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Contribution of Murine Double Minute 2 Genotypes to Colorectal Cancer Risk in Taiwan

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Abstract. *Background/Aim: The genomic role of human mouse double minute 2 (MDM2) in colorectal cancer (CRC) is unclear, therefore, our study aimed to evaluate the contribution of MDM2 genotype to the risk of CRC in Taiwan. Materials and Methods: In this case–control study, MDM2 SNP309 T to G (rs2279744) genotypes were determined and their association with CRC risk were investigated among 362 patients with CRC and 362 age- and gender-matched healthy controls in central Taiwan. In addition, the interaction of MDM2 SNP309 genotypes with personal behaviors and clinicopathological features were also examined. Results: The percentage of variant GG for the MDM2 SNP309 genotype was 30.9% in the CRC group and 24.0% in the control group, respectively (odds ratio (OR)=1.78, 95% confidence interval (CI)=1.25-2.86, p=0.0057). The allelic frequency distribution analysis showed that the variant G allele of MDM2 SNP309 conferred a significantly increased susceptibility to CRC compared with the wild-type T allele (OR=1.32, 95% CI=1.14-1.69, p=0.0062). As for the gene-lifestyle interaction, there was an obvious joint effect of MDM2 SNP309 GG genotype on the risk of CRC among ever-smokers and non-alcohol drinkers, but not non-smoker or alcohol drinker subgroups. No statistically significant correlation was observed between MDM2 SNP309 genotypic distributions and age, gender, tumor size, location or metastasis status. Conclusion: The genotypes of MDM2 SNP309 may allow forr early detection of and predictor for CRC risk, especially among smokers and non-alcohol drinkers, but not for prognosis. The combined effects of MDM2 SNP309 and other genes (such as matrix metalloproteinases) on CRC susceptibility and prognosis, should also be taken into consideration in the era of precision medicine.*

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Worldwide, colorectal cancer (CRC) is the third most common cancer and the fourth most common cause of death from cancer (1), and its incidence and mortality rates vary markedly across the globe with regional differences that can sometimes be 10-fold (2, 3). Genetic epidemiology and twin studies [cited in (4)] demonstrated that upwards of 35% of CRC cases may be due to inherited factors, which indicates the importance of inherited genetic susceptibility in carcinogenesis. In addition to genetic

background, the mechanisms underlying the development of CRC are complex and lifestyle or environmental factors may also play an important role in the occurrence and progression of CRC (5). In Taiwan, the incidence and mortality of CRC has occupied the first and third places among the common types of cancer for many years and its high incidence has been proposed to be closely associated with dietary changes to Western food style and decrease in the consumption of dietary fiber or grain-derived foods. From the epidemiological viewpoint, studies have attributed more than 85% of CRC etiology to lifestyle/environmental risk factors, particularly meat consumption, cigarette smoking, and exposure to carcinogenic aromatic amines, such as arylamines and heterocyclic amines (6, 7). Statistically, 15-20% of CRC cases have strong familial cancer history that have led molecular epidemiologists to be interested in additional genomic susceptibility factors (8-10). In Taiwan, although specific biomarkers for early detection of CRC have been examined during recent years (11-16), useful genomic markers are still in urgent need and the mechanisms underlying them are largely unknown.

A well-known functional polymorphism of human mouse double minute 2 (*MDM2*) (SNP309, rs 2279744) is a novel T to G substitution located at the 309th nucleotide in the first intron, showing a genomic determiner for binding affinity of its coding protein with the transcription factor Sp1 (17). Therefore, it was rationally hypothesized that the genomic variant may have an impact on the expression level of MDM2 and affect an individual's susceptibility to carcinogenesis. Mounting studies have investigated the association of *MDM2* SNP309 genotypes with various tumor types, but their results are controversial and inconclusive (18-20). Some studies have reported a direct connection between *MDM2* SNP309 and CRC risk (21-23); however, others have shown the opposite (24, 25).

In 2012, Zhang and colleagues reported that there was no significant association between *MDM2* SNP309 genotype and CRC, instead, a combined effect of *TP53* Arg72Pro and *MDM2* SNP309 variant genotype conferred an increased CRC risk in a Chinese population (26). Considering that the meta-analysis conclusion mentioning the contribution of *MDM2* genotype to CRC risk and evaluating the differences between Asia and Western countries were only based on two investigations in China with limited sample sizes (26, 27), more studies are urgently needed to validate these findings. Therefore, in the current case–control study, we aimed to genotype *MDM2* SNP309 and evaluate its association with CRC risk in a Taiwanese population.

