Identification of microRNA-135b in stool as a potential non-invasive biomarker for colorectal cancer and adenoma

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Keywords: microRNA; stool marker; colorectal cancer; adenoma; diagnosis.

Abbreviations
APC, adenomatous polyposis coli; IBD, inflammatory bowel disease; miRNA, microRNA; CRC, colorectal cancer; CTC, CT colonography; FIT, fecal immunochemical test; FOBT, fecal occult blood test; gFOBT, the guaiac fecal occult blood test; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; ROC, receiver operating characteristic.

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Statement of translational relevance:
Colorectal cancer (CRC) screening allows the detection and removal of early stage lesions, and has been demonstrated to reduce both CRC morbidity and mortality. Compared to DNA, microRNA (miRNA) represents an emerging class of biomolecule being utilized as stool based marker for CRC screening. In the current study, we demonstrated for the first time, in a large cohort of patients with CRC or advanced adenoma that miR-135b, elevated in tumor tissue and stool samples, can be used as stool-based biomarker for CRC as well as adenomas. We revealed that the detection of miR-135b in stool has a sensitivity of 78% for CRC and 73% for advanced adenoma, thus stool-based miR-135b can be used as a potential non-invasive biomarker for the diagnosis of CRC and adenoma.
ABSTRACT

Purpose: Detecting microRNA (miRNA) in stool is a novel approach for colorectal cancer (CRC) screening. This study aimed to identify stool-based miRNA as non-invasive biomarkers for detection of CRC and adenoma.

Experimental Design: A miRNA expression array covering 667 human miRNAs was performed on five pairs of CRC and two pairs of advanced adenoma tissues. The most up-regulated miRNAs were validated in 40 pairs of CRC tissues, 16 pairs of advanced adenoma tissues and 424 stool samples including 104 CRCs, 169 adenomas, 42 inflammatory bowel diseases (IBD), and 109 healthy controls. miRNA levels were followed-up after removal of lesions.

Results: In an array analysis, miR-31 and miR-135b were the most up-regulated miRNAs in CRC and advanced adenoma as compared with their adjacent normal tissues (> 13-fold increase). In stool samples, level of miR-135b was significantly higher in subjects with CRC (P < 0.0001) or adenomas (P < 0.0001), but not in IBD patients compared with controls. miR-135b showed a significant increasing trend across the adenoma to cancer sequence (P < 0.0001). Levels of miR-31 were not significantly different among groups. The sensitivity of stool mR-135b was 78% for CRC, 73% for advanced adenoma, and 65% for any adenoma, respectively, with a specificity of 68%. No significant difference in miR-135b level was found between proximal and distal colorectal lesions. Stool miR-135b dropped significantly upon removal of CRC or advanced adenoma (P < 0.0001).

Conclusion: Stool-based miR-135b can be used as a non-invasive biomarker for the detection of CRC and advanced adenoma.
INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide (1). Although the incidence of CRC is declining in the West, it remains the second most common overall cause of cancer death in the West. Both the incidence and death rates from CRC are increasing rapidly in Asian countries (2). CRC screening allows the detection and removal of early stage lesions, and has been demonstrated to reduce both CRC morbidity and mortality (3, 4). However, mass screening efforts have been hindered by variable public acceptance and the limitations of existing tools. Colonoscopy although considered a gold standard test for CRC screening is associated with high cost and relatively low patient acceptance rate. The fecal occult blood test (FOBT) is the most widely adopted screening method but is compromised by a low sensitivity and specificity and poor patient adherence. The newer fecal immunochemical tests (FITs) have demonstrated a higher sensitivity for CRC, but sensitivity remains low for premalignant precursor lesions (5). CT colonography (CTC) is less invasive and accurate for the detection of CRC and advanced adenomas but is associated with high cost (6-9). There is therefore a need for better screening tools to avoid non-therapeutic colonoscopies for adenomas or colonoscopies to confirm CRC.

Testing for molecular aberrations in the stool has emerged as a promising non-invasive approach for CRC screening. Among stool-based molecular tests, DNA testing is the most established test (10-12). A DNA panel which combined 4 methylation markers, 7 reference mutations, β-actin and a hemoglobin assay, achieved a sensitivity of 85% for CRC, and 54% for adenoma ≥ 1cm. Each gene typically yielded an area under the
curve (AUC) value ranging from 0.61 to 0.75 towards CRC.

miRNAs are short non-coding RNAs that regulate gene translation (13). Most tumor types, including CRC, are found to have altered miRNA expression profiles (14-17). As tumor cells shed from CRC tumor surface into the lumen, aberrantly expressed miRNA levels can be detected in the stool. We have previously demonstrated that stool miRNAs (miR-21 and miR-92a) are stable and reproducible in a small number of the samples using an optimized RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) protocol (18). This provides a rationale of utilizing stool miRNA for the detection of CRC and adenomas. So far, four other studies have reported the use of stool based miRNA as a screening tool for CRC (19-22). Most of these studies involved small cohorts and were limited to the investigation of CRC.

