Functional Polymorphism in the MicroRNA-367 Binding Site as a Prognostic Factor for Colonic Cancer

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Abstract. *Background: As microRNAs play important roles in cancer development and progression by regulating the expressions of oncogenes and tumor suppressor genes though interacting with the 3' untranslated region (UTR) of target genes, we aimed to evaluate the association between genetic variants of miRNAs and their binding sites and prognosis in patients with colorectal cancer (CRC). Materials and Methods: Three miRNA variants and four variants in the miRNA binding sites were selected based on allelic frequencies, while their potential impact has been described in previous studies. DNA was extracted from fresh-frozen tissues of 344 patients with CRC who underwent curative surgery and genotyping analyses were performed using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. Results: Among seven target variants, rs1044129 at the miR-367 binding site of calcium channel ryanodine receptor gene 3 (RYR3) was associated with relapse-free survival (RFS) for colon cancer patients as a recessive model in a univariate analysis. Moreover, a multivariate analysis revealed that patients carrying the GG genotype had poor RFS, compared to those with the AA or AG genotype (hazard ratio, HR=2.864; p=0.005), yet there was only a marginal trend for disease-specific survival (HR=2.226; p=0.087)*

regardless of patient and tumor characteristics. Conclusion: The current study suggests that the functional variant (rs1044129) in the miR-367 binding site of RYR3 may be a potential marker for prognosis in patients following curative surgery for CRC.

Colorectal cancer (CRC) is a leading cause of death and is annually responsible for more than 500,000 deaths worldwide. To date, the main prognostic factor used in clinical practice is the tumor stage, yet several molecules and genetic alterations have also been introduced as potential markers. Constitutional host-related biological features, including genetic variation, have long been suspected to explain why some patients treated for CRC experience relapse while others do not, despite their having similar baseline characteristics. For CRC, polymorphisms in the genes involved in tumor progression, apoptosis, and angiogenesis have already been extensively studied for their association with cancer susceptibility and prognosis (1-7).

MicroRNAs (miRNAs), a class of small, endogenous, non-coding RNAs, are able to regulate gene expression by translational repression or mRNA degradation of the target, thereby affecting critical functions in various physiological processes, ranging from cell proliferation to apoptosis (8, 9). Moreover, some recent studies have demonstrated a relationship between the aberrant expression of miRNAs and CRC susceptibility, prognosis, and responsiveness to treatment (10-12). In particular, most miRNAs bind to target sequences located within the 3'-untranslated region (3'UTR) of mRNAs, resulting in the cleavage of the target mRNAs or repression of their translation (13). Thus, polymorphisms residing within miRNAs or within the miRNA-binding sites of the target genes that are implicated in cancer or function as tumor suppressors or oncogenes, could contribute to carcinogenesis or progression by altering the miRNA–mRNA interaction and thereby affecting the expression of the miRNA targets, as shown in previous studies (14, 15).

Accordingly, the present study selected seven target variants of miRNAs or miRNA binding sites based on web-

based data and investigated whether those variants might be associated with the prognosis for Korean patients with CRC who underwent curative surgery.

Materials and Methods

Study population. All the tissues investigated in this study were obtained from 344 consecutive Korean patients who had undergone a curative resection between March, 2003 and August, 2006 at the Kyungpook National University Hospital (Daegu, Korea). The diagnosis and staging of CRC were assessed according to the WHO classifications (16) and TNM classifications set out by the American Joint Committee on Cancer (AJCC) (17). Written informed consent for the current study was received from all the patients before surgery, and the study was approved by the Institutional Research Board at Kyungpook National University Hospital.

Selection of polymorphisms. We selected seven previously identified single nucleotide polymorphisms (SNPs) including three variants (rs12976445, rs41275794, and rs11614913) of two miRNAs (miR-125a and miR-192a2) and four (rs1044129, rs3134615, rs4245739, and rs5186) in the 3'UTR miRNA-binding sites for four genes [*ryanodine receptor 3* (*RYR3*), *myc myelocytomatosis viral oncogene homolog 1* (*MYCL1*), mouse double minute 4 (MDM4), and *angiotensin II receptor type 1* (*AGTR1*)], whose potential cancer association has been indicated in previous studies (18-24) and which passed the selection criteria of a minor allelic frequency >0.01 based on SNP databases (HapMap data: http://hapmap.ncbi.nlm.nih.gov).

