# A *miR-146a* Polymorphism (rs2910164) Predicts Risk of and Survival from Colorectal Cancer

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**Abstract.** *Background: Recent evidence suggests that the rs2910164 variant of miR-146a is associated with the development of certain types of cancer. Therefore, the aim of this study was to investigate the association of this genetic variant with susceptibility and prognosis in patients with colorectal cancer (CRC). Materials and Methods: Genotyping analyses of miR-146a rs2690164 for risk and survival in CRC were performed in a case–control study (n=967) using a polymerase chain reaction (PCR)-restriction fragment length polymorphism assay. Results: The C allelic frequency of miR-146 rs2690164 in the 399 patients and 568 controls was 61.9% and 53.9%, respectively. In the case–control study, those who possessed the CC genotype had a higher risk of CRC compared to those with the CG or GG genotype (odds ratio=1.569; 95% confidence interval=1.196-2.059; p=0.001), regardless of the tumor site. In the survival analysis of 343 patients with CRC who underwent curative surgery, those with CC genotype had a worse survival outcome compared with those with CG or GG genotype in a Kaplan–Meier survival analysis. Moreover, a multivariate analysis showed that the CC genotype of miR-146a rs2910164 was associated with worse relapse-free and disease-specific survival compared to the CG or GG genotype in a recessive model of the C allele, adjusted for patient and tumor characteristics (hazard ratio=2.120 and 2.349, p=0.005 and 0.007, respectively). Conclusion: The current study provides evidence that the miR-146a rs2690164 polymorphism, as the dominant model of the G allele, is associated with the susceptibility and prognosis of CRC.*

Colorectal cancer (CRC) is a leading cause of death and annually responsible for more than 500,000 deaths worldwide. To date, although the main prognostic factor used in clinical practice is the tumor stage, several molecules and genetic alterations have also been introduced as potential markers. In particular, constitutional host-related biological features have long been suspected on explaining why some patients treated for CRC will experience relapse while others will not, despite similar baseline characteristics and polymorphisms in the genes involved in tumor progression. Thus, apoptosis and angiogenesis have been widely studied for their association with CRC susceptibility and prognosis (1-8).

MicroRNAs (miRNAs), a class of small, endogenous, non-coding RNAs, can regulate gene expression by translational repression or mRNA degradation of the target, affecting critical functions in various physiological processes, including cell proliferation and apoptosis, and tumor development (9, 10). Previous studies have already demonstrated the relationship of the expression of miRNAs with prognosis or responsiveness to treatment in CRC (11-13). Moreover, some variants of miRNAs have recently been introduced as potential biomarkers of CRC.

The *miRNA-146a* polymorphism rs2910164 causes a change from a G:U pair to a C:U mismatch in the stem structure of the miR-146a precursor, leading to process variation and lower expression of the mature sequence. Recent evidence also suggests that the rs2910164 single-nucleotide polymorphism (SNP) in *miR-146a* is associated with the development and prognosis of various types of cancer (14-21). However, its effect on cancer is still

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*Key Words:* Colorectal cancer, *miR-146a* polymorphism, rs2910164, risk, prognosis.

controversial due to inconsistencies between studies, and no data is currently available for CRC in the Korean population.

Accordingly, the present study investigated whether the *miR-146a* rs2910164 polymorphism is associated with the risk and prognosis of CRC in the Korean population. A genotyping analysis of *miR-146* rs2690164 C/G in the miRNA region was performed, and then its association with susceptibility was evaluated in a case–control study with 399 patients with CRC and 568 cancer-free controls, while its association with prognosis was evaluated using a survival analysis based on 343 patients with CRC who underwent curative surgery.

## Materials and Methods

*Study population*. For the case–control study, 399 patients newly-diagnosed with CRC between December 2002 and June 2005 at Kyungpook National University Hospital (Daegu, Korea) and a cancer-free control population of 568 was selected, excluding any with a family history suggestive of hereditary CRC. The diagnosis and staging of the colorectal cancer were assessed according to the WHO (22) and TNM (23) classifications, respectively. The controls were randomly selected from a pool of healthy volunteers (n=5,578) who visited the general health check-up center at Kyungpook National University Hospital during the same period, as described for our previous studies (24, 25). All the participants enrolled in this study (cases and controls) were ethnic Koreans who resided in Daegu City or the surrounding regions. For the survival analysis, we selected 343 cases who underwent curative surgery but did not receive neoadjuvant therapy before surgery among patients enrolled in a case–control study. Each patient was examined every 3-6 months for the first five years following the diagnosis of CRC, and every year thereafter, in accordance with the national guidelines. Written informed consent for the current study was received from each participant, and the study was approved by the Institutional Research Board at Kyungpook National University Hospital (KNUHBIO_08-1001).

