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ORIGINAL ARTICLE

Prospective Study

Polymorphisms of glutathione S-transferase genes and survival of resected hepatocellular carcinoma patients

Kai Qu, Su-Shun Liu, Zhi-Xin Wang, Zi-Chao Huang, Si-Nan Liu, Hu-Lin Chang, Xin-Sen Xu, Ting Lin, Ya-Feng Dong, Chang Liu

Kai Qu, Su-Shun Liu, Zhi-Xin Wang, Zi-Chao Huang, Si-Nan Liu, Hu-Lin Chang, Xin-Sen Xu, Ting Lin, Chang Liu, Department of Hepatobiliary Surgery, The First Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China

Ya-Feng Dong, Department of Obstetrics and Gynecology, University of Kansas School of Medicine, Kansas City, KS 66160, United States

Author contributions: Qu K and Liu C designed the research and wrote this paper; Liu SS, Wang ZX and Huang ZC performed the statistical analysis; Liu SN, Chang HL and Xu XS were involved in collecting patient data; Qu K and Lin T took part in literature searches; Dong YF edited the manuscript.

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Correspondence to: Chang Liu, MD, PhD, Department of Hepatobiliary Surgery, the First Affiliated Hospital of Medical College, Xi'an Jiaotong University, No. 277 Yanta West Road, Xi'an 710061, Shaanxi Province, China. liuchangdoctor@163.com
Telephone: +86-29-82654746
Fax: +86-29-82654746

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Abstract

AIM: To investigate the effects of single nucleotide polymorphisms (SNPs) in glutathione S-transferase (GST) genes on survival of hepatocellular carcinoma (HCC) patients.

METHODS: Twelve tagging SNPs in *GST* genes (including *GSTA1*, *GSTA4*, *GSTM2*, *GSTM3*, *GSTO1*, *GSTO2* and *GSTP1*) were genotyped using Sequenom MassARRAY iPLEX genotyping method in a cohort of 214 Chinese patients with resected HCC. The Cox proportional hazards model and log-rank test were performed to determine the SNPs related to outcome. Additionally, stratified analysis was performed at each level of the demographic and clinical variables. An SNP-gene expression association model was further established to investigate the correlation between SNP and gene expression.

RESULTS: Two SNPs (*GSTO2*: rs7085725 and *GSTP1*: rs4147581) were significantly associated with overall survival in HCC patients (P = 0.035 and 0.042, respectively). In stratified analysis, they were more significantly associated with overall survival in patients with younger age, male gender and cirrhosis. We further investigated cumulative effects of these two SNPs on overall survival in HCC patients. Compared with the patients carrying no unfavorable genotypes, those carrying 2 unfavorable genotypes had a 1.70-fold increased risk of death (P < 0.001). The cumulative effects were more significant in those patients with younger age, male gender and cirrhosis (HR = 2.00, 1.94 and 1.97, respectively; all P < 0.001). Additionally, we found that heavy smoking resulted in a significantly worse overall survival in those patients carrying variant

alleles of rs7085725 (HR = 2.07, 95%CI: 1.13-3.76, *P* = 0.018). The distributions of *GSTO2*: rs7085725 and *GSTP1*: rs4147581 genotypes were associated with altered gene expression and contributed to influences on overall survival.

CONCLUSION: Our study provides the first evidence that *GSTO2* and *GSTP1* gene polymorphisms may serve as independent prognostic markers for HCC patients.

Key words: Glutathione S-transferase; Polymorphism; Hepatocellular carcinoma; Clinical outcome; Surgery

Core tip: To determine the prognostic value of single nucleotide polymorphisms (SNPs) in *GST* genes in hepatocellular carcinoma (HCC) after liver resection, we analyzed the association between 12 tagging SNPs in *GST* genes and outcome of 214 HCC patients who underwent liver resection. Our data showed two SNPs (*GSTO2*: rs7085725 and *GSTP1*: rs4147581) to be significantly associated with overall survival in HCC patients. These two SNPs used in combination were more powerful prognostic markers for HCC patients. Our study provides the first evidence that *GSTO2* and *GSTP1* gene polymorphisms may serve as independent prognostic markers for HCC patients.

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INTRODUCTION

Glutathione S-transferases (GSTs) are a broadly expressed family of phase Ⅱ isoenzymes that protect normal cells against damage induced by hepatitis virus or aflatoxin-related hepatocarcinogen[1]. Recently, emerging evidence indicates that the detoxifying properties of the GSTs are genetically determined[2]. In human, several classes of *GST* genes exist: α (*GSTA*), μ (*GSTM*), π (*GSTP*), θ (*GSTT*), and ω (*GSTO*) with one or more genes in each class[3,4]. Altered expression of these *GST* genes has been suggested to increase the risk of cancer[4-6]. Polymorphisms in these genes have been shown to produce significant alterations in the metabolism of many substrates, including carcinogens and chemotherapeutic agents[7]. As a result, these polymorphisms also have been suggested to be associated with the risk of many malignances[2,8-16]. Some of them have also been suggested to affect the survival status of cancer patients[8-10,14].

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer mortality, resulting in about 500000 deaths per year. Half of HCC cases and deaths were estimated to occur in China[17]. Most of these cases in China arise due to the chronic infection with hepatitis virus, the common carcinogen to HCC[18]. Not only hepatitis virus infection, but also other environmental carcinogens, such as aflatoxin or chemical carcinogens, would contribute to hepatic carcinogenesis[19]. The metabolizing enzymes for detoxification or activation of these carcinogens to lower toxicity, potentially affect the individual cancer risk and progression of HCC. Evidence has shown that some SNPs of *GSTs* were associated with HCC risk[12]. However, none of these polymorphisms are investigated in relation to HCC survival.

In this study, we assessed the effects of 12 tagging SNPs in seven *GST* genes *GSTA1*, *GSTA4*, *GSTM2*, *GSTM3*, *GSTO1*, *GSTO2* and *GSTP1* on 5-year survival in a cohort of 214 Chinese HCC patients receiving surgical treatment. To the best of our knowledge, this is the first study to investigate the predictive value of SNPs in these *GST* genes for HCC prognosis in Chinese population.

MATERIALS AND METHODS

Study population

A total of 214 Han Chinese HCC patients were recruited at the Department of Hepatobiliary Surgery, the First Affiliated Hospital of Xi'an Jiaotong University in Xi'an, China. These patients were recruited between January 2009 and October 2013. HCC diagnosis was based on the National Comprehensive Cancer Network (NCCN) clinical practice guidelines in oncology. All subjects included in this study had histopathologically confirmed HCC. All cases received surgical treatment and had no previous history of other cancers or cancer-related treatments. There were no recruitment restrictions on age at diagnosis, gender and tumor stage. Participants received other treatments (such as chemotherapy, interventional therapy or biological target therapy) after surgical operation were excluded for further analysis. Ethics committee of the First Affiliated Hospital of Xi'an Jiaotong University approved this study. Written informed consent was obtained from each participant before enrollment.

Another 86 HCC patients derived from Queen Mary Hospital, the University of Hong Kong, were recruited as a validated cohort (Hong Kong Cohort). All genetic and SNP data were obtained from two microarray series (GSE28127 and GSE22058) in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (http://www.

Table 1 Demographic and clinical characteristics of hepatocellular carcinoma patients

Variable	Our cohort		Hong Kong cohort	
	n	%	*n*	%
Total	214	100	401	100
Age (yr)				
≤ 55	108	50.5	NA	NA
> 55	106	49.5	NA	NA
Gender				
Male	176	82.2	322	80.3
Female	38	17.8	79	19.7
Smoking behavior				
Yes	70	32.7	223	55.6
No	144	67.3	178	44.4
Cirrhosis				
Present	126	58.9	229	57.1
Absent	88	41.1	172	42.9
Child-Pugh score				
A	172	80.4	390	97.3
B	42	19.6	11	2.7
AFP level (ng/mL)				
< 200	100	46.7	NA	NA
≥ 200	114	53.3	NA	NA
Tumor size				
< 5 cm	102	47.7	NA	NA
≥ 5 cm	112	52.3	NA	NA
TNM stage				
Ⅰ + Ⅱ	119	55.6	177	44.1
Ⅲ	95	44.4	224	55.9
Outcome				
Dead	111	51.9	272	67.8
Alive	103	48.1	129	32.2

ncbi.nlm.nih.gov/geo/).

