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Association between TP53 genetic polymorphisms and the methylation and expression of miR-34a, 34b/c in colorectal cancer tissues

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Abstract. Colorectal cancer (CRC) is one of the most common types of cancers, as evidenced by the >1.2 million patient diagnoses and 600,000 mortalities globally each year. Recently, the microRNA (miR/miRNA)-34 miRNA precursor family was revealed to participate in the tumor protein (TP)-53 pathway, which is frequently involved in CRC. Furthermore, the expression of miR-34 is reportedly regulated by DNA methylation. Accordingly, the present study investigated the correlation between the methylation status of miR-34 miRNAs and miR-34 expression in paired CRC tumor and normal tissues. The methylation status of miR-34a and miR-34b/c was determined using the MethyLight assay, and the expression of miR-34a and miR-34b/c in the same paired tissues was analyzed by reverse transcription-quantitative polymerase chain reaction. The results revealed significantly elevated miR-34a (P=0.012) and miR-34b/c (P<0.0001) methylation levels in tumor tissues when compared with normal tissues, whereas only the expression of miR-34b/c differed (P=0.005) between the paired tissues. In addition, an association between TP53 haplotypes and miR-34 family expression levels was observed. The miR-34a methylation levels in the TP53 PIN A1A1 (48.56±36.49) and TP53 MSP GG (49.00±36.44) genotypes were increased in the tumor tissues when compared with normal tissues. In conclusion, it was determined that miR-34 promoter methylation and TP53 polymorphisms may be associated with CRC pathogenesis.

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Introduction

Colorectal cancer (CRC) is one of the most common cancers, affecting more than 1.2 million patients and resulting in 600,000 deaths annually (1,2). According to the National Cancer Information Center of Korea, CRC has the third highest incidence rate in both males and females, and the second highest mortality rate in females (3). Established CRC risk factors include exposure to processed meats and alcohol, smoking, and gamma radiation; however, recent studies have likewise investigated the effects of the microRNA (miR/miRNA)-34 miRNA precursor family (1,2).

MicroRNAs (miRNAs, miRs) are small, 22-24 nucleotide non-coding RNAs that negatively regulate the translation of messenger RNA to protein via base pairing to a partially complementary sequence in the open reading frames and 3'-untranslated regions (3'-UTR) (4,5). Further, miRNAs are encoded across the entire genome, including exonic, intronic, and intergenic regions. However, almost all miRNAs are found in intronic regions (6). The RNA polymerases RNase II or III transcribe miRNAs as long primary transcripts (pri-miRNAs), which are then processed into stem-loop structure miRNA precursor molecules (pre-miRNAs) in the nucleus by a nuclear complex consisting of Drosha, a member of the ribonuclease III family (RNase III), and its cofactors (DGCR8) (7,8). The pre-miRNAs are exported to the cytoplasm by exportin-5 (XPO5) and processed into mature, 18-25 nucleotide (nt) miRNAs following cleavage of the double-stranded portion of the hairpin by the RNase III enzyme Dicer (9,10). Altered miR expression resulting from deregulation occurs in several human diseases including cancer. As well, miRs act as either tumor suppressor genes

or oncogenes, resulting in the dissemination of cancer (11-13). The deregulation of miR via the epigenetic silencing of miR expression is likewise associated with CpG island methylation and repressive histone modifications (11).

The miRNA-34 family, comprised of miR-34a, miR-34b, and miR-34c, is part of the p53 network, which induces the expression of miR-34 miRNAs in response to DNA damage or oncogenic stress (14-16). Located on chromosome 1p36, miR-34a is associated with glioma, neuroblastoma, pancreatic cancer, and chronic myelogenous leukemia (17-20), and is the key regulator of cell cycle progression (E2F3) and apoptosis (BCL2) (14,15,20). Reduced miR-34a expression is frequently observed in pancreatic tumors and neuroblastomas (18,21). Located on chromosome 11q23, miR-34b and miR-34c are co-transcribed from one transcription unit (22). Reduced miR-34b/c expression is seen in non-small cell lung cancer (20). The methylation of miR-34b/c CpG sites has been found in CRC (23) and oral squamous cell carcinoma (24) as well as malignant melanoma, in which miR-34b/c CpG site methylation is likewise correlated with metastatic potential (25). The cancer-related downregulation of members of the miR-34 family indicates that these miRs function as tumor-suppressor genes, suggesting a potential prognostic marker role (26).

Several mechanisms, including gene amplification, deletion, epigenetic alterations, and single-nucleotide substitutions, have been implicated as potential regulators of miR expression; however, to date, no studies have actually demonstrated a precise regulatory role for miR expression (26,27). Although single nucleotide polymorphisms (SNPs) in miRs are not considered functionally important, nucleotide variations in primary (pri)- or precursor (pre)-miRs may affect miR processing and result in modified miR expression (28). Recently, studies reported that rs4938723, a potentially functional SNP in the promoter region of pri-miR-34b/c, may contribute to susceptibility to hepatocellular carcinoma (29), CRC (30), endometrial cancer (31), and decreased breast cancer survival (32). However, reports on the relationship between SNPs in the miR-34b/c promoter and the subsequent risk and prognostic significance in CRC patients are limited.

