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Association of Matrix Metalloproteinase-9 rs3918242 Promoter Genotypes With Colorectal Cancer Risk

MING-HSIEN WU[1,2,3*], HUEY-EN TZENG[4,5,6*], CHENG-NAN WU[7*], TE-CHENG YUEH[1,2,3], YEN-CHUN PENG[8], CHUN-HAO TSAI[1,2], YUN-CHI WANG[2], TAO-WEI KE[2], JEN-SHENG PEI[9], WEN-SHIN CHANG[1,2,7], CHIA-WEN TSAI[1,2,7] and DA-TIAN BAU[1,2,10]

[1]*Graduate Institute of Biomedical Sciences, China Medical University, Taichung, Taiwan, R.O.C.;*
[2]*Terry Fox Cancer Research Laboratory, Translational Medicine Research Center, China Medical University Hospital, Taichung, Taiwan, R.O.C.;*
[3]*Taichung Armed Forces General Hospital, Taichung, Taiwan, R.O.C.;*
[4]*Ph.D. Program for Cancer Molecular Biology and Drug Discovery, College of Medical Science and Technology, Taipei Medical University, Taipei, Taiwan, R.O.C.;*
[5]*Division of Hematology and Oncology, Department of Medicine, Taipei Medical University Hospital, Taipei, Taiwan, R.O.C.;*
[6]*Graduate Institute of Cancer Biology and Drug Discovery, College of Medical Science and Technology, Taipei Medical University, Taipei, Taiwan, R.O.C.;*
[7]*Department of Medical Laboratory Science and Biotechnology, Central Taiwan University of Science and Technology, Taichung, Taiwan, R.O.C.;*
[8]*Division of Gastroenterology and Hepatology, Taichung Veterans General Hospital, Taichung, Taiwan, R.O.C.;*
[9]*Department of Pediatrics, Taoyuan General Hospital, Ministry of Health and Welfare, Taoyuan, Taiwan, R.O.C.;*
[10]*Department of Bioinformatics and Medical Engineering, Asia University, Taichung, Taiwan, R.O.C.*

Abstract. *Background/Aim: Matrix metalloproteinase-9 (MMP-9) is responsible for modifying extracellular components and plays a crucial role in the metastatic behavior of cancer. This study aimed at examining the role of MMP-9 rs3918242 genotypes on colorectal cancer (CRC) risk. Materials and Methods: A total of 362 CRC patients and 362 healthy subjects in Taiwan, were examined by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methodology. Results: The MMP-9 rs3918242 TT genotype carriers had a slightly increased risk of CRC compared to CC carriers (p=0.1642, OR=1.88, 95% CI=0.84-4.16). Patients of CT/TT genotypes were on significantly higher risk of metastasis (p=0.0027) than those of CC genotype. No obvious association was found between MMP-9 genotype and CRC risk among ever-smokers, non-smokers, non-alcohol drinkers or alcohol drinkers. No significant correlation was observed between MMP-9 genotypic distributions with age, gender, tumor size or location. Conclusion: MMP-9 rs3918242 genotypes may interact with BMI to serve as a predictor for higher CRC risk, and independently as a predictor for metastasis.*

*These Authors contributed equally to this study.

Correspondence to: Da-Tian Bau, Chia-Wen Tsai and Wen-Shin Chang, Terry Fox Cancer Research Laboratory, Translational Medicine Research Center, China Medical University Hospital, 2 Yuh-Der Road, Taichung, 404 Taiwan, R.O.C. Tel: +886 422053366 (Ext. 5805), e-mail: artbau2@gmail.com (Bau DT); wenwen816@gmail.com (Tsai CW); halittlemelon@hotmail.com (Chang WS)

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Colorectal cancer (CRC) is the third most prevalent and fourth most death-causing cancer worldwide (1). Based on the annual data of Taiwan, the incidence and mortality of CRC have been ranked as the first and third among the common types of cancer for many years (2). From the viewpoint of epidemiology, the extremely high incidence of CRC has been closely associated with modern dietary habits of the Western world and the rapid decrease in the consumption of dietary fiber or grain-derived foods. However, the definitive mechanisms of CRC carcinogenesis remain largely unknown. Mounting evidence has suggested that CRC is the result of the interaction among individual genomic and environmental factors, which are waiting to be revealed in the era of precise medicine (3-5). This hypothesis is supported by epidemiological studies, that have attributed indirect evidence for the involvement of specific lifestyle and environmental

factors in the etiology of more than 85% of CRC cases, particularly meat consumption, cigarette smoking, and exposure to carcinogenic aromatic amines, such as arylamines and heterocyclic amines (6, 7). In addition, 15-20% of CRC cases have a history of familial cancer that have led molecular epidemiologists to search for genomic susceptibility factors that can serve as cancer predictors (8-10). In Taiwan, although several biomarkers for early detection of CRC have been revealed and published during the past decade (11-16), their phenotype and value in clinical practice is yet unknown.