Demographic and clinical data

Detailed demographic and clinical information was collected through in-person interview, medical chart review, or consultation with treating physicians. The demographic data included the date of diagnosis, smoking behavior, liver cirrhosis, Child-Pugh score, serum alpha-fetoprotein (AFP), and tumor-node-metastasis (TNM) stage. To calculate pack-years of smoking, the average of number of cigarettes smoked per day was divided by 20 to give packs per day and multiplied by the total number of years of smoking. At 3-mo intervals, follow-up information on death of patients was updated by a trained clinical specialist through on-site interview, direct calling or medical chart review. The latest follow-up data in this analysis were obtained in January 2014. Demographic and clinical characteristics of HCC patients are shown in Table 1.

Tagging SNP selection and genotyping

Tagging SNP selection was based on data from the NIEHS (National Institute of Environmental Health Sciences, http://snpinfo.niehs.nih.gov/snpinfo/snptag.htm). Together with SNPs within a gene, we also considered polymorphisms in the 5'- and 3'-flanking regions up to 5 kb. We chose common SNPs with a minor allele frequency (MAF) ≥ 0.05 in a population of Chinese Han in Beijing (CHB) and an SNP design score cutoff ≥ 0.6 (indicating a higher probability for a successful assay). To select tagging SNPs, we used a pairwise tagging approach with r^2 ≥ 0.8. Finally, a total of 12 potentially tagging SNPs in 7 genes were selected, including *GSTA1*: rs6917150; *GSTA4*: rs385636; *GSTM2*: rs638820; *GSTM3*: rs1109138, rs7483; *GSTO1*: rs2282326, rs17116779; *GSTO2*: rs156699, rs7085725, rs157077; *GSTP1*: rs4147581, rs2370141. Information of selected tagging SNPs is shown in Table 2 and Figure 1.

Table 2 Summarized information of selected tagging SNPs in *GST* genes

Gene	Tagging SNPs	bp position	Ref SNP alleles	MAF_CHB
GSTA1	rs6917150	Chr 6: 52760414	T/C	0.338
GSTA4	rs385636	Chr 6: 52948896	A/G	0.174
GSTM2	rs638820	Chr 1: 110011429	T/C	0.344
GSTM3	rs1109138	Chr 1: 110079472	G/A	0.167
	rs7483	Chr 1: 110081224	A/G	0.244
GSTO1	rs2282326	Chr 10: 106010388	A/C	0.244
	rs17116779	Chr 10: 106021185	G/A	0.078
GSTO2	rs156699	Chr 10: 106043631	T/C	0.222
	rs7085725	Chr 10: 106050199	T/C	0.122
	rs157077	Chr 10: 106027884	A/G	0.378
GSTP1	rs4147581	Chr 11: 67108161	G/C	0.273
	rs2370141	Chr 11: 67105105	A/G	0.181

MAF (minor allele frequency) was derived from CHB (Chinese Han people in Beijing) population in HapMap website (http://hapmap.ncbi.nlm.nih.gov/).

Genomic DNA extraction and genotyping

Genomic DNA was extracted from peripheral blood samples using E.Z.N.A. Blood DNA Midi Kit (Omega Bio-Tek, Norcross, GA, United States) according to the manufacture's instructions. Genotyping was performed using Sequenom MassARRAY iPLEX genotyping system (Sequenom Inc, San Diego, CA, United States). Laboratory personnel conducting genotyping were blinded to patients' information.

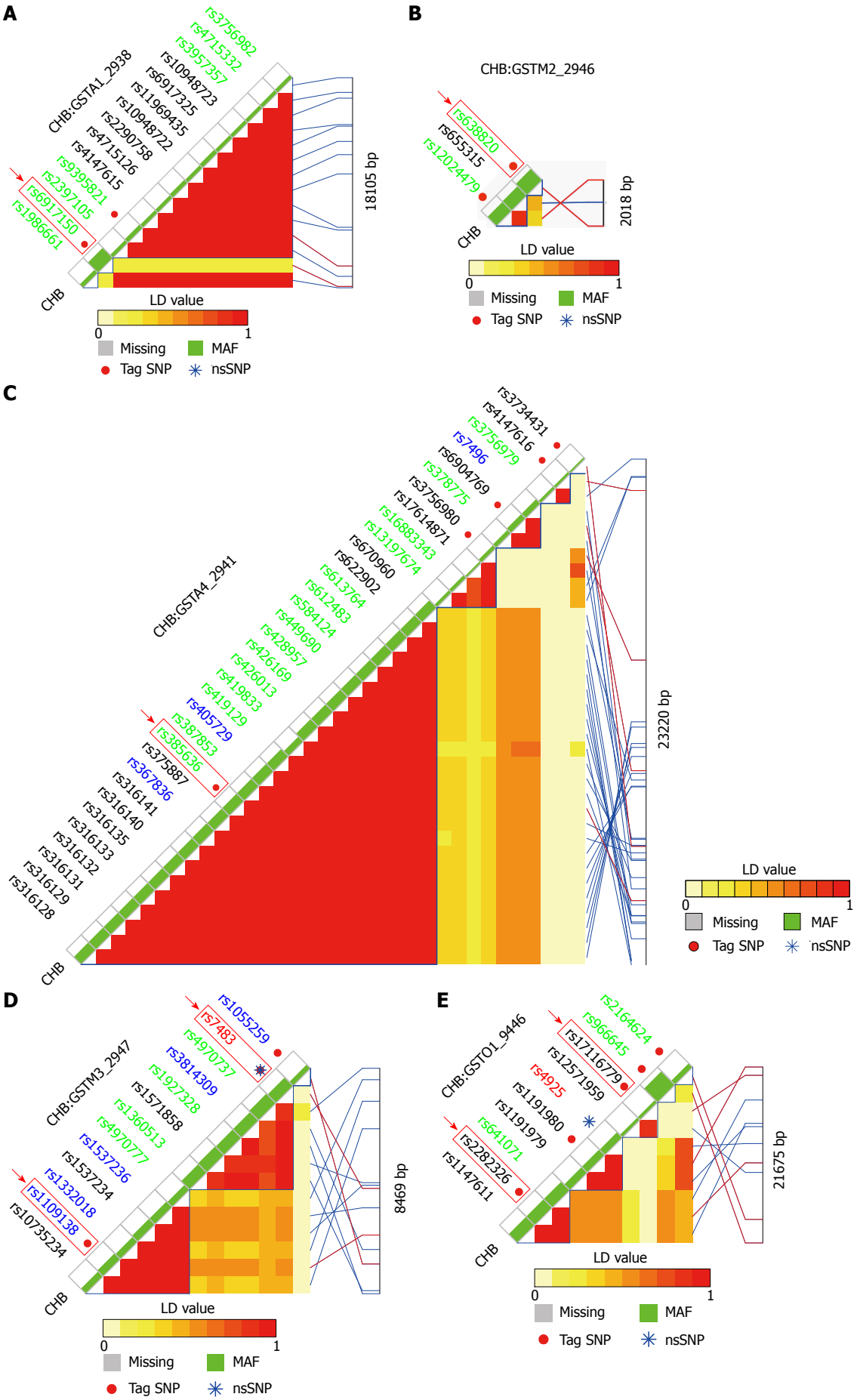
Statistical analysis

All statistical analyses were performed using the SPSS 21.0 statistical package for Microsoft Windows (SPSS, Chicago, IL, United States). The survival time was defined as the period from the date of first treatment to the date of death or last follow-up. Hazard ratios (HRs) and 95%CIs were estimated from a Cox proportional hazards model. Kaplan-Meier curve and log-rank test were also used to assess the differences of overall survival. All statistical tests were two-sided and $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of the study population