Genotyping of polymorphisms. Genomic DNA was extracted from fresh colorectal mucosal tissue at the time of surgery using a Wizard genomic DNA purification kit (Promega, Madison, WI, USA). The seven selected SNPs (rs12976445, rs41275794, rs11614913, rs1044129, rs3134615, rs4245739 and rs5186) were determined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). For quality control, the genotyping analysis was performed blind as regards the subjects. The selected PCR-amplified DNA samples (n=2 for each genotype) were also examined by DNA sequencing to confirm the genotyping results.

Statistical analysis. The SNP genotype was analyzed as a three-group categorial variable (referent model), and also grouped according to the dominant and recessive model. The Hardy–Weinberg equilibrium for the polymorphism was analyzed using a χ^2-test. For the survival analysis, the outcome measures included relapse-free survival (RFS), defined as the time to disease recurrence, and disease-specific survival (DSS), defined as the time-to-death as a result of CRC. The differences in the RFS or DSS according to the genotype were compared using log-rank tests. The Cox's proportional hazard regression model was used for the multivariate survival analyses adjusted for stage, age (≤60 *vs*. >60 years), sex, site of the primary disease (colon *vs*. rectum), carcinoembryonic antigen (CEA) level (normal *vs*. elevated), pathological differentiation (well to poor), and type of adjuvant treatment. A cut-off *p*-value of 0.05 was adopted for all statistical analyses. The statistical data were obtained using an SPSS software package (SPSS 11.5, SPSS Inc., Chicago, IL, USA).

Table I. *Patients' characteristics (n=344).*

Variables	N (%)
Mean age, years (SD)	61.1 (±10.2)
Median age, years (range)	63.0 (30-79)
Age, years	
>60	206 (59.9)
≤60	138 (40.1)
Gender	
Male	219 (63.7)
Female	125 (36.3)
Site	
Colon	182 (52.9)
Rectum	159 (46.2)
Both	3 (0.9)
Histological grade	
Well	63 (18.3)
Moderate	262 (76.2)
Poor or signet ring cell type	19 (5.5)
LVI	226 (65.7)
Elevated CEA	67 (19.5)
AJCC stage	
I	64 (18.6)
IIA	98 (28.5)
IIB	13 (3.8)
IIIA	16 (4.7)
IIIB	97 (28.2)
IIIC	56 (16.3)
Surgery	
Open	109 (31.7)
Laparoscopic	235 (68.3)
Adjuvant chemotherapy	
None	68 (19.8)
Oral 5-FU[†]	181 (52.6)
Mayo	78 (22.7)
FOLFOX	17 (4.9)
Adjuvant radiotherapy[‡]	42 (25.9)
Recurrence	74 (21.5)
Death	71 (20.7)
Disease-specific	59
Other	12
Other cancer	2
Stroke	3
Unknown	2
Suicide	2
Pneumonia	1
COPD	1
CRF	1
Mean duration of follow-up, months	52.6±19.1
Median duration of follow-up, months	48.8

LVI, Lymphovascular invasion; COPD, chronic obstructive pulmonary disease; CRF, chronic renal failure; 5-FU, 5-fluorouracil; FOFLOX: 5-fluorouracil/Leucovorin/Oxaliplatin. [†]8 Cycles of capecitabine (n=20) or doxifluridine for one year (n=161); [‡]among patients (n=162) with rectal or combined cancer.