In this study, we aimed to identify miRNA markers with higher sensitivity, particularly for the diagnosis of precancerous colorectal lesions in additional consecutive stool samples particularly from subjects with advanced adenomas. We investigated the miRNA expression profile in CRCs and in advanced adenomas to identify the most up-regulated miRNAs. Candidate miRNA markers were validated in 40 paired primary CRC tumors and 16 paired advanced adenoma tissues, and then in a large cohort of 424 stool samples of 104 CRC, 169 adenomas, 42 inflammatory bowel disease IBD as disease control and 109 healthy subjects as normal control. We identified and characterized that stool-based miR-135b accurately detects colorectal cancer and adenoma in this large case-control study.
MATERIALS AND METHODS

Subjects and stool sample collection

Stool samples were collected from 424 subjects including 104 patients with CRC (mean age, 66.8 ± 11.9 years), 59 patients with advanced adenomas (62.1 ± 9.5 years), 110 subjects with adenomas of less than 1 cm in size (58.9 ± 6.9 years), 42 subjects with IBD (48.2 ± 11.6 years), and 109 individuals who had a normal colonoscopy (60.4 ± 7.0 years) (Table 1). We have included IBD patients as a control group to demonstrate that the markers are specific to CRC and precancerous adenoma but not to common inflammatory intestinal diseases. The mean age of the CRC group was significantly older than the control group (P < 0.0001), whereas subjects with IBD were significantly younger than the control group (P < 0.0001). There were more males in the CRC than control group (58% versus 46%; P < 0.0001) (Table 1). Exclusion criteria included subjects with a family history of familial adenomatous polyposis or hereditary non-polyposis CRC, previous colonic surgery, or adjuvant therapy for CRC before surgery. Any patient who were passing liquid stool were also excluded (Supplementary Figure1).

All patients were recruited from Prince of Wales Hospital and Alice Ho Miu Ling Nethersole Hospital, Hong Kong. All participants had signed informed consent for obtaining stool or tissue samples. The study protocol was approved by the institutional review board of the Chinese University of Hong Kong and the Hong Kong Hospital Authority.

All cancer stool samples were collected 7 days after colonoscopy. All normal and adenoma stool samples were collected before colonoscopy. Fresh human stool samples
were collected from patients using a 30 ml universal sample container (Height: 93mm, cap diameter: 30.1mm) with spoon cap. The container was aseptically manufactured under clean room condition to exclude microbiological contamination. All samples were stored at 4°C immediately and transferred to -80°C within 24 hours. Stools were collected before bowel purgation and colonoscopy or 1 week after colonoscopy (but before resection of CRC or removal of advanced adenoma).

To investigate the changes in stool miRNA levels after removal of CRC or advanced adenomas, repeat stool collection was performed at least one month after surgical removal of CRC or at least 7 days after removal of the advanced adenoma. Advanced adenomas were defined as adenomas ≥ 1cm in diameter, adenomas with villous or tubulovillous features or high-grade dysplasia. Proximal lesions included lesions at or proximal to the splenic flexure, and distal lesions were lesions distal to the splenic flexure.

**Tissue collection**

Forty pairs of CRC tissues and 16 pairs of advanced adenoma tissues were collected (Supplementary Table 1). CRC and advanced adenoma tissues and their respective adjacent normal tissues (at least 4 cm apart from the lesion) were biopsied during the initial colonoscopy or during surgical resection. “Paired lesions” refers to a lesion paired with adjacent normal mucosal sample. Tissue samples were snap frozen upon collection and stored in -80°C freezer.
miRNA extraction in tissue and stool samples

Frozen tissue of 10 to 20 µg was added into 0.5 ml Trizol reagent (Invitrogen, Carlsbad, CA) in a 1.5 ml tube. The tissue was homogenized by RNase-free pestles. Chloroform of 200µl was added to the 1.5 ml tube.

