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The Impact of *GPC3 rs2267531* Polymorphism on Hepatocellular Carcinoma Susceptibility

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Running Title: *GPC3 rs2267531* Polymorphism and Hepatocellular Carcinoma

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Objective: To evaluate the association between the *GPC3 rs2267531* polymorphism and hepatocellular carcinoma (HCC) in Mongolians. **Method:** This study included 270 participants, consisting of 110 males and 160 females, with a mean age of 61±9.5 years. Serum samples were collected from all participants, who were categorized into three groups: 90 patients with hepatocellular carcinoma (HCC), 90 individuals in the risk group (RG), and 90 healthy controls. The collected samples were analyzed using allele-specific polymerase chain reaction (PCR). **Results:** No significant differences in genotype and allele frequencies were observed between the HCC group and the risk or control groups. However, the frequency of the *GPC3* C allele was significantly higher in females in the HCC group. Additionally, the protective GG genotype of *GPC3* was found to have a significantly lower frequency in females in the HCC group. In contrast, HCV-positive individuals exhibited a higher frequency of the GC genotype. **Conclusion:** The *GPC3* C allele frequency is associated with HCC in females, while the heterozygote GC genotype is linked to HCV infection in both genders. These findings suggest that the *GPC3 rs2267531* polymorphism may play a role in HCC susceptibility, particularly in relation to gender and HCV status.

Keywords: HCC, Liver cancer, SNP, Genotype, Hepatitis virus (B, C, D)

Introduction

Chronic infections with hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis D virus (HDV) are well-established as the primary risk factors for HCC. In developing countries, viral

hepatitis is present in approximately 75-90% of HCC cases.[1,2]

Notably, in Mongolia, 93% of all HCC cases are attributed to viral hepatitis, with HBV alone accounting for 34.5%.[3] This highlights a distinctive aspect of HCC epidemiology in the region. Moreover, the prevalence of HDV co-infection and superinfection is particularly high in Mongolia.[4] The combined infection of HBV and HDV increases the risk of HCC by up to 30-fold compared to HBV mono-infection.[2]

HCV affects approximately 185 million individuals worldwide and is responsible for an estimated 350,000 deaths annually. Up to 80% of infected individuals develop chronic infection, which can progress to severe liver fibrosis and cirrhosis, with 30–50% of cirrhosis cases ultimately advancing to hepatocellular carcinoma (HCC).[5]

In addition to infectious etiologies, several non-infectious factors also contribute to hepatic carcinogenesis. These include metabolic disturbances such as dysregulation of lipid and carbohydrate metabolism, chronic alcohol consumption, autoimmune liver diseases, and exposure to hepatotoxic substances.[2,6]

Liver cancer remains one of the leading causes of cancer-related mortality worldwide. According to estimates by the World Health Organization (WHO), 905,677 new cases of liver cancer were reported in 2020, resulting in 830,180 deaths.[7] Hepatocellular carcinoma (HCC), the most common form of primary liver cancer, is highly prevalent in East Asia and Africa, where it leads in both incidence and mortality. Notably, a rising trend in HCC incidence and mortality has also been observed in Europe and the United States.[8,9] Epidemiological studies further indicate a pronounced sex disparity, with HCC incidence rates being two to four times higher in men than in women.[10] In Mongolia, 6,702 new cancer cases were diagnosed in 2020, of which 2,183 cases (32.6%) were attributed to HCC, underscoring its significant public health burden in the region.[11]

The glypican-3 (*GPC3*) gene, located on the long arm of the X chromosome (Xq26), encodes a membrane-bound heparan sulfate proteoglycan involved in regulating cell proliferation, migration, and differentiation. *GPC3* plays a critical role in both tumor suppression and oncogenesis, depending on the cellular context. Notably, its expression is markedly elevated in the majority of HCC cases but remains low or undetectable in benign liver conditions such as cirrhosis and non-malignant liver tumors, highlighting its potential as a diagnostic and therapeutic target in liver cancer.[12,13]