Mutations in p53 reportedly initiate or participate in early events in several diverse cancers types. Prior reports likewise indicate that the tumorigenicity is related to the deregulation of p53-mediated transcription (28). Of the variations in the *TP53* gene, Arg72Pro is the most widely investigated. The 72Arg allele induces apoptosis more efficiently than the 72Pro allele (32). Prior reports have indicated that Pro homozygosity in *TP53*Arg72Pro is a potential risk factor for lung, esophageal, stomach, breast, nasopharynx, urothelium, and prostate cancers (33,34). However, the results of a meta-analysis of CRC, performed to estimate the effect of the *TP53*Arg72Pro polymorphism on CRC risk, failed to identify a significant association (35). Conversely, we detected a negative relationship between the *TP53*Arg72Pro polymorphism and CRC risk in the Korean population. Transcriptional silencing of CpG methylation represents an important mechanism for the inactivation of key tumor suppressor genes (25,36), and approximately 60% of CpG islands are located in promoter regions. However, only CpG islands located in promoter regions exhibit methylation of cytosine at position 5 and the inactivation of surrounding chromatin via the recruitment of histone deacetylases following proteins binding to methylated CpG residues (37). Therefore, the aim of this study has investigated the differences in the degree of methylation according to SNPs in the DNA promoter region (Fig. 1) and confirmed the changes in the expression pattern of the miR-34 family according to the gene mutation of p53.

Materials and methods

Tissue samples and genomic DNA isolation. Paired tumor and normal tissues were retrospectively selected from 104 CRC patients treated at the CHA Bundang Medical Center (Seongnam, Korea) between March 2010 and March 2012. Genomic DNA (gDNA) isolation was performed according to an established protocol (38). Briefly, 50 μl of solution 1 (420 μl STE buffer and 10% SDS) was added to the tissues followed by the addition of 30 μl proteinase K and overnight incubation at 50˚C. After incubation, 500 μl of solution 2 (phenol:chloroform:isoamyl alcohol=25:24:1) was added to the tissue samples and the supernatants were collected following centrifugation. Next, 500 μl of solution 3 (chloroform:isoamyl alcohol=24:1) was added, the supernatants were collected after centrifugation, and 25 μl 3 M sodium acetate and 900 μl 100% EtOH were added. The samples were centrifuged again, and the resulting supernatants were quenched on ice for 1 h. The resulting gDNA samples were dissolved in the rehydration buffer. The present study was approved by the Institutional Review Board of CHA Bundang Medical Center; IRB no. 2009-08-077-010) and written informed consent was provided by all patients.

Genetic analysis. Three TP53 single nucleotide polymorphisms (SNPs), TP53 codon 72G>C (rs1042522, Arg>Pro, exon 4), TP53 *MSP*I A>G (rs1625895, intron 6), and TP53 PIN (rs17878362, intron 6), and one miR-34b/c SNP, miR-34bc T>C (rs4938723, promoter) were selected from the human genome SNP database (dbSNP, www.ncbi.nlm.nih.gov/snp). The genotypes were determined using a polymerase chain reaction (PCR)-restriction fragment length polymorphism assay. The PCR primers for the TP53 codon 72G>C polymorphism were forward 5'-TTGCCGTCCCAAGCAATG GATGA-3' and reverse 5'-TCTGGGAAGGGACAGAAG ATGAC-3'. The PCR conditions included an initial 5 min of denaturation at 94˚C followed by 35 cycles of denaturing at 94˚C for 30 sec, annealing at 56˚C for 30 sec, and extension at 72˚C for 30 sec followed by a final extension at 72˚C for 5 min. The PCR products were cut using the *Bst*UI restriction enzyme (New England Biolabs, Beverly, MA, USA) at 60˚C for 16 h.

The PCR primers for the TP53 MSPI A>G polymorphism were forward 5'-ATAGTGTGGTGGTGCCCTAT-3' and reverse 5'-CCTTAGCCTCTGTAAGCTTCA-3', and the primers for the TP53 PIN polymorphism were forward: 5'-GACTGACTTTCTGCTCTTGTCTT-3' and reverse 5'-ATC GTCCGG-3'. The PCR conditions included an initial 5 min of denaturation at 94˚C followed by 35 cycles of denaturing at 94˚C for 30 sec, annealing at 60˚C for 30 sec, and extension at 72˚C for 30 sec followed by a final extension at 72˚C for 5 min. The PCR products were cut using the *MSP*I restriction enzyme (New England Biolabs) at 37˚C for 16 h.