Fresh human stool sample (20-30 gram) was collected with a 50ml specimen cup and stored in -80°C. Four categories of stool consistency were defined: ‘firm’ (the stool has clear-cut edges, maintains its own shape during handling but deforms with pressure), ‘soft’ (the stool has a uniform consistency but few or less apparent natural edges, it maintains its own shape but deforms with minimal handling), ‘loose’ (the stool has a semi-solid consistency and can take over the shape of the container), ‘watery’ (no solid pieces, completely liquid). Only ‘firm’, ‘soft’ and 'loose' stools were analyzed. More than 90% stool samples in each group belong to soft or loose. Stool sample of 200 mg to 300 mg (wet weight) was added to 1 ml Trizol LS reagent in a 2 ml tube (Invitrogen), and homogenized mechanically by RNase-free pestles (USA Scientific, Woodland, CA) to deform it completely. Chloroform of 300µl was added to the 2 ml tube.

Total RNA was extracted from the Trizol-chloroform mixture using the miRNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA was eluted in 50µl nuclease free water. RNA concentration was measured by Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE) (the range of the initial RNA concentration RNA concentration was 300 to 500 ng/µl).

Reverse transcription and microRNA microarray in CRC and advanced adenoma
tissues

Reverse transcription (RT) for microRNA microarray was carried out using Megaplex Primer pools, Human Pools A and B v2.1 kit (Applied Biosystems, Foster City, CA). Briefly, 2 ng total RNA was used in one RT reaction with a total volume of 3 μl. The RT product was diluted 4-fold by adding 9 ul nuclease free water (18).

Initial miRNA profiling was performed on 14 tissue samples from five pairs of CRC and two pairs of advanced adenoma. The characteristics of these patients are shown in Supplementary Table 2. Quantitation of 667 miRNAs in each of these samples was carried out using TaqMan Human MicroRNA Array Set version 2.0 (Applied Biosystems). In the array, miRNAs were normalized to mammalian U6 small RNA expression as based on the manufacturer’s guide (Applied Biosystems). Real-time quantitative PCR (qRT-PCR) was performed using Applied Biosystems 7900HT Real-Time PCR System. Results were analyzed by the SDS RQ Manager 1.2 software (Applied Biosystems) (Supplementary Table 3).

miRNA quantitation by qRT-PCR

qRT-PCR of individual miRNA was performed using TaqMan miRNA Reverse Transcription Kit (Applied Biosystems) and TaqMan Human MiRNA Assay (Assay ID: RNU6B: 001093; miR-31: 002279; miR-135b: 002261). The quantitation of miRNA was based on standard curve plotted by known input amongst all of the miRNAs, and normalized to per nanogram (ng) of input RNA (18). Assays were performed in a blinded fashion. Based on standard curves plotted from known amount of synthetic miR-135b, a
technical detection limit of 3 copies of miR-135b would approximately give a Ct of 42. Therefore, we assigned all Ct larger than 42 as “0”. For samples with no amplification of miR-135b at all, as long as that sample could be amplified with at least another miRNA tested (such as miR-18a, miR-20a and miR-221), the sample was regarded to have legitimate quality for qRT-PCR. Therefore, instead of excluding un-amplifiable samples, we assigned the sample as “0” in the analysis of miR-135b.

**Sample size and Statistics**

Given the exploratory aspect of this initial miRNA profiling and limitations of resources, we did not use a formal statistical test to choose a sample size, and we analyzed five pairs of CRC samples and two pairs of advanced adenoma sample for initial miRNA profiling. In our validation study, we analyzed 40 pairs of CRCs and 16 pairs of advanced adenoma tissue samples. Differences in miRNA expression between paired lesion tissues and adjacent normal tissues were evaluated by the Wilcoxon matched-pairs test. Differences in stool miRNA levels between groups were analyzed by Mann-Whitney U test. Receiver operating characteristics (ROC) curves were generated based on the comparison with the control group. Differences in miRNA levels before and after removal of the CRC or advanced adenoma were determined by Wilcoxon matched-pairs test. Significance in trend was tested by Jonckheere-Terpstra test. Two cutoff values were selected using ROC curves for reference, based on a high sensitivity or a high specificity. $P < 0.05$ was taken as statistically significant. Jonckheere-Terpstra test was done by SPSS 13.0 (SPSS Inc., Chicoga, Illinois). All other statistical tests were performed using Graphpad Prism 5.0 (Graphpad Software Inc., San Diego, CA).
RESULTS

miRNA profiling in CRC and advanced adenoma tissues

miRNA expression profiles were performed in five pairs of CRC samples and two pairs of advanced adenoma samples. The miRNA expression levels were compared between the tumor and their adjacent non-tumorous tissues in each case (Table 2). Amongst 667 miRNAs detected in each sample, miR-31 and miR-135b were identified to be the most up-regulated miRNAs in both CRC (miR-31: 42.28-fold increase; miR-135b: 13.00-fold increase) and advanced adenomas (miR-31: 106.36-fold; miR-135b: 13.32-fold) (Table 2). We have therefore focused on these two miRNA in subsequent experiments.

