

Retrospective Cohort Study

MTNR1B polymorphisms with CDKN2A and MGMT methylation status are associated with poor prognosis of colorectal cancer in Taiwan

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Abstract**BACKGROUND**

Identifying novel colorectal cancer (CRC) prognostic biomarkers is crucial to helping clinicians make appropriate therapy decisions. Melatonin plays a major role in managing the circadian rhythm and exerts oncostatic effects on different kinds of tumours.

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Informed consent statement:

Written informed consent was obtained from all patients before enrollment into the study to evaluate their prognosis.

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AIM

To explore the relationship between *MTNR1B* single-nucleotide polymorphism (SNPs) combined with gene hypermethylation and CRC prognosis.

METHODS

A total of 94 CRC tumour tissues were investigated. Genotyping for the four *MTNR1B* SNPs (rs1387153, rs2166706, rs10830963, and rs1447352) was performed using multiplex polymerase chain reaction. The relationships between the *MTNR1B* SNPs and CRC 5-year overall survival (OS) was assessed by calculating hazard ratios with 95% CIs.

RESULTS

All SNPs (rs1387153, rs2166706, rs10830963, and rs1447352) were correlated with decreased 5-year OS. In stratified analysis, rs1387153, rs10830963, and rs1447352 risk genotype combined with *CDKN2A* and *MGMT* methylation status were associated with 5-year OS. A strong cumulative effect of the four polymorphisms on CRC prognosis was observed. Four haplotypes of *MTNR1B* SNPs were also associated with the 5-year OS. *MTNR1B* SNPs combined with *CDKN2A* and *MGMT* gene methylation status could be used to predict shorter CRC survival.

CONCLUSION

The novel genetic biomarkers combined with epigenetic biomarkers may be predictive tool for CRC prognosis and thus could be used to individualise treatment for patients with CRC.

Key Words: Colorectal cancer; Melatonin; Hypermethylation; Polymorphism; Prognosis; Biomarker

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Core Tip: In this retrospective cohort study, we found that *MTNR1B* single-nucleotide polymorphism were associated with a significantly increased risk of colorectal cancer (CRC) 5-year overall survival. A strong cumulative effect of the four polymorphisms on CRC prognosis was observed. This study indicated the novel genetic biomarkers, *MTNR1B*, combined with *CDKN2A* and *MGMT* gene methylation statuses, maybe a predictive tool for CRC prognosis.

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INTRODUCTION

Colorectal cancer (CRC) is the third most newly diagnosed cancer and second most frequent cause of cancer-related deaths worldwide[1]. The sudden increase in the incidence of CRC in Taiwan may be associated with obesity, a sedentary lifestyle, and unhealthy dietary habits resulting from improvement of the economy[2]. However, these well-known risk factors cannot wholly account for the increased incidence of CRC. Extensive studies have demonstrated that genetic and epigenetic variations influence personal CRC susceptibility and prognosis[3-6]. Finding novel CRC prognostic biomarkers is crucial because it would help clinicians in making appropriate decisions. Melatonin, which plays a central role in the management of circadian rhythm, has been identified in the pineal retina, lymphocytes, bone marrow, and gastrointestinal tract[7]. Several epidemiological studies have indicated that melatonin exerts oncostatic effects, including antioxidant activity, stimulation of apoptosis, regulation of prosurvival signalling and tumour metabolism, inhibition of

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angiogenesis, metastasis, and induction of epigenetic alteration[8] on different types of tumours[9-11]. Melatonin prevents metastases, increases the 1-year longevity of patients with resected CRC, and enhances myelotoxicity, lymphocytopenia, and other undesired haematological and immunological side effects. Furthermore, melatonin reduces neurotoxicity, weakness, insomnia, and psychological stress. These effects have been reported when melatonin is administered as a single pharmaceutical agent or coadministered with the usual first-line and second-line schedules for radiotherapy and chemotherapy[12]. The growing interest in understanding the association between melatonin and colorectal carcinogenesis has led to thorough studies regarding the presence of unique melatonin binding sites in the human colon's intestinal mucosa and submucosa. The melatonin receptor MT2, one of the largest superfamilies of G-protein linked receptors and encoded by *MTNR1B*, is generally responsible for mediating the downstream effects of melatonin[13]. In 2006, Ekmekcioglu *et al*[14] reported that MT2 was involved in the antiproliferative action of melatonin. Some research demonstrated that the levels of melatonin are regulated through its biosynthesis from the amino acid tryptophan, which is mediated by MT2[15]. Research analyses have demonstrated reduced expression of MT2 in tumour mucosa compared with the normal mucosa in patients with CRC[16,17]. This may be caused by MT2 downregulation, which reduces protection against CRC and facilitates the development of CRC tumours. In vitro findings indicate that melatonin triggers p53 phosphorylation through the activation of MT2[18].

Studies have reported an association between *MTNR1B* gene polymorphisms and impaired insulin secretion, higher fasting glucose level, increased risk of type 2 diabetes, and gestational diabetes[19,20]. However, the effect of *MTNR1B* gene polymorphisms on CRC sensitivity is little understood. Many studies have demonstrated that *CDKN2A* is more frequently methylated in poorly differentiated, lymphatic metastasis of CRC[21]. In addition, the hypermethylation state of DNA repair genes, *MGMT* and *MLH1*, which are silenced, and have been shown to be correlated with specific mutations in tumor DNA, such as *KRAS* mutations or microsatellite instability, respectively[22]. Furthermore, aberrant promoter methylation of the *CDKN2A*, *MGMT*, and *MLH1* genes has been reported to be related to adenoma-carcinoma sequence and could serve as a diagnostic prognostic marker of CRC[23,24].