This expression pattern highlights its value as both a diagnostic and therapeutic target in liver cancer. The protein can be detected in serum and tissue samples, and its diagnostic utility has been demonstrated through immunohistochemical analysis and biomarker assays. Mechanistically, *GPC3* contributes to tumor progression in HCC by enhancing oncogenic signaling pathways. It promotes the expression of c-Myc, a critical transcription factor in the Wnt/β-catenin signaling cascade, which is known to drive tumor growth. Additionally, *GPC3* interferes with apoptotic pathways by modulating key regulatory proteins such as Bax, Bcl-2, cytochrome c, and caspase-3, facilitating both cellular proliferation and resistance to apoptosis.[14,15]

The promoter region of a gene plays a critical role in regulating gene expression and may influence susceptibility to cancer and other diseases. Single nucleotide polymorphisms (SNPs) within the promoter region of the *GPC3* gene are hypothesized to affect transcription factor binding, thereby altering gene expression levels.[16] Promoter region SNPs are particularly relevant in disease pathogenesis due to their capacity to modulate transcriptional activity, making *rs2267531* a biologically plausible candidate for genetic association studies. Previous research has reported a significant association between *rs2267531* and hepatocellular carcinoma (HCC), particularly in the Egyptian population.[17] Building upon these findings, the present study aimed to investigate the role of *rs2267531* in HCC susceptibility among the Mongolian population, further exploring its potential contribution to disease risk in different ethnic contexts.

Material and Methods

This case–control study was conducted at the Central Scientific Research Laboratory, Institute of Medical Sciences of Mongolian National University of Medical Sciences. The case group consisted of 90 individuals diagnosed with HCC at the National Cancer Center. The sample size was calculated considering several critical parameters, including the case-control study design, a significance level (α) of 0.05, an anticipated odds ratio (OR) of 4.0, a 95% confidence interval, and a statistical power of 80%. Based on these criteria, a total of 270 participants was deemed necessary to achieve adequate representativeness and statistical reliability. A total of 270 serum samples were collected between October 2022 and March 2023, comprising three groups: the HCC

group (n = 90), a risk group (RG) (n = 90), and a healthy control group (n = 90). The risk group included individuals with chronic hepatitis, toxic hepatitis, alcohol-related liver disease, and other liver disorders. The average age of participants was 61 ± 9.5 years. Of the total participants, 110 (40.7%) were male and 160 (59.3%) were female. All participants provided written informed consent and completed a standardized questionnaire prior to inclusion in the study. Participants were included in the study based on the following inclusion and exclusion criteria. Inclusion Criteria: 1. Voluntarily agreed to participate in the study 2. Over 18 years old 3. Diagnosed with hepatocellular carcinoma (HCC) at the National Cancer Center. Exclusion Criteria: 1. Declined to participate in the study, 2. Diagnosed with a disease other than hepatocellular carcinoma based on pathological examination 3. Presence of other comorbid diseases.

Blood Sample Collection

Peripheral venous blood samples (4 mL) were collected from each participant using vacuum tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant and disposable sterile needle. The collected samples were immediately processed and stored at -80°C until further analysis.

DNA Isolation and Gel Electrophoresis

Genomic DNA was extracted from whole blood samples using Qiagen's blood DNA extraction kit (Catalog No.51194, Germany), by the manufacturer's instructions. The concentration and purity of the extracted DNA were evaluated by gel electrophoresis to ensure the integrity of the samples for downstream analysis.

Primer Design

The *GPC3* gene sequence was retrieved from the National Center for Biotechnology Information (NCBI) database. Allele-specific primers for the detection of the *rs2267531* polymorphism were designed using SnapGene software. To ensure specificity for each SNP allele, a deliberate mismatch was introduced at the third nucleotide from the 3′ end of the primer sequence. Additionally, the primer lengths were optimized to allow clear differentiation of allele-specific PCR products based on amplicon size.

GPC3 primer sequences:

Forward	5′-ACACAAAATCCACCTCCTCATTC TACTCTCTGAGGAGCTTCCTGG-3′, 5′-CTCTCTGAG GAGCTTCCTGC-3′
Reverse	3′-CGTTTATCAGCGCAAGTCCC-5′

The *rs2267531* SNP was selected for analysis due to its location within the promoter region of the GPC3 gene.

