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BRIEF ARTICLE

Association between MDM2-SNP309 and hepatocellular carcinoma in Taiwanese population

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Abstract

AIM: To investigate the risk association and compare the onset age of hepatocellular carcinoma (HCC) patients in Taiwan with different genotypes of MDM2-SNP309.

METHODS: We analyzed MDM2-SNP309 genotypes from 58 patients with HCC and 138 cancer-free healthy controls consecutively. Genotyping of MDM2-SNP309 was conducted by restriction fragment length polymorphism assay.

RESULTS: The proportion of homozygous MDM2-SNP309 genotype (G/G) in cases and cancer-free healthy controls was similar (17.2% *vs* 16.7%). Multivariate analysis showed that the risk of G/G genotype of MDM2-SNP309 *vs* wild-type T/T genotype in patients with HCC was not significant (OR = 1.265, 95% CI = 0.074-21.77) after adjustment for sex, hepatitis B or C virus infection, age, and cardiovascular disease/diabetes. Nevertheless, there was a trend that GG genotype of MDM2-SNP309 might increase the risk in HCC patients infected with hepatitis virus (OR = 2.568, 95% CI = 0.054-121.69). Besides, the homozygous MDM2-SNP309 genotype did not exhibit a significantly earlier age of onset for HCC.

CONCLUSION: Current data suggest that the association between MDM2-SNP309 GG genotype and HCC is not significant, while the risk may be enhanced in patients infected by hepatitis virus in Taiwan.

Key words: MDM2 protein; Hepatocellular carcinoma; Taiwan; Tumor suppressor protein p53

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a prevalent type of cancer. It represents the fifth most prevalent cancer worldwide, and accounts for the top three causes of death in the Asia-Pacific region[1,2]. The risk factors associated with HCC include age, sex, alcohol, diet, and infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV)[2]. Most newly diagnosed HCC is reported in Asia (> 70%), in which chronic HBV infection accounts for 75% of cases worldwide[3]. Significantly, 55% of HCC cases are reported from the Chinese population[4]. Although the incidence rate of HCC is plausibly linked to geographic area and geo-economic conditions, it is possible that gene polymorphism may be associated with the risk of HCC[5,6].

Single nucleotide polymorphism (SNPs) occur frequently in human genomes. SNPs can be detected once per 1000 bp in DNA sequences, on average, and may affect gene transcription and amino acid composition if they are located in gene regions. Several lines of evidence have shown that SNPs in genes such as small inducible cytokine B14 precursor (*SCYB14*), glial cell-derived neurotrophic factor family receptor α 1 (*GFRA1*), corticotropin-releasing hormone receptor 2 (*CRHR2*), glucose-regulated protein 78 (*GRP78*), heat shock protein A1B (*HSPA1B*), DNA-methyltransferase-3B (*DNMT3B*), α-fetoprotein (*AFP*) and *p53 R72P* are associated significantly with HCC[6-12]. These SNPs localize in promoter regions, coding sequences, or even introns of individual genes, which suggests that the expression level and functions of affected genes can influence the incidence rate of HCC.

MDM2 oncoprotein is a direct negative regulator for the p53 tumor suppressor protein, which accounts for 50% of human cancers if deleted or with loss-of-function[13,14]. Overexpression of MDM2 by up to fourfold in transgenic mice that harbor wild-type p53 leads to complete tumorigenesis[15]. MDM2 overexpression also is associated with poor survival and is a useful predictive factor for poor prognosis in humans with liver cancer[16,17]. A genetic polymorphism located in intron 1 of the MDM2 gene, so called MDM2-SNP309 (a change from T to G, rs2279744) can enhance the binding of Sp1 general transcription factor to this promoter region and increase *MDM2* gene transcription[18]. It has been suggested that this SNP is associated with the risk and early onset age of various human cancers[19]. Some studies have shown that MDM2-SNP309 is associated with the risk of HCC in Japanese and Moroccan patients with chronic hepatitis C, and Korean patients with chronic hepatitis B[20-22]. Although > 50% of HCC cases are reported from Asia, it remains largely unknown whether MDM2-SNP309 influences the risk and onset age of HCC in other countries in this region, except for Japan and Korea.