In the present study, a hospital-based retrospective cohort study was conducted to evaluate the effects of *MTNR1B* gene polymorphisms rs1387153, rs2166706, rs10830963, and rs1447352 and their haplotypes on the 5-year overall survival (OS) of patients with CRC and to analyse interactions based on the methylation status of the *CDKN2A* and *MGMT* genes. We hypothesised that the influence of *MTNR1B* gene variation combined with the hypermethylation of *CDKN2A* and *MGMT* genes would predict the prognosis and provide clinical recommendations for optimal treatment of CRC.

MATERIALS AND METHODS

Samples and DNA extraction

A retrospective cohort study, described in detail elsewhere[24-27], was conducted to predict the OS of patients with CRC in Taiwan. Among this cohort, 94 tumour tissues were collected from patients with CRC, which was diagnosed in the Tri-Service General Hospital (TSGH), Taiwan, from 2006 to 2010. The 5-year prognosis was assessed using the tumour tissues. The TSGH Colon and Rectum Division's clinical practice guideline requires enrollees to return once every 3 mo in the first year after surgical resection and once every 3-6 mo thereafter. Written informed consent was obtained from all patients before participation in the study. The TSGH Institutional Review Board approved this study (TSGHIRB approval number: 098-05-292 and 2-105-05-129). Data regarding registered patients—including sex, surgical age (permanent variable), adjuvant chemotherapy, histologic grade and location of the tumour, and survival—were collected from the TSGH's cancer registry database and analyzed to investigate the association with *MTNR1B* genotyping. All methods were performed in accordance with the relevant guidelines and regulations. OS was defined as the time from the date of surgery to the date of death from any cause or the last follow-up date before December 31, 2010. Cellulose-coated magnetic beads were employed to extract genomic DNA from the tumour tissues stored at -80 °C in a liquid nitrogen tank by using the MagCore Compact Automated Nucleic Acid Extractor (catalogue no. MCA0801; RBC Bioscience, Taipei, Taiwan) and Genomic DNA Tissue Kit (catalogue

no. 69504; Qiagen, Taipei, Taiwan).

***MTNR1B* genotyping**

The *MTNR1B* gene polymorphisms rs1387153, rs2166706, rs10830963, and rs1447352 were screened using the Agena MassARRAY platform with iPLEX gold chemistry (Agena, San Diego, CA, United States). The detailed genotyping protocols have been reported elsewhere[28,29]. Following the manufacturer guide, the specific polymerase chain reaction (PCR) primer and extension primer sequences were designed using the Assay Designer software package (v.4.0). A 1- μ L genomic DNA sample (10 ng/ μ L) was employed in multiplex PCR in 5- μ L volumes containing 1 unit of Taq polymerase, 500 nmol of each PCR primer mix, and 2.5 mmol/L of each dNTP (Agena, PCR accessory and Enzyme kit). Thermocycling was performed at 94 °C for 4 min, which was followed by 45 cycles at 94 °C for 20 s, 56 °C for 30 s, 72 °C for 1 min, and then 72 °C for 3 min. Unincorporated dNTPs were deactivated using 0.3 U of shrimp alkaline phosphatase. Single-base extension reaction was performed using iPLEX enzyme, terminator mix, and extension primer mix; this was followed by 94 °C for 30 s, 40 cycles at 94 °C for 5 s, 5 inner-cycles at 56 °C for 5 s, 80 °C for 5 s, and finally 72 °C for 3 min (Agena, iPLEX gold kit). After cation exchange resin was added to remove residual salt from the reaction, 7 nL of the purified primer extension reaction was loaded onto the matrix pad of a SpectroCHIP (Agena). The SpectroCHIPS were analysed using a MassARRAY Analyzer 4, and clustering analysis was performed using TYPER 4.0 software.

Methylation-specific-PCR

We analyzed *CDKN2A*, *hMLH1*, and *MGMT* DNA methylation in the promoter regions through methylation-specific polymerase chain reaction (MS-PCR), as described in our earlier study[25]. Perform MS-PCR with 1.2- μ L aliquots of forward and reverse primers, with 12.5 μ L HotStart Taq Premix (RBC Bioscience) and bisulfite-converted DNA according to the manufacturer's protocol. The sequences, annealing temperature of individual primer used for amplification, and MS-PCR product sizes are illustrated in Table 1. The MS-PCR procedures were in accordance with the previous study[25]: first, 10 min at 95 °C; then, 35 cycles of 30-s denaturation at 95 °C, 30-s annealing, and 48-s extension at 72 °C; finally, 4-min extension at 72 °C. After the amplification, MS-PCR products were mixed with a loading buffer, electrophoresed on 2% agarose gel by using 0.2- μ L gel-stained dye for 25 min, and visualized using an ultraviolet transilluminator.

Statistical analysis

Student's *t*-tests were performed to analyse continuous variables, and χ^2 tests were performed for statistical analyses of categorical variables (IBM SPSS Statistics 22). The existence of a Hardy-Weinberg equilibrium per single-nucleotide polymorphism (SNP) was assessed using two goodness-of-fit tests. Linkage disequilibrium (LD) among genotyped SNPs was obtained using the Haploview 4.2 programme. The minor allele frequencies of four *MTNR1B* gene polymorphisms (rs1387153, rs2166706, rs10830963, and rs1447352) are higher than 5% in an ethnic Chinese population according to data in the dbSNP database.

The relationships between the *MTNR1B* SNPs and 5-year OS of patients with CRC and between the cumulative effect of *MTNR1B* SNPs and 5-year OS of CRC were assessed using adjusted hazard ratios (aHRs) with 95% CIs, calculated using Cox proportional-hazards analyses and adjusted for all the aforementioned patient-level and hospital-level characteristics.