For the 214 patients included, the median age at the


A
CHB:GSTA1_2938
rs3756982
rs4715332
rs3957357
rs10948723
rs6917325
rs11969435
rs10948722
rs2290758
rs4715126
rs4147615
rs9395821
rs2397105
rs6917150
rs1986661
18105 bp
CHB
LD value
0
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nsSNP
B
CHB:GSTM2_2946
rs638820
rs655315
rs12024479
2018 bp
CHB
LD value
0
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Missing
MAF
Tag SNP
nsSNP
C
CHB:GSTA4_2941
rs3734431
rs4147616
rs3756979
rs7496
rs6904769
rs378775
rs3756980
rs17614871
rs16883343
rs13197674
rs670960
rs622902
rs613764
rs612483
rs584124
rs449690
rs428957
rs426169
rs426013
rs419833
rs419129
rs405729
rs387853
rs385636
rs375887
rs367836
rs316141
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rs316135
rs316133
rs316132
rs316131
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rs316128
23220 bp
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CHB:GSTM3_2947
rs1055259
rs7483
rs4970737
rs3814309
rs1927328
rs1571858
rs1360513
rs4970777
rs1537236
rs1537234
rs1332018
rs1109138
rs1073534
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rs966645
rs17116779
rs12571959
rs4925
rs1191980
rs1191979
rs641071
rs2282326
rs1147611
21675 bp
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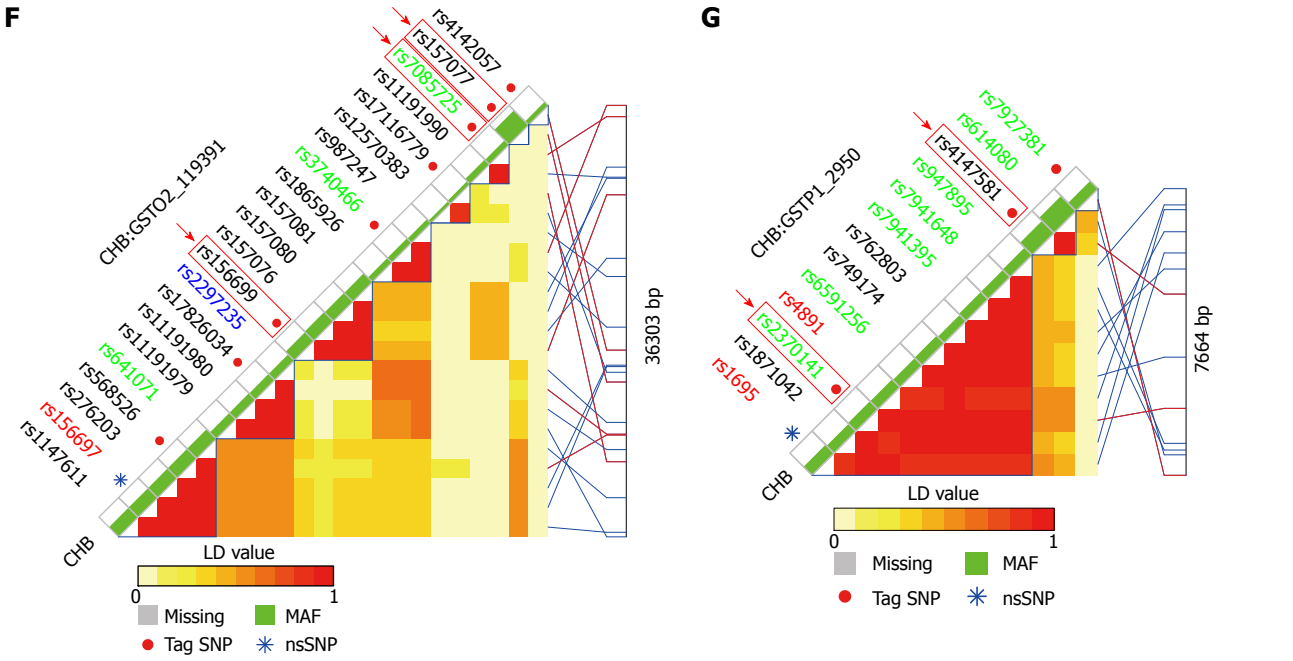


Figure 1 Relevant pictures of polymorphisms in seven *GST* genes. A: *GSTA1* (Gene ID: 2946); B: *GSTM2* (Gene ID: 2946); C: *GSTA4* (Gene ID: 2941); D: *GSTM3* (Gene ID: 2947); E: *GSTO1* (Gene ID: 9446); F: *GSTO2* (Gene ID: 119391); G: *GSTP1* (Gene ID: 2950). Genotype data were obtained from HapMap database (http://hapmap.ncbi.nlm.nih.gov) and SNPs were sorted by LD similarity using NIEHS tools (http://snpinfo.niehs.nih.gov). Twelve selected SNPs were labeled by red block.

time of HCC diagnosis was 55 years (range: 20-84 years). The majority of patients (82.2%, 176/214) were men and 32.7% (70/214) of all patients had smoking behavior. About 58.9% (126/214) of patients had cirrhosis and 80.4% (172/214) had Child-Pugh score A. There were 52.3% (112) of patients with tumor size ⩾ of 5 cm and 53.3% (114) with significantly increased serum AFP (200 ng/mL). The percentages of patients with TNM stage Ⅰ/Ⅱ or stage Ⅲ disease were 55.6% (119/214) and 44.4% (95/214), respectively. The median follow-up time was 24 mo (ranging from 3 to 60 mo). By the last follow-up, 51.9% (111/214) of patients were dead, and the median survival time for the entire cohort of patients was 33.6 mo.

Association between individual SNPs and overall survival in HCC patients

We assessed the effect of 14 tagging SNPs in seven *GST* genes on death of HCC patients using the Cox regression model. When the dominant genetic model was tested, our data showed that SNPs rs7085725 in *GSTO2* and rs4147581 in *GSTP1* gene were significantly associated with the overall survival of HCC patients (Table 3). Patients carrying at least one variant allele of rs7085725 (WV + VV genotypes) had a significantly increased risk of death (HR = 1.60; 95%CI: 1.03-2.47), meanwhile, those carrying WV + VV genotypes of rs4147581 had a decreased risk of death (HR = 0.68; 95%CI: 0.46-0.99), when compared with those carrying homozygous wild-type alleles (WW genotype) (P = 0.035 and 0.042, respectively). Kaplan-Meier analysis showed a significantly shorter median survival time in patients with WV + VV genotypes of SNP rs7085725 than those with WW genotype (19.8 mo *vs* 36.6 mo; log rank P = 0.03) (Figure 2A). In contrast, patients with WV + VV genotypes of SNP rs4147581 had a longer median survival time than those with WW genotype (33.9 mo *vs* 26.8 mo; log rank P = 0.04) (Figure 2B).