Validation of miRNA candidates in CRC and advanced adenoma tissues

In a validation set of 40 pairs of CRCs and 16 pairs of advanced adenoma tissue samples, miR-135b was demonstrated to be 555.4-fold higher in CRCs ($P < 0.0001$) and 33.1-fold higher in advanced adenoma tissues ($P = 0.0003$); whereas miR-31 was 105.1-fold higher in CRCs ($P < 0.0001$) and 86.1-fold higher in advanced adenoma ($P = 0.0003$) compared to their corresponding normal tissues (Table 3).

miR-135b is a potential non-invasive stool marker for CRC and adenoma

We then examined the levels of miR-135b and miR-31 in 424 stool samples which included 104 CRC, 169 adenomas, 42 IBD, and 109 controls. Stool-based miR-135b level measured in number of copies/ng stool extracted RNA was significantly higher in subjects with CRC [median: 67.9, Interquartile range (IQR): 16.1-182.7, $P < 0.0001$] and
adenomas (median: 28.4, IQR: 0.2-79.7, \( P < 0.0001 \)) compared with controls (median: 0, IQR: 0-30.8) (Figure 1A). In contrast, there was no significant difference in the level of stool miR-135b in subjects with IBD (median: 7.57, IQR: 0-60). The AUC values for miR-135b were 0.79 and 0.71 for the detection of CRC and adenomas, respectively (Figure 1B). As shown in Table 4, two cutoff values demonstrated the performance of this marker: a cutoff of 14 copies/ng of stool RNA provided the maximum sum of sensitivity and specificity; miR-135b has a sensitivity of 78% for CRC, 73% for advanced adenoma, 61% for adenoma < 1cm in diameter, 65% for any adenoma and a specificity of 68%. A cutoff of 38 copies/ng of stool RNA reflects its performance at a relatively high specificity (80%) level for reference, and the sensitivity was 44%, 46% and 64% for adenoma < 1cm, advanced adenoma and CRC, respectively (Table 4). However, there were no significant differences in the levels of stool miR-31 among CRC (median: 1583, IQR: 574.5-3364), adenomas (median: 1647, IQR: 661.9-3148), IBD (median: 1642, IQR: 1066-3345) and controls (median: 1293, IQR: 721-2612) (Figure 1C).

**Stool miR-135b level is significantly reduced after removal of neoplasm**

We repeated miRNA measurement in a subgroup of 28 patients (8 CRC, 20 advanced adenomas) after the removal of CRC or advanced adenomas. Upon removal of the primary lesions, levels of stool miR-135b dropped significantly compared to their initial levels (\( P < 0.0001 \), Figure 2A). These findings suggested that the initial high levels of stool miR-135b were derived from the primary neoplasms.

**Stool miR-135 level increases across the adenoma to cancer sequence**
As shown in Figure 2B, expression of stool miR-135 showed a significantly increasing trend across the disease transition from adenoma with diameter < 1cm (n = 110), advanced adenoma (n = 59), TNM stage I and II cancer (n = 24) to TNM stage III and IV cancer (n = 76) sequence (P < 0.0001). In keeping with this, an increasing sensitivity for stool-based miR-135 detection was demonstrated with 61% for adenomas with diameter < 1cm, 73% for advanced adenoma, 67% for TNM stage I and II cancer, and 80% for TNM stage III and IV cancer, and a specificity of 58.4%. Based on Table 3, the increase fold change of miR-135b in CRC tissue samples is approximately 17-fold higher than in advanced adenoma tissue samples.

**Stool miR-135 level is not associated with the location of CRC or advanced adenoma**

We compared the level of stool miR-135 between distal and proximal lesions. No significant difference was found in miR-135b levels for CRC or advanced adenomas located in proximal or distal colon (Figure 2C), although at a specific cutoff value (14 copies/ng RNA), miR-135b showed better sensitivity in detecting distal advanced adenoma (81%) compared to proximal ones (65%), with specificity of 68% for both distal and proximal advanced adenoma (Table 4).

**DISCUSSION**

As CRC demonstrates high homogeneity in miRNA alteration, stool miRNA could represent a useful non-invasive tool for screening CRC and its precancerous lesions (18, 22, 23). For maximum benefit, a CRC screening tool should be effective in detecting
CRC in its early stage and premalignant precursors throughout the entire colorectum. In this study, we performed systematical examinations with the aim to identify the best miRNA biomarker for the screening of CRC and advanced adenomas.

