miR-194 as a Predictor for Adenoma Recurrence in Patients with Advanced Colorectal Adenoma after Polypectomy

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Abstract
microRNAs (miRNA) are promising predictors in colorectal cancer (CRC). We investigated whether miRNAs could predict adenoma recurrence in patients with advanced colorectal adenoma (ACRA) after polypectomy. miRNA expression profiling was performed by miRNA microarray to identify recurrence-related miRNAs. Candidate miRNAs extracted from formalin-fixed paraffin-embedded blocks of patients with ACRA were measured using real-time PCR. Logistic regression analysis was conducted to investigate whether validated miRNA expression profiles were independent from other known adenoma recurrence risk factors. The prognostic values of six miRNAs and three independent risk factors were assessed by the area under the receiver operating characteristic (ROC) curve analysis. The expressions of six candidate miRNAs were significantly decreased from levels in normal colorectal tissue compared with ARCA with adenoma recurrence (RACRA) in this retrospective cohort. However, only miRNA (miR)-194 emerged as a practical predictor. The sensitivity and specificity of miR-194 as a predictor were 71.0% and 78.0%, respectively, at a cutoff value of 0.1311 in the retrospective cohort. Sensitivity and specificity were 76.1% and 77.2%, respectively, in the prospective cohort using the same cutoff value. Low expression levels of miR-194, adenoma size ≥2 cm, and ≥3 adenomas were independent risk factors for adenoma recurrence. Moreover, low expression of miR-194 was a better predictor of adenoma recurrence than the adenoma size and numbers according to ROC curve analysis. miR-194 may be an independent predictor for adenoma recurrence in patients with ACRA after polypectomy. Cancer Prev Res; 7(6): 607–16. ©2014 AACR.

Introduction
Advanced colorectal adenomas (ACRA) are precursors of colorectal cancer (CRC), and their removal is central for reducing incidence and mortality rates (1). Treatment strategies for removal of ACRA rely on their prior detection by colonoscopy and follow-up surveillance (2). In the United States, patients with ACRA are recommended to have 3-year follow-up colonoscopies (3). The removal of adenomas lowers the risk of CRC; however, CRC is still the third most common cancer and the second leading cause of death from cancer in the United States (4). This is because the recurrence of adenomas (46.7%), especially ACRA (11.8%), after polypectomy is common during a median follow-up period of 47.2 months (5). The study suggested that aggressive recolonoscopies may be more effective in preventing CRC. Consequently, postpolypectomy surveillance has been increased to occupy about 25% of total colonoscopy capacity (6) and poses a substantial burden on healthcare systems. Therefore, recognizing patients who have the potential for adenoma recurrence to undergo individualized surveillance is a key step to solve this dilemma. To date, the prediction of adenoma recurrence has remained difficult, even though there have been several trials exploring adenoma recurrence–related molecules during food or drug interventions (7–12). Therefore, predictors associated with adenoma recurrence are urgently required to distinguish high-risk patients for repetitive surveillance.

microRNAs (miRNA) are 18 to 25 nucleotide noncoding RNA molecules that regulate the expression of target genes by binding to partially complementary recognition sequences of target mRNAs (13). Changes in miRNA expression play a crucial role in human cancer (14). miRNAs have been shown to be a practical predictor associated with prognosis in CRC (15–17), and to be superior to mRNA, due to its small size, stability, and resistance to RNase. 

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degradation (18). Considering the potential prognostic value in CRC, we explored differentially expressed miRNAs from ACRA with adenoma recurrence (RACRA) compared with ACRA with no adenoma recurrence (NRACRA) to establish a predictor for adenoma recurrence.