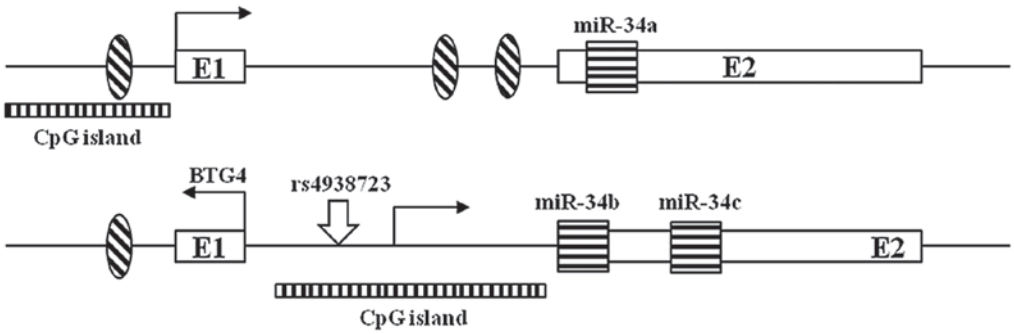

Figure 1. Structure of the miR-34a and miR-34b/c genes. The hatched oval shapes indicate p53-binding sites and the white boxes represent exons. Black striped boxes indicate miR hairpins. CpG islands are indicated as the long black striped rectangles. miR, microRNA.

The PCR primers for the miR-34b/c T>C polymorphism were forward 5'-CCTCTGGGAACCTTCTTTGACCAAT-3' and reverse 5'-TGAGATCAAGGCCATACCATTCAAGA-3'. The PCR conditions included an initial 5 min of denaturation at 94˚C was followed by 35 cycles of denaturing at 94˚C for 30 sec, annealing at 55˚C for 30 sec, and extension at 72˚C for 30 sec followed by a final extension at 72˚C for 5 min. The PCR products were cut using the *Tsp*509I restriction enzyme (New England Biolabs) at 65˚C for 16 h.

All of the resulting PCR products were analyzed by gel electrophoresis. In this study, the designated A1 and A2 alleles for the TP53 PIN polymorphism indicated a 16-base pair (bp) deletion and insertion in intron 6, respectively.

gDNA bisulfite modification and methylation analysis. The DNA was treated with bisulfite using an EZ DNA methylation kit (Zymo Research, Irvine, CA, USA). The modified DNA was then eluted in a final volume of 10 μl and 1 μl was used for the real-time methylation PCR (MethyLight). Typically, methylation-specific PCR (MSP) is used to measure DNA methylation, but we opted to use the semi-Quantitative MethyLight technology (39). In the MethyLight technology, the discrimination is made during the PCR amplification step using primers and probes that specifically anneal to either the converted methylated or converted unmethylated sequence. Real-time PCR was performed in triplicate to confirm the results, and the average value was used. The EpiTect® PCR control DNA set was also used (Qiagen, Valencia, CA, USA). The resulting data were analyzed using the Cmeth method (EpiTect® MethyLight PCR Handbook; Qiagen) (40).

RNA extraction and reverse transcriptase reaction. Total RNA was extracted from each tumor and normal tissue sample using the TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and treated with RNase-free DNase (Promega, Madison, WI, USA). The concentration of the RNA was measured using a spectrophotometer and the samples were stored at -80˚C until analysis. Reverse transcription (RT) of the total RNA was performed using an Invitrogen RT kit according to the manufacturer's instructions.

RT-quantitative polymerase chain reaction (RT-qPCR) analysis. The expression of miR was measured by qPCR using the EvaGreen® Master Mix, primers for each gene, and the Rotor-Gene RG-3000 thermal cycler (Corbett Research). The primer sequences were as follows (forward and reverse): ACTB (213 bp) 5'TGACATTAAGGAGAAGCTGTGCTAC3' and 5'GAGTTGAAGGTAGTTTCGTGGATG3'; miR-34a (128 bp) 5'CGTCACCTCTTAGGCTTGGA3' and 5'CAT TGGTGTCGTTGTGCTCT3'; and miR-34b/c (84 bp) 5'-GTG CTCGGTTTGTAGGCAGT3' and 5'-GTGCCTTGTTTTGAT GGCAG3' (7,41). The expression results were calculated using the -ΔΔCq method and expressed graphically using ACTB (β-actin) as the reference gene (42). The experiments were performed in triplicate and the average values were used.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA, USA) and MedCalc version 12.1.4 (MedCalc Software bvba, Ostend, Belgium). The genotype and allele frequencies were calculated to investigate deviations from Hardy-Weinberg equilibrium. The expression pattern of the miRNA identified in CRC tissues was analyzed using Student's t-test and one-way analysis of variance with Student-Newman-Keuls post hoc test. The allele combinations were estimated with SNPAlyze version 5.1 (Dynacom Co., Ltd., Nakase, Japan) and HapStat version 3.0 (dlin.web.unc.edu/). The results are presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Of the 104 CRC patients, 54 were colon cancer patients and 50 were rectal cancer patients. The mean age was 64 years. Other clinical characteristics including sex, TNM stage, tumor location, and tumor size are summarized in Table I. The relationship between miR-34 methylation level and clinical characteristics was further evaluated. A significant association was found between miR-34a methylation level and age in tumor tissues (>64 vs. ≤64 years; $P=0.035$). However, no significant association was detected between miR-34 methylation level and sex, location of the primary tumor, TNM stage, tumor size, or BMI (Table I).