Materials and Methods

Investigated population. The target investigated population consisted of 724 individuals, 362 patients with CRC and 362 controls. The patients diagnosed with CRC were recruited at the outpatient clinics of general surgery during the period of 2002 and 2008 at the China Medical University Hospital in central Taiwan by surgical teams under the supervision of LB Jeng and MD Yang. The clinical characteristics of patients, including histological details, were all graded and defined by expert surgeons (12-16, 28). All 724 participants completed a self-administered questionnaire and provided a 5-ml sample of peripheral blood for genotyping examination. Matched for age, gender and some habits, 362 non-cancer healthy volunteers were selected as controls after initial random sampling from the Health Examination Cohort of the Hospital with the help of colleagues at the Department of Family Medicine. The exclusion criteria of the controls included previous malignancy, metastasized cancer from other or unknown origin and any familial or genetic diseases. This study was approved by the Institutional Review Board of the China Medical University Hospital (IRB project identification coding number: DMR99-IRB-108) and written informed consent was obtained from all the participants with the help of Tissue Bank of China Medical University Hospital. The selective demographic information for the 724 participants in this study is summarized in Table I.

Genotyping conditions. Genomic DNA was extracted from peripheral blood leukocytes with a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan, ROC), stored long-term at −80˚C, then diluted and aliquoted for genotyping as a working stock at −20˚C as per routine practice (12, 13). The *MDM2* genotyping methodology regarding the designing of the primer pairs and the selection of restriction enzyme were modified from previously published articles (24, 29). In brief, the polymerase chain reaction (PCR) cycling conditions were: one cycle at 94˚C for 5 min; 35 cycles of 94˚C for 30 s, 59˚C for 30 s and 72˚C for 30 s, and a final extension at 72˚C for 10 min. The genotyping PCR for *MDM2* SNP309 was conducted using 5'-GTTTTGTTGGACTGGGGCTA-3' and 5'-CTGCGATCATCCGGACCT-3' as the forward and reverse primer pairs, respectively. After amplification, the PCR products were subject to enzyme digestion by *Msp*A II restriction endonuclease (New England Biolabs, Beverly, MA, USA) according to the manufacturer's instructions and separation using 3% agarose gel electrophoresis. All the genotypic processing was repeated by two expert researchers independently and blindly, and their results were 100% concordant. In addition, the success rate of PCR-restrictive fragment length polymorphism (RFLP) was 100%, and the genotypes of 5% of the participants in both the control and patient groups were analyzed by PCR direct sequencing (Genomics BioSci & Tech Co. The concordance between direct sequencing and PCR-RFLP methods was 100%.

Statistical analyses. Student's *t*-test was applied for the comparison of continuous variables such as age between the CRC case and control groups. Pearson's Chi-square was applied to compare the distribution of the *MDM2* genotypes among the subgroups. The associations between *MDM2* genotypes and CRC risk were estimated by computing odds ratios (ORs) and their 95% confidence intervals (CIs) from logistic regression analysis. Statistically, any difference between the two groups at $p<0.05$ was taken as significant.

Results

The frequency distributions of selected demographic characters, including age and gender for the 362 patients with CRC and 362 non-cancer healthy controls, are summarized and compared in Table I. In addition, tumor size, location, and lymph node metastasis status are also

Table I. *Summary of selected data of the 362 patients with colorectal cancer and 362 matched non-cancer healthy controls.*

Characteristic	Controls (n=362) n (%)	Cases (n=362) n (%)	*p*-Value[a]
Age (years)			
≤60	93 (25.7%)	95 (26.2%)	0.8654
>60	269 (74.3%)	267 (73.8%)	
Gender			
Male	209 (57.7%)	203 (56.1%)	0.6525
Female	153 (42.3%)	159 (43.9%)	
Tumor size (cm)			
<5		195 (53.9%)	
≥5		167 (46.1%)	
Location			
Colon		257 (71.0%)	
Rectum		105 (29.0%)	
Lymph node metastasis			
Negative		210 (58.0%)	
Positive		152 (42.0%)	

SD, Standard deviation; [a]based on Chi-square test without Yates' correction.

reported in Table I. Since we applied frequency matching strategies focusing on age and gender to recruit the 362 non-cancer healthy individuals as controls, the data showed that there was no difference in respect of the distributions of age and gender between the two groups (p=0.8654 and 0.6525, respectively) (Table I).