We first identified that miR-135b and miR-31 were the two most up-regulated miRNAs both in CRCs and adenomas by miRNA expression arrays. In subsequent validation, miR-135b and miR-31 were also found to be significantly up-regulated in CRC and advanced adenoma as compared to their adjacent normal tissues (Table 3). This is consistent with previous studies showing that miR-135b (24, 25) and miR-31 (14, 26-29) are up-regulated miRNAs in CRC. The levels of miR-135b and miR-31 were therefore tested in a large cohort of stool samples. Significantly higher level of miR-135b, but not miR-31, was detected in the stool samples of CRC ($P < 0.0001$) and advanced adenoma ($P < 0.0001$) compared with healthy controls (Figure 1A). In addition, miR-135b level correlated positively with stages of lesions, with more advanced lesions having the highest miRNA level. A significant increasing trend across the histological sequence was observed from small adenoma, advanced adenomas, TNM stage I and II, to TNM stage III and IV ($P < 0.0001$, Figure 2B). Areas under the ROC curve were 0.79 for the detection CRC, and 0.71 for adenoma (Figure 1B). At a cutoff of 14 copies/ng RNA, miR-135b had a sensitivity of 73% and 78% for the detection of advanced adenomas and CRC respectively, with a specificity of 68%. We have included IBD patients as a control group to demonstrate that the markers are specific to CRC and precancerous adenoma but not to common inflammatory intestinal diseases. The significant drop in stool miR-135b level upon removal of advanced adenoma and CRC,
and the relatively low level of miR-135b detected in stool of IBD patients indicated that the up-regulation of miR-135b is a specific biomarker for CRC and its precancerous lesion. In this study, we did not use RNU6B as internal control for stool miRAN detection. The choice and rationale of normalizing stool based miRNA have been discussed in our previous study (18), which is mainly because RNU6B could only be detected in 83.3% (25/30) of stool samples; whilst the candidate miRNAs could be detected in 100% samples (18). In addition, under equal amount of input RNA and detection threshold, RNU6B was detected with a much lower abundance compared to candidate miRNAs (18). Thus, RNU6B may not be an ideal internal control for stool-based miRNA. In this regard, absolute quantitation with standard curve calibration was adopted for miRNA quantitation in the current study. Other upregulated miRNA candidates including miR-18a, miR-20a and miR-221 from our miRNA array assay were also upregulated in CRC tissues compared to adjacent normal controls. As these miRNA candidates were not able to discriminate patients with adenoma from healthy individuals in stool samples (Supplementary Table 4), and the sensitivities and specificities of these miRNAs were lower compared to miR-135b for the stool detection, we did not included these miRNAs in this study. miR-135b was demonstrated to be the most discriminating marker for the detection of both advanced adenoma and CRC.

miR-135b originates from 1q32.1, a region of frequent copy number gain in CRC tumorigenesis. miR-135b targets the 3’UTR of the adenomatous polyposis coli (APC) gene, a well-known tumor suppressor, and suppresses its expression (24). APC down-regulation activates the Wnt signaling pathway, an important oncogenic pathway in
regulating cell proliferation and apoptosis in CRC. Up-regulation of miR-135b is consistent with its role in regulating APC gene, whose loss-of-function is well-established to be an early event of the adenoma-cancer sequence in colorectum.

Results from this study have several clinical implications. First, effective detection of proximal colon neoplasms is an important criteria for an effective screening tool (30). Current screening modalities appear less sensitive for proximal than distal colonic neoplasms, and interval CRC is more likely to be found in the proximal colon (11, 31). In this study, miR-135b level has comparable efficacy for the detection of both proximal and distal CRC and advanced adenoma. Secondly, miR-135b represents a stool-based test that is non-invasive, avoids the unpleasant bowel preparation, and allows the possibility of off-site sample collection. Therefore, miR-135b may act as a potential non-invasive diagnostic biomarker for CRC and its precancerous lesion (advanced adenoma), but more studies in different populations to validate miR-135b as a biomarker for CRC screening are required before it can be applied clinically. Ultimately test adoption will depend on test performance in the screening setting, availability, affordability, and user appeal in a large population-based programme. For instance, screening using fecal DNA markers demonstrated a higher sensitivity than the FOBT, although the two tests yielded similar specificities (10). There has been no direct comparison of stool DNA versus RNA tests in the screening setting. The evidence for the effectiveness of gFOBTs in reducing CRC incidence and mortality is strong, and is based on randomised controlled trials in large number of average risk populations. FIT is a more sensitive test for screen-relevant neoplasia than FOBT; FITs processing and
interpretation are automated and objective. Compared with gFOBT and FIT, miRNA lacks a large scale validation. Only if this validation is conducted, the possible advantage of miRNA in detecting more proximal neoplasms than fecal blood tests will be known.