Significant association of rs7085725 and rs4147581 with overall survival in HCC patients

We further analyzed the effect of SNPs rs7085725 and rs4147581 on the overall survival in HCC patients stratified by demographic and clinicopathological characteristics. As shown in Table 4, the significantly increased death risk conferred by SNP rs7085725 was observed in younger patients [HR (95%CI) = 1.86 (1.02-3.38); P = 0.044], male patients [HR (95%CI) = 1.83 (1.14-2.94); P = 0.013], smoking patients [HR (95%CI) = 2.59 (1.27-5.27); P = 0.09], and those with cirrhosis [HR (95%CI) = 2.00 (1.11-3.59); P = 0.020], Child-Pugh score A [HR (95%CI:) = 2.28 (1.32-3.93); P = 0.003] and AFP ⩾ 200 ng/mL [HR (95%CI) = 2.40 (1.34-4.29); P = 0.003]. Meanwhile, the significantly decreased death risk conferred by SNP rs4147581 was observed in younger patients [HR (95%CI) = 0.52 (0.30-0.92), P = 0.024], male patients [HR (95%CI) = 0.64 (0.42-0.99), P = 0.042], non-smoking patients [HR (95%CI) = 0.58 (0.36-0.93); P = 0.024], and those with cirrhosis [HR (95%CI) = 0.50 (0.29-0.85); P = 0.011], Child-Pugh score B [HR (95%CI) = 0.45 (0.22-0.90); P = 0.024],

Table 3 Associations between polymorphisms of tagging SNPs in *GST* genes and overall survival in hepatocellular carcinoma patients

Gene	Tagging SNP	Genotype	Outcome, *n* (%)		HR (95%CI)	*P* value
			Dead	Alive		
GSTA1	rs6917150	WW	48 (51.6)	45 (48.4)	1 (ref)	
		WV + VV	63 (52.1)	58 (47.9)	1.01 (0.69-1.48)	0.961
GSTA4	rs385636	WW	82 (55.4)	66 (44.6)	1 (ref)	
		WV + VV	29 (43.9)	37 (56.1)	0.71 (0.46-1.09)	0.117
GSTM2	rs638820	WW	42 (51.9)	39 (48.1)	1 (ref)	
		WV + VV	69 (51.9)	64 (48.1)	1.13 (0.77-1.67)	0.526
GSTM3	rs1109138	WW	85 (54.1)	72 (45.9)	1 (ref)	
		WV + VV	26 (45.6)	31 (54.4)	0.81 (0.52-1.26)	0.353
	rs7483	WW	63 (56.2)	49 (43.8)	1 (ref)	
		WV + VV	48 (47.1)	54 (52.9)	0.94 (0.64-1.38)	0.760
GSTO1	rs2282326	WW	60 (56.1)	47 (43.9)	1 (ref)	
		WV + VV	51 (47.7)	56 (52.3)	0.92 (0.63-1.35)	0.675
	rs17116779	WW	99 (51.0)	95 (49.0)	1 (ref)	
		WV + VV	12 (60.0)	8 (40.0)	0.95 (0.52-1.75)	0.869
GSTO2	rs156699	WW	64 (50.0)	64 (50.0)	1 (ref)	
		WV + VV	47 (54.7)	39 (45.3)	1.10 (0.75-1.62)	0.612
	rs7085725	WW	81 (46.0)	95 (54.0)	1 (ref)	
		WV + VV	30 (78.9)	8 (21.1)	1.60 (1.03-2.47)[a]	0.035[a]
	rs157077	WW	50 (58.1)	36 (41.9)	1 (ref)	
		WV + VV	61 (47.7)	67 (52.3)	0.78 (0.54-1.14)	0.198
GSTP1	rs4147581	WW	57 (55.3)	46 (44.7)	1 (ref)	
		WV + VV	54 (48.6)	57 (51.4)	0.68 (0.46-0.99)[a]	0.042[a]
	rs2370141	WW	74 (52.1)	68 (47.9)	1 (ref)	
		WV + VV	37 (51.4)	35 (48.6)	0.90 (0.61-1.34)	0.597

[a]$P < 0.05$ was considered statistically significant. WW: Homozygous wild-type genotype; WV: Heterozygous genotype; VV: Homozygous variant genotype; SNP: Single nucleotide polymorphisms.

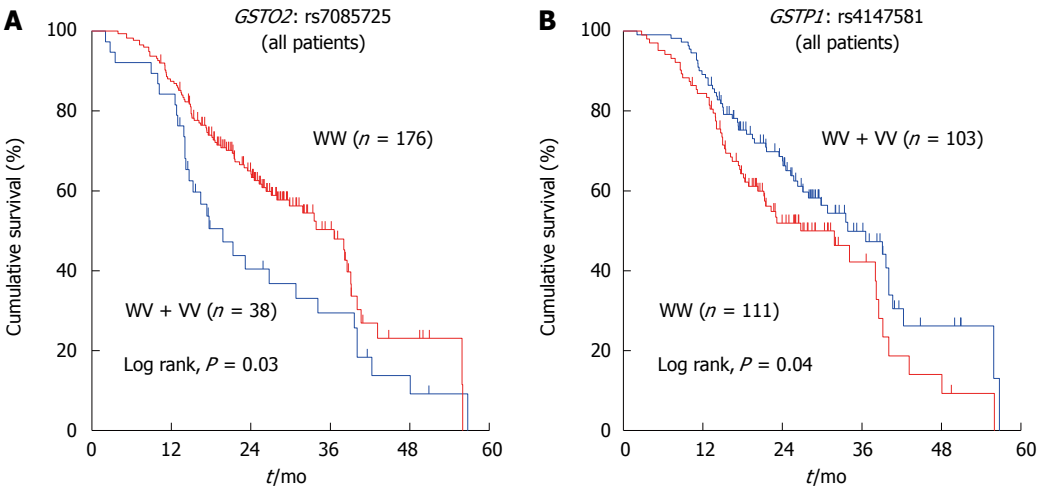


Figure 2 Kaplan-Meier curves for hepatocellular carcinoma patients carrying genetic variants of *GSTO2*: rs7085725 (A) and *GSTP1*: rs4147581 (B). WW: Homozygous wild-type genotype; WV: Heterozygous genotype; VV: Homozygous variant genotype.

AFP < 200 ng/mL [HR (95%CI) = 0.51 (0.29-0.89); $P = 0.018$] and tumor size ⩾ 5 cm [HR (95%CI) = 0.60 (0.37-1.00); $P = 0.048$]. Interestingly, both of the two SNPs were significantly associated with overall survival in patients with younger age, male gender and cirrhosis. Log-rank test further indicated significant differences in overall survival between WV + VV and WW genotypes of SNPs rs7085725 or rs4147581 in those patients with above-mentioned factors (Figure 3).