Haplotype frequencies for these SNPs combinations were first estimated using haplo.stats (version 12.1) for the R statistical package and then verified using Haploview 4.2. These software programmes employ expectation-maximisation algorithms when constructing the haplotypes. All statistical tests were two-sided, and $P < 0.05$ was considered statistically significant.

RESULTS

In this study, 94 CRC tumor samples from the TSGH tumor bank were analyzed. LD was evaluated for all *MTNR1B* SNP pairs. The Lewontin's *D'* values of the pairs were 1.00 (rs1387153: rs2166706), 0.90 (rs1387153: rs10830963), and 0.88 (rs1387153: rs1447352); and the *R*² values were 0.98, 1.00, and 1.00, respectively. Four haplotypes with frequencies of 0.034 (T-C-G-A), 0.011 (C-T-C-A), 0.011 (C-T-C-G), and 0.472 (C-T-

Table 1 Primer sequences, annealing temperature and product size for methylation-specific polymerase of target genes

Genes		Forward primer (5'→3')	Annealing temperature (oC)	Product size (bp)
<i>CDKN2A</i>	M	F: TTATTAGAGGGTGGGGCGGATCGC R: GACCCCGAACCGGACCGTAA	62	150
	U	F: TTATTAGAGGGTGGGGTGGATTGT R: CAACCCCAAACCACAACCATAA	62	151
<i>MLH1</i>	M	F: ACGTAGACGTTTTATTAGGGTCGC R: CCTCATCGTAACTACCCGCG	60	118
	U	F: TTTTGATGTAGATGTTTTATTAGGGTGTG R: ACCACCTCATCATAACTACCCACA	60	124
<i>MGMT</i>	M	F: TTTTCGACGTTTCGTAGTTTTTCGC R: GCACTCTCCGAAAACGAAACG	53	81
	U	F: TTTGTGTTTTGATGTTTGTAGGTTTTTGT R: AACTCCACACTCTTCCAAAAACAAAACA	53	93

MSP: Methylation-specific polymerase chain reaction; M: Methylation; U: Unmethylation.

G-A) were selected for the haplotype association analysis. The relationship between the *MTNR1B* genotype and the demographic and clinicopathological features of patients with CRC was evaluated. Among the patients with CRC, 41.5% were men; the mean age at surgery was 64.2 ± 13.8 years; 51.0% were at stage III-IV; and 19.1% of the patients died during the study period. Table 2 and Table 3 show that certain possible CRC risk factors, such as age, sex, tumor-node-metastasis stage, and *CDKN2A*, *MLH1* and *MGMT* genes methylation status, were not significantly associated with the *MTNR1B* genotype. However, tumor location and survival were associated with a *MTNR1B* polymorphism. The relationships between each *MTNR1B* genotype were analyzed to determine their association with 5-year OS in patients with CRC using Cox proportional-hazards models adjusted for age, sex, stage, adjuvant chemotherapy, tumor location, and the methylation status of the *CDKN2A*, *MLH1* and *MGMT* genes (Table 4). All SNPs were associated with lower 5-year OS. The variant types of rs1387153 (TT vs CC + CT: aHR = 6.23, 95%CI = 2.01-19.3), rs2166706 (CC vs TT + TC: aHR = 6.40, 95%CI = 2.21-18.6), rs10830963 (GG vs CC + CG: aHR = 7.43, 95%CI = 2.63-21.1), and rs1447352 (AA vs GG + GA: aHR = 4.28, 95%CI = 1.32-13.9) decreased the 5-year OS in patients with CRC.

We further examined the relationship between each SNP and the 5-year OS, with data stratified by the methylation status of the *CDKN2A* and *MGMT* genes (Table 5). Particularly, 5-year OS was significantly reduced in the rs1387153 and rs1447352 polymorphism subgroups of unmethylation of the *MGMT* gene (aHR = 8.57, 95%CI = 1.67-44.1; aHR = 19.4, 95%CI = 2.94-128, respectively) compared with the opposite subgroups. In contrast, we determined that the rs1447352 polymorphism was related to a higher risk of mortality in the subjects with methylation of the *CDKN2A* gene (aHR = 9.40, 95%CI = 1.02-86.8). Besides, rs10830963 exhibited a significant association with 5-year OS in the subgroups with hypermethylation of the *CDKN2A* gene (aHR = 27.2, 95%CI = 3.12-233). According to the small number of hypermethylation *MLH1* gene, we could not perform the *MLH1* gene methylation-stratified analysis.

The cumulative effects of four SNPs were then evaluated. We selected polymorphism to classify the risk based on genotypes, in accordance with the findings summarised in Table 4: TT vs CC + CT for rs1387153, CC vs TT + TC for rs2166706, GG vs CC + CG for rs10830963, and AA vs GG + GA for rs1447352. The subjects were classified into five groups on the basis of their genotypical risk score (0, 1, 2, 3, and 4), and the significance of the linear trend was then evaluated. The risk of poor CRC prognosis significantly increased with an increase in the SNP risk genotypes ($P_{\text{trend}} < 0.001$, Table 6). Furthermore, patients were divided into two groups on the basis of the number of risk genotypes, forming the < 2 and ≥ 2 SNP risk genotypes groups. The 5-year OS was significantly different between the group with two or more SNP risk genotypes and the comparison group (aHR = 5.81, 95%CI = 2.03-16.6).