Stool miR-31 levels did not differ significantly among controls, adenoma and CRC (Figure 1C), and it did not correlate with stool miR-135b level. We excluded the possibility that the aberrant up-regulation of miR-31 was restricted to the submucosal regions given that the up-regulation was also present in CRC epithelial cells (14). The reason why stool miR-31 is not discriminating remains to be elucidated; however, this demonstrates the practical fact that abnormalities at the tissue level do not necessarily translate into a clinically useful marker in the stool. As a non-invasive marker, empirical testing in large number of stool samples is required to establish its usefulness.

This study has several limitations. Most patients were recruited from only two centers and some patients were symptomatic, results might not be representative of the screening setting, although we did not detect significant difference in miRNA results when patients were divided into asymptomatic screening or symptomatic surgical cohorts. More late stage CRC patients were identified in the study (n = 24 for TNM I and II vs. n = 76 for TNM III and IV) and the stool samples from CRC used in the study might be biased to late stage. Prompted by these findings, larger scale validation across multiple centers and different populations will be conducted. In addition, there were more male subjects in the CRC group and they were significantly older than the control
group. Nonetheless when we took into account age and gender in the data analysis for stool miR-135b level, our results remained valid and were independent of age and gender. We have not assessed the effect of time point of sampling (before versus after colonoscopy) on miRNA results. In all patients with advanced adenomas, stool was collected one week before colonoscopy whereas in subjects with colorectal cancer, stool samples were collected one week after colonoscopy but before surgery. Lastly there are no head to head comparisons of miRNA and fecal occult blood tests.

In conclusion, this study demonstrates that stool-based miR-135b appears to be a potential non-invasive biomarker for the detection of CRC and advanced adenoma. Further validation in multiple cohorts of patients, and comparison with the established tests are needed before it can be used as a biomarker in routine clinical practice.

ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

Chung Wah Wu, Siew Chien Ng and Jun Yu contributed to study concept, design, analysis and interpretation of data and drafting of the manuscript. Francis Ka Leung Chan and Joseph Jao Yiu Sung revised the manuscript for important intellectual content.
Simon Siu Man Ng, Yu Juan Dong, Wing Wa Leung, Wai Tak Law and Tung On Yau contributed to acquisition of samples, data and technical support, Linwei Tian contributed to statistical analyses.

REFERENCES


Table 1. Clinicopathological characteristics of subjects

<table>
<thead>
<tr>
<th>Category</th>
<th>Normal</th>
<th>Adenoma &lt;1cm</th>
<th>Advanced Adenoma*</th>
<th>Colorectal Cancer</th>
<th>Inflammatory Bowel Disease</th>
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<tbody>
<tr>
<td>No. of cases</td>
<td>109</td>
<td>110</td>
<td>59</td>
<td>104</td>
<td>42</td>
</tr>
<tr>
<td>Age at enrollment, Years</td>
<td>Mean±SD</td>
<td>60.4 ± 7.0</td>
<td>58.9 ± 6.9</td>
<td>62.1 ± 9.5</td>
<td>66.8 ± 11.9</td>
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<td>Gender, number (%)</td>
<td>Female</td>
<td>59 (54.1)</td>
<td>51 (46.4)</td>
<td>29 (49.2)</td>
<td>44 (42.3)</td>
</tr>
<tr>
<td>Location†, number (%)</td>
<td>Proximal</td>
<td>32 (29.1)</td>
<td>26 (44.1)</td>
<td>29 (27.9)</td>
<td>-</td>
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<tr>
<td></td>
<td>Distal</td>
<td>65 (59.1)</td>
<td>31 (52.5)</td>
<td>75 (72.1)</td>
<td>-</td>
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<td></td>
<td>Both</td>
<td>13 (11.8)</td>
<td>2 (3.4)</td>
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<td>Tumor histology, number (%)</td>
<td>Adenocarcinoma</td>
<td>-</td>
<td>-</td>
<td>97 (93.3)</td>
<td>-</td>
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<tr>
<td></td>
<td>Mucinous adenocarcinoma</td>
<td>-</td>
<td>-</td>
<td>6 (5.8)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Signet ring cell and mucinous</td>
<td>-</td>
<td>-</td>
<td>1 (0.9)</td>
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<tr>
<td>TNM stage‡, number (%)</td>
<td>I and II</td>
<td>-</td>
<td>-</td>
<td>24 (23.1)</td>
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<tr>
<td></td>
<td>III and IV</td>
<td>-</td>
<td>-</td>
<td>76 (73.1)</td>
<td>-</td>
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*Advanced Adenoma is defined as adenoma 1 cm or greater in diameter, adenoma with more than 25% villous feature, or adenoma with high-grade dysplasia.
†Proximal lesions include tumors at or proximal to the splenic flexure, and distal lesions are those distal to the splenic flexure.
‡Tumor-node-metastasis (TNM) stage data of four patients were unavailable.
Table 2. Top 10 most up-regulated microRNAs identified by profiling of 667 microRNAs in five CRC patients and two advanced adenoma patients