*Genotyping*. Genomic DNA was extracted from normal tissues of patients with CRC, while blood samples were obtained from the controls. The *miR-146a* rs2910164 genotypes for the patients and the controls were determined using a polymerase chain reaction (PCR)-restriction fragment length polymorphism assay. The PCR reactions were performed in a total volume of 20 μl, containing 100 ng of genomic DNA, 0.2 mM dNTPs, 1 unit of Taq polymerase (New England BioLabs, Beverly, MA, USA), 1x reaction buffer [10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 0.1% Triton X-100], and the following primers: forward 5'-TGG GTT GTG TCA GTG TCA GAG C-3' and reverse 5'-TGC CTT CTG TCT CCA GTC TTC C-3'. For the rs2910164 genotyping, the PCR products were digested overnight using a *Ban*II restriction enzyme (New England BioLabs Inc., Ipswich, MA, USA) at 37˚C. The digested PCR products were then resolved on a Multi-NA microchip electrophoresis system (Shimadzu, Tokyo, Japan). To confirm the genotyping results, selected PCR-amplified DNA samples (n=10 for each genotype) were examined by DNA sequencing, and the results were shown to match 100%.

Table I. *Selected characteristics of patients with colorectal cancer and controls*.

| Variables | Patients* (n=399) | Controls (n=568) | *p*-Value† |
|---|---|---|---|
| Mean age±SD, years | 62.4±11.4 | 61.7±9.0 | 0.314 |
| Age years | | | |
| >60 | 263 (65.9) | 362 (63.7) | 0.495 |
| ≤60 | 136 (34.1) | 206 (36.3) | |
| Gender | | | |
| Male | 218 (54.6) | 337 (59.3) | 0.147 |
| Female | 181 (45.4) | 231 (40.7) | |
| Genotype, rs2910164‡ | | | |
| GG | 61 (15.3) | 121 (21.3) | 0.002 |
| CG | 182 (45.6) | 282 (49.6) | |
| CC | 156 (39.1) | 165 (29.0) | |
| Site | | | |
| Colon | 221 (55.4) | | |
| Rectum | 175 (43.9) | | |
| Both | 3 (0.8) | | |
| Histological grade | | | |
| Well | 79 (20.0) | | |
| Moderate | 302 (75.7) | | |
| Poor or signet ring cell type | 18 (4.5) | | |
| Stage | | | |
| I | 79 (19.8) | | |
| IIA | 118 (29.6) | | |
| IIB | 14 (3.5) | | |
| IIIA | 10 (2.5) | | |
| IIIB | 81 (20.3) | | |
| IIIC | 44 (11.3) | | |
| IV | 53 (13.3) | | |

*Among whom, 343 patients underwent surgery with curative intent. †Two-sided $\chi^2$-test. ‡Each genotype frequency was under Hardy–Weinberg equilibrium (HWE, *p*-value=0.511 and 0.980 for patients and controls, respectively).

*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 (HWE) for the polymorphism was analyzed using a $\chi^2$-test.

For the case–control study, a $\chi^2$-test was also used to evaluate the differences between the patients and the controls

Table II. *Allelic and genotype frequencies of miR-146a rs2910164.*

| | Genotype | n (%) | | Adjusted OR* | 95% CI | *p*-Value |
|---|---|---|---|---|---|---|
| | | Patients (n=399) | Controls (n=568) | | | |
| Total | G allele | 304 (38.1) | 524 (46.1) | 1.000 | | |
| | C allele | 494 (61.9) | 612 (53.9) | 1.388 | 1.154-1.670 | 0.001 |
| | GG | 61 (15.3) | 121 (21.3) | 1.000 | | 0.002 |
| | CG | 182 (45.6) | 282 (49.6) | 1.079 | 0.885-1.821 | 0.195 |
| | CC | 156 (39.1) | 165 (29.0) | 1.865 | 1.278-2.722 | 0.001 |
| | CG or GG | 243 (60.9) | 403 (71.0) | 1.000 | | |
| | CC | 156 (39.1) | 165 (29.0) | 1.569 | 1.196-2.059 | 0.001 |
| Colon† | GG | 38 (17.2) | 121 (21.3) | 1.000 | | 0.008 |
| | CG | 93 (42.1) | 282 (49.6) | 1.042 | 0.674-1.609 | 0.854 |
| | CC | 90 (40.7) | 165 (29.0) | 1.724 | 1.103-2.695 | 0.017 |
| | CG or GG | 131 (59.3) | 403 (71.0) | 1.000 | | |
| | CC | 90 (40.7) | 165 (29.0) | 1.675 | 1.210-2.320 | 0.002 |
| Rectum‡ | GG | 23 (13.1) | 121 (21.3) | 1.000 | | 0.023 |
| | CG | 87 (49.4) | 282 (49.6) | 1.600 | 0.963-2.658 | 0.069 |
| | CC | 66 (37.5) | 165 (29.0) | 2.092 | 1.232-3.553 | 0.006 |
| | CG or GG | 110 (62.5) | 403 (71.0) | 1.000 | | |
| | CC | 66 (37.5) | 165 (29.0) | 1.473 | 1.032-2.101 | 0.033 |