Table 2 Clinical characteristics of colorectal cancer patients and *MTNR1B* genotypes (rs1387153 and rs2166706)

No of subjects	Total	rs1387153 (C>T)				rs2166706 (T>C)							
		CC (%)	CT (%)	TT (%)	<i>P</i> value	CC + CT (%)	<i>P</i> value	TT (%)	TC (%)	CC (%)	<i>P</i> value	TT + TC (%)	<i>P</i> value
Sex													
Male	39 (41.5)	8 (34.8)	20 (41.7)	10 (47.6)	0.69	28 (39.4)	0.62	8 (36.4)	20 (40.8)	11 (47.8)	0.73	28 (39.4)	0.63
Female	55 (58.5)	15 (65.2)	28 (58.3)	11 (52.4)		43 (60.6)		14 (63.6)	29 (59.2)	12 (52.2)		43 (60.6)	
Age at surgery													
mean ± SD (yr)	64.2 ± 13.8	66.8 ± 13.8	61.7 ± 13.8	67.0 ± 13.3	0.20	63.3 ± 13.9	0.29	67.4 ± 13.8	61.8 ± 13.8	65.6 ± 13.5	0.24	63.6 ± 13.9	0.54
< 65	50 (53.2)	12 (52.2)	27 (56.2)	10 (47.6)	0.80	39 (54.9)	0.62	11 (50.0)	27 (55.1)	12 (52.2)	0.92	38 (53.5)	1.00
≥ 65	44 (46.8)	11 (47.8)	21 (43.8)	11 (52.4)		32 (45.1)		11 (50.0)	22 (44.9)	11 (47.8)		33 (46.5)	
Stage													
I	12 (12.8)	2 (8.7)	6 (12.5)	4 (19.0)	0.31	8 (11.3)	0.12	2 (9.1)	6.1 (2.2)	4 (17.4)	0.24	8 (11.3)	0.08
II	34 (36.2)	9 (39.1)	18 (37.5)	6 (28.6)		27 (38.0)		8 (36.4)	19 (38.8)	7 (30.4)		27 (38.0)	
III	30 (31.9)	10 (43.5)	16 (33.3)	4 (19.0)		26 (36.6)		10 (45.5)	16 (32.7)	4 (17.4)		26 (36.6)	
IV	18 (19.1)	2 (8.7)	8 (16.7)	7 (33.3)		10 (14.1)		2 (9.1)	8 (16.3)	8 (34.8)		10 (14.1)	
Adjuvant chemotherapy ^a													
No	23 (24.5)	5 (22.7)	12 (25.5)	6 (33.3)	0.74	17 (24.6)	0.55	5 (23.8)	12 (25.5)	6 (30.0)	0.90	17 (25.0)	0.77
Yes	65 (69.1)	17 (77.3)	35 (74.5)	12 (66.7)		52 (75.4)		16 (76.2)	35 (74.5)	4 (70.0)		51 (75.0)	
Tumor location ^a													
Colon	74 (78.7)	21 (95.5)	41 (87.2)	11 (61.1)	< 0.001	62 (89.9)	< 0.001	20 (95.2)	41 (87.2)	13 (65.0)	0.02	61 (89.7)	0.01
Rectum	14 (14.9)	1 (4.5)	6 (12.5)	7 (38.9)		7 (10.1)		1 (4.8)	6 (12.8)	7 (35.0)		7 (10.3)	
<i>CDKN2A</i> gene													
Unmethylation	43 (45.7)	13 (56.5)	19 (39.6)	10 (47.6)	0.40	32 (45.1)	1.00	12 (54.5)	21 (42.9)	10 (43.5)	0.64	33 (46.5)	0.99
Methylation	51 (54.3)	10 (43.5)	29 (60.4)	11 (52.4)		39 (54.9)		10 (45.5)	28 (57.1)	13 (56.5)		38 (53.5)	
<i>MLH1</i> gene													
Unmethylation	77 (81.9)	20 (87.0)	38 (79.2)	17 (81.0)	0.73	58 (81.7)	1.00	19 (86.4)	39 (79.6)	19 (82.6)	0.79	58 (81.7)	1.00
Methylation	17 (18.1)	3 (13.0)	10 (20.8)	4 (19.0)		13 (18.3)		3 (13.6)	10 (20.4)	4 (17.4)		13 (18.3)	
<i>MGMT</i> gene													

Unmethylation	46 (48.9)	8 (34.8)	26 (54.2)	12 (57.1)	0.24	34 (47.9)	0.62	8 (36.4)	25 (51.0)	13 (56.5)	0.37	33 (46.5)	0.55
Methylation	48 (51.1)	15 (65.2)	22 (45.8)	9 (42.9)		37 (52.1)		14 (63.6)	24 (49.0)	10 (43.5)		38 (53.5)	
Death in 5 yr													
No	76 (80.9)	21 (91.3)	41 (85.4)	13 (61.9)	0.03	62 (87.3)	0.02	20 (90.9)	42 (85.7)	14 (60.9)	0.02	62 (87.3)	0.01
Yes	18 (19.1)	2 (8.7)	7 (14.6)	8 (38.1)		9 (12.7)		2 (9.1)	7 (14.3)	9 (39.1)		9 (12.7)	

^aThe total number of patients with colorectal cancer does not correspond because of missing data. CRC: Colorectal cancer.

Haplotype analysis was performed to determine the relationship between haplotypes of the studied SNPs (rs1387153, rs2166706, rs10830963, and rs1447352) and the 5-year OS. Four haplotypes were screened and two demonstrated significance. The T-C-G-A haplotype contributed to reduced 5-year OS (aHR = 2.75, 95%CI = 1.82–11.2), whereas the C-T-C-G haplotype reduced the risk of mortality (aHR = 0.21, 95%CI = 0.06–0.71, Table 7). No significant relationship with the 5-year OS was evident for the C-T-G-A haplotype (aHR = 1.96, 95%CI = 0.44–8.66) or the C-T-C-A haplotype (aHR = 0.70, 95%CI = 0.28–1.72).