<table>
<thead>
<tr>
<th>Rank</th>
<th>microRNA</th>
<th>Accession Number</th>
<th>Chromosomal location</th>
<th>Average fold increase†</th>
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<tr>
<td>1</td>
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<td>MI0000089</td>
<td>9p21.3</td>
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<td>MI0000301</td>
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<td>MI0000298</td>
<td>Xp11.3</td>
<td>3.95</td>
</tr>
<tr>
<td>8</td>
<td>miR-21</td>
<td>MI0000077</td>
<td>17q23.1</td>
<td>3.39</td>
</tr>
<tr>
<td>9</td>
<td>miR-223</td>
<td>MI0000300</td>
<td>Xq12</td>
<td>3.18</td>
</tr>
<tr>
<td>10</td>
<td>miR-20a</td>
<td>MI0000076</td>
<td>13q31.3</td>
<td>3.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rank</th>
<th>microRNA</th>
<th>Accession Number</th>
<th>Chromosomal location</th>
<th>Average fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>miR-31</td>
<td>MI0000089</td>
<td>9p21.3</td>
<td>106.36</td>
</tr>
<tr>
<td>2</td>
<td>miR-135b</td>
<td>MI0000810</td>
<td>1q32.1</td>
<td>13.32</td>
</tr>
<tr>
<td>3</td>
<td>miR-20a-3p</td>
<td>MIMAT0004493</td>
<td>13q31.3</td>
<td>4.30</td>
</tr>
<tr>
<td>4</td>
<td>miR-182</td>
<td>MI0000272</td>
<td>7q32.2</td>
<td>3.99</td>
</tr>
<tr>
<td>5</td>
<td>miR-649</td>
<td>MI0003664</td>
<td>22q11.21</td>
<td>3.93</td>
</tr>
<tr>
<td>6</td>
<td>miR-26a-1-3p</td>
<td>MIMAT0004499</td>
<td>3p22.2</td>
<td>3.33</td>
</tr>
<tr>
<td>7</td>
<td>miR-625</td>
<td>MI0003639</td>
<td>14q23.3</td>
<td>3.13</td>
</tr>
<tr>
<td>8</td>
<td>miR-18a</td>
<td>MI0000072</td>
<td>13q31.3</td>
<td>2.78</td>
</tr>
<tr>
<td>9</td>
<td>miR-20a</td>
<td>MI0000076</td>
<td>13q31.3</td>
<td>2.75</td>
</tr>
<tr>
<td>10</td>
<td>miR-552</td>
<td>MI0003557</td>
<td>1p34.3</td>
<td>2.17</td>
</tr>
</tbody>
</table>

†Average fold change in colorectal tumor was expressed as ratio of the microRNA expression in tumor to adjacent non-tumorous tissue. Average fold change in advanced adenoma was expressed as ratio of microRNA expression in advanced adenoma to adjacent non-adenomatous tissue.
Table 3. Expression of miR-135b and miR-31 in colorectal tumor or advanced adenoma

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Percentage of samples with elevated expression in tumor or advanced adenoma</th>
<th>Average fold increase</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colorectal cancer (n = 40)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-135b</td>
<td>92.5% (37/40)</td>
<td>555.4</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>miR-31</td>
<td>87.5% (35/40)</td>
<td>105.1</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>Advanced adenoma (n = 16)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-135b</td>
<td>93.8% (15/16)</td>
<td>33.1</td>
<td>0.0003</td>
</tr>
<tr>
<td>miR-31</td>
<td>87.5% (14/16)</td>
<td>86.1</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

*P values were estimated by Wilcoxon matched pairs test
Table 4. Stool-based miR-135b sensitivities and specificities