In this study, we initiated a hospital-based case-control study to investigate the risk association between MDM2-SNP309 and HCC in Taiwanese patients. We also examined whether HCC onset was earlier in patients with homozygous MDM2-SNP309 (G/G) compared to wild-type MDM2-SNP309 (T/T).

MATERIALS AND METHODS

Patients and cancer-free healthy controls

We studied 58 patients with HCC diagnosed by cancer specialists, and 138 cancer-free healthy adults enrolled from Taipei City Hospital Ren Ai Branch, Taiwan during 2007. All volunteers signed the consent form and filled out the structured questionnaire before providing their blood samples. All patients and 50 cancer-free healthy controls were tested for hepatitis B and C by anti-HBsAg, HBsAg, Anti-HBc IgG, and anti-HCV. Several risk factors associated with HCC were included in the questionnaire, including age, sex, alcohol intake, frequency of exercise, and cardiovascular diseases/diabetes. There was no other cancer type diagnosed in each patient. The subjects were born in the Taiwan Island except five patients and four cancer-free healthy controls who had emigrated from mainland China. This study was approved by institutional review committee board of National Yang-Ming University and Taipei City Hospital.

MDM2-SNP309 genotyping

Analysis of MDM2-SNP309 genotyping was conducted by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), as described previously[23]. Genomic DNA was extracted from 200 μL whole blood sample using the Qiagen Mini Blood DNA Extraction kit (Valencia, CA, USA). The DNA fragment that contained the *MDM2* SNP309 was amplified by PCR using the forward (5'-CGGGAGTTCAGGGTAAAGGT-3') and reverse primer (5'-AGCAAGTCGGTGCTTACCTG-3') (Protech Inc., Taipei, Taiwan). Each PCR reaction was conducted using 100 ng genomic DNA, 0.2 μmol/L primer, 200 μmol/L dNTP, 1.5 mmol/L $MgCl_2$, 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl and 1 U Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA). The thermal cycler conditions were 94℃ for 1-5 min; 40 cycles with denaturing at 94℃, annealing at 59℃, and elongation at 72℃ for 30 s each; one cycle at 72℃ for 10 min. Subsequently, 10 μL of the PCR product was digested with 1 U *Msp*A1I restriction enzyme (New England Biolabs, Ipswich, MA, USA) at 37℃ for 30-60 min. The T/T, T/G and G/G genotypes were identified as 233 bp/88 bp, 233 bp/187 bp/88 bp, and 187 bp/88 bp running on the 3% NuSieve agarose gel, respectively. The genotypes were confirmed by direct sequencing of the PCR products by the Sequencing Core Facility of Genomic Research Center in National Yang-Ming University.

Statistical analysis

Whether the frequency of MDM2-SNP309 genotype obeyed the Hardy-Weinberg equilibrium was determined by an on-line public statistical tool (http://www.genes.org.uk/software/hardy-weinberg.shtml). Two-sample *t* test was used to evaluate the difference in age and body mass index (BMI) between cases and controls. Differences in sex, hepatitis frequency, HBV and HCV infection, alcohol intake, exercise, and incidence of cardiovascular diseases and diabetes between cases and controls were determined by Fisher's exact test. Multivariate logistic regression analysis was used to calculate the OR and 95% CI to determine the association between HCC risk and MDM2-SNP309 genotypes. The Kaplan-Meier survival analysis was used to describe the onset age of HCC, and the log-rank test was used to compare the median onset age between patients with GG and those with TT genotypes in MDM2 SNP309. $P < 0.05$ was considered statistically significant in all tests. All statistical analyses were performed with Statistical Analysis System ver. 9.1 (SAS Institute, Cary, NC, USA).