The distributions of the *MDM2* SNP309 genotypes among the 362 non-cancer healthy controls and the 362 patients with CRC are presented and analyzed in Table II. The genotypes of *MDM2* SNP309 were differently distributed between the case and control groups (p for trend=0.0215) (Table II). In detail, the *MDM2* SNP309 homozygous GG genotype was associated with increased CRC risk compared with the wild-type TT genotype (adjusted OR=1.78, 95% CI=1.25-2.86, p=0.0057). However, the heterozygous TG genotype was not associated with altered CRC risk, compared to the wild-type TT genotype (adjusted OR=1.34, 95%CI=0.96-2.03, p=0.0670). In the dominant model, there was a significant association between G allele carriers of *MDM2* SNP309 and CRC risk compared with TT wild-type genotype (adjusted OR=1.53, 95%CI=1.12-2.28, p=0.0158).

In order to confirm the results in Table II, analysis of allelic frequency distributions for MDM2 SNP309 was also conducted and the results are presented in Table III. Supporting the findings that genotype of MDM2 SNP309 was associated with CRC risk, the frequency of variant allele G was 56.2% in the case group, significantly higher than that of 49.0% in the control group (adjusted OR=1.32, 95%CI=1.14-1.69, p=0.0062) (Table III).

Table II. *Distributions of human mouse double minute 2 (MDM2) SNP309 genotypic frequencies among the patients with colorectal cancer and healthy controls.*

Genotype	Cases, n (%)	Controls, n (%)	Adjusted OR (95% CI)[a]	*p*-Value[b]
TT	67 (18.5)	94 (26.0)	1.00 (Reference)	
TG	183 (50.6)	181 (50.0)	1.34 (0.96-2.03)	0.0670
GG	112 (30.9)	87 (24.0)	**1.78 (1.25-2.86)**	**0.0057**
TG+GG	295 (81.5)	268 (74.0)	**1.53 (1.12-2.28)**	**0.0158**
p_{trend}				**0.0215**

OR, Odds ratio; CI, confidence interval; [a]Data adjusted for confounding factors: age, gender, smoking, alcohol and betel quid consumption; [b]Based on Chi-square test without Yates' correction. Significant p-values (p<0.05) are shown in bold.

Table III. *Allelic frequencies for human mouse double minute 2 (MDM2) SNP309 polymorphisms among the patients with colorectal cancer and healthy controls.*

Allele	Cases, n (%) (n=724)	Controls, n (%) (n=724)	Adjusted OR (95% CI)[a]	*p*-Value[b]
T	317 (43.8)	369 (51.0)	1.00 (Reference)	
G	407 (56.2)	355 (49.0)	**1.32 (1.14-1.69)**	**0.0062**

OR, Odds ratio; CI, confidence interval. [a]Data adjusted for confounding factors: age, gender, smoking, alcohol and betel quid consumption. [b]Based on Chi-square test without Yates' correction. Significant p-values (p<0.05) are shown in bold.

Since smoking and alcohol drinking habits are well-known risk factors for CRC in Taiwan, we further investigated the interactions between the genotype of *MDM2* SNP309 and personal cigarette smoking and alcohol drinking behaviors, and the results presented in Tables IV and V. Firstly, among smokers, those with homozygous GG genotype at *MDM2* SNP309 were at 3.20-fold odds of having CRC (95% CI=1.35-7.58, p=0.0071) conferring a risky effect, while this was not the case for the heterozygous TG genotype (95% CI=0.98-4.84, p=0.0544). After adjusting for age, gender and alcohol drinking status, statistical significance still existed at a similar level for GG but not TG genotype (Table IV, right panel). On the other hand, a non-significant effect was found among the non-smokers (Table IV, left panel). Secondly, among alcohol drinkers, those with AG and GG genotypes at *MDM2* SNP309 were at non-significantly increased risk of having CRC (95%CI=0.53-5.13 and 0.96-10.49, p=0.3876 and 0.0549, respectively), while there was a significantly increased risk for those with GG genotype among non-

Table IV. *Odds ratios for association of human mouse double minute 2 (MDM2) SNP309 genotype with colorectal cancer after stratification by smoking status.*