In humans, 28 subtypes of matrix metalloproteinases (MMPs), that are in charge of maintaining the components of the extracellular matrix (ECM) and controlling the metastatic behavior of cancer cells, have been detected and identified (17-21). Among the MMPs, matrix metalloproteinases-9 (MMP-9), located at the human chromosome 20q12-13, is one of the most important enzymes to breakdown extracellular matrix, which plays a crucial role in various types of cancer as a member of MMPs family (21). Among the single-nucleotide polymorphisms (SNPs) constituting substitutions of single bases being found in the promoter regions of *MMP-9* gene, *MMP-9* C-1562T (rs3918242) is the one most frequently studied. *MMP-9* rs3918242 genotypes have been reported to serve as genomic marker for prediction of the personal risk for several types of cancers, including gastric (22, 23), lung cancer (24), prostate cancer (25), and breast cancer (26). As for CRC, there are only few reports investigating the contribution of *MMP-9* rs3918242 genotypes to CRC risk in China (27) and Brazil (28), but never in Taiwan. Therefore, the current case–control study, aimed to investigate the distribution of genotypes of *MMP-9* rs3918242 polymorphism, evaluate their association with CRC risk in a Taiwanese population, and concisely summarize their significance among the current and other studies.

Materials and Methods

Studied population. The investigated population was composed of 724 individuals, including 362 patients identified with CRC and the same number of control subjects. The 362 patients with CRC were invited at the general surgery outpatient clinics at the China Medical University Hospital (CMUH) by the surgical teams under the supervision of LB Jeng, MD Yang and TW Ke. The clinical characteristics for the investigated CRC patients, including the histological profiles, were all identified and recorded by the well-trained surgeons (12, 16, 29). Well-matched for age (no different than 5 years), gender (most of them were the same) and some behavioral habits (such as cigarette smoking and alcohol consumption), the same amount of cancer-free healthy subjects were selected as control group from the Health Examination Cohort of the Hospital with the help of colleagues at the Department of Family Medicine. At the same time, the exclusion criteria for recruiting the control subjects included any previous malignancy or metastasized cancer from other or unknown origin in addition to any familial or genetic disease. All the 724 recruited participants completed a self-administered questionnaire and provided a 5-ml sample of peripheral blood for genotyping. This study has been approved by the Institutional Review Board of CMUH (DMR99-IRB-108) and written informed consent was obtained from each individual with the expert help of Tissue-Bank of CMUH. Several important demographic information for 362 CRC patients and 362 controls is presented in Table I.

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

Character	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)	
Smoking status			
Non-smokers	278 (76.8)	271 (74.9)	
Smokers	84 (23.2)	91 (25.1)	0.5434
Drinking Status			
Non-drinkers	311 (85.9)	318 (87.8)	
Drinkers	51 (14.1)	44 (12.2)	0.4410
BMI (kg/m^2)			
<23	135 (37.3)	104 (28.7)	
23~25	121 (33.4)	107 (29.6)	0.4585
>25	106 (29.3)	151 (41.7)	**0.0007***
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. Significant values (p<0.05) are shown with a star and in bold.