RESULTS

We investigated 58 HCC patients and 138 cancer-free healthy controls to evaluate the risk association between

Table 1 The demography of HCC patients and cancer-free healthy controls from Taiwan *n* (%)

Characteristics[1]	Cases (58)	Controls (138)	*P* value
Age (yr)			
mean	65.90	40.20	< 0.0001
SD	10.14	15.24	
Gender			
Male	45 (77.6)	42 (30.2)	< 0.0001
Female	13 (22.4)	96 (69.8)	
Hepatitis			
Yes	49 (84.5)	10 (7.2)	< 0.0001
No	9 (15.5)	79 (57.25)	
Unknown	0	49 (35.5)	
HBV[2]			
+	29	10	0.0002
-	28	40	
HCV[3]			
+	20	0	< 0.0001
-	37	50	
Alcohol intake			
Yes	26 (44.8)	81 (58.7)	0.0851
No	32 (55.2)	57 (41.3)	
Cardiovascular diseases and/or disorders			
Yes	23 (39.7)	26 (18.7)	0.0035
No	35 (59.6)	116 (81.3)	
BMI	22.7 ± 3.13	22.59 ± 3.44	0.8341

[1]Age and BMI (Body Mass Index) were determined by *t* test. Other parameters were analyzed by Fisher's exact test; [2,3]Two HCC cases were infected with both HBV and HCV. HCC: Hepatocellular carcinoma.

Table 2 The risk evaluation of MDM2 SNP309 genotypic frequencies on the development of HCC *n* (%)

SNP309	Cases	Controls	OR (95% CI)[1]	*P* value
TT	11 (19)	35 (25.3)	1	
TG	37 (63.8)	80 (58)	1.016 (0.152-6.8)	0.99
GG	10 (17.2)	23 (16.7)	1.265 (0.074-21.767)	0.87
TG+GG	47 (81)	103 (57.5)	1.037 (0.757-6.862)	0.97
TT+TG	48 (82.8)	115 (83.3)	1	
GG	10 (17.2)	23 (16.7)	1.25 (0.123-12.66)	0.85

[1]The multivariate logistic regression model was used to calculate odds ratio adjusted for gender, infection of hepatitis virus (B or C type), age, cardiovascular disease and/or diabetes.

Table 3 Estimation of odds ratio for different genotypes of MDM2-SNP309 in hepatitis viral infected patients with HCC *n* (%)

Hepatitis[1]	SNP309	Cases	Controls	OR[2]	95% CI	*P* value
Positive	TT	7 (14.3)	1 (10)	1.000		
	TG	32 (65.3)	7 (70)	2.340	0.11-49.655	0.59
	GG	10 (20.4)	2 (20)	2.568	0.054-121.687	0.63
	TG+GG	42 (85.7)	9 (90)	2.376	0.115-48.896	0.57
Negative	TT	4 (44.4)	19 (24)	1.000		
	TG	5 (55.6)	45 (57)	0.512	0.046-5.712	0.59
	GG[3]	0 (0)	15 (19)	-	-	-
	TG+GG	5 (55.6)	60 (76)	0.470	0.041-5.387	0.54

[1]Include HBV or HCV infected patients with HCC; [2]OR was adjusted for age, gender and cardiovascular diseases and/or diabetes; [3]OR was not estimated because the number in case was zero.

MDM2-SNP309 and HCC. The characteristics of these blood donors are summarized in Table 1. The mean ages were significantly different between cases and controls (65.9 ± 10.14 years *vs* 40.2 ± 15.24 years) at the time that they joined this study. These participants were enrolled consecutively without pre-selection for age, therefore, this difference may confirm that HCC usually is found in elderly individuals. In HCC patients, > 80% had been infected with HBV or HCV, and two cases were infected with both HBV and HCV. In addition, the frequency of HCC patients infected with HBV was slightly higher than that of those infected with HCV. Other factors that may affect the incidence of HCC are also summarized in Table 1. Except for BMI and alcohol intake, other confounding factors exhibited a significant difference between HCC cases and cancer-free healthy controls. They were adjusted for the multivariate logistic regression model thereafter.