Allele-Specific PCR

Genomic DNA was amplified using allele-specific polymerase chain reaction (PCR) with the Takara EX Taq Perfect Mix Kit. The PCR conditions were as follows: an initial denaturation at 95°C for 5 minutes, followed by 32 cycles of denaturation at 94°C for 1 minute, annealing at 57°C and 64°C for 30 seconds, extension at 72°C for 40 seconds, and a final extension at 72°C for 5 minutes. The amplified products were resolved on a 4-5% agarose gel and electrophoresis was performed at 100 V for 30 to 45 minutes to visualize the PCR products.

Ethics

This study was approved by the Institutional Ethics Committee of the Mongolian National University of Medical Sciences (MNUMS) and has followed the principles outlined in the Declaration of Helsinki (approval number: 2022/3-05, 2022.05.20).

Statistical Analysis

Variations between groups were analyzed by Chi-square test for categorical variables and Student's t-test for numerical variables. ANOVA was used to assess differences among the three groups. The statistical significance of differences in genotype frequencies was evaluated using the Chi-square (χ^2) test and Fisher's exact test. The ORs, Risk Ratio (RRs), and 95% CIs were estimated for association analysis by logistic regression. The Breslow-Day test was used to test for heterogeneity between ORs, and was calculated with the Cochran-Mantel-Haenszel (CMH) test. A P-value of less than 0.05 for a two-tailed test was considered statistically significant.

Results

Detection of the *rs2267531* polymorphism in the *GPC3* gene

A total of 270 participants (110 males and 160 females, aged 33-85 years, with a mean age of 61 ± 9.5 years) were evaluated for the *GPC3 rs2267531* polymorphism. The allele-specific PCR products were resolved on a 4% agarose gel, where the guanine allele (G) and cytosine allele (C) were detected as amplicons of 112 bp and 87 bp, respectively (Figure 1).

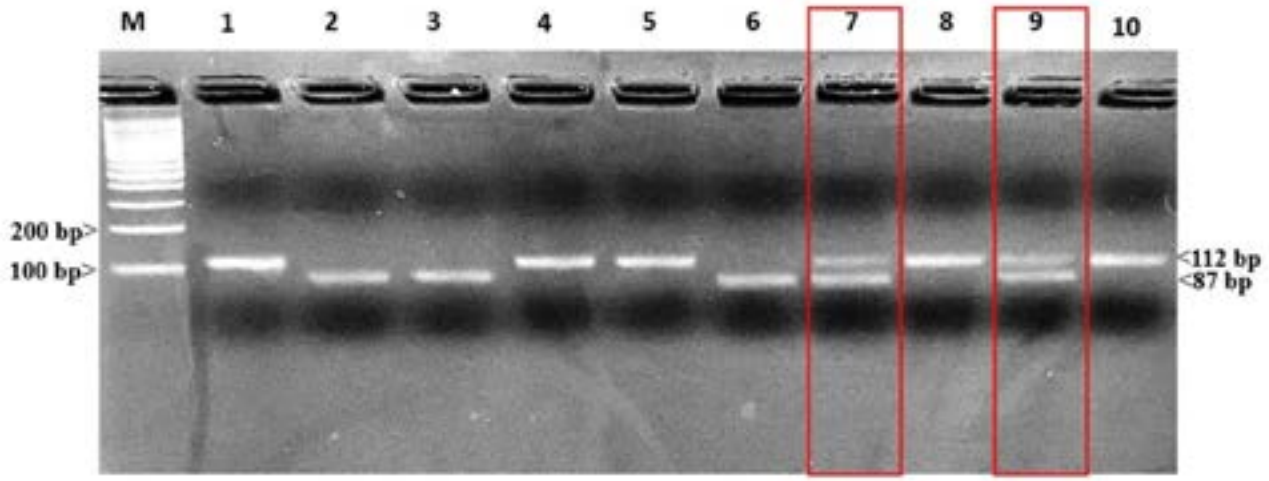

Figure 1. Confirmation of the PCR product by gel electrophoresis: In samples 1, 4, 5, and 7-10, the guanine allele is present, as indicated by the band at 112 base pairs (bp), which corresponds to the guanine allele of the *GPC3* gene. In samples 2, 3, 6, 7, and 9, the cytosine allele is present, with the band at 87 bp representing the cytosine allele. Samples 7 and 9, highlighted in red, are heterozygous, exhibiting both guanine and cytosine alleles. 'M' represents the molecular marker, and samples 1–10 correspond to the study participants. *bp - base pairs, M - marker

The genotype distributions and allele frequencies for the HCC group, the risk group (RG), and the control group are summarized in Figure 2A, B.