Materials and Methods

Clinical specimen collection

All nine samples (miRNA microarray cohort) were obtained in 2007 from Shanghai 1st Hospital (Shanghai, China) for miRNA microarray analysis. Six fresh adenoma
Figure 2. A, heatmap of miRNA microarray expression data from fresh tissue samples of normal colorectal tissues, NRACRA tissues, and RACRA tissues. The expression of miRNA is hierarchically clustered on the y-axis, and samples from three groups are hierarchically clustered on the x-axis. The legend on the right indicates the miRNA represented in the corresponding row. The relative miRNA expression is depicted according to the color scale shown on the right. Red, upregulation; green, downregulation. B, miRNA microarray showed that the expression of miR-10a, miR-141, miR-146a, miR-151, miR-194, and miR-3607-3p levels decreased with the progression from normal colorectal tissues to RACRA tissues through NRACRA tissues with respect to increasing carcinogenesis of the colorectal tissue.
tissue samples were obtained from patients with ACRA who underwent polypectomy during surveillance intervals of 22 to 24 months; three with adenoma recurrence (RACRA) and three with no adenoma recurrence (NRACRA). Three fresh samples of normal colorectal tissue were obtained from healthy volunteers. Formalin-fixed paraffin-embedded (FFPE) samples were obtained from 37 healthy volunteers and 227 patients with ACRA who underwent polypectomy from a retrospective cohort recruited at Renji Hospital (Shanghai, China), in addition to 158 patients with ACRA after polypectomy in prospective cohort at Shanghai 1st Hospital. A summary of the characteristics of the study participants and adenomas is shown in Supplementary Table S1.

**Study design**

The study was divided into three parts. In the first study, miRNA microarray analyses were performed on samples from the miRNA microarray cohort. The second study was based on the retrospective study (Fig. 1A), involving the 37 healthy volunteers and 227 patients with ACRA between 2006 and 2007 at Renji Hospital. Of the 227 patients, 100 experienced adenoma recurrence and 127 had no recurrence of their adenoma during surveillance periods of 22 to 24 months. The third study was based on a prospective study, which included the 158 patients with ACRA in 2010 at the Shanghai 1st Hospital (Fig. 1B). Data collected during the study interval (January 1, 2006 to December 31, 2012) included adenoma characteristics, patients’ clinical characteristics, and outcomes of surveillance colonoscopy. For inclusion in the study, patients had to meet the following criteria: (1) ACRA, defined as a tubular adenoma with a diameter of ≥10 mm, at least 25% villous elements, or the presence of high-grade intraepithelial neoplasia; (2) complete colonoscopy in which the bowel was prepared adequately, the cecum was visualized, and all visualized lesions were removed by polypectomy; (3) at least one surveillance colonoscopy was performed within 6 months after polypectomy to remove missed adenomas; and (4) no history of CRC, familial adenomatous polyposis, inflammatory bowel disease, and bowel resection excluding appendectomy. All adenomas found during subsequent colonoscopies within 6 months of the initial colonoscopy were regarded as missed adenomas. An adenoma detected at the previous polypectomy site was regarded as a local residual adenoma. Recurrent adenoma was defined if at least three adenomas were found during the surveillance colonoscopy, irrespective of size, or at least one metachronous adenoma that measured ≥6 mm in diameter.

To ensure a standardized pathologic diagnosis, two pathologists independently examined one slide from each polyp. Discrepancies were resolved by the local pathologist. For individuals with more than one adenoma, the highest degree of villous component or intraepithelial neoplasia grade was used for classification purposes. For individuals with more than one resected adenoma, the largest was used for classification of size and for miRNA expression analysis. Adenoma location was classified as distal colon and rectum (rectum, rectosigmoid, sigmoid colon, descending colon, splenic flexure) or proximal colon (transverse colon, hepatic flexure, ascending colon, and cecum). For individuals with more than one adenoma located in both sites, a separate category was created.

**RNA isolation and real-time quantitative reverse transcription PCR**

Total RNA was extracted from FFPE tissues using RecoverAll Total Nucleic Acid Isolation Kit (cat. no: AM1975, Ambion). Total RNA was eluted in 50 μl nuclease-free water. The RNA concentration was measured with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

For miRNA-based real-time quantitative reverse transcription PCR (qRT-PCR) assays, 2.0 μg total RNA from FFPE samples was reverse transcribed in a total reaction volume of 25 μl, using the All-in-One miRNA Q-PCR Detection Kit (cat. no: AOMD-Q050, GeneCopoeia), according to the manufacturer’s instructions. A dilution factor of 1:5 for the RT products was used as a template for the qRT-PCR stage. Real-time qRT-PCR for mature miRNAs and RNU16B (internal control) was performed in triplicate according to the manufacturer’s instructions, using specific primers provided by GeneCopoeia (miR-10a: miRQP0032; miR-141: miRQP0184; miR-146a: miRQP0196; miR-151-3p: miRQP0211; miR-194: miRQP0280; miR-3607-3p: miRQP1901; RNU16B: miRQP9001). All qRT-PCR assays were performed on an Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems). The miRNA expression analyses were conducted using the comparative Ct (threshold cycle) method to calculate the relative expression level, using the equation: \( \text{log}_{10} (2^{\Delta C_t}) \), where \( \Delta C_t = (\text{Ct} \text{miR-CTRNU16B}) \).