The data analysis was performed using the Cmeth method and the EpiTect® PCR control DNA set (Qiagen). The correlation of methylation status and miR-34 expression levels between normal and tumor tissue are shown in Fig. 2. The tumor tissue exhibited higher miR-34a (normal tissue: 35.93±3.53 vs. tumor tissue: 48.99±3.57; $P=0.012$) and miR-34b/c (normal tissue: 13.08±1.94 vs. tumor tissue: 55.63±3.35; $P<0.0001$)

Table I. Associations between miR-34 methylation level and clinical characteristics of colorectal cancer patients.

Characteristic	Total n (n=104), n (%)	Normal tissue				Tumor tissue			
		miR-34a	P-value[a]	miR-34b/c	P-value[a]	miR-34a	P-value[a]	miR-34b/c	P-value[a]
Sex									
Male	61 (58.7)	37.07±37.42	0.851	12.40±19.17	0.628	46.46±36.30	0.554	57.25±31.54	0.407
Female	43 (41.3)	35.71±35.18		14.31±20.11		50.77±36.70		51.71±34.73	
Age, years									
>64	55 (52.9)	36.76±34.93	0.897	11.73±18.55	0.381	56.93±35.08	0.035[c]	54.60±35.96	0.864
≤64	49 (47.1)	35.84±37.15		15.12±20.63		41.92±36.45		53.47±31.27	
TNM stage									
1	5 (4.8)	34.82±41.51	0.967[b]	12.05±15.42	0.178[b]	63.02±42.83	0.728[b]	64.40±34.64	0.073[b]
2	23 (22.1)	33.17±34.05		21.17±25.92		46.07±37.16		59.42±36.58	
3	66 (63.5)	37.06±36.15		11.89±18.19		47.88±36.92		55.16±31.65	
4	10 (9.6)	38.95±41.42		7.43±8.54		56.05±30.79		28.70±29.55	
Tumor site									
Colon	54 (51.9)	35.08±34.57	0.726	16.60±22.83	0.097	51.15±37.46	0.532	53.57±33.07	0.892
Rectum	50 (48.1)	37.57±37.69		10.20±15.06		46.66±35.49		54.46±34.09	
Tumor size, cm									
<5	38 (36.5)	42.41±37.19	0.188	13.56±19.01	0.989	54.69±36.71	0.227	51.61±35.91	0.582
≥5	66 (63.5)	32.74±35.02		13.50±20.17		45.70±36.12		55.38±32.07	
BMI, kg/m^2									
≤25	80 (76.9)	38.77±36.25	0.198	13.34±19.94	0.863	49.24±35.92	0.899	53.15±33.62	0.638
>25	24 (23.1)	27.96±34.35		14.14±19.08		48.16±38.82		56.84±33.22	

[a]P-value calculated by paired t-test; [b]P-value calculated by one-way analysis of variance test; [c]P<0.05. miR, microRNA.

