Prognostic significance of microRNA gene polymorphisms in patients with surgically resected colorectal cancer

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Abstract. MicroRNAs (miRNAs) are small 19- to 22-nucleotide sequences of RNA that participate in the regulation of cell differentiation, cell cycle progression and apoptosis. Although single-nucleotide polymorphisms (SNPs) in miRNA regions are considered unlikely to be functionally important, nucleotide variations within the sequences of primary (pri)- or precursor (pre)-miRNAs may affect miRNA processing and ultimately result in the modification of miRNA expression. The aim of this study was to investigate associations between four SNPs in pre-miRNA genes and the survival of colorectal cancer patients. A total of 407 colorectal patients were consecutively enrolled. DNA was extracted from blood specimens, and the *hsa-mir-146a*C>G, *hsa-mir-149*C>T, *hsa-mir-196a2*C>T and *hsa-mir-499*A>G polymorphisms were genotyped by PCR-RFLP. We were unable to identify independent prognostic SNPs for colorectal cancer. However, the heterozygous TC genotype of the *196a2*C>T polymorphism was a significant risk factor for the overall survival of rectal cancer patients (HR=3.554, 95% CI 1.296-9.747, p=0.014). Further large-population studies are warranted to define the *196a2*C>T polymorphism as a prognostic factor of rectal cancer.

Introduction

Colorectal cancer is the third most frequently diagnosed cancer and the second leading cause of cancer-related death in Western countries (1). The prognosis of colorectal cancer patients depends on the tumor stage at the time of diagnosis. However, over 57% have regional or distant spread of tumor cells at the time of diagnosis (2). Therefore, it is a significant public health problem worldwide, with specific mortality rates approaching 33% in developed countries (3). In colorectal carcinogenesis, the molecular basis and genetic changes result in a specific phenotype often associated with varying tumor behaviors relevant to the prognosis and the response to specific therapies. As a result, colorectal cancer no longer refers to a single disease, but a heterogeneous group of diseases caused by a differential genetic/epigenetic background. In this respect, many ongoing studies are aimed at assessing biomarkers as potential predictors of prognosis or response to therapy, which will most likely lead to individualized management for patients.

RNA has long been thought to play a relatively passive role in carcinogenesis. However, increasing interest in non-coding genomic sequences has revealed the unappreciated involvement of several classes of non-coding RNA (ncRNA), including antisense RNA (4), small nucleolar RNA (5) and microRNA (miRNA) in carcinogenesis. Among these ncRNAs, many researchers have focused on miRNA due to its frequent dysregulation in cancer. Several studies have revealed that miRNAs, short non-coding RNAs that hybridize to their target mRNAs and repress the expression of encoded proteins, are involved in biological processes, such as cellular differentiation, proliferation, apoptosis and metastasis, through their interactions with intracellular signaling networks (6). miRNAs play critical roles in carcinogenesis by regulating the expression of proto-oncogenes or tumor-suppressor genes (7,8).

The precise mechanisms regulating miRNA expression have not been elucidated, but previous studies strongly support an association between altered miRNA expression and cancer (7,9). Several mechanisms, including gene amplification, deletion, epigenetic alterations and single-nucleotide substitution, have been implicated in altered miRNA expression (10,11). Although single-nucleotide polymorphisms (SNPs) in miRNA regions are considered unlikely to be functionally important (12), nucleotide variations within the sequences of primary (pri)- or precursor (pre)-miRNAs may affect miRNA processing and ultimately result in the modification of miRNA expression (13). Previous studies have reported that nucleotide variations within the seed sequence of the miRNA may affect miRNA processing and lead to reduced miRNA expression (10,13).

However, there were few associations identified for SNPs in pre-miRNA with prognostic significance in colorectal cancer patients (14-16). In the present study, we investigated

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whether polymorphisms in pre-miRNA genes influence the outcome of patients with surgically resected colorectal cancer. Common (i.e., minor allele frequency >0.05) SNPs located in pre-miRNA and their surrounding regions were surveyed using *in silico* approaches in the public database, miRBase (17). We then selected four pre-miRNA SNPs (*hsa-mir-146a* rs2910164 C→G, *hsa-mir-196a2* rs11614913 C→T, *hsa-mir-499* rs3746444 G→A and *hsa-mir-149* rs2292832 C→T) and evaluated their prognostic significance in patients with surgically resected colorectal cancer.