DISCUSSION

In this retrospective cohort study, we examined the associations between four *MTNR1B* gene polymorphisms (rs1387153, rs2166706, rs10830963, and rs1447352) and CRC outcomes in terms of OS. Correlations between all SNPs and the 5-year OS were identified. In stratified analysis, the rs1387153 and rs1447352 risk genotypes were determined to be associated with 5-year OS in the unmethylation *MGMT* gene subgroup. In contrast, the rs10830963 and rs1447352 risk genotypes with hypermethylation *CDKN2A* gene had a higher risk of death in five years. Four haplotypes of *MTNR1B* SNPs were also determined to be associated with increased risk of mortality. This study is one of a few that has reported an association between *MTNR1B* SNPs and the 5-year OS in patients with CRC. The *MTNR1B* gene location of both rs1387153 and rs2166706 is > 11 kb upstream. The variant rs10830963 is located in an intronic region, whereas the variant rs1447352 is located at approximately 4.5 kb from the *MTNR1B* gene[30]. Qiu *et al*[31] indicated that these SNPs may influence the *MTNR1B* expression, causing a functional deficiency of melatonin. De Luis *et al*[32] demonstrated that rs10830963 was associated with an increased *MTNR1B* mRNA expression and the expression of other genes that may affect the energy balance role of melatonin[33]. However, the potential role and regulation of the other three SNPs in the *MTNR1B* expression are poorly understood[34].

Table 3 Clinical characteristics of colorectal cancer patients and *MTNR1B* genotypes (rs10830963 and rs1447352)

Variables	Total	rs10830963 (C>G)				rs1447352 (A>G)							
		CC (%)	CG (%)	GG (%)	P value	CC + CG (%)	P value	GG (%)	GA (%)	AA (%)	P value	GG + GA (%)	P value
Sex													
Male	39 (41.5)	7 (33.3)	22 (44.9)	10 (43.5)	0.66	29 (41.4)	1.00	4 (66.7)	16 (43.2)	19 (38.0)	0.40	20 (46.5)	0.53
Female	55 (58.5)	14 (66.7)	27 (55.1)	13 (56.5)		41 (58.6)		2 (33.3)	21 (56.8)	31 (62.0)		23 (53.5)	
Age at surgery													
mean ± SD (yr)	64.2 ± 13.8	67.0 ± 14.0	61.4 ± 13.9	66.8 ± 13.1	0.16	63.1 ± 14.0	0.26	69.7 ± 14.1	64.4 ± 14.0	63.0 ± 13.8	0.53	65.1 ± 14.0	0.47
< 65	50 (53.2)	11 (52.4)	28 (57.1)	11 (47.8)	0.75	39 (55.7)	0.63	3 (50.0)	18 (48.6)	29 (58.0)	0.68	21 (48.8)	0.41
≥ 65	44 (46.8)	10 (47.6)	21 (42.9)	12 (52.2)		31 (44.3)		3 (50.0)	19 (51.4)	21 (42.0)		22 (51.2)	
Stage													
I	12 (12.8)	2 (9.5)	6 (12.2)	4 (17.4)	0.56	8 (11.4)	0.30	0 (0)	4 (10.8)	8 (16.0)	0.54	4 (9.3)	0.60
II	34 (36.2)	8 (38.1)	18 (36.7)	7 (30.4)		26 (37.1)		1 (16.7)	15 (40.5)	17 (34.0)		16 (37.2)	
III	30 (31.9)	9 (42.9)	16 (32.7)	5 (21.7)		25 (35.7)		4 (66.7)	12 (32.4)	14 (28.0)		16 (37.2)	
IV	18 (19.1)	2 (9.5)	9 (18.4)	7 (30.4)		11 (15.7)		1 (16.7)	6 (16.2)	11 (22.0)		7 (16.3)	
Adjuvant chemotherapy ^a													
No	23 (24.5)	5 (23.8)	12 (25.5)	6 (30.0)	0.90	17 (25.0)	0.77	1 (16.7)	8 (22.2)	14 (30.4)	0.61	9 (21.4)	0.47
Yes	65 (69.1)	16 (76.2)	35 (74.5)	14 (70.0)		51 (75.0)		5 (83.3)	28 (77.8)	32 (69.6)		33 (78.6)	
Tumor location ^a													
Colon	74 (78.7)	20 (95.2)	41 (87.2)	13 (65.0)	0.02	61 (89.7)	0.01	6 (100)	34 (94.4)	34 (73.9)	0.02	40 (95.2)	< 0.001
Rectum	14 (14.9)	1 (4.85)	6 (12.8)	7 (35.0)		7 (10.3)		0 (0)	2 (5.6)	12 (26.1)		2 (4.8)	
<i>CDKN2A</i> gene													
Unmethylation	43 (45.7)	12 (57.1)	17 (34.7)	13 (56.5)	0.10	29 (41.4)	0.31	4 (66.7)	16 (43.2)	22 (44.0)	0.55	20 (46.5)	0.97
Methylation	51 (54.3)	9 (42.9)	32 (65.3)	10 (43.5)		41 (58.6)		2 (33.3)	21 (56.8)	28 (56.0)		23 (53.5)	
<i>MLH1</i> gene													
Unmethylation	77 (81.9)	18 (85.7)	39 (79.6)	19 (82.6)	0.83	57 (81.4)	1.00	5 (83.3)	31 (83.8)	40 (80.0)	0.90	36 (83.7)	0.79
Methylation	17 (18.1)	3 (14.3)	10 (20.4)	4 (17.4)		13 (18.6)		1 (16.7)	6 (16.2)	10 (20.0)		7 (16.3)	
<i>MGMT</i> gene													