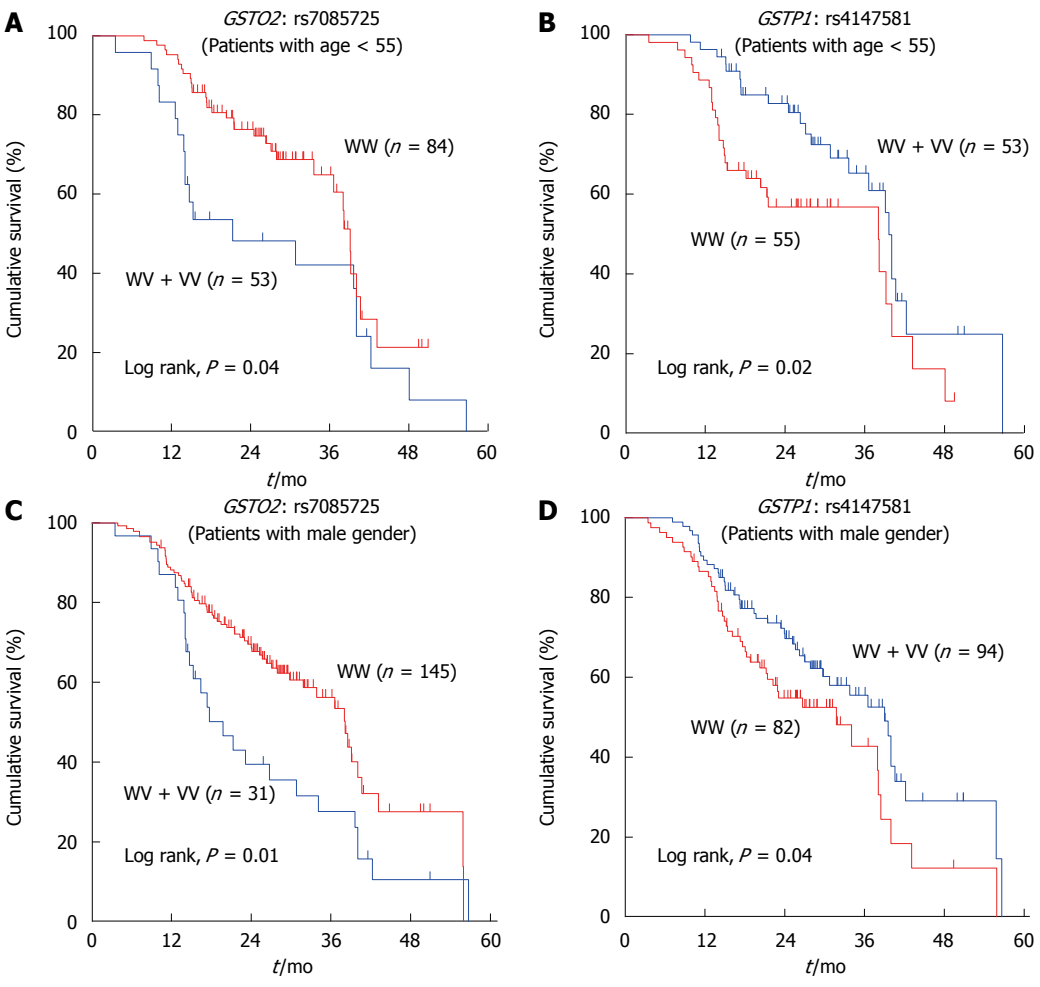
Cumulative effect of unfavorable genotypes on overall survival

To further assess the cumulative effects of genetic variants of SNPs rs7085725 and rs4147581 on overall survival in HCC, we did a joint analysis by including the two SNPs (Table 5). The unfavorable genotypes were defined as WV or VV for rs7085725 and WW for rs4147581. When using group 1 (with 0 unfavorable genotype) as reference, HCC patients in group 3

Table 4 Stratified analysis on the association between *GSTO2*: rs7085725 and *GSTP1*: rs4147581 genotype and overall survival of hepatocellular carcinoma patients

Variable	GSTO2: rs7085725				GSTP1: rs4147581			
	Dead/Alive		HR (95%CI)	*P* value	Dead/Alive		HR (95%CI)	*P* value
	WW	WV + VV			WW	WV + VV		
Age (yr)								
≤ 55	32/52	19/5	1.86[a] (1.02-3.38)	0.044[a]	28/25	23/32	0.52[a] (0.30-0.92)	0.024[a]
> 55	49/43	11/3	1.71 (0.89-3.31)	0.110	29/21	31/25	0.92 (0.55-1.54)	0.755
Gender								
Male	62/83	26/5	1.83[a] (1.14-2.94)	0.013[a]	44/38	44/50	0.64[a] (0.42-0.99)	0.042[a]
Female	19/12	4/3	0.76 (0.22-2.64)	0.663	13/8	10/7	1.06 (0.45-2.50)	0.902
Smoking behavior								
No	52/67	19/6	1.35 (0.78-2.37)	0.287	35/26	36/47	0.58[a] (0.36-0.93)	0.024[a]
Yes	29/28	11/2	2.59[a] (1.27-5.27)	0.009[a]	22/20	18/10	1.06 (0.56-1.99)	0.870
Cirrhosis								
Absent	41/30	12/5	1.21 (0.62-2.35)	0.579	23/17	30/18	0.91 (0.52-1.59)	0.746
Present	40/65	18/3	2.00[a] (1.11-3.59)	0.020[a]	34/29	24/39	0.50[a] (0.29-0.85)	0.011[a]
Child-Pugh score								
A	61/87	17/7	2.28[a] (1.32-3.93)	0.003[a]	39/44	39/50	0.83 (0.53-1.30)	0.422
B	20/8	13/1	0.97 (0.47-2.03)	0.940	18/2	15/7	0.45[a] (0.22-0.90)	0.024[a]
AFP level (ng/mL)								
< 200	39/40	14/7	1.09 (0.57-2.11)	0.793	26/17	27/30	0.51[a] (0.29-0.89)	0.018[a]
≥ 200	42/55	16/1	2.40[a] (1.34-4.29)	0.003[a]	31/29	27/27	0.85 (0.51-1.44)	0.552
Tumor size								
< 5 cm	37/50	10/5	1.51 (0.74-3.06)	0.254	23/26	24/29	0.77 (0.43-1.38)	0.379
≥ 5 cm	44/45	20/3	1.59 (0.90-2.80)	0.109	34/20	30/28	0.60[a] (0.37-1.00)	0.048
TNM stage								
Ⅰ + Ⅱ	44/60	11/4	1.24 (0.61-2.53)	0.555	27/26	28/38	0.64 (0.37-1.10)	0.104
Ⅲ	37/35	19/4	1.66 (0.94-2.93)	0.081	30/20	26/19	0.75 (0.43-1.30)	0.311

[a]*P* < 0.05 was considered statistically significant. WW: Homozygous wild-type genotype; WV: Heterozygous genotype; VV: Homozygous variant genotype.



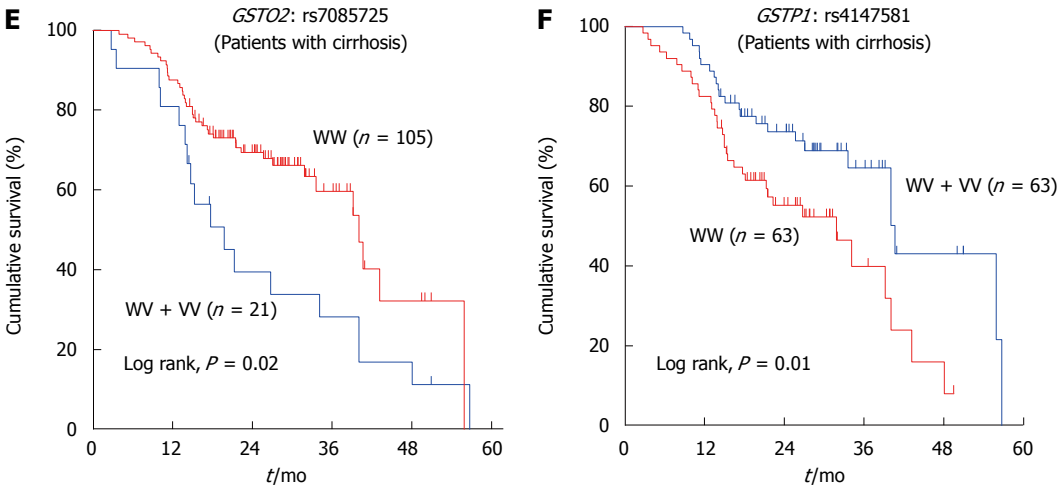


Figure 3 Kaplan-Meier curves for hepatocellular carcinoma patients carrying genetic variants of *GSTO2*: rs7085725 and *GSTP1*: rs4147581 in stratified subgroups. A and B: In patients with age < 55 years; C and D: In patients with male gender; E and F: In patients with cirrhosis. WW: Homozygous wild-type genotype, WV: Heterozygous genotype; VV: Homozygous variant genotype.