Materials and methods

Study population. Between June 1996 and January 2009, blood samples were collected from 446 consecutive Korean patients who had undergone surgical resection at Bundang CHA Medical Center (Seongnam, Korea). The study only included patients who had undergone surgical resection with a curative intent and who had histologically proven adenocarcinomas [407 patients (91.3% of all colorectal cancer patients)]. The American Joint Committee on Cancer: Classification and Stage Groupings 6th edition was used for tumor assessment. We retrospectively obtained information concerning the date of diagnosis, pathological stage, relapse and death. All study subjects provided written consent and were all ethnic Koreans. The study protocol was approved by the Institutional Review Board of Bundang CHA Medical Center, Seongnam, Republic of Korea.

Genotyping. DNA was extracted from leukocytes using a G-DEX™ II Genomic DNA Extraction kit (Intron Biotechnology, Seongnam, Korea), according to the manufacturer's instructions. The *hsa-mir-146a*C>G, *hsa-mir-149*C>T, *hsa-mir-196a2*C>T and *hsa-mir-499*A>G polymorphisms were analyzed by PCR-RFLP assays. Primer sequences for amplification were *hsa-mir-146a*C>G, forward 5'-CAT GGG TTG TGT CAG TGT CAG AGC T-3' and reverse 5'-TGC CTT CTG TCT CCA GTC TTC CAA-3'; *hsa-mir-149*C>T, forward 5'-TGT CTT CAC TCC CGT GCT TGT CC-3' and reverse 5'-TGA GGC CCG AAA CAC CCG TA-3'; *hsa-mir-196a2*C>T, forward 5'-CCC CTT CCC TTC TCC TCC AGA TA-3' and reverse 5'-CGA AAA CCG ACT GAT GTA ACT CCG-3', and *hsa-mir-499*A>G, forward 5'-CAA AGT CTT CAC TTC CCT GCC A-3' and reverse 5'-GAT GTT TAA CTC CTC TCC ACG TGA TC-3' [contains a mismatch sequence (underlined)]. The annealing temperature was 58˚C. The *hsa-mir-146a*C>G, *hsa-mir-149*C>T and the *hsa-mir-196a2*C>T polymorphisms were digested at 37˚C for 16 h with *Sac*I, *Alu*I and *Msp*I, respectively, and *499*A>G polymorphism was digested at 50˚C for 16 h with *Bcl*I (New England BioLabs, Beverly, MA, USA). The reaction product (12 μl) was run on a 3.0% agarose gel, stained with ethidium bromide and directly visualized under ultraviolet illumination. Approximately 10% of the PCR reactions for the four miRNA polymorphisms were randomly repeated, and the results were checked for concordance with DNA sequencing using an automatic sequencer (ABI3730xl DNA Analyzer; Applied Biosystems, Foster City, CA, USA). The concordance of the quality control samples was 100%.

Statistical analysis. The genotypes for each SNP were analyzed as a three group categorical variable (reference model) and were also grouped according to the dominant and recessive model. The genotype specific risks were estimated as odds ratios (OR) with associated 95% confidence intervals (95% CI) by logistic regression analysis and were adjusted for age, gender, differentiation, tumor site, chemotherapy and stage. The overall survival (OS) and relapse-free survival (RFS) were compared using the Kaplan-Meier method, and the potential variables were verified by multivariate analysis using a Cox regression model. All tests were two-tailed, and a p-value of <0.05 was taken to indicate a significant difference. All statistical calculations were performed using SPSS software (ver. 17; SPSS Inc., Chicago, IL, USA).

Table I. Baseline characteristics of study subjects (n=407).