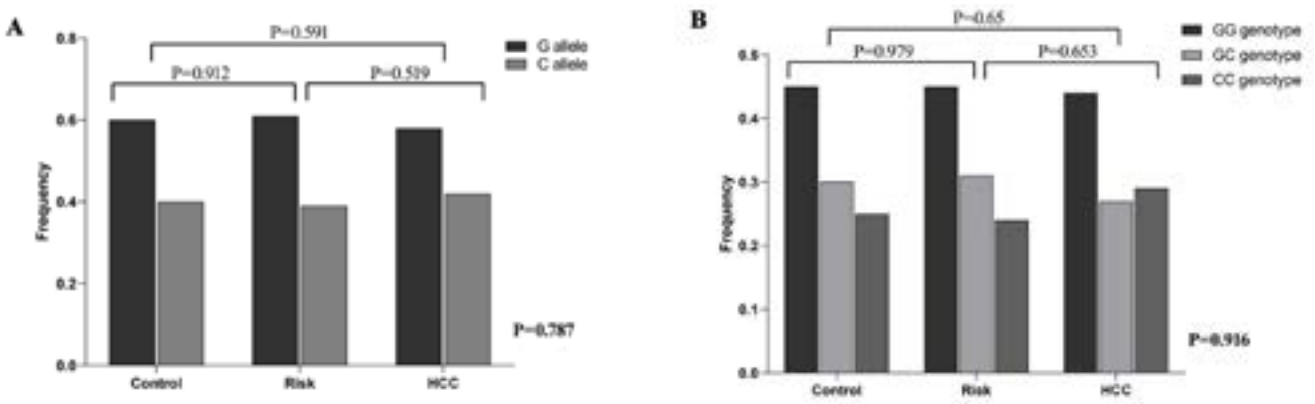

Figure 1. A, B. Frequency distribution of alleles and genotypes for *GPC3 rs2267531* in study groups *P-value

The genotype distributions in the three study groups were as follows: 45.2% (122) GG, 25.5% (69) CC, and 29.3% (79) G/C. Regarding allele frequencies, the guanine allele was present in 59.8% (162) of samples, while the cytosine allele was observed in 40.2% (108) of samples. No significant difference was found between the HCC group and either the risk group (RG) or the control group (P = 0.844).

Association of SNP *rs2267531* Alleles and Genotypes with Demographic and Clinical Characteristics of the Participants

Tables 1 and 2 present a comparison of allele and genotype distributions with the demographic and clinical characteristics of the participants. The statistical significance of differences in genotype frequencies was evaluated using the chi-square (χ^2) test and Fisher's exact test.

A significant difference in genotype distribution between

females and males was observed across all groups (P = 0.000). Specifically, the GC genotype was notably more prevalent in females compared to males in the control (26 vs 1 (n)), HCC (22 vs 2 (n)), and risk groups (28 vs 0(n)). Furthermore, genotype distribution was significantly associated with HCV status (P = 0.049), with HCV-negative individuals displaying a

Table 1. Correlation between SNP *rs2267531* genotypes and demographic and clinical characteristics

Groups	Variables	Genotype	GG	GC	CC	P-value
Control (n=90)	Age	≤ 60	23	15	15	0.594
		> 60	18	12	7	
	Gender	Male	20	1	14	0.000
		Female	21	26	8	
	Age	≤ 60	21	18	10	0.436
		> 60	20	10	11	
	Gender	Male	16	0	14	0.000
		Female	25	28	7	
Risk (n=90)	HBV	Yes	11	9	3	0.354
		No	30	19	18	
	HCV	Yes	16	10	10	0.691
		No	25	18	11	
	Cirrhosis	Yes	8	9	3	0.282
		No	33	19	18	
	Steatosis	Yes	21	16	14	0.508
		No	20	12	7	
HCC (n=90)	Age	≤ 60	23	6	10	0.033
		> 60	17	18	16	
	Gender	Male	28	2	15	0.000
		Female	12	22	11	
	HBV	Yes	23	10	12	0.423
		No	17	14	14	
	HCV	Yes	14	16	12	0.049
		No	26	8	14	
	Cirrhosis	Yes	29	19	18	0.721
		No	11	5	8	
	Steatosis	Yes	20	17	15	0.263
		No	20	7	11	