The rest of experimental methods are described in detail in the online Supplementary Material. The project was approved by the Institutional Review Board of Renji Hospital and Shanghai 1st Hospital.

**Results**

**Identification of candidate miRNAs**

Global miRNA expression profiling of normal colorectal tissues (normal), NRACRA tissues, and RACRA tissues was performed by miRNA microarray analysis to identify potential adenoma recurrence–related miRNAs. Selection criteria of candidate miRNAs were based on two principles:
(1) candidate miRNAs increased or decreased expression from normal colorectal tissues, NRACRA, and RACRA successively with respect to decreasing or increasing grades of carcinogenesis. (2) The threshold value used to define upregulation or downregulation of miRNAs between NRACRA and RACRA was a fold change of >1.5, with significance set at \( P < 0.05 \), calculated by Student t test. The preliminary selections were miRNA (miR)-10a, miR-141, miR-146a, miR-151, miR-194, and miR-3607-3p based on their decreased expression from normal colorectal tissues, NRACRA, and RACRA in parallel with increasing carcinogenesis (Fig. 2A and B and Supplementary Tables S2 and S3). However, none of the miRNAs showed increased levels of expression when correlated against carcinogenesis progression. Therefore, these six miRNAs were selected for further validation and analysis.

**Validation of the six candidate miRNAs**

The expression levels of the six candidate miRNAs (miR-10a in Fig. 3A, a1; miR-141 in Fig. 3A, a2; miR-146a in Fig. 3A, a3; miR-151 in Fig. 3A, a4; miR-194 in Fig. 3A, a5; and miR-3607-3p in Fig. 3A, a6) in FFPE samples of normal colorectal tissues (\( n = 37 \)), NRACRA tissues (\( n = 127 \)), and RACRA tissues (\( n = 100 \)) from the retrospective cohort, were investigated by real-time qRT-PCR (Fig. 3A). In normal colorectal tissues, the expression levels \( \log_{10} (2^{-}\Delta C_{t}) \) mean ± SEM of miR-10a, miR-141, miR-146a, miR-151, miR-194, and miR-3607-3p were \( -0.405 \pm 0.0350, -0.406 \pm 0.08481, -0.3589 \pm 0.1113, -0.4284 \pm 0.09397, 0.6322 \pm 0.08677, \) and \( 0.5999 \pm 0.09928 \), respectively; in NRACRA tissues, the expression levels were \( -0.5308 \pm 0.06439, -0.3774 \pm 0.05234, -0.6110 \pm 0.05903, -0.8192 \pm 0.06558, 0.4484 \pm 0.06556, \) and \( 0.2879 \pm 0.08477 \), respectively; in RACRA tissues, the expression levels were \( -1.005 \pm 0.08165, -0.8962 \pm 0.06793, -0.9736 \pm 0.07229, -1.207 \pm 0.07867, -0.1601 \pm 0.08431, \) and \( -0.04026 \pm 0.09734 \), respectively. These data suggest that the expression levels of all six candidate miRNAs were significantly decreased in RACRA and NRACRA tissues compared with control samples; furthermore, these six miRNA candidates were expressed at significantly lower levels in RACRA tissues compared with NRACRA tissues (Mann–Whitney U test; \( P < 0.001 \)).

Receiver operating characteristic (ROC) curve analyses were conducted to investigate the potential adenoma recurrence prediction value of the six candidate miRNAs (Fig. 3B). The area under the ROC curve (AUC) cutoff value, sensitivity, and specificity for six miRNAs (miR-10a in Fig. 3B, b1; miR-141 in Fig. 3B, b2; miR-146a in Fig. 3B, b3; miR-151 in Fig. 3B, b4; miR-194 in Fig. 3B, b5; and miR-3607-3p in Fig. 3B, b6) are shown in Table 1. miR-194 is demonstrated to be a practical predictor, with an AUC of 0.755. The optimal sensitivity and specificity were 71.0% and 78.0%, respectively, at a cutoff value of 0.1311. As the AUCs of the other candidate miRNAs were all less than 0.70, their prognostic value is likely to be limited. Therefore, the other five miRNAs were excluded from further prospective cohort validations.

