypes	Cases (%) ($N = 132$)	Controls (%) ($N = 141$)	OR (95% CI)	p Value
GSTM1				
Present	48 (36.4)	73 (51.77)	1.0	
Null	84 (63.6)	68 (48.23)	1.88 (1.16–3.05)	0.011*
GSTT1				
Present	93 (70.5)	91 (64.50)	1.0	
Null	39 (29.5)	50 (35.50)	0.76 (0.46–1.27)	0.298
NAT2				
Fast acetylator	20 (15.15)	41 (29.08)	1.0	
Slow acetylator	112 (84.85)	100 (70.92)	2.30 (1.26–4.18)	0.006**

GSTM1: Glutathione S-transferase mu 1; *GSTT1*: Glutathione S-transferase theta 1; NAT2: N-acetyltransferase 2; OR: Odds ratios; CI: Confidence intervals; *$p < 0.05$; **$p < 0.01$.

Table 3. Risk of HCC associated with *GSTM1/T1* and *NAT2* stratified with smoking status.

	Genotypes	Cases/cONTROLS	OR (95% CI)[a]	p Value
Non smokers	*GSTM1*			
	Present	19/37	1.0	
	Null	26/32	1.58 (0.74–3.38)	0.236
	GSTT1			
	Present	33/50	1.0	
	Null	12/19	0.96 (0.41–2.23)	0.919
	NAT2			
	Fast acetylator	05/16	1.0	
	Slow acetylator	40/53	2.42 (0.82–7.15)	0.105
Smokers	*GSTM1*			
	Present	29/36	1.0	
	Null	58/36	2.00 (1.05–3.8)	0.034*
	GSTT1			
	Present	60/41	1.0	
	Null	27/31	0.60 (0.31–1.14)	0.118
	NAT2			
	Fast acetylator	15/25	1.0	
	Slow acetylator	72/47	2.55 (1.22–5.34)	0.012*

[a]OR adjusted for tobacco consumption. *GSTM1*: Glutathione S-transferase mu 1; *GSTT1*: Glutathione S-transferase theta 1; *NAT2*: N-acetyltransferase 2; OR: Odds ratios; CI: Confidence intervals; *$p < 0.05$.

Table 4. Effect of gene-gene interaction of *GSTM1*, *GSTT1* and *NAT2* slow acetylator on the development of HCC.

Genotypes interaction	Frequency cases (%)	Frequency controls (%)	OR(95% CI)	p Value
GSTM1 null/slow acetylator	55.30	35.46	2.27 (1.29–4.01)	0.005**
GSTM1 null/*GSTT1* null	15.91	15.60	1.00 (0.47–2.13)	1.000
GSTT1 null/slow acetylator	23.48	25.53	0.85 (0.45–1.62)	0.623

GSTM1: Glutathione S-transferase mu 1; *GSTT1*: Glutathione S-transferase theta 1; OR: Odds ratios; CI: Confidence intervals; **$p < 0.01$.

(XMGs) may regulate an individual's predisposition to chemical-induced carcinogenesis including HCC. We report herein for the first time, the genetic polymorphism of three XMGs (*NAT2*, *GSTM1*, *GSTT1*) and susceptibility to developing HCC in the Algerian population. The choice of these allelic variants is justified for each variant's potential as a marker of susceptibility to different types of cancer (Medjani et al. 2020).

GST and NAT2 are important detoxifying phase II enzymes involved in the biotransformation of xenobiotic and carcinogenic substances. Increasing evidence suggests that XMG polymorphisms are related to susceptibility to numerous diseases, including hepatocellular carcinoma, due to alteration of detoxifying enzyme activity (Autrup 2000). Therefore, understanding the contribution of polymorphic genes and their interactions with other relevant factors may improve screening diagnostic assays for HCC (Zacharakis et al. 2018).

The epidemiological characteristics of our sample showed a predominance of male patients (83.33%) whose age is over 60 years. This result is quite similar to what has been reported in the literature (Llovet et al. 2003; De Sanctis et al. 2020). The predominance of males was corroborated by other survey evidence and it may be related to lifestyle, as men are more exposed to the habits of smoking as compared to women (Fattovich et al. 2004), or the hormonal status (De Sanctis et al. 2020).

The results of genotyping analysis show that homozygous deletion of *GSTM1* was more frequent in patients (63.6%) than in controls (48.23%) with an association between this genotype and an increased risk of HCC (OR = 1.88, 95% CI 1.16–3.05, $p < 0.011$). Similar associations have also been described in Asian populations (Dai Long et al. 2006; Asim et al. 2010; Wang et al. 2010; Li et al. 2019) but little or no associations were reported for the Brazilian, Caucasian and African populations (Ladero et al. 2006; Wang et al. 2010; Li et al. 2019; Ferreira et al. 2021). Studies on other types of cancer have also reported positive associations between GSTM1 polymorphisms and an increased risk of colorectal cancer in the Brazilian population (Rodrigues-Fleming et al. 2018), bladder cancer in the Mongolian population (Avirmed et al. 2021), and breast cancer in the Mizoram population (Kimi et al. 2016).