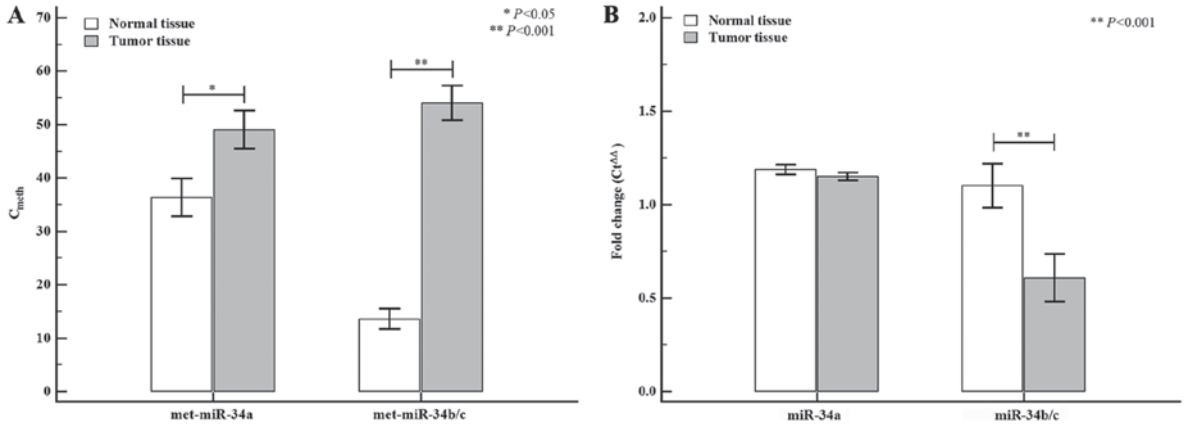

Figure 2. Methylation and expression levels of the miR-34 family between normal and tumor tissues. (A) The methylation status (C_{meth}) of miR-34 miRNAs. The miR-34a methylation status was significantly different in normal and tumor tissues (P=0.012). In addition, the methylation of miR-34b/c was lower in normal tissues (13.08±1.94) than in tumor tissues (55.63±3.35). (B) Of the different miR-34 family, only miR-34b/c exhibited significantly different expression between normal and tumor tissues (P=0.005). *P<0.05 and **P<0.01, as indicated. miR/miRNA, microRNA; met, methylated.

methylation levels than the normal tissue, whereas differences in miR-34 miRNA expression levels between normal and tumor tissues were only detected for miR-34b/c (normal tissue: 1.10±1.19 vs. tumor tissue: 0.60±1.56; P=0.005). The correlation of the methylation status of miR-34 miRNAs between colon and rectal tumors is shown in Fig. 3. When the tumor tissue was stratified by tumor location (colon or rectum), the methylation level of miR-34a was higher in colon tumors (normal tissue: 35.66±4.78 vs. tumor tissue: 51.30±5.12; P=0.041) than in rectal tumors (normal tissue: 35.39±5.32 vs. tumor tissue: 45.24±5.10; P=0.141), but the differences were not statistically significant. However, the methylation level of miR-34b/c in colon tumors was significantly higher (normal tissue: 16.48±3.12 vs. tumor tissue: 55.34±4.63;

Table II. TP53 and miR-34b/c genotype frequencies and miR-34 methylation status in normal and tumor tissues.

		miR-34a			miR-34b/c		
Genotypes	CRC (n=104), n (%)	Normal tissue	Tumor tissue	P-value[b]	Normal tissue	Tumor tissue	P-value[b]
TP53 72 codon							
GG	46 (44.2)	35.40±34.22	46.96±34.69	0.111	14.44±20.76	55.14±35.65	<0.001[e]
GC	42 (40.4)	31.58±36.90	43.82±38.06	0.138	14.55±21.09	54.60±32.81	<0.001[e]
CC	16 (15.4)	51.11±36.62	68.38±32.43	0.168	8.19±10.44	49.15±29.60	<0.001[e]
P-value[a]		0.177	0.062		0.502	0.820	
TP53 PIN							
A1A1	96 (92.3)	35.93±36.00	48.56±36.49	0.017[c]	13.39±20.01	53.60±33.89	<0.001[e]
A1A2	7 (6.7)	39.83±40.48	51.91±40.01	0.585	16.42±16.60	55.58±29.02	0.009[d]
A2A2	1 (1.0)	44.14	69.38		6.00	81.30	
P-value[a]		0.941	0.833		0.862	0.710	
TP53 MSP							
GG	95 (91.3)	35.60±36.05	49.00±36.44	0.012[c]	13.32±20.10	53.51±34.05	<0.001[e]
AG	8 (7.7)	43.24±38.70	46.32±40.27	0.878	16.86±15.42	56.41±26.97	0.003[d]
AA	1 (1.0)	44.14	69.38		6.00±0.00	81.30	
P-value[a]		0.829	0.839		0.827	0.698	
miR-34b/c							
TT	55 (52.9)	41.55±38.35	46.73±36.61	0.471	14.50±19.90	59.64±31.19	<0.001[e]
TC	41 (39.4)	32.15±34.12	51.61±35.64	0.014[c]	13.44±20.57	45.51±35.27	<0.001[e]
CC	8 (7.7)	21.11±20.91	51.14±42.82	0.096	7.26±12.72	58.76±33.27	0.001[e]
P-value[a]		0.208	0.801		0.627	0.112	

[a]P-value calculated by one-way analysis of variance test; [b]P-value calculated by paired t-test; [c]P<0.05; [d]P<0.01; [e]P<0.001. miR, microRNA; TP53, tumor protein 53; MSP, methylation-specific polymerase chain reaction; CRC, colorectal cancer; PIN, polymorphism within intron.