Genotype	Non-smokers, n		OR (95% CI)[a]	aOR (95% CI)[b]	*p*-Value	Smokers, n		OR (95% CI)[a]	aOR (95% CI)[b]	*p*-Value
	Controls	Cases				Controls	Cases			
TT	69	54	1.00 (ref)	1.00 (ref)		25	13	1.00 (ref)	1.00 (ref)	
TG	143	140	1.25 (0.81-1.91)	1.19 (0.84-1.78)	0.3020	38	43	2.18 (0.98-4.84)	2.21 (0.97-4.68)	0.0544
GG	66	77	1.49 (0.92-2.42)	1.34 (0.95-2.36)	0.1058	21	35	3.20 (1.35-7.58)	3.48 (1.53-6.89)	**0.0071**
Total	278	271				84	91			
p_{trend}					0.2700					**0.0256**

[a]Multivariate logistic regression analysis; [b]multivariate logistic regression analysis after adjusting for age, gender and alcohol drinking status; CI, confidence interval; aOR, adjusted odds ratio. Significant *p*-values ($p<0.05$) are shown in bold.

Table V. *Odds ratios for human mouse double minute 2 (MDM2) SNP309 genotype and colorectal cancer after stratification by alcohol drinking status.*

Genotype	Non-drinkers, n		OR (95% CI)[a]	aOR (95% CI)[b]	*p*-Value	Drinkers, n		OR (95% CI)[a]	aOR (95% CI)[b]	*p*-Value
	Controls	Cases				Controls	Cases			
TT	81	61	1.00 (ref)	1.00 (ref)		13	6	1.00 (ref)	1.00 (ref)	
TG	156	164	1.40 (0.94-2.08)	1.35 (0.95-1.89)	0.0999	25	19	1.65 (0.53-5.13)	1.24 (0.47-3.21)	0.3876
GG	74	93	1.67 (1.06-2.62)	1.79 (1.24-2.53)	**0.0257**	13	19	3.17 (0.96-10.49)	2.86 (0.88-7.45)	0.0549
Total	311	318				51	44			
p_{trend}					0.0780					0.1334

[a]Multivariate logistic regression analysis; [b]multivariate logistic regression analysis after adjusting for age, gender and smoking status; CI, confidence interval; aOR, adjusted odds ratio. Significant *p*-values ($p<0.05$) are shown in bold.

drinkers (95% CI=1.06-2.62, $p=0.0257$) (Table V). After adjusting for age, gender and smoking status, the trends were still the same for each sub-group (Table V).

The correlations between genotypes of *MDM2* SNP309 and clinicopathological features of 362 patients with CRC were further stratified and analyzed in Table VI. No statistically significant correlation was observed between *MDM2* SNP309 genotypic distributions and age, gender, tumor size, location or metastasis status (all $p>0.05$) (Table VI).

Discussion

MDM2 protein was originally identified as an oncoprotein which binds to p53 and inhibits p53-mediated transactivation and MDM2 is reported to be overexpressed in many tumor sites such as sarcomas, osteosarcomas and esophageal sarcomas (30, 31). As originally identified, MDM2 can directly bind to p53 and down-regulate its function as a tumor suppressor. Therefore, the oncogenic properties of MDM2 are thought to be p53-dependent. However, some scientists have reported that MDM2 may form complexes with other tumor-suppressor proteins independently of p53 in p53-deficient cells, which indicated that the oncogenic function of MDM2 was p53-independent (32, 33). Most importantly, although *MDM2* SNP309 is located at the p53-response intronic promoter region, the p53-independent overexpression of MDM2 was still observed (34). In a few reports, it was found that MDM2 amplification might be regulated at the post-transcriptional level (35, 36). All the aforementioned findings indicate that complex mechanisms underlie the regulation of *MDM2* gene during tumorigenesis and which are largely unknown. Considering that *MDM2* SNP309 may closely regulate the expression level of MDM2 protein, it is of great value to evaluate its association with cancer risk, such as CRC risk in Taiwan.

In the present study, we firstly found that GG genotype at *MDM2* SNP309 was associated with increased risk of CRC in this Taiwanese population (Table II). In the literature on genomic studies of CRC, whether *MDM2* SNP309 has a direct effect on carcinogenesis is still controversial. Similarly, some reported findings that the TG genotype is associated with increased CRC risk compared with the TT genotype (21-23), whereas other reports have provided negative results with no significant association (24, 25).