Characteristics	n (%)
Age, mean±SD in years	61.4±12.4
Gender, male/female	227/180
Primary tumor site	
Colon	238 (58.5)
Rectum	169 (41.5)
Histological differentiation	
Well	31 (7.6)
Moderate	353 (86.7)
Poor	23 (5.7)
Stage	
0/I	38 (9.3)
II	180 (44.2)
III	149 (36.6)
IV	40 (9.8)
Adjuvant chemotherapy	
No	59 (14.5)
Yes	348 (85.5)
Relapse-free survival rate (4-year)	78.0
Overall survival rate (4-year)	79.1

Results

Patient characteristics. The 407 patients were comprised of 227 men and 180 women, and the mean age was 61.4 years (SD=12.4). Two hundred and thirty-eight patients (58.5%) had colon cancer and 169 patients (70.7%) had rectal cancer. Regarding histological differentiation, 353 patients (86.7%) had moderately differentiated cancer, 31 patients (7.6%) had well-differentiated cancer and 23 patients (5.7%) had poorly differentiated cancer. Pathological staging after curative resection was as follows: stage I for 38 (9.3%), stage II for 188 (44.2%), stage III for 149 (36.6%) and stage IV for 40 (9.8%) patients. Three hundred and forty-eight (85.5%) patients received adjuvant chemotherapy, while 59 (14.5%) patients had non-adjuvant chemotherapy. At the median follow-up duration of 41 months (range 4-182), the estimated 4-year OS and RFS rates for all patients were 79.1 and 78.0%, respectively. A summary of the baseline characteristics of the study subjects is shown in Table I.

Table II. Survival rate according to genotype in colorectal cancer.

Genotype	OS			RFS		
	n (%)	Adjusted HR[a] (95% CI)	p-value	n (%)	Adjusted HR[a] (95% CI)	p-value
*hsa-mir-146a*C>G						
CC	137 (33.7)	1.000 (Ref.)		135 (34.5)	1.000 (Ref.)	
GC	217 (53.3)	1.203 (0.746-1.939)	0.452	205 (52.5)	1.128 (0.702-1.813)	0.621
GG	53 (13.0)	0.854 (0.384-1.899)	0.701	51 (13.0)	1.494 (0.752-2.970)	0.255
CC	137 (33.7)	1.000 (Ref.)		135 (34.5)	1.000 (Ref.)	
GC/GG	270 (66.3)	1.141 (0.715-1.822)	0.582	256 (65.5)	1.186 (0.751-1.874)	0.466
CC/GC	354 (87.0)	1.000 (Ref.)		340 (87.0)	1.000 (Ref.)	
GG	53 (13.0)	0.757 (0.365-1.571)	0.457	51 (13.0)	1.386 (0.748-2.568)	0.302
*hsa-mir-149*C>T						
TT	194 (47.7)	1.000 (Ref.)		185 (47.3)	1.000 (Ref.)	
TC	168 (41.2)	1.008 (0.640-1.588)	0.973	163 (41.7)	1.201 (0.759-1.900)	0.438
CC	45 (11.1)	0.990 (0.475-2.062)	0.978	43 (11.0)	0.903 (0.427-1.908)	0.790
TT	194 (49.7)	1.000 (Ref.)		185 (47.3)	1.000 (Ref.)	
TC/CC	213 (52.3)	1.004 (0.654-1.542)	0.985	206 (52.7)	1.134 (0.731-1.761)	0.576
TT/TC	362 (88.9)	1.000 (Ref.)		348 (89.0)	1.000 (Ref.)	
CC	45 (11.1)	0.986 (0.487-1.995)	0.969	43 (11.0)	0.822 (0.405-1.665)	0.588
*hsa-mir-196a2*C>T						
TT	111 (27.3)	1.000 (Ref.)		107 (27.4)	1.000 (Ref.)	
TC	195 (47.9)	1.080 (0.631-1.850)	0.780	189 (48.3)	0.763 (0.445-1.311)	0.330
CC	101 (24.8)	1.070 (0.574-1.993)	0.832	95 (24.3)	0.998 (0.555-1.794)	0.995
TT	111 (27.3)	1.000 (Ref.)		107 (27.4)	1.000 (Ref.)	
TC/CC	296 (72.7)	1.077 (0.649-1.788)	0.776	284 (72.6)	0.845 (0.514-1.387)	0.507
TT/TC	306 (75.2)	1.000 (Ref.)		296 (75.7)	1.000 (Ref.)	
CC	101 (24.8)	1.017 (0.611-1.692)	0.948	95 (24.3)	1.187 (0.732-1.923)	0.490
*hsa-mir-499*A>G						
AA	259 (63.6)	1.000 (Ref.)		250 (63.9)	1.000 (Ref.)	
AG	137 (33.7)	1.020 (0.641-1.624)	0.933	130 (33.3)	0.939 (0.576-1.531)	0.801
GG	11 (2.7)	1.135 (0.275-4.682)	0.862	11 (2.8)	1.707 (0.524-5.562)	0.377
AA	259 (63.6)	1.000 (Ref.)		250 (63.9)	1.000 (Ref.)	
AG/GG	148 (36.4)	1.027 (0.652-1.619)	0.909	141 (36.1)	0.983 (0.613-1.578)	0.944
AA/AG	396 (97.3)	1.000 (Ref.)		380 (97.2)	1.000 (Ref.)	
GG	11 (2.7)	1.127 (0.276-4.600)	0.869	11 (2.8)	1.752 (0.547-5.616)	0.348