Table 5 Cumulative effects of unfavorable genotypes on overall survival of hepatocellular carcinoma patients

Group (number of unfavorable genotypes[1])	Death/Total	HR (95%CI)	*P* value
In all patients			
Group 1 (0)	44/95	1 (ref)	
Group 2 (1)	47/97	1.08 (0.71-1.63)	0.730
Group 3 (2)	20/22	1.70[a] (1.30-2.22)	< 0.001[a]
P for trend			0.004[a]
In patients with age ⩽ 55			
Group 1 (0)	18/47	1 (ref)	
Group 2 (1)	26/45	1.01 (0.52-1.95)	0.975
Group 3 (2)	14/16	2.00[a] (1.40-3.86)	< 0.001[a]
P for trend			0.003[a]
In patients with male gender			
Group 1 (0)	36/82	1 (ref)	
Group 2 (1)	34/75	0.97 (0.60-1.56)	0.898
Group 3 (2)	18/19	1.94[a] (1.45-2.59)	< 0.001[a]
P for trend			0.002[a]
In patients with cirrhosis			
Group 1 (0)	20/56	1 (ref)	
Group 2 (1)	24/56	1.29 (0.71-2.35)	0.410
Group 3 (2)	14/14	1.97[a] (1.39-2.79)	< 0.001[a]
P for trend			0.001[a]

[1]Unfavorable genotypes: *GSTO2*: rs7085725 (WV or VV) and *GSTP1*: rs4147581 (WW). [a]$P < 0.05$ was considered statistically significant. WW: Homozygous wild-type genotype; WV: heterozygous genotype; VV: Homozygous variant genotype.

(with two unfavorable genotypes) had a 1.70-fold increased risk of death (95%CI: 1.30-2.22; $P < 0.001$), when compared with those in the reference group. A significant dose-response trend was observed (P for trend = 0.004). Furthermore, the death risk of carriers of two unfavorable genotypes was significantly increased among HCC patients with younger age, male gender or cirrhosis, when compared with the reference group (HR = 2.00, 1.94 and 1.97, respectively; $P < 0.001$). Kaplan-Meier analysis showed that there was significantly decreased overall survival in patients carrying two unfavorable genotypes, when compared with others, either in all patients or in stratified subgroups (Figure 4).

Modulating effects of smoking on overall survival in HCC patients by rs7085725

Previous report from our center showed that cigarette smoking was associated with an increased risk of death in HCC[20]. As a metabolizing enzyme, *GSTP1* has been extensively investigated in the activation and detoxification of pro-carcinogens in tobacco smoke[11,21]. In line with this evidence, we further assessed the modulating effects on the association between smoking and overall patient survival, stratified by SNPs rs7085725 and rs4147581 (Table 6). We observed that the survival time of those patients carrying variant alleles of rs7085725 were adversely affected by smoking exposure [HR (95%CI) = 1.52 (1.02-2.26); $P = 0.042$]. This significant adverse effect on patient survival conferred by smoking was also observed in smoking quantity-stratified analysis [HR (95%CI) = 2.07 (1.13-3.76); $P = 0.018$]. Kaplan-Meier analysis further suggested that smoking exposure adversely affected overall survival of variant allele carriers of rs7085725, either in smoking behavior- or in smoking quantity-stratified analysis (Figure 5).

Genotype distributions of rs7085725 and rs4147581 in HCC patients with different gene expression

To further investigate the association between SNP and gene expression, we developed an SNP-gene expression model by integrating genotyping and gene expression data from Hong Kong Cohort. We selected two SNPs rs11191994 and rs614080 to predict genotypes of rs7085725 and rs4147581, respectively. Both of the two SNPs were completely linked with rs7085725 or rs4147581 [linkage disequilibrium (LD): $r^2 = 1.000$, D' = 1.000]. As shown in Figure 6, there

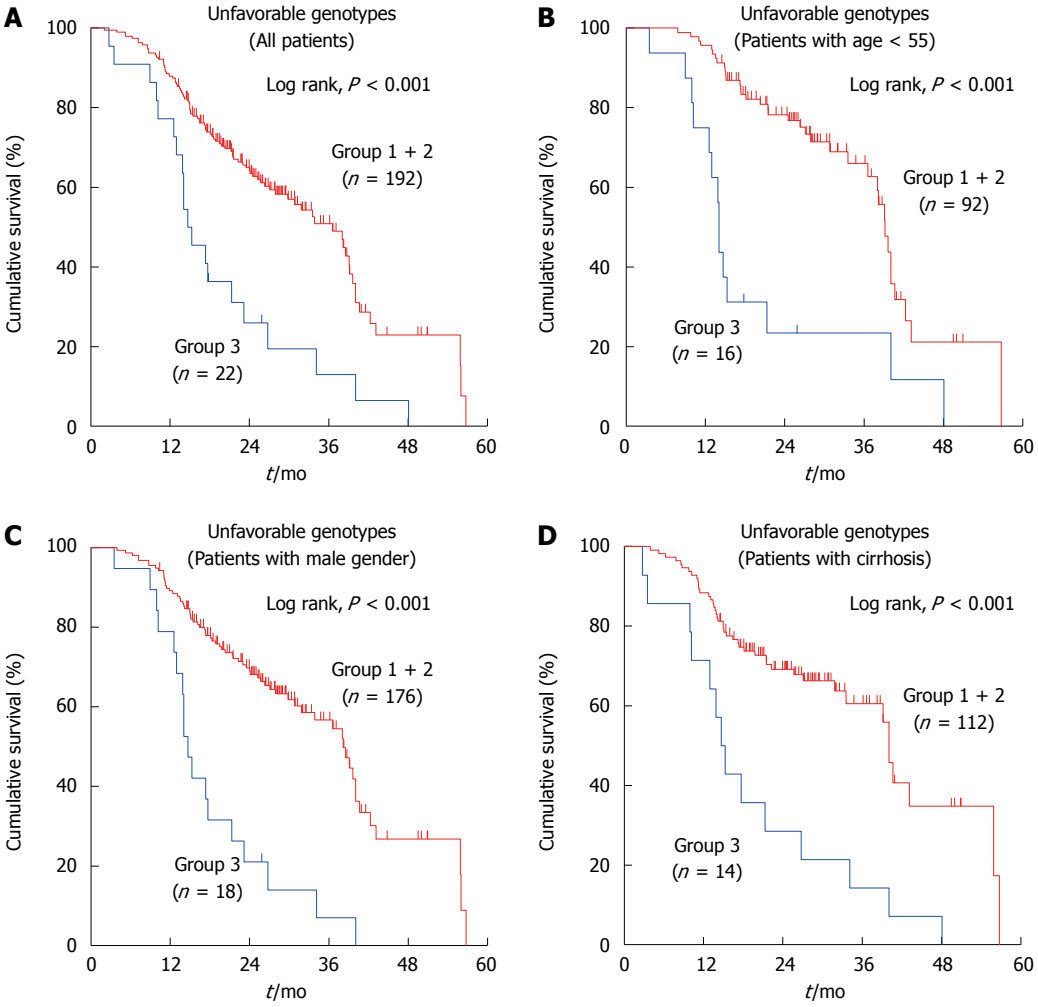


Figure 4 Kaplan-Meier curves for hepatocellular carcinoma patients carrying unfavorable genotypes. A: In all patients; B: In patients with age < 55 years; C: In patients with male gender; D: In patients with cirrhosis. Patients were classified into two groups, according to number of unfavorable genotypes carried. Group 1 + 2 carried 0 or 1 unfavorable genotype, while Group 3 carried 2 unfavorable genotypes. Unfavorable genotypes were *GSTO2*: rs7085725 (WV or VV) and *GSTP1*: rs4147581 (WW).