Unmethylation	46 (48.9)	8 (38.1)	26 (53.1)	12 (52.2)	0.50	34 (48.6)	0.95	4 (66.7)	18 (48.6)	24 (48.0)	0.68	22 (51.2)	0.92
Methylation	48 (51.1)	13 (61.9)	23 (46.9)	11 (47.8)		36 (51.4)		2 (33.3)	19 (51.4)	26 (52.0)		21 (48.8)	
Death in 5 yr													
No	76 (80.9)	19 (90.5)	43 (87.8)	13 (56.5)	< 0.001	62 (88.6)	< 0.001	6 (100)	33 (89.2)	36 (72.0)	0.06	39 (90.7)	0.03
Yes	18 (19.1)	2 (9.5)	6 (12.2)	10 (43.5)		8 (11.4)		0 (0)	4 (10.8)	14 (28.0)		4 (9.3)	

^aThe total number of patients with colorectal cancer does not correspond because of missing data. CRC: Colorectal cancer.

Mechanisms involving the oncostatic effect of melatonin binding to MT1 and MT2 receptors in CRC have been reported in numerous studies. An *in vitro* study conducted by Karasek *et al*[35] determined that both MT1 and MT2 were part of the oncostatic action of melatonin on Colon 38 adenocarcinoma cells. Furthermore, activation of the tumour suppressor *p53* gene by melatonin is reportedly directly controlled by MT1 and MT2. Melatonin's suppression of cell proliferation and clonogenic activity is impaired because of the lack of either receptor[18]. León *et al*[16] demonstrated an association between reduced MT1 and MT2 expression and increased malignancy in CRC in 54 Spanish patients with CRC. Moreover, expression of the tumour markers CD44 and CD133 was negatively correlated with MT1 and MT2 expression in patients with CRC[17]. Furthermore, the role of the MT1 receptor in gastric adenocarcinoma was demonstrated in patients over the age of 50 years[36]. In the present study, we determined that four *MTNR1B* gene polymorphisms were significantly associated with the 5-year OS of patients with CRC, with a cumulative effect on prediction for poorer prognosis. Few studies have assessed the relationship between *MTNR1B* gene polymorphisms and CRC prognosis. However, some type 2 diabetes susceptibility genes are correlated with metastasis development[37]. Nasrabadi *et al*[38] indicated that high expression of MT2 was associated with gastric adenocarcinoma, because MT2 receptors enhance the secretion of bicarbonate by stimulating calcium release into the mucosa of enterochromaffin cells. Numerous reports have indicated that *MTNR1B* SNPs are associated with fasting glucose level, obesity, carbohydrate disorders, and type 2 diabetes, which are crucial metabolic risk factors for CRC[39,40]. Indeed, Johnson *et al*[41] reviewed the association between colorectal cancer and type 2 diabetes and indicated that there is a positive and observational correlation. Besides, the GG genotype of the variant rs10830963 was discovered to significantly increase the risk of breast cancer than the CC genotype[39,42]. Moreover, the AA genotype of the variant rs10765576 was correlated with lower risk of breast cancer compared with the GG or GA major allele among Chinese women[13].

Data concerning the effect of gene methylation modification on the association between *MTNR1B* gene polymorphisms and CRC prognosis are scarce. The case-only analysis performed by Das *et al*[43] assessed the potential interactions and associations between epigenetics, genetics, and the risk of oesophageal cancer. Das *et al*[43]

Table 4 Relationship between *MTNR1B* single-nucleotide polymorphism and 5-year overall survival of colorectal cancer patients

	No. of subjects	No. of cases (%)	Crude		Adjusted ^a	
			HR	95%CI	HR	95%CI
rs1387153 (C>T)						
CC	23	2 (11.8)	1.00	Referent	1.00	Referent
CT	48	7 (41.2)	1.65	(0.34 to 7.95)	1.98	(0.40 to 9.82)
TT	21	8 (47.1)	6.03	(1.28 to 28.4)	10.6	(1.87 to 59.5)
TT vs CC + CT			4.18	(1.61 to 10.9)	6.23	(2.01 to 19.3)
rs2166706 (T>C)						
TT	22	2 (11.1)	1.00	Referent	1.00	Referent
TC	49	7 (38.9)	1.55	(0.32 to 7.47)	1.91	(0.39 to 9.45)
CC	23	9 (50.0)	5.74	(1.24 to 26.6)	10.5	(1.96 to 56.4)
CC vs TT + TC			4.15	(1.65 to 10.5)	6.40	(2.21 to 18.6)
rs10830963 (C>G)						
CC	21	2 (11.1)	1.00	Referent	1.00	Referent
CG	49	6 (33.3)	1.19	(0.24 to 5.91)	1.40	(0.27 to 7.22)
GG	23	10 (55.6)	5.79	(1.27 to 26.5)	9.46	(1.90 to 47.1)
GG vs CC + CG			5.09	(2.01 to 12.9)	7.43	(2.63 to 21.1)
rs1447352 (A>G)						
AA	50	14 (77.8)	1.00	Referent	1.00	Referent
GA	37	4 (22.2)	0.36	(0.12 to 1.08)	0.31	(0.10 to 0.99)
GG	6	0 (0)	N/A	N/A	N/A	N/A
AA vs GG + GA			3.37	(1.11 to 10.2)	4.28	(1.32 to 13.9)

^aAdjusted for age, sex, stage, adjuvant chemotherapy, tumor location and the methylation status of *CDKN2A*, *MLH1* and *MGMT* gene. HR: Hazard ratio; N/A: Not applicable.