In contrast, the *GSTT1* deletion polymorphism does not show any significant difference between the patient and control groups, without association with the risk of HCC. This corroborates the results found in Asian populations (Asim et al. 2010; Li et al. 2012), unlike the results found in Brazilian and Italian populations (Boccia et al. 2015; Araujo et al. 2021).

In a meta-analysis (combined the results of 46 studies including 6124 cases and 9781 controls), Li et al. (2019) reported that *GSTM1* null genotype (OR = 1.37, 95% CI = 1.18–1.59) and *GSTT1* null genotype (OR = 1.43, 95% CI = 1.23–1.66) were correlated with an increased risk of HCC. The subgroup analysis showed more evident associations between GST gene polymorphism and HCC risk in Asian ethnic groups than in Caucasian or African groups, which corroborate the loss of association between null *GSTT1* and HCC risk in our population. These inconsistent data amongst the different ethnicities may indicate different effects of the GSTM1 and GSTT1 polymorphisms on HCC risk in different ethnic genetic backgrounds (Wang et al. 2010).

Since glutathione-S-transferases provide protection against carcinogens, including those in tobacco smoke, an aetiological role of smoking in HCC has therefore been suggested (Staretz et al. 1997; Wang et al. 1998). In line with this, our results confirm this relationship and suggest a direct link between the *GSTM1* null genotype and increased risk of HCC in smokers (OR = 2.0, 95% CI 1.05–3.8, $p < 0.034$). Homozygous *GSTM1* and *GSTT1* deletions have been associated with reduced detoxification function and increased susceptibility to cancer (Li et al. 2012). However, the association of tobacco use with HCC is not always clearly established. Several studies conducted on HCC cancer have found no association between *GSTM1* deletion and smoking status (Yu et al. 1999; Munaka et al. 2003; Gelatti et al. 2005). This inconsistency in results is probably due to differences in the studied populations, genetic backgrounds, ethnicity and geographical variation as well as in the social and healthcare environments (Wild and Hall 2000; Wang et al. 2010).

Depending on the allelic combination, humans display three different phenotypes characterising the *NAT2* gene activity. Individuals were classified as rapid, intermediate and slow acetylators if they possessed zero, one or two slow acetylation *NAT2* haplotypes, namely *NAT2**5, *NAT2**6, *NAT2**7 and *NAT2***14* (Agúndez et al. 1996; Agúndez and García-Martín 2018). Previous studies have been reported the higher frequencies of the slow acetylator phenotypes in North African populations (Jebabli et al. 2010; Khelil et al. 2010; Guaoua et al. 2014). A positive association was observed between the *NAT2* acetylation status and certain diseases in smokers such as bladder cancer (Zhu et al. 2015), colorectal cancer (Da Silva et al. 2011), and breast cancer (Ambrosone et al. 1996; Chang-Claude et al. 2002). Consistent with this, our results show that *NAT2* slow-acetylator genotypes are highly frequent in patients than in controls (84.8% vs. 70.9% respectively), and significantly associated with an increased risk of HCC (OR= 2.30, 95% CI 1.26-4.18, $P < 0.01$).

Many studies showed that *NAT2* slow acetylator alleles are related to a reduced ability to detoxify the xenobiotics, especially those found in tobacco, which is associated with a higher risk of developing this cancer (Agúndez et al. 1996; Farker et al. 2003). In line with this, our results show a higher risk associated with smoking among *NAT2 slow acetylator* phenotypes. The same observations have been confirmed with other cancers such as bladder cancer (Avirmed et al. 2021), in contrast to breast cancer (Kocabaş et al. 2004).

Since the small sample size could limit the statistical power for detecting significant results, we conducted a power analysis for our sample size ($n = 273$). The result revealed a statistical power of 71.2% to detect a significant effect (Hwang et al. 1994) and provided evidence that our study is not underpowered.

In conclusion, our results suggest that *GSTM1*, *NAT2* gene polymorphisms are strongly associated with the

development of HCC in tobacco smokers, whereas *GSTT1* does not seem to play any role in the aetiology of this cancer. Nevertheless, it is advisable to conduct further studies with large sample size to confirm the present study's findings and reach at better conclusions.

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Informed consent

All authors have read and approved the content and agree to submit it for consideration for publication in your journal.

Author contributions

LC, KB, designed and performed the experiments, analysed the data; LC, KB, AF prepared the tables and wrote the manuscript; FD, FZS, DH, KC, helped design the experiments, read the manuscript and critically reviewed it. KB conceived and supervised the study.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that supported the findings of this study are available from the corresponding author LC upon request.

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