Table 6 Modulating effects of smoking on hepatocellular carcinoma overall survival by *GSTO2*: rs7085725 or *GSTP1*: rs4147581 genotypes

SNP and variables	Death/total	HR (95%CI)	*P* value
In patients with WW genotype of *GSTO2*: rs7085725			
Smoking behavior			
No smoking	52/119	1 (ref)	
Smoking	29/57	1.04 (0.82-1.31)	0.757
Smoking quantity (packs-year)[1]			
0	52/119	1 (ref)	
> 0 and < 20	20/45	1.06 (0.82-1.37)	0.667
⩾ 20	9/12	0.99 (0.76-1.30)	0.956
In patients with WV + VV genotypes of *GSTO2*: rs7085725			
Smoking behavior			
No smoking	19/25	1 (ref)	
Smoking	11/33	1.52 (1.02-2.26)[a]	0.042[a]
Smoking quantity (packs-year)[1]			
0	19/25	1 (ref)	
> 0 and < 20	9/11	1.42 (0.93-2.16)	0.106
⩾ 20	2/2	2.07 (1.13-3.76)	0.018[a]
In patients with WW genotype of *GSTP1*: rs4147581			
Smoking behavior			
No smoking	35/61	1 (ref)	
Smoking	22/42	0.95 (0.72-1.24)	0.697
Smoking quantity (packs-year)[1]			
0	35/61	1 (ref)	
> 0 and < 20	15/33	0.97 (0.71-1.31)	0.824
⩾ 20	7/9	0.95 (0.71-1.28)	0.754
In patients with WV + VV genotypes of *GSTP1*: rs4147581			
Smoking behavior			
No smoking	36/83	1 (ref)	
Smoking	18/28	1.27 (0.95-1.69)	0.101
Smoking quantity (packs-year)[1]			
0	36/83	1 (ref)	
> 0 and < 20	14/23	1.27 (0.93-1.74)	0.128
⩾ 20	4/5	1.16 (0.81-1.66)	0.426

[1]To calculate pack-years of smoking, the average of number of cigarettes smoked per day was divided by 20 to give packs per day and multiplied by the total number of years of smoking. [a]$P < 0.05$ was considered statistically significant. WW: Homozygous wild-type genotype; WV: Heterozygous genotype; VV: Homozygous variant genotype; SNP: Single nucleotide polymorphisms.

was a significant difference in the genotype distribution of rs7085725 between patients with high and median GSTO2 expression levels ($P = 0.042$). Additionally, the

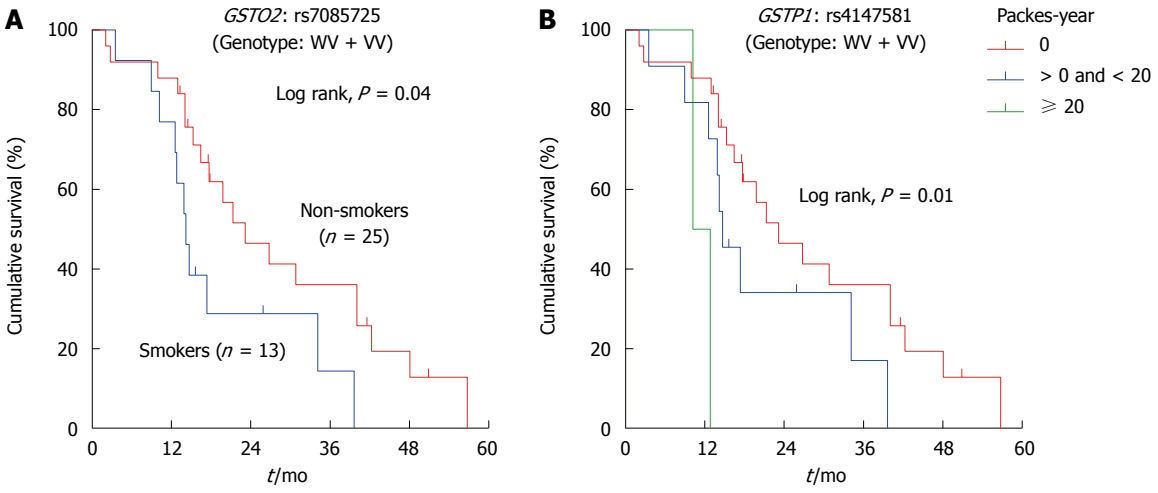


Figure 5 Kaplan-Meier curves for hepatocellular carcinoma patients carrying WV + VV genotypes in different smoking subgroups. A: HCC patients were classified by smoking behavior; B: HCC patients were classified by smoking quantity. WV: Heterozygous genotype; VV: Homozygous variant genotype.

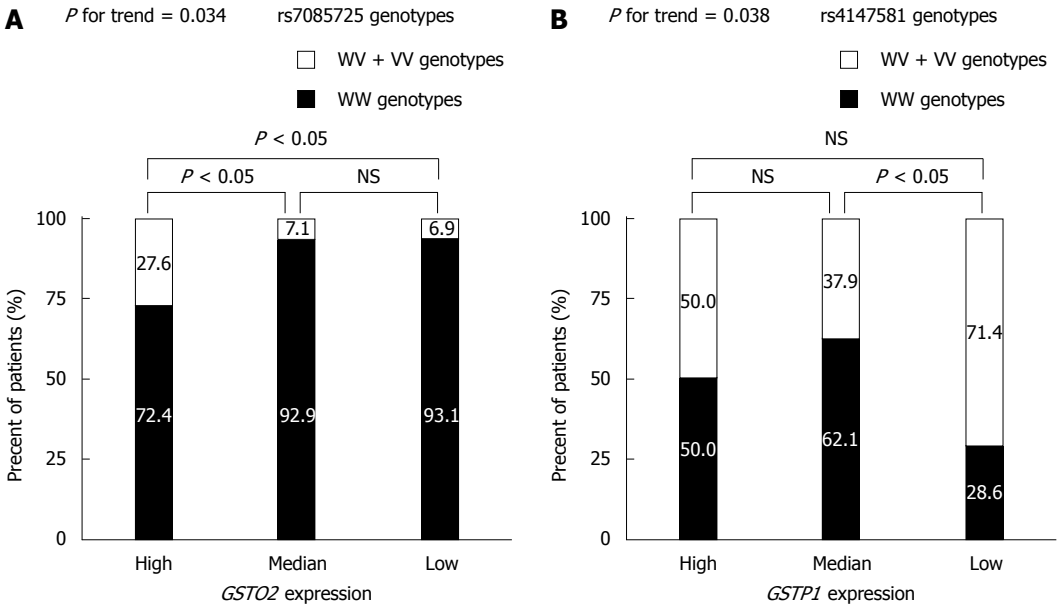


Figure 6 Comparison of genotype distribution in hepatocellular carcinoma patients with different *GSTO2* and *GSTP1* expression. A: rs7085725 genotype distribution in different *GSTO2* expression groups. rs7085725 genotype was predicted according to rs11191994 (LD r^2 = 1.000, D' = 1.000); B: rs4147581 genotype distribution in different *GSTP1* expression groups. rs4147581 genotype was predicted according to rs614080 (LD r^2 = 1.000, D' = 1.000). WV: Heterozygous genotype; VV: Homozygous variant genotype; NS: Not significant.

genotype distributions of rs4147581 in low and median *GSTP1* expression groups were also significantly different (P = 0.011). We found that patients carrying WV + VV genotypes of rs7085725 were more frequent in high *GSTO2* expression subgroup, while rs4147581 WW allele carriers were dominant in low *GSTP1* expression subgroup (Figure 6).

DISCUSSION

In this study, we evaluated the prognostic value of 12 tagging polymorphisms from seven *GST* genes in Chinese HCC patients. The most important finding was that SNPs *GSTO2:* rs7085725 and *GSTP1*: rs4147581 were significantly associated with the overall survival of HCC patients (P = 0.035 and P = 0.042, respectively). Our data indicated that SNPs *GSTO2:* rs7085725 and *GSTP1*: rs4147581 used alone or in combination were potential prognostic markers for HCC patients, especially in those patients with younger age, male gender and cirrhosis. If further validated, the two SNPs may potentially be developed as simple non-invasive predictive biomarkers for HCC patients.