determined that *CDKN2A* methylation and the *p53* polymorphism were significantly associated with oesophageal cancer risk. DNA methylation can regulate gene expression by modifying chromatin complexes and recruiting methyl-CpG domain-binding proteins around CpG islands, and this is the most common epigenetic alteration. There was a clinical study had revealed the feasibility of using specific gene methylation statuses as biomarkers for CRC prognosis[44]. For instance, the hypermethylation of *CDKN2A* and *MGMT* promoters has been suggested to be independently correlated with poorer prognosis (including metastasis, recurrence, and mortality) in patients with CRC[45,46]. Our findings demonstrated that the risk of mortality in *CDKN2A* hypermethylation patients with rs10830963 or rs1447352 risk genotype was higher than that in the opposing subgroups. However, SNPs-rs1387153 and rs1447352 with unmethylation of the *MGMT* gene were associated with poorer CRC prognosis. All these findings regarding the correlation with gene promoter methylation status and polymorphisms suggest an overlap and crosstalk between the involved pathways, adversely affecting cancer prognosis, and indicate a strong correlation between genetic and epigenetic factors in the Taiwanese population. Therefore, *CDKN2A* and *MGMT* methylation status and *MTNR1B* SNPs may be used as molecular targets for predicting CRC prognosis. The efficiency of any single polymorphic site for risk detection is usually limited because of the multistep model of colorectal carcinogenesis. The benefits of using a combination of several SNPs are well-documented[47]. Our analysis revealed a significant cumulative effect, which was observed as *MTNR1B* SNPs correlating with the 5-year OS, which indicates that using more risk genotypes may improve the accuracy of CRC prognoses. Furthermore, the risk of mortality in individuals with the TT genotype rs1387153, CC genotype rs2166706, and GG genotype rs10830963 may be 2.75-fold higher than that in other haplotypes.

Table 5 Stratified effect between gene promoter region methylation and *MTNR1B* genotypes for 5-year overall survival of colorectal cancer patients

		No. of subjects	No. of cases (%)	Crude		Adjusted ^a	
				HR	95%CI	HR	95%CI
rs1387153 (C>T)							
TT vs CC + CT	CDKN2A						
	U	10	4 (40.0)	5.01	(1.33 to 18.9)	10.4	(1.17 to 92.4)
	M	11	4 (36.4)	3.83	(0.96 to 15.3)	8.86	(1.08 to 72.8)
	MGMT						
	U	12	5 (41.7)	3.63	(1.05 to 12.6)	8.57	(1.67 to 44.1)
	M	9	3 (33.3)	4.93	(1.10 to 22.1)	3.05	(0.33 to 28.0)
rs2166706 (T>C)							
CC vs TT + TC	CDKN2A						
	U	10	4 (40.0)	5.02	(1.33 to 19.0)	10.4	(1.17 to 92.4)
	M	13	5 (38.5)	3.96	(1.06 to 14.7)	10.1	(1.49 to 68.0)
	MGMT						
	U	13	5 (38.5)	3.07	(0.89 to 10.7)	6.28	(1.54 to 25.7)
	M	23	9 (39.1)	6.25	(1.56 to 25.1)	8.28	(0.95 to 72.3)
rs10830963 (C>G)							
GG vs CC + CG	CDKN2A						
	U	13	5 (38.5)	4.63	(1.24 to 17.3)	8.47	(1.57 to 45.6)
	M	10	5 (50.0)	5.61	(1.51 to 20.9)	27.2	(3.12 to 233)
	MGMT						
	U	12	5 (41.7)	3.47	(1.00 to 12.0)	8.50	(1.98 to 36.5)
	M	11	5 (45.5)	7.91	(1.89 to 33.2)	9.80	(1.42 to 67.5)
rs1447352 (A>G)							
AA vs GG + GA	CDKN2A						
	U	22	6 (27.3)	2.05	(0.51 to 8.22)	2.28	(0.51 to 10.2)
	M	28	8 (28.6)	7.34	(0.92 to 58.7)	9.40	(1.02 to 86.8)
	MGMT						
	U	24	8 (33.3)	3.94	(0.84 to 18.6)	19.4	(2.94 to 128)
	M	26	6 (23.1)	2.77	(0.56 to 13.7)	2.04	(0.35 to 12.0)

^aAdjusted for age, sex, stage, adjuvant chemotherapy, tumor location and the methylation status of *CDKN2A* and *MLH1* or *MGMT* and *MLH1* gene. U: Unmethylation; M: Methylation; HR: Hazard ratio.

Certain limitations to the present study should be considered. First, limitations are inherent in any retrospective cohort study. Second, our sample size was not sufficiently large enough to provide a more precise estimate of the association between *MTNR1B* SNPs and CRC prognosis. Third, our preliminary retrospective cohort study was not designed to clarify the pathophysiology of how the risk genotype of the *MTNR1B* gene reduces postoperative survival. In addition, data regarding registered patients were collected from the TSGH's cancer registry database. Other potential risk factors, such as dietary habits, obesity, and combined primary diseases were unavailable from the database. Furthermore, this study did not include normal colorectal tissues, *i.e.* we could not describe *MTNR1B* polymorphic variants in normal colorectal tissues. The results of the present study should be verified using a large-scale study that controls for confounding variables, such as lifestyle, carcinogen exposure, and diet, among others.