Table VI. *Correlation between human mouse double minute 2 (MDM2) SNP309 genotype and clinicopathological features of 362 patients with colorectal cancer.*

Characteristic	Cases, n	Genotype, n (%)			*p*-Value[a]
		TT	TG	GG	
Age					
≤60 Years	95	16 (16.8)	49 (51.6)	30 (31.6)	0.8882
>60 Years	267	51 (19.1)	134 (50.2)	82 (30.7)	
Gender					
Male	203	35 (17.2)	106 (52.2)	62 (30.6)	0.7126
Female	159	32 (20.1)	77 (48.4)	50 (31.5)	
Tumor size					
<5 cm	195	33 (16.9)	95 (48.7)	67 (34.4)	0.2933
≥5 cm	167	34 (20.4)	88 (52.7)	45 (26.9)	
Location					
Colon	257	46 (17.9)	128 (49.8)	83 (32.3)	0.6695
Rectum	105	21 (20.0)	55 (52.4)	29 (27.6)	
Lymph node metastasis					
Negative	210	42 (20.0)	105 (50.0)	63 (30.0)	0.6791
Positive	152	25 (16.5)	78 (51.3)	49 (32.2)	

[a]Based on Chi-square test without Yates's correction.

Recently, Zhang and colleagues reported a combined effect of *TP53* Arg72Pro and *MDM2* SNP309 in a dose–response manner, increasing CRC risk in a population from central China, but still no association between *MDM2* SNP309 alone and CRC risk was found (26). In our study population, the *MDM2* SNP309 GG carriers, but not those with TG, were found to have an increased CRC risk compared to those carrying TT genotype (Table II). Interestingly, a meta-analysis published in 2012 also found an increased CRC risk among individuals with TG genotype, especially among Asians, when compared to the TT genotype (37). However, no association was found between *MDM2* SNP309 and CRC risk among Europeans. Considering the frequencies of the *MDM2* SNP309 G allele among the cases and controls were different by ethnicity (minor allelic frequency: 0.47 in Asians and 0.39 in Europeans), this indicates a possible ethnic difference in genetic background.

Long-term smoking has been reported as a risk factor for CRC (38), thus we are interested in the interaction of *MDM2* genotype and smoking on CRC risk. Not among the non-smokers, but the smokers, we found that GG genotype was associated with an increased risk of CRC in Taiwanese (Table IV). Similarly, Zhang and colleagues also reported that the interaction between *TP53* Arg72Pro and *MDM2* SNP309 was associated with elevated CRC risk among smokers but not among non-smokers (26). In addition to smoking, alcohol consumption is also associated with CRC risk (39), and thus we were interested in the interaction of *MDM2* genotype and alcohol drinking on CRC risk. We found that t GG genotype was associated with an increased risk of CRC among non-alcohol drinkers but not drinkers in Taiwanese (Table V).

We also examined the correlations between genotypes of *MDM2* SNP309 and clinicopathological features of investigated patients with CRC in Taiwan. The influence of *MDM2* SNP309 on CRC risk was previously found to be more pronounced among older people, which may reflect the cumulative effects of risk factors, such as prolonged red meat consumption (40); we found no significant association with age. The second factor analyzed was gender, but no significant difference was found (Table VI). In a study of a Japanese population, increased CRC risk found to be associated with *MDM2* SNP309 only in men but not in women (22). A significant earlier age of onset was observed to be associated with *MDM2* SNP309 for several tumor types (17). In addition, several studies showed the association of MDM2 SNP309 with CRC risk especially in women, but not in men (24, 41). The biological explanation for the inconsistence of our finding with others may be that CRC among Taiwanese is very prevalent, no matter in men or women, and has a trend for early onset. Regarding tumor size, location and lymph node metastasis, we did not find any determinant effect of *MDM2* SNP309 on CRC in the patients we investigated (Table VI).

From the viewpoint of molecular interaction, *MDM2* SNP309 variants might lead to a relatively lower activity of p53, and thereby increase the possibility that some colon cells may be more vulnerable to damage by tobacco carcinogens or alcohol and might escape apoptosis triggered

by p53. Therefore, smokers carrying *MDM2* SNP309 are expected to have a higher risk of CRC, but further validation is still needed.

In conclusion, we provide evidence showing that *MDM2* SNP309 was associated with increased CRC risk in a Taiwanese population. Additionally, in the stratified analyses, we found that increased risk was more pronounced among smokers and non-alcohol drinkers. Moreover, earlier age of cancer onset or gender difference among patients carrying *MDM2* SNP309 variant genotypes was not found in our study unlike others. We hope to validate the combined effects of *p53* and *MDM2* on CRC risk in Taiwanese, and further validation of large population-based studies in different ethnicities are urgently encouraged and needed.

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