GSTP1, an extensively studied member of GSTs, acts as a part of the protection system against a wide range of potentially harmful cytotoxic compounds. It was observed that altered expression of *GSTP1* existed in liver cancer cell lines[22], and in more than 77.8% of

HBV-associated HCC tissues[23]. Previous studied have extensively demonstrated that *GSTP1* polymorphisms would contribute to the increased risk and poor outcome of many cancers, including esophageal cancer[24], colorectal cancer[8], glioma[10], breast cancer[2,25], prostate cancer[26] and ovarian cancer[9,27]. However, little is known to date about their effect on HCC risk and prognosis. Chen *et al*[13] reported that *GSTP1* gene polymorphisms might be considered as factors increasing the susceptibility to HCC in Taiwanese aged ⩽ 57 years. In the present study, we similarly identified a tagging SNP of *GSTP1* (rs4147581) as a predictive biomarker in HCC patients aged ⩽ 55 years. Besides younger age, we additionally identified that *GSTP1*: rs4147581 polymorphism was associated with the overall survival in those patients with male gender, non-smoking behavior, liver cirrhosis, Child-Pugh score B, AFP < 200 ng/mL, or tumor size ⩾ 5 cm, all of which suggested this SNP as a more powerful predictor than previous SNP markers.

The function of *GSTP1*: rs4147581 to date remains to be unclear. In the present study, we first reported that genotype distribution of rs4147581 was associated with GSTP1 expression. It has been reported that *GSTP1*: rs4147581 was associated with DNA methylation[28], which has been clearly demonstrated to play a critical role in down-regulating *GSTP1* expression in HCC[6,22,23]. Considering that loss of *GSTP1* expression has been suggested to increase the risk of DNA damage[6], we put forward the hypothesis that rs4147581 may control *GSTP1* expression by influencing DNA methylation, and consequently induce high risk of carcinogenesis and cancer progression.

GSTO2 gene encodes omega class GST member GSTO2, which exhibits dehydroascorbate (DHA) reductase activity in addition to novel thioltransferase, and monomethylarsonate reductase activities[29,30]. GSTO2 has 70-100 times higher DHA reductase (DHAR) activity than GSTO1, another GST omega class GST member, and is considered to be the most active DHAR in mammalian cells[30]. Previous studies have extensively demonstrated that *GSTO2* polymorphisms might contribute to the increased risk of colorectal cancer[31], gastric cancer[32], ovarian cancer[33] and leukemia[34]. This finding suggested the hypothesis that *GSTO2* gene polymorphisms might contribute to the increased risk and outcome of HCC. Although Marahatta *et al*[35] did not find a difference in genotypic distribution for *GSTO2* polymorphism between HCC patients and controls, limited number of patients (only 28 cases of HCC and 98 controls) recruited in their study might contribute to this negative result. In our study, we identified that *GSTO2*: rs7085725 polymorphism was associated with overall survival of HCC patients. When combined with *GSTP1*: rs4147581, the predictive power of *GSTO2*: rs7085725 increased, especially in those younger, male and cirrhotic patients.

Recently, emerging evidence has shown that *GSTO2* gene polymorphism is associated with lung function modified by smoking exposure[36], and contributes to the development of lung cancer[37]. These reports suggest the critical role of *GSTO2:* rs7085725 in smoking related HCC risk. Our data indicated the significant association between *GSTO2*: rs7085725 and smoking behavior, which always predicts a relatively poor survival[21,38]. We further investigated the interaction between SNP *GSTO2:* rs7085725 and smoking exposure in modulating HCC patient survival. Interestingly, these variant allele carriers of *GSTO2:* rs7085725 were more susceptible to smoking exposure, when compared with wild type allele carriers. All the above results clearly demonstrated that the population carrying variant alleles of *GSTO2:* rs7085725 might obtain more benefit of smoking cessation, which may guide those patients to make better decision.

It has been reported that the polymorphisms in the *GSTO2* gene affect its expression levels in Alzheimer and Parkinson disease[39]; however, the influence of rs7085725 on *GSTO2* expression in HCC remains unclear. Our data showed that rs7085725 minor allele carriers were significantly increased in high *GSTO2* expression subgroup, when compared with median and low expression subgroups. Although the biological function of *GSTO2* has not been clearly demonstrated, it has been observed that the expression of *GSTO1*, which was an important paralog gene of *GSTO2* and exhibited very similar biological function to *GSTO2*, was over-expressed in chemo- and radio-resistant cancer cells[40,41]. Besides, elevation of GSTO1 protein in colorectal cancer cells was also involved in cell invasion and metastasis[42]. Above evidence suggested that high *GSTO2* expression might contribute to tumor progression. This hypothesis might partly explain why rs7085725 WV + VV allele carriers always had worse overall survival. To date there is no report of regulating mechanism of rs7085725 on *GSTO2* expression. The functional prediction indicates that rs7085725 is located in the 3'-UTR of the *GSTO2* gene. Previous evidence showed that SNP sequences in 3'-UTRs of many other genes are clearly involved in the regulation of mRNA expression either by providing mutated binding sites for proteins and microRNAs to alter mRNA stability, or by forming hairpin loop structures to stabilize and thus slow down mRNA degradation. This suggests a possibility of elevated expression regulation on the *GSTO2* gene by rs7085725. However, all given explanations are speculative and merit further research.

There are several strengths in this study. The patients analyzed in this study were enrolled from Xi' an and adjacent area, and these patients were all surgically treated to remove the primary tumors by the same medical center. The highly homogenous patient characteristics and treatments, as well as low rate of patient loss to follow-up, greatly reduced the confounding effects of the heterogeneous therapeutics

modalities in many other similar biomarker studies of HCC prognosis. The limitations of our study include the generalizability issue, because our study was restricted to Han Chinese. Further evaluation is necessary to determine whether these findings can be generalized to other ethnic groups. Moreover, the moderate sample size limited the validity of some stratified analyses. Following validation using larger independent populations is warranted.

In conclusion, our study presents the first epidemiological evidence supporting a role of *GSTO2:* rs7085725 and *GSTP1*: rs4147581 in the prognosis prediction in a Chinese population of HCC patients.

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COMMENTS

Background

Previous studies have revealed that several *GST* genes are highly polymorphic, with particular genotypes shown to be associated with cancer risk and progression. Evidence has shown that some single nucleotide polymorphisms (SNPs) of *GST* were associated with HCC risk. However, none of these polymorphisms are investigated in relation to survival of patients with hepatocellular carcinoma (HCC).

Research frontiers

HCC is one of the most common malignancies worldwide, especially in China, and it largely remains an incurable disease. Traditional clinicopathological features such as tumor stage, histological grade and concentration of serum AFP seem to be insufficient to predict clinical outcomes in HCC patients after surgical treatment. Therefore, it is extremely urgent to explore novel biomarkers to discriminate patient with different clinical outcomes and direct future treatment for HCC patients.

Innovations and breakthroughs

In recent decades, widespread efforts have been made to investigate tumor tissue and serological markers that would predict survival of resected HCC patients. However, to date there have been very few studies to explore the prognostic value of genetic markers. This study presents the first epidemiological evidence supporting a role of genetic markers in the prognosis prediction in a Chinese population of HCC patients.

Applications

By establishing the novel prognostic predictive genetic markers, this study may represent a future strategy for cancer prediction in the follow-up of patients with HCC.

Terminology

The term "genetic polymorphism" refers to genetic variants within the population that allow evolution by natural selection. It is defined by the occurrence in the same population of multiple discrete allelic states of which at least two have a high frequency (conventionally of 1% or more). Genetic polymorphism as a predictive marker for clinical outcomes has been extensively investigated.

Peer-review

The manuscript investigates the effects of SNPs in *GST* genes on survival of HCC patients, and suggests that the *GSTO2* and *GSTP1* gene polymorphisms may serve as independent prognostic markers for HCC patients. This study may be very useful for a large number of hospitals worldwide.

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