Table 6 Cumulative effect of *MTNR1B* single-nucleotide polymorphism associated with 5-year overall survival of colorectal cancer patients

	No. of subjects	No. of cases (%)	Crude		Adjusted ^a	
			HR	95%CI	HR	95%CI
No. of SNP risk genotypes						
0	41	3 (7.3)	1.00	Referent	1.00	Referent
1	25	4 (16.0)	2.27	(0.51 to 10.1)	2.60	(0.55 to 12.2)
2	7	3 (42.9)	6.40	(1.29 to 317)	6.89	(1.16 to 41.0)
3	1	0 (0)	N/A	N/A	N/A	N/A
4	18	7 (38.9)	7.60	(1.96 to 29.5)	14.0	(2.94 to 66.3)
<i>P</i> for trend				< 0.001		< 0.001
≥ 2 of SNP risk genotypes	26	10 (38.5)	4.00	(1.58 to 10.1)	5.81	(2.03 to 16.6)

^aAdjusted for age, sex, stage, adjuvant chemotherapy, tumor location and the methylation status of *CDKN2A*, *MLH1* and *MGMT* gene. SNP: Single-nucleotide polymorphism; U: Unmethylation; M: Methylation; HR: Hazard ratio; N/A: Not applicable.

Table 7 Relationship between haplotypes of *MTNR1B* single-nucleotide polymorphism and 5-year overall survival of colorectal cancer patients

Haplotypes	Survival group (%)	Death group (%)	Crude		Adjusted ^a	
			HR	95%CI	HR	95%CI
T-C-G-A	42.0	68.4	2.58	(1.27 to 5.28)	2.75	(1.82 to 11.2)
C-T-G-A	2.7	5.3	1.74	(0.42 to 7.26)	1.96	(0.44 to 8.66)
C-T-C-G	30.0	7.9	0.23	(0.07 to 0.76)	0.21	(0.06 to 0.71)
C-T-C-A	22.0	15.8	0.73	(0.30 to 1.76)	0.70	(0.28 to 1.72)

^aAdjusted for age, sex, stage, adjuvant chemotherapy, tumor location. The reference is the set of all the other haplotypes when one haplotype is regarded as an analyzed item. HR: Hazard ratio.

CONCLUSION

In summary, we conducted a retrospective cohort study to investigate the association between *MTNR1B* SNPs and CRC prognosis in those with different gene methylation statuses. All polymorphisms were correlated with 5-year OS. Three SNPs (rs1387153, rs10830963, and rs1447352) were associated with enhanced mortality risk when combined with different *CDKN2A* or *MGMT* gene methylation status. Furthermore, we observed a strong cumulative effect of *MTNR1B* SNPs on the 5-year OS of patients with CRC. Our findings indicate that *MTNR1B* SNPs combined with *CDKN2A* and *MGMT* gene methylation statuses may be predictive biomarkers for CRC prognosis. This study offers insights into novel genetic and epigenetic biomarkers for the prediction of CRC prognosis, and the findings could be used to individualise the treatment of patients with CRC.

ARTICLE HIGHLIGHTS

Research background

Melatonin plays a central role in the management of circadian rhythm and was identified in the gastrointestinal tract. Epidemiological studies demonstrated that melatonin has oncostatic effects including induction of epigenetic alteration on different types of tumours. The melatonin receptor MT2 encoded by *MTNR1B* is generally responsible for mediating the downstream effects of melatonin. The expression of MT2 in tumour mucosa is lower than the normal mucosa in patients with

colorectal cancer (CRC).

Research motivation

Growing studies have investigated the association between melatonin and CRC carcinogenesis. However, the relationship between *MTNR1B* gene polymorphisms and CRC sensitivity is not clear. To analyze the effects of *MTNR1B* gene polymorphisms on CRC prognosis and evaluate the interactions with aberrant promoter methylation of the *CDKN2A* and *MGMT* genes will be of great significance.

Research objectives

In our study, we aimed to explore the association between *MTNR1B* single-nucleotide polymorphism (SNPs) and the 5-year overall survival (OS) of CRC patients. To further assess the interaction between *MTNR1B* SNPs and *CDKN2A* and *MGMT* gene methylation, we examined the relationship between each SNP and the 5-year OS, with data stratified by the methylation status of the *CDKN2A* and *MGMT* gene.

Research methods

Ninety four CRC patients from Taiwan were enrolled to evaluate the association between *MTNR1B* SNPs, *CDKN2A*, *MGMT* gene hypermethylation and 5-year OS. The *MTNR1B* gene polymorphisms were screened using the Agena MassARRAY platform with iPLEX gold chemistry. The promoter methylation status of *CDKN2A* and *MGMT* was assessed using methylation-specific polymerase chain reaction. Associations of the genetic and epigenetic effect and 5-year OS were assessed using the Cox proportional hazards regression model.

Research results

In this retrospective cohort study, we found that *MTNR1B* SNPs was associated with a significantly increased risk of CRC 5-year OS. A strong cumulative effect of the four polymorphisms on CRC prognosis was observed. In stratified analysis, rs1387153, and rs1447352 risk genotype were determined to be associated with 5-year OS in the unmethylation *MGMT* gene subgroup. In contrast, rs10830963 and rs1447352 risk genotype with hypermethylation *CDKN2A* gene had a higher risk of death in five years. Four haplotypes of *MTNR1B* SNPs were also determined to be associated with increased risk of mortality.

Research conclusions

This study is one of few reports which demonstrated the association between *MTNR1B* SNPs and the 5-year OS in patients with CRC. Our data identified these novel genetic biomarkers combined with *CDKN2A* and *MGMT* methylation status for the prediction of CRC prognosis, and the findings could be used to individualise the treatment of patients with CRC.

Research perspectives

Based on our findings, the novel genetic biomarkers, *MTNR1B*, combined with *CDKN2A* and *MGMT* gene methylation statuses could be a predictive tool for CRC prognosis. The new set of markers may help physicians make treatment decisions based on the prognostic information and would improve the OS of patients with CRC. This study warrant further investigation of the underlying mechanisms related to oncogenic effects of *MTNR1B* on CRC.

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