OR: Odds ratio; CI: confidence interval. *Adjusted for age and gender. †n=221; ‡n=175.

as regards the frequency of the variables, including age, sex, and variant genotype; the association between the genotype and the risk of CRC was also estimated using the odds ratio (OR) and 95% confidence interval (CI) calculated using a multivariate logistic regression analysis. 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 rs2910164 genotype were compared using log-rank tests. 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), and pathological differentiation (well to poor). The hazard ratio (HR) and its 95% CI were also estimated. A cut-off *p*-value of 0.05 was adopted for all the statistical analyses. The statistical data were obtained using an SPSS software package (SPSS 15.0, SPSS, Chicago, IL, USA).

## Results

*Characteristics of the study population (case–control study).* Among the 399 patients with CRC, the primary tumor was colonic in 221 cases (55.4%) and rectal in 175 cases (43.9%), 53 (13.3%) patients had stage IV disease at diagnosis. The baseline characteristics were similar in age and sex between the patients and the controls (Table I).

*Genotypic distribution of miR-146a rs2910164 and risk of CRC (case–control study).* The allelic frequencies in the controls were similar to those in the HapMap Asian population, and the genotypic distributions in the patients and controls were under the HWE ($\chi^2$=0.432 and 0.0006; $p$=0.511 and 0.980, respectively).

The *miR-146a* rs2910164 polymorphism was significantly associated with the risk of CRC according to the $\chi^2$-test ($p$=0.002). After adjusting for potential variables (age and sex), individuals carrying CC had a higher risk of CRC compared to those carrying CG or GG separately ($p_{trend}$=0.002) and in the recessive model for the C allele (OR=1.569, 95% CI=1.196-2.059; $p$=0.001); the C allele was also identified as a risk factor for CRC (OR=1.388, 95% CI=1.154-1.670; $p$=0.001) regardless of the tumor site (Table II).

*Association of miR-146a rs2910164 with survival (survival analysis).* The characteristics and outcomes for the 343 patients who underwent curative surgery are listed in Table III. The American Joint Committee on Cancer (AJCC) pathological stages were as follows: stage I (n=79, 21.0%), stage II (n=131, 38.2%), and stage III (n=133, 38.8%). Adjuvant chemotherapy was administered to 261 (76.1%) patients. Among the 168 patients with rectal cancer, 36 (22.0%) patients received radiotherapy. For a median follow-up of 42.3 months, 286 patients were alive at the last follow-up, yet there were 65 cases of relapse and 57 deaths, including 46 CRC-specific deaths. The estimated 5-year RFS and OS for all the patients was 78.8% (95% CI=74.8% to 84.8%) and

76.1% (95% CI=69.1% to 83.1%), respectively. In the univariate analysis, the histological grade, lymphovascular invasion, pathological stage, and serum CEA were all significantly associated with both RFS and DSS ($p<0.05$ for each). In the Kaplan–Meier survival analysis, the CC genotype of rs2910164 was associated with a worse survival outcome compared with the CG or GG genotype in the recessive model of the C allele (Figure 1). Moreover, the multivariate analysis showed that the CC genotype of the *miR-146a* rs2910164 was associated with a worse RFS and DSS compared to the G allele in the recessive model, adjusted for patient and tumor characteristics (HR=2.120 and 2.349; $p=0.005$ and 0.007, respectively; Table IV). There was no statistical difference in the clinicopathological characteristics according to the genotype of *miR-146a* rs2910164.