On the basis of miR-194 expression levels, the 158 patients with ACRA were assigned to high-expression group \( [\text{miR-194} \log_{10} (2^{-}\Delta C_{t}) > 0.1311; n = 93] \) or low-expression group \( [\text{miR-194} \log_{10} (2^{-}\Delta C_{t}) \leq 0.1311; n = 65] \). During the surveillance intervals of 22 to 24 months, 11 patients dropped out, including seven from the low-expression group and four from the high-expression group. Final surveillance colonoscopy results showed adenoma recurrence in 35 patients from the low-expression group (\( n = 58 \)) and in 11 patients from the high-expression group (\( n = 89 \); Fig. 1B). The optimal sensitivity and specificity with respect to a cutoff value of 0.1311 were 76.1% [95% confidence interval (CI), 61.2%–87.4%] and 77.2% (95% CI, 67.8%–85.0%), respectively. These data indicate that low expression of miR-194 may be a predictor for adenoma recurrence.

**Low expression of miR-194 as an independent factor for adenoma recurrence**

Logistic regression analysis was conducted to investigate whether the prognostic value of miR-194 was independent of other potential adenoma recurrence–related factors. Table 2 presents the results of logistic regression analysis for adenoma recurrence at colonoscopy surveillance among patients with ACRA at baseline in the two combined cohorts. In the univariate analysis, male gender, low expression of miR-194, adenoma size \( \geq 2 \) cm, number of adenomas \( \geq 3 \), adenomas in both locations or in proximal regions, villous components \( \geq 50 \%), and high-grade intraepithelial neoplasia were associated with adenoma recurrence. In the multivariate analysis, the
following factors were independently associated with adenoma recurrence: low expression of miR-194, adenoma size \( \geq 2 \) cm, and number of adenomas \( \geq 3 \).

**Evaluation for prognostic value for independent adenoma recurrence–related factor**

We performed ROC curve analyses to compare the prognostic value of the three independent adenoma recurrence–related factors (low expression of miR-194, adenoma size \( \geq 2 \) cm, and \( \geq 3 \) adenomas). The AUC, sensitivity, and specificity for three recurrence-related factors are shown in Fig. 4A–C. These analyses confirmed that low expression of miR-194 is better for predicting adenoma recurrence than adenoma size \( \geq 2 \) cm (\( P < 0.001 \)) and \( \geq 3 \) adenomas (\( P < 0.001 \); Fig. 4D).

**Discussion**

Recognition of adenoma recurrence after ACRA polypectomy is the key to reducing the incidence of CRC. Currently, the prediction of adenoma recurrence relies on morphology and histopathology of the tumor (5); however, the underlying molecular mechanisms and carcinogenetic potential can be very different in otherwise morphologically or pathologically similar tumors. Although there have been several trials investigating adenoma recurrence–related molecules (7–12), all of these studies were food or drug prevention trials, which suggested that these molecules only predict the efficacy of bioactive substance interventions, rather than for use in patients with ACRA.

Given the potential prognostic value of miRNAs in CRC (15–17), the miRNA profiles were analyzed to examine their
potential roles in ACRA prognosis. The six candidate miRNAs were selected for further validation based on their potential anti-oncogenic effects. Only miR-194 demonstrated a higher ability for predicting adenoma recurrence in the retrospective cohort. Moreover, the expression profile of miR-194 was validated in the prospective cohort, which confirmed its predictive value. The presence of multiple adenomas, large adenomas, proximal adenomas, old age, male sex, or obesity have been demonstrated to be related to the recurrence of adenoma in a pooled analysis comprising 9,167 postpolypectomy patients (5). Therefore, this study evaluated whether the prognostic value of low miR-194 expression was independent of the above known factors. Our multivariate analyses confirmed the importance of low expression of miR-194 independently, as well as adenoma size ≥2 cm and ≥3 adenomas for determining the risk. However, the latter two factors have very limited predictive ability with both AUCs <0.60. According to European guidelines (19), three or more adenomas with at least one ≥1 cm in size detected at any single examination indicate high risk and an extra examination should be undertaken at 12 months before returning to 3-yearly surveillance colonoscopies. In our study, 374 patients completed surveillance colonoscopy, in which 33 patients had more than three adenomas with at least one ≥1 cm. In those patients, 22 cases of recurrent adenoma occurred. The combination of adenoma size ≥1 cm and ≥3 adenomas showed a sensitivity and specificity of 15.1% (95% CI, 9.7%–22.0%) and 95.2% (95% CI, 91.6%–97.6%), respectively. Our study suggests that although these two parameters are major risk factors for adenoma recurrence, many cases of adenoma recurrence are still missed with 2 yearly surveillance colonoscopies. The low expression
of miR-194 has improved our ability to predict recurrence, over and above morphologic characteristics of the adenoma in surveillance colonoscopies.