The frequency of MDM2-SNP309 distributed in wild-type (TT), heterozygous (TG) and homozygous (GG) genotypes are shown in Table 2. The genotype distributions in HCC cases and cancer-free healthy controls slightly departed from Hardy-Weinberg equilibrium (HCC, $\chi^2 = 4.426$, $P = 0.035$; controls, $\chi^2 = 3.907$, $P = 0.048$). It is impossible to predict the genotype of each blood donor, therefore, it is expected that this inconsistency may have been due to the small sample size. Although the frequency of wild-type MDM2-SNP309 genotype was lower in HCC cases than in cancer-free healthy controls, there was no significant difference found in the frequency of homozygosity between these two groups (Table 2). To determine the risk association between HCC and MDM2-SNP309 genotypes, multivariate logistic regression analysis showed that the OR of heterozygous (TG) and homozygous (GG) SNP309 genotypes was 1.016 (95% CI = 0.152-6.8, P = 0.987) and 1.265 (95% CI = 0.074-21.767, P = 0.87) compared to wild-type SNP309 genotype, respectively (Table 2). These OR values were adjusted for age, sex, infection with HBV or HCV, cardiovascular disease and/or diabetes. Comparison of homozygous MDM2-SNP309 (GG) genotype with common (TT) or heterozygous (TG) MDM2-SNP309 carriers showed that the adjusted OR was 1.247 (95% CI = 0.123-12.66, P = 0.852). The TG or GG genotype of MDM2-SNP309 verses TT variant form exhibited an adjusted OR of 1.037 (95% CI = 0.757-6.682, P = 0.97) (Table 2). These OR values are lower than those reported previously in Japanese, Korean and Moroccan populations[20-22]. Thus, current statistical results showed that risk association between MDM2-SNP309 genotype and HCC was not significant in the Taiwanese population.

We next investigated whether HBV or HCV infection influenced the effects of MDM2-SNP309 genotypes on patients with HCC. As shown in Table 1, 49 cases infected with HBV or HCV were analyzed by the logistic regression model. It revealed that the homozygous (GG) MDM2-SNP309 genotypes exhibited increased risk over the common (TT) genotype of MDM2-SNP309 (adjusted OR = 2.568, 95% CI = 0.054-121.687, P = 0.632) (Table 3). The adjusted OR of TG or GG of MDM2-SNP309 genotype was 2.376 compared to TT genotype (95% CI =

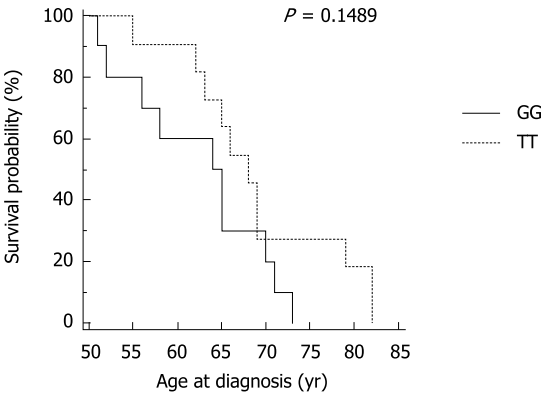

Figure 1 Comparison of age at diagnosis in HCC patients with wild-type (T/T) and homozygous (G/G) MDM2-SNP309 by the Kaplan-Meier method and the log-rank test. The survival fraction was the cumulative case-free survival rate against age at diagnosis of patients with HCC with different MDM2-SNP309 genotypes.

0.115-48.896, $P = 0.575$). The broad range of 95% CI was due to the small sample size, while it seems plausible that the risk association between MDM2-SNP309 and patients with HCC may have been enhanced by viral hepatitis.

We compared the age at diagnosis for HCC patients who had different MDM2-SNP309 genotypes, and the median ages for TT, TG and GG genotypes were 68 (range 55-82), 66 (range 38-87) and 64.5 (range 51-73) years old, respectively. The arithmetic mean ages and SD for TT, TG and GG were 69.1 ± 8.23, 64.3 ± 11.1, and 62.5 ± 7.88 years old, respectively. Although the mean age of patients with homozygous MDM2-SNP309 genotype (GG) was 6.6 years lower than that of patients with wild-type MDM2-SNP309 genotype (TT), there was no significant difference between them by *t* test ($P = 0.0844$, 95% CI = -0.9835-14.1654). Besides, the Kaplan-Meier survival analysis showed that comparison of the age at diagnosis for HCC patients with GG and TT genotypes was not significantly different by log-rank test ($P = 0.1489$, Figure 1). Therefore, the current results suggest that MDM2-SNP309 is not associated with onset age of HCC in the Taiwanese population. However, a larger sample size may be necessary to confirm this observation.