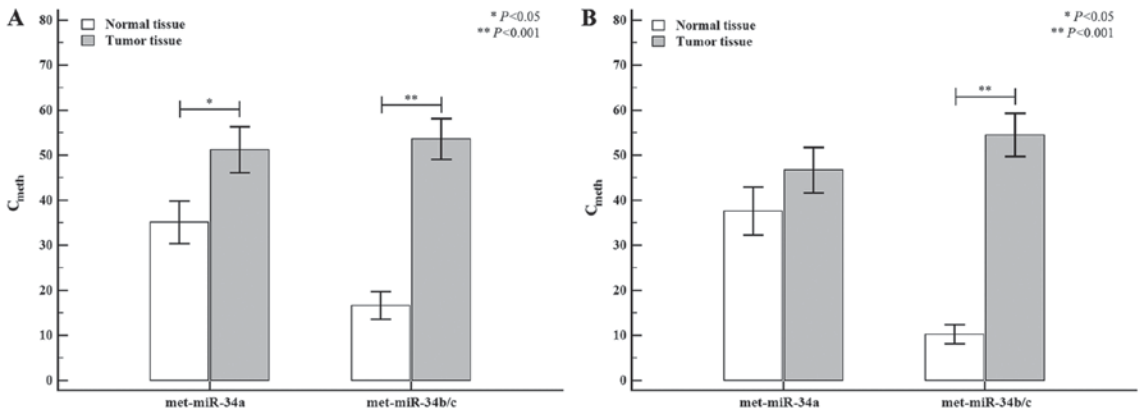

Figure 3. Differential methylation status of miR-34 family between normal and tumor tissues in the colon and rectal region. (A) The methylation status of miR-34 family in the colon. The methylation levels of miR-34a and miR-34b/c were elevated in the colon tumor tissues compared with normal tissues, and the methylation of miR-34b/c was particularly high. (B) The methylation status of miR-34b/c was increased in rectal tumor tissues when compared with normal tissues. Unlike the differences observed in the colon tumors, no differences were found in the miR-34a methylation levels between normal and tumor tissues. *P<0.05 and **P<0.01, as indicated. miR/miRNA, microRNA; met, methylated.

P<0.0001) than the corresponding methylation levels observed in rectal tumors (normal tissue: 10.07±2.23 vs. tumor tissue: 54.46±4.99; P<0.0001).

The gene polymorphism of TP53 showed a significant correlation with the methylation of the miR-34a and -34b/c promoter region in the analysis of the correlation between the polymorphisms and DNA methylation levels (data not shown). Therefore, we were performed that analysis of variance for the methylation amount of miR-34a and miR-34b/c, the results of the TP53 and mir-34bc polymorphism frequency analysis are shown in Table II. No statistically significant differences in genotype frequencies were found when the normal and tumor tissues were compared. However, the methylation levels of the miR-34 miRNAs were significantly increased in the tumor

Table III. *TP53* and *miR-34b/c* genotype frequencies and miR-34 family expression levels between normal and tumor tissue.

		miR-34a			miR-34b/c		
Genotypes	CRC (n=104)	Normal tissue	Tumor tissue	P-value[b]	Normal tissue	Tumor tissue	P-value[b]
TP53 72 codon							
GG	46 (44.2)	1.16±0.24	1.14±0.19	0.801	0.92±1.13	0.68±1.50	0.386
GC	42 (40.4)	1.22±0.30	1.14±0.22	0.201	1.09±1.18	0.73±1.03	0.150
CC	16 (15.4)	1.19±0.18	1.19±0.25	0.992	1.64±1.35	0.14±0.99	0.001[d]
P-value[a]		0.546	0.749		0.117	0.259	
TP53 PIN							
A1A1	96 (92.3)	1.19±0.27	1.16±0.22	0.336	1.08±1.22	0.63±1.29	0.016[c]
A1A2	7 (6.7)	1.05±0.04	1.06±0.08	0.747	1.38±1.05	0.46±0.75	0.080
A2A2	1 (1.0)	1.39	1.01		0.94	-0.62	
P-value[a]		0.258	0.394		0.807	0.586	
TP53 MSP							
GG	95 (91.3)	1.19±0.26	1.16±0.22	0.436	1.09±1.22	0.62±1.29	0.011[c]
AG	8 (7.7)	1.13±0.23	1.06±0.07	0.430	1.21±1.08	0.66±0.89	0.280
AA	1 (1.0)	1.39	1.01		0.94	-0.62	
P-value[a]		0.594	0.345		0.956	0.622	
miR-34b/c							
TT	55 (52.9)	1.19±0.26	1.18±0.25	0.883	1.24±1.36	0.74±0.95	0.032[c]
TC	41 (39.4)	1.18±0.26	1.10±0.15	0.125	0.96±0.95	0.44±1.66	0.089
CC	8 (7.7)	1.22±0.24	1.19±0.21	0.836	0.84±1.12	0.51±0.75	0.499
P-value[a]		0.948	0.241		0.435	0.532	

[a]P-value calculated by one-way analysis of variance test; [b]P-value calculated by paired t-test; [c]P<0.05; [d]P<0.001. miR, microRNA; TP53, tumor protein 53; MSP, methylation-specific polymerase chain reaction; CRC, colorectal cancer; PIN, polymorphism within intron.

tissues compared with normal tissue. The miR-34a methylation levels in the TP53 PIN A1A1 (48.56±36.49), TP53 MSP GG (49.00±36.44), and miR-34b/c TC (51.61±35.64) genotypes were increased in the tumor tissue compared with normal tissue; however, the methylation levels of the miR-34 miRNAs were not significantly different between the genotypes in each polymorphism. Interestingly, the miR-34b/c methylation levels were significantly elevated in tumor tissue compared with normal tissue across all of the polymorphisms, with the miR-34b/c GG genotype (59.64±31.19; P<0.001) exhibiting the highest degree of methylation (Table II).