Results

Patients' characteristics and genotypic frequency. The median age of the 344 patients was 63 (range=30-79) years, and 219 (63.7%) patients were male. One hundred and

Table II. *Allelic frequencies and p-values for Hardy−Weinberg equilibrium (HWEs) for selected single nucleotide polymorphisms (SNPs).*

	SNP	Related miRNA	Related gene	Genotype	N	%	HWE *p*-Value	Minor allelic frequency
miRNA	rs12976445	miR-125a	Variable	CC	278	83.7	0.699	0.086
				TC	51	15.4		
				TT	3	0.9		
	rs41275794	miR-125a	Variable	GG	261	75.9	0.464	0.126
				GA	79	23.0		
				AA	4	1.2		
	rs11614913	miR-196a	Variable	TT	87	25.3	0.511	0.488
				CT	178	51.7		
				CC	79	23.0		
miRNA binding site	rs1044129	miR-367	*RYR3*	AA	85	24.7	0.448	0.493
				AG	179	52.0		
				GG	80	23.3		
	rs3134615	miR-1827	*MYCL1v*	GG	312	90.7	0.805	0.048
				GT	31	9.0		
				TT	1	0.3		
	rs4245739	miR-191	*MDM4*	AA	300	87.2	0.712	0.064
				CA	42	12.2		
				CC	1	0.3		
	rs5186	miR-155	*AGTR1*	AA	307	89.2	0.292	0.054
				CA	37	10.8		
				CC	0	0		

RYR3, Ryanodine receptor 3; *MYCL1v*, myelocytomatosis viral oncogene homolog 1; *MDM4*, mouse double minute 4, p53−binding protein homolog; *AGTR1*, angiotensin II receptor type 1.

eighty-two (52.9%) patients were diagnosed with colonic cancer. Laparoscopic surgery was performed on 235 (68.3%) patients, while the others underwent an open colorectal resection. The pathological stages after the surgical resection were as follows: stage I, n=64 (18.6%), stage II, n=111 (32.3%), and stage III, n=169 (49.1%). Among the 280 patients with stage II or III disease, 276 received adjuvant chemotherapy with six cycles of 5-fluorouracil/leucovorin (Mayo regimen) with/without radiotherapy (n=78), 12 cycles of 5-fluorouracil/Leucovorin/Oxaliplatin (FOLFOX-4) (n=17), eight cycles of capecitabine (n=20), or doxifluridine for one year (n=161) (Table I). At the time of last analysis (October 2011), 74 patients had experienced a disease relapse and 59 patients had died as a result of colorectal cancer. However, the deaths of 12 patients were not related to colorectal cancer.

The frequencies of each genotype are shown in Table II, and are conformed to the Hardy−Weinberg equilibrium (p>0.05).

Survival analysis. At a median follow-up duration of 48.8 months, the estimated 5-year DSS and RFS for all patients was 80.3±2.7% and 75.7±2.6%, respectively, and the survival differed according to the stage (*p*<0.001, Figure 1). Among the seven target variants, a univariate analysis revealed that rs1044129 in the miR-367-binding site of the *RYR3* was associated with the RFS of the patients with colon cancer (n=182) in the recessive model, although no

association was observed between any of the variants and the survival for all enrolled patients with CRC (Table III). Moreover, a multivariate analysis revealed that patients carrying the GG genotype had a poor RFS when compared to those with the A allele (hazard ratio, HR=2.864; 95% confidence interval (CI)=1.363-6.016; *p*=0.005), yet only a marginal trend for DSS (HR=2.226; 95% CI=0.890-5.568; *p*=0.087) regardless of patient and tumor characteristics (Table IV; Figure 2). In addition, no significant difference in clinicopathological parameters was observed according to the genotype or allele of rs1044129.

Discussion

Among seven selected variants of miRNAs or miRNA-binding sites previously identified as potential biomarkers, the current study identified functional variant rs1044129 at the miR-367 binding site in the 3'UTR of the *RYR3* as a prognostic factor for estimating recurrence after curative surgery in patients with colonic cancer. Given the homogenous ethnic background of Korean patients, any potential confounding effect due to ethnicity is likely to be small in the current study.