OS, overall survival; RFS, relapse-free survival; HR, hazard ratio. [a]Percentage of patients adjusted for age, gender, tumor site, differentiation, chemotherapy and stage.

Survival rate according to genotype in colorectal cancer. The four pre-miRNA SNPs of the pre-miRNA-related genes were successfully amplified in all patients. The genotype frequencies of the miRNA polymorphisms also conformed to Hardy-Weinberg equilibrium (p>0.05). The associations between the genotypes of the four pre-miRNA SNPs in pre-miRNAs and the survival of all colorectal cancer patients are shown in Table II. In the univariate analysis of the OS, the four SNPs were not shown to have a significant effect on the 4-year OS. The multivariate Cox proportional analysis produced the same results. When we analyzed the OS by the dominant and recessive model, there were no significant associations of OS with the four pre-miRNA SNPs. In the multivariate Cox proportional analysis of the RFS, none of the four pre-miRNA SNPs were shown to have a significant effect on RFS. When we analyzed the RFS by the dominant and recessive model, there was no significant association of RFS with the four pre-miRNA SNPs.

Survival rate according to genotype in rectal cancer. Table III shows the association between survival of rectal cancer patients and the four pre-miRNA SNPs. No significant association of OS and RFS with the *hsa-mir-146a*, *hsa-mir-149* or *hsa-mir-499* was detected. However, *hsa-mir-196a2* TC was associated with a significantly unfavorable OS rate (HR=3.554, 95% CI 1.296-9.747, p=0.014). Also, when the OS was analyzed by the dominant model (TT vs. TC+CC), there were significant associations of unfavorable OS with the *hsa-mir-196a2* C allele (HR=3.093, 95% CI 1.139-8.399, p=0.028). In the multivariate Cox proportional analysis of the RFS, none of the four pre-miRNA SNPs were shown to have a significant effect on RFS.

Discussion

In the present study, we investigated whether four pre-miRNA SNPs are related to the prognosis of colorectal cancer in

Table III. Survival rate according to genotype in rectal cancer.