Genotyping methods for MMP-9 rs3918242. Genomic DNA was extracted within 12 h after getting the blood from the peripheral blood leukocytes using QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan, ROC), long-term stored at −80˚C, diluted and aliquoted for *MMP-9* rs3918242 genotyping as a working stock at −20˚C as per our routine practice (30-32). The primer pairs for *MMP-9* rs3918242 PCR-RFLP genotyping methodology are forward: 5'-TGGTCAACGTAGTGAAA CCCCATCT-3'; reverse: 5'-TCCAGCCCCAATTATCACACTTAT-3', respectively. The PCR-based genotyping conditions for *MMP-9* rs3918242 were: originally one cycle at 94˚C for 5 min; then 35 cycles at 94˚C for 30 sec, then 59˚C for 30 sec and 72˚C for 30 sec, and a terminal extension at 72˚C for 10 min. After the PCR amplification, those SNP-containing DNA amplicons for each subject were ready for overnight digestion by the restriction endonuclease, *Sph*I (New England Biolabs, Taipei, Taiwan, ROC) in recognition the SNP site of *MMP-9* rs3918242 at 37˚C for 2 h. Since the C-allele and T-allele DNA adducts were non-digestible and digestible to *Sph*I, respectively, the DNA adducts of CC, CT and TT genotypes were of 386 bp only, 386+320+66 multiple bps, and 320+66 bps, respectively. Following

Table II. *Distribution of matrix metalloproteinase-9 rs3918242 genotypic frequencies among the patients with colorectal cancer and healthy controls.*

Genotype	Cases, n (%)	Controls, n (%)	Adjusted OR (95%CI)[a]	*p*-Value[b]
CC	263 (72.7)	272 (75.1)	1.00 (Reference)	
CT	86 (23.7)	83 (22.9)	1.14 (0.78-1.35)	0.6952
TT	13 (3.6)	7 (2.0)	1.88 (0.84-4.16)	0.1642
CT+TT	99 (27.3)	90 (24.9)	1.23 (0.81-1.59)	0.4463
p_{trend}				0.3670

OR: Odds ratio; CI: confidence interval; [a]Data adjusted for confounding factors: age, gender, smoking and alcohol consumption; [b]Based on Chi-square test without Yates' correction.

Table III. *Allelic frequencies for matrix metalloproteinase-9 rs3918242 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]
C	612 (84.5)	627 (86.6)	1.00 (Reference)	
T	112 (15.5)	97 (13.4)	1.25 (0.89-1.53)	0.2620

OR: Odds ratio; CI: confidence interval. [a]Data adjusted for confounding factors: age, gender, smoking and alcohol consumption. [b]Based on Chi-square test without Yates' correction.

enzyme digestion, each sample was immediately subject to 2.8% agarose gel electrophoresis. All the *MMP-9* rs3918242 genotypic processing was repeated independently and blindly by two well-trained researchers, and the results were 100% concordant to each other. Additionally, the overall success rate of PCR-RFLP was 100%. Furthermore, 5% of the participants in both the control and patient groups were selected according to the *MMP-9* rs3918242 genotypes and sent for direct sequencing (Genomics BioSci & Tech Co, Taipei, Taiwan, ROC). The results showed 100% concordance between the direct sequencing and PCR-RFLP identification.

Statistical analysis. The *Student's t*-test was used for comparison of the continuous variables such as age among the CRC cases and controls. The Pearson's Chi-square was used to compare the distribution of the *MMP-9* rs3918242 genotypes among the investigated subjects. The associations between *MMP-9* rs3918242 genotypes and CRC risk were estimated with the indexes of computing odds ratios (ORs) and their 95% confidence intervals (CIs) using logistic regression analysis. Similar to typical cancer genomic studies, any difference between the two groups at $p<0.05$ was regarded as statistically significant.

Results

The records of several demographic characteristics, including age, gender, smoking status, alcohol drinking status and BMI for the 724 subjects, including 362 patients with CRC and 362 non-cancer healthy controls, are presented and compared between the two groups in Table I. Additionally, several tumor indexes of each patient such as the size, location, and lymph node metastasis status are also presented in Table I. Since we have strategies in matching the case and control groups with similar age, gender, smoking and alcohol drinking habits in recruiting the same number of non-cancer healthy individuals as controls, there was no difference in respect to these distributions of age, gender, smoking and alcohol drinking habits between the two groups (p=0.8654, 0.6525, 0.5434 and 0.4410 respectively) (Table I). Also, there was no difference in respect to the distributions of BMI between slim people (BMI<23) and normal BMI people (BMI=23~25), while those with BMI>25 may have a higher risk of CRC than those with BMI<23 in Taiwan (Table I).