We confirmed the expression of miRNAs by genetic polymorphism, unfortunately, we did not find any significant effect on polymorphisms (Table III). However, when the miRNA expression levels analyzed according to haplotype of TP53 polymorphisms, we could confirm difference for the expression of miR-34a and miR-34b/c (Table IV). In particular, the expression pattern of miR-34b/c was increased in TP53 PIN/MSP haplotypes, which were confirmed in both normal and tumor tissues. In addition, this haplotype also confirmed the increased expression of miR-34a in tumor tissues.

Discussion

CRC is an age-related disease and a multistage process involving both genetic and epigenetic changes (43). Recently, several miRNA profiling studies demonstrated that miRNAs are distinctively and differentially expressed in cancer tissues compared with normal adjacent tissues (44). Hence, epigenetic mechanisms appear to influence the deregulation of miRNAs in cancer pathogenesis (4).

The miR-34 miRNA family is comprised of miR-34a, miR-34b, and miR-34c, which are encoded by two different genes. Although miR-34a is encoded by its own transcript in chr.1p36.23, miR-34b and miR-34c share a common primary transcript in chr.11q23. The members of the miR-34 family are reportedly direct targets of TP53, which induces apoptosis, cell cycle arrest, and senescence, indicating a potential tumor suppressor role of the miR-34 miRNAs (6,13). In particular, miR-34 miRNAs enable TP53 to regulate numerous proteins, despite the prior synthesis of their transcripts (6). As well, several studies have shown that miR-34 miRNAs are silenced by CpG methylation (45,46).

According to prior *in vitro* studies, miR-34a overexpression causes the decreased proliferation and activation of apoptosis in multiple tumor cells, indicating that miR-34a could play a role in tumor suppression. In addition, miR-34a is downregulated in several tumor types (7,17,19,47-49). To date, over 77 miR-34 targets have been validated, including factors that control the cell cycle (CDK4, CDK6, c-Myc, and E2F3), regulators of apoptosis (BCL2, survivin, and CREB), proteins involved in invasion (c-Met, AXL receptor, and the RAS-oncogene homolog RRAS), factors related to epithelial mesenchymal transition (EMT-inducing transcription factor

Table IV. Associations between miR-34 family expression levels and the TP53 haplotype in normal and tumor tissues.

	miR-34a expression			miR-34b/c expression		
Allele combination model	Normal tissue	Tumor tissue	P-value[b]	Normal tissue	Tumor tissue	P-value[b]
TP53 codon 72/PIN/MSP						
G-A1-G	1.36±1.88	2.29±3.42	0.015[c]	2.53±3.62	2.56±5.35	0.963
G-A1-A	2.30±2.24	4.96±5.34	0.288	6.32±6.84	11.45±15.95	0.485
C-A1-G	1.25±2.13	2.17±2.94	0.069	2.88±3.60	3.41±7.10	0.639
C-A2-G	2.25±2.50	5.22±5.93	0.331	6.25±7.64	10.71±17.71	0.619
P-value[a]	0.479	0.075		0.031[c]	0.003[d]	
TP53 codon 72/PIN						
G-A1	1.41±1.90	2.43±3.56	0.008[d]	2.75±3.93	3.09±6.64	0.664
C-A1	1.25±2.13	2.17±2.94	0.069	2.88±3.60	3.41±7.10	0.639
C-A2	1.87±2.14	4.43±5.02	0.239	4.97±6.61	7.74±15.33	0.669
P-value[a]	0.711	0.270		0.362	0.269	
TP53 codon 72/MSP						
G-G	1.36±1.88	2.29±3.42	0.015[c]	2.53±3.62	2.56±5.35	0.963
G-A	2.30±2.24	4.96±5.34	0.288	6.32±6.84	11.45±15.95	0.485
C-G	1.33±2.16	2.44±3.33	0.038[c]	3.19±4.13	4.08±8.57	0.491
P-value[a]	0.694	0.338		0.124	0.024[c]	
TP53 PIN/MSP						
A1-G	1.32±1.96	2.25±3.26	0.002[d]	2.65±3.60	2.85±6.00	0.727
A1-A	2.30±2.24	4.96±5.34	0.288	6.32±6.84	11.45±15.95	0.485
A2-G	2.25±2.50	5.22±5.93	0.331	6.25±7.64	10.71±17.71	0.619
P-value[a]	0.304	0.032[c]		0.013[c]	0.001[e]	

[a]P-value calculated by one-way analysis of variance test; [b]P-value calculated by paired t-test; [c]P<0.05; [d]P<0.01; [e]P<0.001. miR, microRNA; TP53, tumor protein 53; MSP, methylation-specific polymerase chain reaction; PIN, polymorphism within intron.