DISCUSSION

HCC commonly occurs in the Asia-Pacific region, and it also accounts for high morbidity and mortality in this area[2]. Although the incidence rate of male HCC patients is high in the Asia-Pacific region (14-36 per 100 000 men), it exhibits significant variance in different countries. The highest incidence rate of male HCC occurs in China and Taiwan, which is 58 and 53 per 100 000 men, respectively[2]. The main etiological agents that result in high incidence of HCC in the Asia-Pacific region include high infection rate with HBV and HCV, cirrhosis, family history, environmental contamination, diet, and α-fetoprotein[4,24-26]. MDM2-SNP309 is a novel predictor for Japanese and Korean patients with HCC infected by HCV and HBV, respectively[20,21]. In this study, we examined the risk association between Taiwanese HCC patients and MDM2-SNP309. Although the multivariate analysis showed that the association between MDM2-SNP309 and HCC in the Taiwanese population was not significant, there was a trend that homozygous (GG) or heterozygous (TG) MDM2-SNP309 genotype exhibited a higher risk in the subset of HCC patients infected with HBV or HCV. This is consistent with previous reports in Japanese and Korean patients with HCC, at least in part. Besides, our data failed to prove that MDM2-SNP309 could accelerate the development of HCC, although the median age at diagnosis of patients with homozygous MDM2-SNP309 genotype (GG) was 3.5 years lower than that of patients with the common genotype (TT). This result also agrees with the studies in the Japanese and Moroccan but not in the Korean population[20-22]. Therefore, the risk association and the effects of age onset between MDM2-SNP309 genotypes and HCC are likely to be dependent on the selected patient subgroups.

MDM2 negatively regulates p53 tumor suppressor protein. It is reasonable to expect that over-produced MDM2 will repress p53 function on cancer prevention. This hypothesis has been demonstrated in an MDM2 overexpression transgenic mouse model that develops systemic tumors. The putative effect of MDM2-SNP309 is to enhance the transcription of the MDM2 gene, and to affect the p53 regulatory pathway for tumor development. Several lines of evidence have demonstrated an association between MDM2-SNP309 and various sporadic or hereditary human cancers, including risk and earlier age onset. Nevertheless, non-supportive data are also reported to disagree that there is a risk association between MDM2-SNP309 and human cancers, even when the same cancer types are studied[27-36]. One of the reasons is likely to be the different subgroups of patients and races. For instance, a recent meta-analysis has demonstrated that the G allele of MDM2-SNP309 may affect breast cancer in the Chinese population rather than non-Chinese population[37]. Although HCC is a common cancer type in the Asia-Pacific region, its association with MDM2-SNP309 has only been reported in Japanese patients with chronic hepatitis C and in Korean patients chronically infected with hepatitis B virus[20,21]. HCC has the second highest cancer mortality in Taiwanese subjects who are vulnerable to be infection with HBV or HCV (http://crs.cph.ntu.edu.tw/). To the best of our knowledge, this is the first study to investigate the Taiwanese population to analyze the effects of MDM2-SNP309 genotypes on HCC development.

The sample size is the primary limitation of our study. The participants were from a single hospital, and most patients who suffered from HCC had a lack of enthusiasm to provide blood samples for analysis. Besides, the age distribution of cases and cancer-free healthy controls was significantly different. The patients were enrolled consecutively, therefore, this bias was not due to selection and was adjusted for in multivariate analysis. Another limitation is that sex distribution and incidence of hepatitis between cases and controls were not comparable. In HCC patients, the ratio of men to women was 3.46, and 84.5% of patients also had hepatitis. The mean ages at diagnosis for HBV- and HCV-infected HCC patients were 59.53 ±

10.72 years and 70.63 ± 6.39 years, respectively ($P = 0.0002$). Male HCC patients were infected mainly by HBV (64.9%), while female HCC patients were infected by HCV (66.7%). Furthermore, the male/female ratio was 6.0 for HCC patients with HBV infection, while it was 1.375 for those with HCV infection. All of these results are similar to a previous study of 18 423 Taiwanese HCC patients enrolled from 1981 to 2001[2,38]. Therefore, the characteristics of HCC patients did not exhibit a significant discrepancy compared to a large cohort study in Taiwan, even though a small sample size was used in this study.