The frequency distributions of the *MMP-9* rs3918242 genotypes among the 362 non-cancer healthy controls and the 362 patients with CRC are collected and analyzed in Table II. First, the distribution of *MMP-9* rs3918242 genotypes among the controls fits well with the Hardy-Weinberg Equilibrium (p for HWE=0.2507). Secondly, the genotypes of *MMP-9* rs3918242 were not differently distributed between the case and control groups (p for trend=0.3670) (Table II). In detail, the *MMP-9* rs3918242 heterozygous and homozygous variant CT and TT genotypes were not associated with altered CRC risk compared with the wild-type CC genotype (adjusted OR=1.14 and 1.88, 95%CI=0.78-1.35 and 0.84-4.16, p=0.6952 and 0.1642, respectively). In addition, in the dominant model, there was no significant association between T-allele carriers (CT+TT) of *MMP-9* rs3918242 and CRC risk compared with the CC genotype (adjusted OR=1.23, 95%CI=0.81-1.59, p=0.4463).

So as to confirm the highlight findings in Table II, the allelic frequency distribution for *MMP-9* rs3918242 was also analyzed, and the results are presented in Table III. Consistent with the finding that the variant genotypes of *MMP-9* rs3918242 are not associated with CRC risk, the frequency of variant allele T was 15.5% in the case group, slightly but non-

Table IV. *Odds ratios for association of matrix metalloproteinase-9 rs3918242 genotype with colorectal cancer after stratification by smoking status.*

Genotype	Non-smokers, n		OR (95%CI)[a]	aOR (95%CI)[b]	*p*-Value[c]	Smokers, n		OR (95%CI)[a]	aOR (95%CI)[b]	*p*-Value[c]
	Controls	Cases				Controls	Cases			
CC	207	197	1.00 (ref)	1.00 (ref)		65	66	1.00 (ref)	1.00 (ref)	
CT	66	64	1.02 (0.69-1.51)	1.13 (0.73-1.46)	0.9260	17	22	1.27 (0.62-2.62)	1.24 (0.67-2.14)	0.5084
TT	5	10	2.10 (0.71-6.26)	2.28 (0.79-5.38)	0.1732	2	3	1.48 (0.24-9.13)	1.35 (0.38-4.26)	0.6729
Total	278	271				84	91			
p_{trend}					0.3953					0.7522

[a]Multivariate logistic regression analysis; [b]multivariate logistic regression analysis after adjusting for age, gender and alcohol drinking status; [c]Chi-square without Yates' correction or Fisher's exact test (when n<5); CI: confidence interval; aOR: adjusted odds ratio.

Table V. *Odds ratios for matrix metalloproteinase-9 rs3918242 genotype and colorectal cancer after stratification by alcohol drinking status.*

Genotype	Non-drinkers, n		OR (95%CI)[a]	aOR (95%CI)[b]	*p*-Value[c]	Drinkers, n		OR (95%CI)[a]	aOR (95%CI)[b]	*p*-Value[c]
	Controls	Cases				Controls	Cases			
CC	237	235	1.00 (ref)	1.00 (ref)		35	28	1.00 (ref)	1.00 (ref)	
CT	69	72	1.05 (0.72-1.53)	1.18 (0.75-1.46)	0.7904	14	14	1.25 (0.51-3.05)	1.31 (0.53-2.87)	0.6237
TT	5	11	2.22 (0.76-6.48)	2.39 (0.89-5.43)	0.1357	2	2	1.25 (0.17-9.44)	1.33 (0.38-8.21)	0.8285
Total	311	318				51	44			
p_{trend}					0.3255					0.8766

[a]Multivariate logistic regression analysis; [b]multivariate logistic regression analysis after adjusting for age, gender and smoking status; [c]Chi-square without Yates' correction or Fisher's exact test (when n<5); CI: confidence interval; aOR: adjusted odds ratio.

significantly higher than that of 13.4% in the control group (adjusted OR=1.25, 95%CI=0.89-1.53, p=0.2620) (Table III).

From the epidemiological viewpoint, personal smoking and alcohol drinking habits are well-known risk factors for CRC in Taiwan. Thus, the interactions between the genotypes of *MMP-9* rs3918242 and personal cigarette smoking and alcohol drinking behaviors of the investigated Taiwanese population were examined, and the results are presented in Tables IV and V. Firstly, among non-smokers, people with CT and TT genotypes at *MMP-9* rs3918242 were at 1.02- and 2.10-fold odds of having CRC (95%CI=0.69-1.51 and 0.71-6.26, p=0.9260 and 0.1732) (Table IV, left panel). After adjusting for confounding factors including age, gender and alcohol drinking status, the statistical results still were maintained at a non-significant level for CT or TT genotypes (Table IV, left panel). Similarly, a non-significant effect was found among the non-smokers (Table IV, right panel). Secondly, among non-alcohol drinkers and alcohol drinkers, those with CT and TT genotypes at *MMP-9* rs3918242 did not have a significantly increased risk of having CRC compared to those with wild-type CC genotype (Table V).