To the best of our knowledge, we have performed the largest study yet to analyze miRNA profiles related to adenoma recurrence in patients with ACRA after polypectomy and the first to use two independent cohorts. Both cohorts showed low miR-194 expression in ACRAs as an independent predictor for adenoma recurrence. Our data are consistent with previous studies that have shown that the downregulation of miR-194 expression promotes carcinogenesis. In these studies, dramatic decreases in miR-194 expression were found in hepatocellular carcinoma (HCC; refs. 20, 21), CRC (22), gastric cancer (23), and multiple myeloma (24), suggesting its tumor-suppressive function. Chiang and colleagues also confirmed miR-194 as a tumor-suppressor gene in patients with CRC (22), whereas another study revealed a proangiogenesis function of miR-194 in HCT116 cells (25). Up to date, miR-192 and miR-215 have been reported to share the same "seed region" in the miRBase and HGNC websites: miR-192 and miR-194-2 are at 11q13.1 on the same chromosome, and miR-194-1 and miR-215 are at 1q41. Furthermore, miR-194 is composed from the mature sequences of miR-194-1 and miR-194-2. Several studies have indicated that miR-194 and its cluster associates, miR-192 and miR-215, may function as tumor suppressors capable of inhibiting cell proliferation and suppressing carcinogenesis through p53 protein upregulation and p21 activation (24, 25). A recent study demonstrated that miR-194 was transcriptionally upregulated by a gastrointestinal tract with enriched hepatic nuclear factor 1α (HNF1-α) during intestinal epithelium differentiation (26). Therefore, we speculated that miR-194 might be relatively specific to the gastrointestinal tract, and aberrations of the nuclear receptor, HNF1-α, may partially account for the downregulation of miR-194. We hypothesized that low expression of miR-194 in the preexisting advanced adenoma may represent altered expression of miR-194 in the entire colon tissues. Therefore, low expression of miR-194 in the preexisting advanced adenoma could be a predictor for adenoma recurrence. This hypothesis was supported by a clinical study performed by Chen and colleagues, in which the low expression of miR-126 in liver tissues was significantly associated with tumor recurrence in patients with HCC who underwent liver transplantation (27). Certainly, we also should investigate whether the expression of miR-194 is downregulated in the normal colon mucosa and recurrent adenoma of the patients. However, the experimental validation is difficult because (i) normal colon mucosa tissue was obtained only by endoscopic mucosal resection (EMR) in surveillance colonoscopy. However, the EMR operation for normal colon mucosa in adenoma patients did not meet clinical ethics principle. (ii) The preexisting adenoma was removed by snare of high-frequency electric resection or endoscopic mucosal resection. However, part of recurrent adenomas (relatively small) in surveillance colonoscopy was removed by argon plasma coagulation. Therefore, the tissues were destroyed and could not be acquired for further experimental procedures.

The detection of FFPE-based miRNAs has several distinct advantages: (i) miRNA expression is very stable in FFPE blocks (28), and has very high concordance in FFPE and fresh-frozen preserved samples (29); (ii) FFPE tissues are the most accessible biologic resources and are easily stored; (iii) the diagnosis of ACRA is established on hematoxylin and eosin-stained slides, which are only sourced from FFPE specimens.

In conclusion, as well as adenoma size ≥2 cm and ≥3 adenomas, the low expression of miR-194 is an independent predictor for adenoma recurrence in patients with ACRA after polypectomy in surveillance colonoscopies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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