## Discussion

The current study demonstrated that the G allele of *miR-146a* rs2910164, as the dominant model, was significantly associated with low risk and better survival in CRC, adjusted for patient characteristics and stage, suggesting a potential role of the *miR-146a* variant in cancer progression, as well as cancer development.

miRNAs have emerged as playing important roles in many biological processes related to tumor development and progression by translational repression or degradation of target mRNAs (26). miR-146a in particular has been actively studied with regards to its role in tumor development and progression; the overexpression of *miR-146a* has also been identified in cancer cell lines and tumor tissues (10, 27). Moreover, as the *miR-146a* variant rs2910164 can alter the processing and expression of miRNA by a G:U to C:U mispairing in the hairpin of miR-146a, as defined in a previous study (28), it has been suggested that the *miR-146a* variant may affect cancer development and its prognosis. Therefore, several studies have recently presented the role of this variant in a variety of cancer types, yet the results are inconsistent according to ethnicity, cancer type, and disease status. For example, some studies demonstrated that the CC genotype was associated with an increased risk of the development of breast cancer in Europeans and gastric cancer in Asians, and glioma in Americans, similar to the current study (14, 17, 18, 29, 30). In particular, Shen et al. demonstrated that the C allele of this variant to be associated with the early onset of breast cancer and displayed increased mature miR-146a production compared with the G allele precursor (17). However, the opposite result has also been shown in relating the expression of miR-146a (28, 31) and cancer susceptibility (28, 31, 32). Other studies failed to show any overall association between this variant and the risk of bladder, kidney, and breast cancer and squamous cell carcinoma of the head and neck in Caucasian or Chinese populations (33-36).

Table III. *Clinical characteristics of 343 patients who underwent curative surgery.*

| Characteristics | n | (%) |
|---|---|---|
| No. patients | 343 | |
| Median age , range (years) | 64 | 21-85 |
| >60 | 214 | (62.4) |
| ≤60 | 129 | (37.6) |
| Gender | | |
| Male | 188 | (54.8) |
| Female | 155 | (45.2) |
| Primary site | | |
| Colon | 187 | (54.5) |
| Rectum | 154 | (44.9) |
| Both | 2 | (0.6) |
| Histological differentiation | | |
| Well | 72 | (21.0) |
| Moderate | 258 | (75.2) |
| Poor or signet ring type | 13 | (3.8) |
| CEA, elevated | 62 | (18.3) |
| Surgery | | |
| Open | 99 | (28.9) |
| Laparoscopy | 244 | (71.1) |
| AJCC stage | | |
| I | 79 | (23.0) |
| II | 131 | (38.2) |
| III | 133 | (38.8) |
| Adjuvant chemotherapy | | |
| None | 82 | (23.9) |
| Oral 5FU | 181 | (52.8) |
| Mayo | 66 | (19.2) |
| FOLFOX | 14 | (4.1) |
| Radiotherapy† | 36 | |

†Only for patients with invasive rectal cancer (n=168). CEA: carcinoembryonic antigen; 5FU: 5-fluorouracil; FOLFOX: 5-fluorouracil/Leucovorin/Oxaliplatin.

Therefore, the inconsistency among such studies suggests that the variant may be tumor-specific in different types of cancers with different etiologies, and is possibly affected by different cancer states, such as carcinogenesis *versus* tumor progression. Unfortunately, there are currently no comparable data for this variant (rs2910164) related to CRC, except for one case–control study assessing its role in the susceptibility of Korean patients to CRC (37). Although the study included a similar number of participants (n=948) as the current study (n=967), no significant relationship was demonstrated between rs2910164 and the risk of CRC, yet this result is questionable as the HWE *p*-value for rs2910164 in the control arm was only 0.063 and the value for rs11614913 was out of the HWE ($p=0.037$) among the four evaluated SNPs. Conversely, in the current study, since the CC genotype showed a consistent effect on risk and prognosis in the same patient population, this finding may have more meaning for Korean patients with CRC.