Table 2. Correlation between SNP *rs2267531* alleles and demographic and clinical characteristics

Groups	Variables	Allele	G	C	P-value
Control (n=90)	Age	≤ 60	61	45	0.594
		> 60	48	26	
	Gender	Male	41	29	0.355
		Female	68	42	
	Age	≤ 60	55	41	0.286
		> 60	55	29	
	Gender	Male	37	23	0.523
		Female	73	47	
Risk (n=90)	HBV	Yes	28	14	0.354
		No	82	56	
	HCV	Yes	43	29	0.758
		No	67	41	
	Cirrhosis	Yes	21	17	0.455
		No	89	53	
	Steatosis	Yes	60	44	0.283
		No	50	26	
HCC (n=90)	Age	≤ 60	52	26	0.05
		> 60	53	49	
	Gender	Male	58	32	0.130
		Female	47	43	
	HBV	Yes	56	34	0.364
		No	49	41	
	HCV	Yes	44	40	0.134
		No	61	35	
	Cirrhosis	Yes	78	54	0.736
		No	27	21	
	Steatosis	Yes	58	46	0.447
		No	47	29	

Association of GPC3 rs2267531 SNP Alleles and Genotypes with Participants' Gender

To investigate potential differences in allele and genotype distributions between gender groups, we examined the association of the rs2267531 SNP alleles and genotypes with gender.

The frequency of the cytosine allele was significantly higher in women in the HCC group compared to the risk group (OR = 1.776, P = 0.044) (Table 3).

Table 3. Association of *rs2267531* SNP alleles with gender across the groups

Cases	Sex	G allele distribution n (%)	C allele distribution n (%)	Allele statistics OR (95% CI), P-value
HCC	Female	46 (51.1%)	44 (48.9%)	1.776[a#] (1.016-3.104), 0.044
	Male	30 (64.4%)	17 (35.6%)	0.647[b#] (0.255-1.644), 0.360
	Combined			1.347[c#] (0.840-2.160), 0.216
Risk	Female	78 (65%)	42 (35%)	0.871[a*] (0.509-1.492), 0.501
	Male	16 (53.3%)	14 (14%)	1.225[b*] (0.461-3.252), 0.683
	Combined			0.930[c*] (0.581-1.486), 0.762
Control	Female	68 (61.8%)	42 (38.2%)	1.548[a&] (0.880-2.723), 0.128
	Male	21 (58.6%)	15 (41.4%)	0.793[b&] (0.325-1.932), 0.610
	Combined			0.797[b*] (0.497-1.281), 0.350
P-value			0.435	

*[a]Females vs female, [b]Male vs male, [c]Combined vs combined. [#]HCC vs Risk, *Risk vs Control[&]*

Moreover, the frequency of the GG genotype was significantly lower in females of the HCC group compared to females in the risk group (OR = 0.305, P = 0.047). However, no significant differences in genotype distributions were observed among the three groups in males (P > 0.05) (Table 4).

Table 4. Association of *rs2267531* SNP genotypes with gender across the groups

Cases	Sex	GG/G	GC	CC/C	Logistic regression statistics OR (95% CI), P-value
HCC	Female	12 (26.6%)	22 (48.9%)	11 (24.5%)	0.305[a#] (0.095-0.985), 0.047
	Male	28 (62.3%)	2 (4.4%)	15 (33.3%)	1.633[b#] (0.630-4.235), 0.313
	Combined				1.269[c#] (0.762-2.114), 0.360
Risk	Female	25 (41.7%)	28 (46.7%)	7 (11.6%)	1.361[a]* (0.423-4.377), 1.361
	Male	16 (53.3%)	0	14 (46.7%)	0.800[b]* (0.297-2.154), 0.659
	Combined				0.995[c]* (0.566-1.609), 0.861
Control	Female	21 (38.2%)	26 (47.3%)	8 (14.5%)	0.461[a&] (0.131-1.318), 0.136
	Male	20 (57.2%)	1 (2.8%)	14 (40%)	1.307[b&] (0.517-3.303), 0.572
	Combined				1.211[b]* (0.730-2.009), 0.458
P-value				0.916	