The correlations between *MMP-9* rs3918242 genotypes and clinicopathological features for the 362 CRC patients were stratified and presented in Table VI. Firstly, the results showed that no statistically significant correlation was observed between *MMP-9* rs3918242 genotypic distributions and age, gender, tumor size or location (all four p>0.05) (Table VI).

Table VI. *Correlation between matrix metalloproteinase-9 rs3918242 genotype and clinicopathological features of 362 patients with colorectal cancer.*

Characteristics	Cases, n	Genotype, n (%)			*p*-Value[a]
		CC	CT	TT	
Age (years)					
≤60	95	71 (74.7)	20 (21.1)	4 (4.2)	
>60	267	192 (71.9)	66 (24.7)	9 (3.4)	0.7367
Gender					
Male	203	149 (73.4)	46 (22.7)	8 (3.9)	
Female	159	114 (71.7)	40 (25.2)	5 (3.1)	0.8078
BMI (kg/m^2)					
<25	211	165 (78.2)	43 (20.4)	3 (1.4)	
>25	151	98 (64.9)	43 (28.5)	10 (6.6)	**0.0037***
Tumor size					
<5 cm	195	146 (74.9)	42 (21.5)	7 (3.6)	
≥5 cm	167	117 (70.1)	44 (26.3)	6 (3.6)	0.5592
Location					
Colon	257	186 (72.4)	61 (23.7)	10 (3.9)	
Rectum	105	77 (73.3)	25 (23.8)	3 (2.9)	0.8907
Lymph node metastasis					
Negative	210	162 (77.1)	46 (21.9)	2 (1.0)	
Positive	152	101 (66.4)	40 (26.3)	11 (7.3)	**0.0027***

[a]Based on Chi-square test without Yates's correction; Significant p-values (p<0.05) are shown in bold.

Interestingly, the *MMP-9* rs3918242 was associated with BMI and metastasis status (p=0.0037 and 0.0027, respectively). As for the BMI, those with CT and CC genotypes were of higher percentages in the large BMI (>25) group than those in the small BMI (<25) group (28.5% *vs*. 20.4%, and 6.6% *vs*. 1.4%, respectively). As for the lymph node metastasis, those with CT and CC genotypes were also of higher percentages in the CRC patients with lymph node metastasis than those in the CRC patients without lymph node metastasis (26.3% *vs*. 21.9%, and 7.3% *vs*. 1.0%, respectively) (Table VI). It seems that the CT and CC genotypes at *MMP-9* rs3918242 are novel markers for CRC patients with larger BMI and metastasis.

Discussion

MMP-9, also known as gelatinase B or type IV collagenase, belongs to the MMP family which teams up in the regulation of ECM components and their metabolism (33). In cancer, MMP-9 plays a central role in carcinogenesis, from angiogenesis, to stromal remodeling, and ultimately in metastasis behaviors of cancer cells (34, 35). The significantly increased expression of MMP-9 has been reported in many types of cancer, such as esophageal (36), gastric (37), breast (38) cancer and in metastatic cancer cells (39). Most important of all, the over-expression of MMP-9 is also found in the tissues of CRC patients, especially those at early stage (40, 41). The concept is supported on the fact that the SNPs on *MMP-9* promoter regions may determine the various expression levels of MMP-9 among investigated citizens (42). However, the contribution of *MMP-9* genotypes to CRC cancer etiology and prediction are seldom examined. Among *MMP-9* SNPs, rs3918242 is the most investigated. The case-control study conducted by Xing and his colleagues was a milestone in colorectal cancer genomics (27). They investigated the contribution of the critical SNP, *MMP-9* rs3918242, located at the promoter region of *MMP-9,* and the alterative substitution from C to T which may cause a dramatic change in the binding capacity between a nuclear protein and the responsive binding sequences (43). They found that the T allele at *MMP-9* rs3918242 was associated with an elevated CRC risk among the elder people (>60 years old), and also the increased risk of lymph node metastasis (27). In agreement, the present study, indicated that CT or TT genotypes at *MMP-9* rs3918242 are not significantly associated with increased risk of CRC in the overall Taiwanese population (Tables II and III). Differently from their study, in age stratified analysis, we did not find that *MMP-9* rs3918242 was associated with an elevated CRC risk among the elder people (>60 years old) (Table VI). But the highlight of both studies is that the variant TT genotype at *MMP-9* rs3918242 is associated with an elevated risk of metastasis (Table VI) (27). Interestingly, we have found that the variant TT genotype at *MMP-9* rs3918242 is associated with an elevated risk of those who have a BMI larger than 25 (Table VI). The detailed mechanisms of how TT genotype at *MMP-9* rs3918242 contribute to their increased risk of CRC need further investigations.