Several potential risk factors were also considered in this study. Frequent alcohol intake is considered to be an etiological agent for the Chinese population (OR = 1.88)[4]. In our study, there was no significant difference between cases and controls regarding alcohol intake. However, identification of alcohol intake is dependent on self-interpretation by participants, and it may lead to apparent bias. It has been reported that metabolic syndrome, such as obesity and diabetes may affect the incidence of HCC[39-41]. We adjusted for these factors in the multivariate logistic regression model to evaluate the risk association between MDM2-SNP309 and HCC in the Taiwanese population. Nevertheless, it remains important to collect more samples to evaluate the potential risk factors for HCC in the presence of various MDM2-SNP309 genotypes in the future.

In summary, this hospital-based case-control study showed that there was no significant association between MDM2-SNP309 and HCC in the Taiwanese population. In the subset with hepatitis B or C, the homozygous or heterozygous MDM2-SNP309 genotype tended to influence the incidence rate of HCC. Homozygous MDM2-SNP309 genotype did not significantly accelerate the development of HCC, even though the median age at diagnosis of patients with homozygous SNP309 was 3.5 years lower than that of patients with wild-type SNP309. In the future, we expect to use a larger sample size to further confirm the effect of MDM2 SNP309 on HCC in the Taiwanese population.

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COMMENTS

Background

The incidence and mortality rate of hepatocellular carcinoma (HCC) are among the highest in Taiwanese cancer patients according to the data from the Taiwan Cancer Registry (http://crs.cph.ntu.edu.tw/main.php?Page=N1). Recently, it has been reported that a single nucleotide polymorphism (SNP) in the promoter region of MDM2 oncogene, MDM2-SNP309, is associated with the risk of HCC in Japanese and Korean patients infected with hepatitis C and hepatitis B virus, respectively. However, it remains unclear whether this observation commonly occurs in other neighboring Asian populations.

Research frontiers

To the best of our knowledge, this is the first study to investigate the association between MDM2-SNP309 and HCC in Taiwanese, a population close to the Han Chinese, which also has a high incidence and mortality of HCC. The data showed that there was no significant association between HCC risk and MDM2-SNP309 in the Taiwanese population, while the association tended to increase in patients with hepatitis B or C virus infection.

Innovations and breakthroughs

The incidence and mortality rate of HCC in Taiwan are comparable to those in Japan and Korea. Previous studies have shown that MDM2-SNP309 is associated with the risk of HCC in Japanese patients with chronic hepatitis C, and Korean patients with chronic hepatitis B. In this study, the authors demonstrated that MDM2-SNP309 genotype may not affect the risk of HCC in the Taiwanese population, except in those with a history of viral hepatitis, regardless of whether hepatitis B or C. This conclusion may be important for previous research groups to reevaluate whether hepatitis can increase the risk effect of MDM2-SNP309 on HCC. It is also an important parameter for other research groups that are dedicated to investigate the association between HCC and genetic polymorphism in the MDM2 gene and its related signaling pathways.

Applications

The results are expected to provide information about MDM2-SNP309 genotyping for routine health and newborn screening for HCC in subjects with or without viral hepatitis.

Terminology

MDM2-SNP309 is a genetic polymorphism that corresponds to nucleotide 309, starting from first nucleotide of intron 1 of the MDM2 gene.

Peer review

This was a well-designed and well-conducted study on the association between polymorphisms in MDM2 gene promoter and HCC. The results are clearly presented and discussed. These results may help dissect the molecular alterations involved in HCC development in Taiwan.

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