SNAIL or the zinc finger 281 protein), proteins involved in the formation of cancer stem cells (Notch1-4, WNT1, WNT3, β-catenin, and CD44), and factors that regulate metabolism (hexokinase 1 and 2, glucose-6-phosphate isomerase, pyruvate dehydrogenase kinase 1, and lactate dehydrogenase A) (50,51). Consequently, the diverse roles of members of the miR-34 family may lead to functional abnormalities such as Wnt signaling, EMT, G1-arrest, or cancer cell progression, leading to carcinogenesis (50). To this end, recent reports suggest that aberrant miR-34b/c promoter methylation is significantly correlated with the metastasis of tumor cells to the lymph nodes (25,49). Finally, miR-34b and miR-34c are directly regulated by promoter hypermethylation and p53 in response to DNA damage or oncogenic stress (14-16,25,52). In fact, p53 regulates the expression of miR-34 via p53 binding sites within miR-34a and miR-34b/c promoter regions. Consequently, the antioncogenic action of miR-34 is regulated by p53.

In a previous study (53), we analyzed the association of SNPs of miR-34b/c and TP53 Arg72Pro with the risk of colon cancer. The previous study presented the association with risk of colon cancer to the TP53 Arg72Pro CC genotype (53), and we considered that this association may be related to the regulation of the miR-34 family expression or methylation in cancer tissues. Therefore, in this study, we showed that the methylation or expression of miR-34 miRNAs and the affected TP53 polymorphisms differ between colorectal tumors and normal tissues. Specifically, the methylation status of miR-34a and miR-34b/c was increased in tumor tissues compared with the normal tissue. As well, we found that miR-34 miRNAs are downregulated in tumor tissues compared with paired normal tissues and that this apparent downregulation is associated with increased methylation of miR-34 miRNAs in colorectal tissues. Furthermore, the haplotypes of TP53 polymorphisms (codon 72-PIN) were shown to influence miR-34b/c expression level in normal and cancer tissues. Interestingly, this is the first report of association with expression and methylation by the SNPs, and this results may be applicated for base data that functional research of CRC. Although it has been established that p53 functions as a tumor suppressor, reports also indicate an emerging role of p53 as an important regulator of metabolic homeostasis, a critical aspect of most major cellular processes (54,55). The roles of p53 are fundamental for cell homeostasis and include metabolic homeostasis, which safeguards against latent cancer, as well as its more classical roles that include genome protection, DNA repair, and programmed cell death (56). Critical mutations in the *TP53* gene are common in most cancers and are major contributors to cancer progression. Previous studies have likewise indicated the regulation of the *TP53* gene by *TP53* haplotypes (50,51,57). In particular, studies have shown that the *TP53* haplotype containing the codon 72-PIN polymorphism affected *TP53* function through

the formation of various haplotypes (50,51), and that *TP53* polymorphisms were presented to linkage disequilibrium (LD) blocks, which were formed many haplotypes and affect to *TP53* abnormality (57). In our previous study, we reported an association between CRC risk and *TP53* polymorphisms combination model (53). Furthermore, the *TP53* haplotypes containing polymorphisms (e.g., codon 72-PIN) influenced miR-34b/c expression levels in normal and cancer tissues, suggesting altered regulation of *TP53*. Interestingly, this is the first report of an association between SNP expression and methylation; thus, these results may be applicable to and serve as the foundation for future CRC research.

In summary, we determined that the hypermethylation of miR-34a and miR-34b/c is a relatively common event in CRC, and that the methylation status affects miR-34 expression. We likewise found that promoter methylation in miR-34 miRNAs and polymorphisms in the TP53 codon 72 are a relatively common event associated with CRC risk. Our study had several limitations. First, all patients were selected from a single institution in Korea and the small sample size could limit the statistical power. Second, further studies are needed to define the relationship between DNA hypermethylation and the pathogenesis of CRC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JWK, KK and NKK conceived and designed the experiments. KHL, JOK, HSP, CSR, JYL and DK performed the experiments. HHJ, JOK, HSP, CSR and JYL analyzed the data and performed statistical analyses. HHJ, KK, KHL, DK and NKK contributed reagents/material/analysis tools. HHJ and KK wrote the manuscript. JWK and NKK prepared the references and managed the data. All authors reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of CHA Bundang Medical Center (Seongnam, Republic of Korea; IRB no. 2009-08-077-010) and written informed consent was provided by all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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