<table>
<thead>
<tr>
<th>Cutoff value*</th>
<th>14 (High sensitivity)</th>
<th>38 (High specificity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivities, % (95% CI)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenoma</td>
<td>65 (57-72)</td>
<td>45 (37-53)</td>
</tr>
<tr>
<td>Adenoma &lt; 1cm</td>
<td>61 (51-70)</td>
<td>44 (34-53)</td>
</tr>
<tr>
<td>Advanced adenoma</td>
<td>73 (60-84)</td>
<td>46 (33-60)</td>
</tr>
<tr>
<td>Size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1cm – 1.9cm</td>
<td>76 (55-91)</td>
<td>40 (21-61)</td>
</tr>
<tr>
<td>≥ 2cm</td>
<td>83 (52-98)</td>
<td>75 (43-95)</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>65 (44-83)</td>
<td>38 (20-59)</td>
</tr>
<tr>
<td>Distal</td>
<td>81 (63-93)</td>
<td>52 (33-70)</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>78 (69-85)</td>
<td>64 (54-74)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNM I, II</td>
<td>67 (45-84)</td>
<td>50 (29-71)</td>
</tr>
<tr>
<td>TNM III, IV</td>
<td>80 (70-89)</td>
<td>67 (55-77)</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>79 (60-92)</td>
<td>59 (39-76)</td>
</tr>
<tr>
<td>Distal</td>
<td>77 (66-86)</td>
<td>67 (55-77)</td>
</tr>
<tr>
<td>Specificity, % (95% CI)</td>
<td>68 (58-77)</td>
<td>80 (71-87)</td>
</tr>
</tbody>
</table>

*Cutoff value was expressed as copies of the microRNA per nanogram of extract stool RNA. Two cutoff values were selected using ROC curves for reference.
FIGURE LEGENDS

Figure 1. Levels of miRNA markers in stool samples. (A) miR-135b and (B) its ROC curve, (C) miR-31 and (D) its ROC curve. Subjects were categorized into 4 groups: individuals of normal colonoscopy results (n = 109), adenoma (n = 72), colorectal cancer, CRC (n = 104) and inflammatory bowel disease, IBD (n = 42) respectively. The miRNA levels were expressed in the number of copies per nanogram of extracted RNA. Open circles represent samples with undetectable miRNA level. The lines denote the medians. P denotes significance measured by Mann-Whitney test. NS denotes no statistical significance. ROC curves were plotted to discriminate CRC or adenoma patients from individuals with normal colonoscopy findings.

Figure 2. Associations between stool miR-135b and clinical features. (A) Changes of stool miR-135b level after removal of tumors (squares) or advanced adenomas (triangles). Samples aligned on the x-axis represent those with undetectable miRNA level. P values indicate significant difference determined by the Wilcoxon matched pairs test. Stool miR-135b level in subjects categorized by (B) lesion stage and (C) lesion location. Based on lesion stage, subjects were categorized into 5 groups: individuals of normal colonoscopy (Normal, n = 109), patients with adenoma of diameter smaller than 1cm (A < 1cm, n = 110), patients with advanced adenoma (AA, n = 59), patients of tumor-node-metastasis (TNM) stages I and II (T 1 2, n=24) and patients of TNM stages III and IV (T 3 4, n = 76), respectively. P value for test for trend denotes significance measured by Jonckheere-Terpstra nonparametric test for trend. Based on lesion location, colorectal cancer (CRC) and advanced adenoma (AA) patients were
categorized into proximal CRC (n = 29) and distal CRC (n = 75), proximal AA (n = 26) and distal AA (n = 31). NS denotes no statistical significance. miRNA levels were expressed in number of copies per nanogram of extracted RNA. Open circles represent samples with undetectable miRNA level. The lines denote the medians.
Figure 1

A

B

C

D

Stool miR-135b level, log_{10}

Stool miR-31 level, log_{10}

Sensitivity

100 - Specificity%

100

80

60

40

20

0

100 - Specificity%

100

80

60

40

20

0

Stool miR-135b level, log_{10}

Stool miR-31 level, log_{10}

Sensitivity

100 - Specificity%

100

80

60

40

20

0

Stool miR-135b level, log_{10}

Stool miR-31 level, log_{10}

Sensitivity

100 - Specificity%

100

80

60

40

20

0

Stool miR-135b level, log_{10}

Stool miR-31 level, log_{10}

Sensitivity

100 - Specificity%

100

80

60

40

20

0

Normal Adenoma CRC IBD

Normal Adenoma CRC IBD

Normal Adenoma CRC IBD

Normal Adenoma CRC IBD

P < 0.0001 P < 0.0001 NS

CRC AUC = 0.79 Adenoma AUC = 0.71 IBD AUC = 0.57

CRC AUC = 0.53 Adenoma AUC = 0.54 IBD AUC = 0.59
Identification of microRNA-135b in stool as a potential non-invasive biomarker for colorectal cancer and adenoma

Chung-Wah Wu, Siew Chien Ng, Yujuan Dong, et al.

Clin Cancer Res  Published OnlineFirst April 1, 2014.

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