Genotype	OS			RFS		
	n (%)	Adjusted HR[a] (95% CI)	p-value	n (%)	Adjusted HR[a] (95% CI)	p-value
*hsa-mir-146a*C>G						
CC	62 (36.7)	1.000 (Ref.)		61 (37.9)	1.000 (Ref.)	
GC	84 (49.7)	1.585 (0.718-3.501)	0.257	78 (48.4)	1.139 (0.558-2.324)	0.723
GG	23 (13.6)	0.638 (0.171-2.378)	0.506	22 (13.7)	0.799 (0.218-2.924)	0.735
CC	62 (36.7)	1.000 (Ref.)		61 (37.9)	1.000 (Ref.)	
GC/GG	107 (63.3)	1.337 (0.618-2.893)	0.463	100 (62.1)	1.089 (0.540-2.196)	0.813
CC/GC	146 (86.4)	1.000 (Ref.)		139 (86.3)	1.000 (Ref.)	
GG	23 (13.6)	0.476 (0.144-1.572)	0.226	22 (13.7)	0.733 (0.220-2.446)	0.615
*hsa-mir-149*C>T						
TT	82 (48.5)	1.000 (Ref.)		77 (47.8)	1.000 (Ref.)	
TC	65 (38.5)	1.094 (0.542-2.210)	0.803	63 (39.1)	1.136 (0.545-2.370)	0.735
CC	22 (13.0)	0.640 (0.212-1.930)	0.430	21 (13.0)	0.664 (0.234-1.885)	0.444
TT	82 (48.5)	1.000 (Ref.)		77 (47.8)	1.000 (Ref.)	
TC/CC	87 (51.5)	0.951 (0.491-1.840)	0.881	84 (52.2)	0.977 (0.486-1.964)	0.949
TT/TC	147 (87.0)	1.000 (Ref.)		140 (87.0)	1.000 (Ref.)	
CC	22 (13.0)	0.615 (0.213-1.777)	0.372	21 (13.0)	0.618 (0.237-1.615)	0.329
*hsa-mir-196a2*C>T						
TT	41 (24.3)	1.000 (Ref.)		39 (24.2)	1.000 (Ref.)	
TC	81 (47.9)	3.554 (1.296-9.747)	0.014	78 (48.5)	1.879 (0.767-4.603)	0.170
CC	47 (27.8)	2.089 (0.642-6.798)	0.224	44 (27.3)	0.997 (0.345-2.885)	0.996
TT	41 (24.3)	1.000 (Ref.)		39 (24.2)	1.000 (Ref.)	
TC/CC	128 (75.7)	3.093 (1.139-8.399)	0.028	122 (75.8)	1.558 (0.647-3.749)	0.325
TT/TC	122 (72.2)	1.000 (Ref.)		117 (72.7)	1.000 (Ref.)	
CC	47 (27.8)	0.780 (0.347-1.750)	0.548	44 (27.3)	0.620 (0.280-1.372)	0.241
*hsa-mir- 499*A>G						
AA	102 (60.4)	1.000 (Ref.)		98 (60.9)	1.000 (Ref.)	
AG	62 (36.6)	0.829 (0.409-1.680)	0.605	58 (36.0)	0.722 (0.345-1.513)	0.391
GG	5 (3.0)	1.206 (0.159-9.152)	0.857	5 (3.1)	1.085 (0.142-8.266)	0.938
AA	102 (60.4)	1.000 (Ref.)		98 (60.9)	1.000 (Ref.)	
AG/GG	67 (39.6)	0.853 (0.430-1.691)	0.650	63 (39.1)	0.744 (0.364-1.524)	0.422
AA/AG	164 (97.0)	1.000 (Ref.)		156 (96.9)	1.000 (Ref.)	
GG	5 (3.0)	1.268 (0.168-9.553)	0.819	5 (3.1)	1.208 (0.160-9.100)	0.855

OS, overall survival; RFS, relapse-free survival; HR, hazard ratio. [a]Percentage of patients adjusted for age, gender, tumor site, differentiation, chemotherapy and stage.

407 colorectal cancer patients whose tumors had been surgically resected with a curative intent. The result showed that the *hsa-mir-146a*C>G, *hsa-mir-149*C>T, *hsa-mir-196a2*C>T and *hsa-mir-499*A>G polymorphisms were not associated with the prognosis of surgically resected colorectal cancer in the Korean population. However, in the subset analysis by tumor site, we found that the heterozygous TC genotype of the *196a2*C>T polymorphism was a significant risk factor for 4-year OS, but not for RFS. To our knowledge, this is the first study to provide evidence that the *196a2*C>T polymorphism is associated with the prognosis of rectal cancer.

Polymorphisms in miRNA-related genes may alter the expression levels of mature miRNAs, with consequences on the regulation of target genes that affect cancer risk or prognosis. Although it is not possible to define the exact mechanisms of these effects, multiple mechanisms, including cell proliferation, differentiation, apoptosis and drug resistance, are involved in the regulation of mature miRNA expression. In a previous study, Hu *et al* (15) reported that survival was significantly decreased in 663 patients with non-small cell lung cancer who were homozygous for *CC* genotype in *hsa-mir-196a2*. Also, in the genotype-phenotype correlation analysis of 23 human lung cancer tissue samples, homozygous *CC* genotype was associated with a statistically significant increase in mature *hsa-mir-196a* expression, but not with changes in the levels of the precursor, suggesting enhanced processing of the pre-miRNA to its mature form. However, this association was not found for the other SNPs, including *hsa-mir-146a*C>G, *hsa-mir-149*C>T and *hsa-mir-499*A>G.