RYRs, a family of high conductance cation channels, play an important role in calcium homeostasis in gut cells based on releasing Ca^{2+} from intracellular stores (25). Furthermore, RYR3, the third isoform of the RYR family, is commonly expressed in cancer cells depending on the histological grade

Table III. *Survival analysis according to the genotype of target variants by a log-rank test.*

SNP	Total N	Total RFS Event	Total RFS p-Value	Total DSS Event	Total DSS p-Value	Colonic N	Colonic RFS Event	Colonic RFS p-Value	Colonic DSS Event	Colonic DSS p-Value	Rectal N	Rectal RFS Event	Rectal RFS p-Value	Rectal DSS Event	Rectal DSS p-Value
rs12976445															
CC	278	58	0.346	45	0.339	150	29	0.425	20	0.354	128	29	0.642	25	0.744
TC or TT†	51/3	14		11		27	7		5		27	7		6	
rs41275794															
GG	261	56	0.820	44	0.841	137	26	0.350	17	0.164	124	30	0.525	27	0.303
GA or AA†	79/4	18		14		48	11		9		38	7		5	
rs11614913															
TT	87	21	0.565	15	0.779	49	11	0.584	8	0.589	38	10	0.853	7	0.831
TC	178	39		31		88	19		13		90	20		18	
CC	79	14		12		45	7		5		34	7		7	
rs1044129															
AA	85	17	0.764	14	0.996	52	8	0.074	6	0.364	33	9	0.444	8	0.399
AG	179	37		30		91	16		12		88	21		18	
GG	80	20		14		39	13		8		41	7		6	
AA or AG†	264	54	0.464	44	0.933	143	24	0.024	18	0.163	121	30	0.258	26	0.236
rs3134615															
GG	312	67	0.849	53	0.951	166	32	0.222	23	0.631	146	35	0.382	30	0.589
GT/TT	31/1	7		5		16	5		3		16	2		2	
rs4245739															
AA	300	66	0.790	52	0.793	155	32	0.924	22	0.829	145	34	0.797	30	0.619
AC or CC†	41/1	8		6		26	5		4		17	3		2	
rs5186															
AA	307	65	0.590	50	0.301	163	34	0.612	24	0.670	144	31	0.202	26	0.060
CA or CC†	37/0	9		8		19	3		2		18	6		6	

RFS, Relapse-free survival; DSS, disease-specific survival; SNP, single nucleotide polymorphism. †Recessive model of the minor allele.

Table IV. *Multivariate survival analysis of patients with colonic cancer (n=182).*

		RFS p-Value	RFS HR	RFS 95% CI	DSS p-Value	DSS HR	DSS 95% CI
Age, >60 years	vs. ≤60	0.005	3.325	1.439-7.681	0.019	3.422	1.225-9.559
Gender, female	vs. male	0.130	1.787	0.842-3.790	0.096	2.241	0.866-5.801
AJCC stage		0.003			0.009		
II	vs. I	0.453	0.340	0.020-5.714	-	-	-
IIIA, B	vs. I	0.541	2.314	0.157-34.195	-	-	-
IIIC	vs. I	0.299	4.352	0.272-69.754	-	-	-
Elevated CEA		0.048	2.150	1.0069-4.592	0.405	1.483	0.587-3.749
Histological grade, G2 or 3	vs. G1	0.785	0.865	0.306-2.450	0.605	0.705	0.188-2.652
LVI		0.061	2.493	0.958-6.489	0.245	1.861	0.654-5.296
Adjuvant chemotherapy		0.353			0.711		
Oral 5-FU	vs. none	0.287	4.264	0.295-61.566	-	-	-
Mayo or FOLFOX	vs. none	0.191	6.089	0.407-91.142	-	-	-
rs1044129, GG	vs. GA or AA	0.005	2.864	1.363-6.016	0.087	2.226	0.890-5.568

RFS, Relapse-free survival; DSS, disease-specific survival; LVI, lymphovascular invasion; 5-FU, 5-fluorouracil; FOFLOX: 5-fluorouracil/Leucovorin/Oxaliplatin; HR, hazard ratio; CI, confidence interval.