The study group of Hoelzle and his colleagues has provided another piece of valuable evidence for the genotype-phenotype correlation of *MMP-9* rs3918242 genotype and its encoded protein (28). They found that the CRC patients had higher levels of MMP-9 in their peripheral blood, independently of their *MMP-9* rs3918242 genotype. More importantly, those with CT genotype at *MMP-9* rs3918242 were of significantly higher levels of MMP-9 than those with CC genotype (28). Overall, the importance of *MMP-9* rs3918242 genotype to CRC etiology was revealed by the current study and previous literature, at least in the metastasis part. The people carrying TT genotypes at *MMP-9* rs3918242 were of higher MMP-9 in not only their circulating blood but also the tumor sites. The elevated MMP-9 in the extracellular microenvironments may direct the local breakage of the cellular membrane and the components of extracellular matrix, which lead to the enhancement for the invasive and metastatic behaviors of the tumor cells.

Long-term smoking behavior has been reported from the epidemiological evidence as an environmental risk factor for CRC (44), and in the current study, the interaction of *MMP-9* rs3918242 genotype and smoking on CRC risk was examined. Among both smokers and non-smokers, variant genotypes at *MMP-9* rs3918242 were not associated with altered risk of CRC in Taiwanese (Table IV). Similarly, the variant genotypes at *MMP-9* rs3918242 were not associated with altered risk of CRC among non-alcohol drinkers or drinkers in Taiwanese (Table V). However, potential interactions between the risk genotype and behaviors may be reflected from the slightly increased ORs among the subgroups of smokers, non-smokers and non-alcohol drinkers, but not alcohol drinkers. In the future, similar studies regrading larger CRC and control populations may provide more detailed evidence regarding which subtypes of citizens are under more risk of CRC, and how we can effectively lower the CRC prevalence based on genotype-environment and genotype-behavior analysis.

In addition, no interaction was found between *MMP-9* rs3918242 genotype and age or gender on CRC risk among the CRC patients. Regarding tumor size or location, no determinant effect of *MMP-9* rs3918242 on CRC was found in the patients investigated (Table VI). The highlight of the current study is that the variant genotypes of *MMP-9* rs3918242 were associated with a higher lymph node metastasis risk, and *MMP-9* rs3918242 genotype may indicate those with BMI >25 to have higher CRC risk (Table VI). The detail mechanisms should be validated by further studies.

In conclusion, solid evidence was provided within a moderate and representative population showing that *MMP-9* rs3918242 was not associated with CRC risk, but with an increased risk of metastasis in a Taiwanese population. The stratified analyses indicated that smokers and alcohol drinkers do not bear additional risk for CRC with a joint effect of

MMP-9 rs3918242 genotypes. Interestingly, *MMP-9* rs3918242 genotype may interact with BMI to increase the risk of CRC. Further validations in other populations and the detailed mechanisms are urgently encouraged and needed.

Conflicts of Interest

We confirmed that each Author declare no conflicts of interest in regard to the current study.

Authors' Contributions

Research Design: Wu MH, Tzeng HE and Wu CN; Patient and Questionnaire Summary: Yueh TC, Wu MH and Ke TW; Experimental Performance: Wang YC and Chang WS; Statistical Analysis: Peng YC, Tsai CH and Pei JS; Manuscript Writing: Tsai CW and Bau DT; Reviewing and Revising: Bau DT, Chang WS and Tsai CW.

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