In colorectal cancer, recent studies have shown an association between SNPs in miRNA-related genes and carcinogenesis, treatment response and prognosis. Landi *et al* (18) found that two SNPs (rs17281995 in *CD86* and rs1051690 in *INSR*) were associated with the risk of colorectal cancer in

a Czech population. Moreover, similar results were shown in a pooled analysis of the Czech and Spanish populations (19). Polymorphisms in miRNA genes were found to be correlated with treatment response in colon cancer patients. SNPs rs7372209 and rs1834306 in pri-miR26a-1 and pri-miR-100, respectively, were correlated with tumor response in metastatic colon cancer patients treated with 5-fluorouracil and irinotecan (20). Moreover, a let-7 miRNA-binding site polymorphism in the 3'-untranslated region of the KRAS gene was also found to be positively related to cetuximab responsiveness in wild-type KRAS patients with metastatic colorectal cancer (21,22). Recently, Lee *et al* (16) investigated the association between survival and 40 polymorphisms of miRNA-related genes in 427 Koreans. This was the first study to evaluate the role of miRNA SNPs in the clinical outcomes of colorectal cancer patients. However, none of the 40 miRNA-related gene SNPs were found to be an independent prognostic marker. The results of the present study are consistent with those results.

Recent studies have reported that the *hsa-mir-196a2* C allele is associated with increased risk of various types of cancers. Hoffman *et al* (23) and Hu *et al* (24) reported that the C allele is significantly associated with increased breast cancer risk. Also, Tian *et al* (25) reported that the *hsa-mir-196a2* CC genotype is significantly associated with increased lung cancer risk in Chinese individuals. Furthermore, other epidemiological studies have reported the association of the C allele with increased risk of congenital heart disease (26), hepatocellular carcinoma (27) and gastric cancer (28). To our knowledge, there have been only two studies that have investigated the prognostic impact of the *hsa-mir-196a2* polymorphism in colorectal cancer (14,16). Consistent with our result, these studies also failed to identify any significance of the *hsa-mir-196a2* C polymorphism as a prognostic biomarker. Notably, in this study we found that the *hsa-mir-196a2* TC genotype was a significant risk factor for 4-year OS in rectal cancer patients. Also, in the dominant model (TT vs. TC+CC), there were significant associations of unfavorable OS with the *hsa-mir-196a2* allele (HR=3.093, 95% CI 1.139-8.399, p=0.028). However, since rectal cancer patients are a relatively small proportion of all colorectal cancer patients, we should be careful when interpreting this result. Larger population studies are warranted to define the association between the *hsa-mir-196a2* polymorphism and the prognosis of rectal cancer.

Several previous studies have shown that the altered expression of certain miRNAs is unique to colorectal cancer. In this respect, there is increased interest in the association between miRNA expression in tumors and prognosis, both with regards to treatment response rate and to survival. Although it is not possible at this time to further define the mechanisms through which these SNPs affect prognosis, several studies have reported a correlation between miRNA expression and the survival of various types of malignancies, including lung, breast, prostate cancer and acute myeloid leukemia (29-32). Particularly for colorectal cancer, miRNA-21 has been correlated with poor survival (33). Furthermore, miRNA-106, miRNA-20, miRNA-106a, miRNA-200c and miRNA-203 were identified as possible prognostic biomarkers (33,34). By contrast, Schepeler *et al* (35) showed that high expression of miRNA-320 and miRNA-498 are associated with longer progression-free survival compared to low expression. Based on this evidence, when we measure miRNA expression in combination with conventional prognostic markers, such as tumor size, metastasis, tumor invasion and differentiation grade, we may more precisely predict the prognosis of colorectal cancer patients. Further study of miRNA expression may provide valuable knowledge on new potential targets for therapy.

In conclusion, we investigated the relationship of four SNPs in pre-miRNA genes with prognosis in surgically resected colorectal cancer patients. We did not identify independent prognostic SNPs for colorectal cancer. However, we found that the heterozygous TC genotype of the *196a2*C>T polymorphism was a significant risk factor for OS in rectal cancer patients. Further large-population studies are warranted to define the *196a2*C>T polymorphism as a prognostic factor for rectal cancer.

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