(26) and has also been shown to modulate tumor cell growth and migration (21, 27). Previous studies revealed that miR-367 regulates the expression of several tumor-related molecules, including RYR3, and moreover, miR-367 expression has been revealed to be a better predictor for survival of patients with brain tumors (21, 28). As microRNAs silence target genes
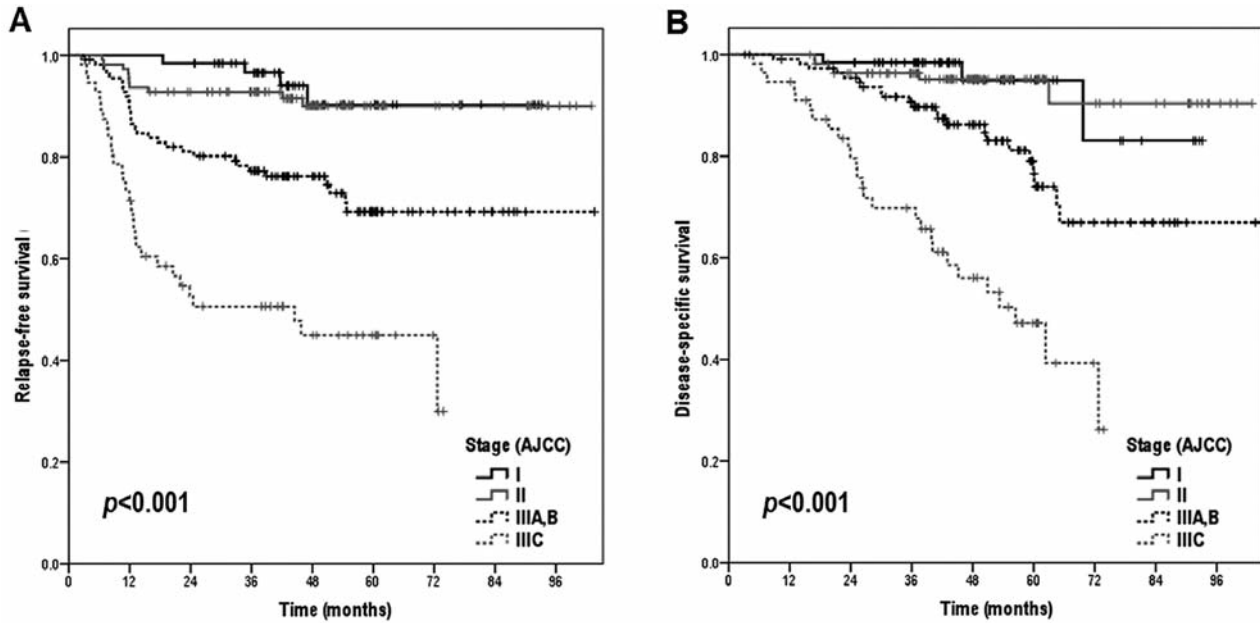
Figure 1. *Survival according to the American Joint Committee on Cancer stage. A: Relapse-free survival; B: disease-specific survival.*
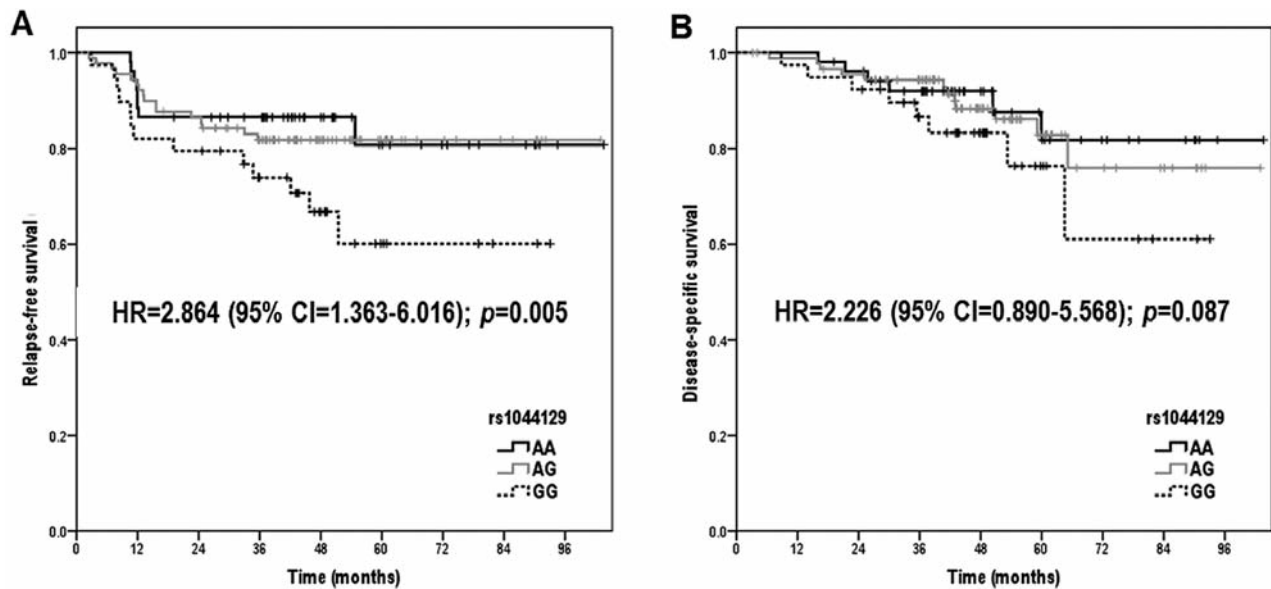