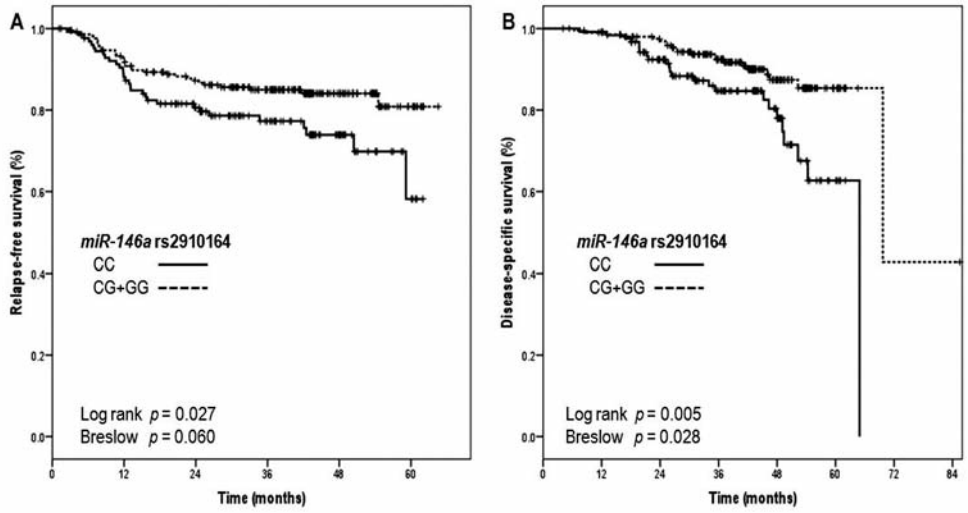


Figure 1. *Kaplan–Meier survival among 343 patients who underwent curative surgery according to genotype of miR-146a rs2910164 as a dominant model of the G allele. A: Relapse-free survival and B: disease-specific survival.*

Table IV. *Multivariate analysis for survival among 343 patients who underwent curative surgery.*

| | | Relapse-free survival | | | | Disease-specific survival | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *p*-Value | HR | 95% CI | | *p*-Value | HR | 95% CI | |
| | | | | Lower | Upper | | | Lower | Upper |
| Age, >60 years | *vs.* ≤60 years | 0.113 | 1.575 | 0.898 | 2.761 | 0.239 | 1.470 | 0.774 | 2.792 |
| Gender, female | *vs.* male | 0.106 | 1.540 | 0.912 | 2.600 | 0.346 | 1.361 | 0.717 | 2.584 |
| Site, rectum | *vs.* colon | 0.638 | 0.878 | 0.511 | 1.509 | 0.975 | 0.990 | 0.519 | 1.887 |
| CEA, elevated | | 0.007 | 2.128 | 1.232 | 3.674 | 0.094 | 1.792 | 0.905 | 3.548 |
| Differentiation, Moderate or poor | *vs.* well | 0.210 | 1.630 | 0.759 | 3.501 | 0.121 | 2.332 | 0.799 | 6.806 |
| Lymphovascular invasion | *vs.* none | 0.820 | 1.083 | 0.543 | 2.161 | 0.289 | 1.635 | 0.659 | 4.053 |
| AJCC stage | | <0.001 | | | | <0.001 | | | |
| II | *vs.* I | 0.514 | 1.555 | 0.413 | 5.855 | 0.672 | 1.426 | 0.276 | 7.358 |
| IIIA,B | *vs.* I | 0.016 | 4.902 | 1.339 | 17.944 | 0.105 | 3.719 | 0.761 | 18.174 |
| IIIC | *vs.* I | <0.001 | 11.230 | 2.930 | 43.047 | 0.002 | 12.481 | 2.464 | 63.226 |
| Adjuvant chemotherapy | | 0.043 | | | | 0.509 | | | |
| Mayo regimen | *vs.* none or oral 5FU | 0.020 | 2.216 | 1.136 | 4.321 | 0.287 | 1.550 | 0.692 | 3.469 |
| FOLFOX/FOLFIRI | *vs.* none or oral 5FU | 0.744 | 0.803 | 0.215 | 2.994 | 0.859 | 0.886 | 0.233 | 3.371 |
| rs2910164, CC | *vs.* GG or CG | 0.005 | 2.120 | 1.257 | 3.574 | 0.007 | 2.349 | 1.257 | 4.390 |

HR: Hazard ratio; CI: confidence interval; CEA: carcinoembryonic antigen; 5FU: 5-fluorouracil; FOLFOX: 5-fluorouracil/leucovorin/oxaliplatin.

Moreover, in the current study, survival analyses revealed for the first time that carrying the C allele of rs2910164 was significantly associated with a poor survival in CRC after curative surgery. This finding is consistent with a recent report of adult glioma showing a similar HR value (0.495 *vs.* 0.487 in the current study), suggesting that the *miR-146a* rs2910164 variant could be a potential prognostic factor in various types of cancer (18).

Since the present analysis is limited to patients of Korean ethnicity, it is unclear whether these results can be generalized to other populations. Therefore, future studies using different populations with various disease statuses and types of cancers are warranted to confirm the role of this variant in cancer.

In summary, the *miR-146a* rs2910164 variant is suggested as a biomarker for predicting the risk and prognosis of CRC

in Koreans. However, as the exact role and tumor specificity of the miR-146a variant has not yet been fully-defined, the present findings need to be confirmed in further studies with other populations of patients with CRC.

## Acknowledgements

This work was supported in part by National Research Foundation of Korea Grant funded by the Korean Government (grant no. KRF-2008-521-E00051), and in part by National Research Foundation of Korea Grant funded by the Korean Government (grant no. 2012-0005226). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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*Received May 7, 2013*
*Revised May 21, 2013*
*Accepted May 22, 2013*