*[a]Females vs female, [b]Male vs male, [c]Combined vs combined. [#]HCC vs Risk, *Risk vs Control[&]*

Discussion

Mongolia has liver cancer incidence and mortality rates that exceed the global average.[18] The present study assessed the association between the *rs2267531* SNP in the promoter region of GPC3 and HCC in Mongolian patients. Our findings indicated no significant difference in genotype distributions or allele frequencies of the *rs2267531* SNP between HCC patients and either the risk or control groups. In contrast, a study by Motawi, et al. reported higher frequencies of the C allele and CC genotype in HCC patients compared to controls.[17]

According to data from the NCBI database, the frequencies

of the guanine and cytosine alleles for the rs2267531 polymorphism in various populations are as follows: in Caribbean countries, 0.4897 and 0.5103; in Europeans, 0.49 and 0.51; in Japanese populations, 0.6974 and 0.3026; and in Chinese populations, 0.66 and 0.33. Notably, the guanine allele was more prevalent in both the Japanese and Chinese populations. In our study, the frequencies of the guanine and cytosine alleles among participants in the three groups were 0.575 and 0.425, respectively. These findings are consistent with previous research indicating a higher prevalence of the guanine allele in Asian populations.[19,20]

Our study revealed a higher prevalence of the GC genotype among female participants compared to male participants. Additionally, the C allele and GC, CC genotypes were more prevalent in females from the HCC group. A previous study identified the CC genotype as a risk factor for HCC, while the GG genotype was found to have a protective role in Egyptian populations.[17]

This suggests that the genetic variation under investigation may be influenced by gender, potentially due to the location of the single-nucleotide polymorphism (SNP) on the X chromosome.[21,22] Although the X chromosome is a compelling target for investigating gender-related disease associations, very few X-linked genes have been reported as disease-related. The findings of this research underscore that the higher C allele in females than in males in the HCC group could be explained by the X-linked genes that may escape X chromosome inactivation at least to some degree, so females can still express many genes from their inactive X chromosomes.[23]

The association between *GPC3* and HCC risk is further supported by studies demonstrating *GPC3* expression in various cancers, including liver, lung, stomach, ovarian cancers, melanoma, and in fetal-stage tumors.[24-29] Notably, comparative studies have shown significantly elevated *GPC3* levels in liver cancer cases.[24,25] *GPC3* also plays crucial roles in several biological processes. During embryonic development, *GPC3* interacts with signaling proteins such as Wnt, fibroblast growth factors (FGFs), and bone morphogenetic proteins (BMPs) to regulate cellular activities including division, differentiation and apoptosis. Therefore, *GPC3* holds promise as a potential biomarker for HCC.[30,31]

In conclusion, while no significant association was found overall, subgroup analyses revealed potential gender-specific trends.

This study has several limitations, including a relatively small sample size and limited access to detailed baseline and clinical characteristics of the participants. Future studies with larger sample sizes and samples from diverse geographical regions are necessary to validate further the association between the *GPC3* SNP and HCC risk.

Conflict of Interest

The authors declare no conflicts of interest.

Authors Contribution

B.B. Mb.B. M.S. and S.J. conceived of the presented idea, M.S. B.Ts supervised the fundings of this work. B.B. O.T. Mts.B. Kh.U. and N.N. collected blood samples from the subjects. Ub.G. and Um.G. contributed to sample collection and preparation and combined the clinical data of patients. B.B. Y.E. U.U. and Y.D. analyzed and interpreted patient data, performed the calculations and carried out the experiment. B.Ts. Mb.B. M.S. and S.J. verified the analytical methods and results. B.B, Kh.U. B.M. and M.S. were major contributors to the writing of the manuscript. S.J. M.S. and

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