Figure 2. *Survival curves according to rs1044129 in patients with colonic cancer (n=182). A: Relapse-free survival; B: disease-specific survival. HR: hazard ratio; CI: confidence interval.*

through binding to the 3'UTR of the target genes, it is also possible that a change in the miRNA−mRNA binding affinity, due to polymorphisms at the binding site, could affect RYR3 expression, thereby altering cancer pathogenesis. Zhang *et al*. (21) found that miR-367 binding affinity varies according to the genotype of rs1044129 at the binding site in 3'UTR of RYR3, where miR-367 has a weaker binding affinity and

thereby increased target gene expression for the G allele. Moreover, the G allele has been identified as bearing a risk for breast cancer development and a poor RFS (22), which is consistent with the results of the current study. Therefore, it is suggested that rs1044129 could be a potential prognostic biomarker for specific types of cancer, such as colonic and breast cancer. Notwithstanding this, although SNPs are

thought to be attractive biomarkers since they are stably inherited, highly abundant and show diversity within and among populations, the application of individual SNPs is limited due to low penetrance and to difficulty involved in identifying their effects. Thus, until the present results are confirmed by replication studies with different populations, caution is warranted in terms of drawing definite conclusions from the current study.

In addition, the current study found that rs1044129 GG was significantly associated with a poor RFS (HR=2.864; *p*=0.005), while only a trend was identified for DSS (HR=2.226; *p*=0.087). This could be explained by the relative low number of individuals as the sub-analysis was performed based on the tumor location. Moreover, the diverse treatment modalities given after recurrence, including metastatectomy and active chemotherapy, could have weakened the impact of this variant on DSS, as previously described in various adjuvant therapy trials for CRC (29, 30). Furthermore, the impact of this variant was not evident in the patients with rectal cancer, which could be related to the controversial issue of molecular differences between colonic and rectal cancer, as specified in previous studies (31, 32).

In summary, *RYR3* 3'UTR rs1044129 was found to be an independent prognostic marker of relapse-free survival in Korean patients with resected colonic cancer when using a recessive model. This finding is consistent with a previous study with breast cancer. However, since the exact mechanism and tumor specificity of the rs1044129 variant have not yet been defined and genetic polymorphisms often vary between different ethnic groups, the present findings need to be confirmed in further studies with other patient populations with CRC in order to clarify the association between these polymorphisms and the prognosis of CRC.

Acknowledgements

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