**International Surgery**

**The expression and Clinical Significance of miRNA-135a and Bach1 in colorectal cancer**

---Manuscript Draft---

| Manuscript Number: | INTSURG-D-20-00026R1 |
|--------------------|-----------------------|
| Full Title:        | The expression and Clinical Significance of miRNA-135a and Bach1 in colorectal cancer |
| Article Type:      | Original Article |
| Keywords:          | miRNA-135a; Bach1, colorectal cancer |
| Corresponding Author: | fei yan Zhu wuxi people's hospital wuxi, Please enter the state or province CHINA |
| Corresponding Author Secondary Information: | |
| Corresponding Author's Institution: | wuxi people's hospital |
| First Author:      | Yang zhi Jiang |
| First Author Secondary Information: | |
| Order of Authors:  | Yang zhi Jiang Qing Guo Tao fei yan Zhu |
| Order of Authors Secondary Information: | |
| Abstract:          | BACKGROUND
AIM
To explore the correlation between the expression of miRNA-135a and Bach1 in colorectal cancer tissue and the patient's clinical information.

Methods
60 patients with colorectal carcinoma were treated as a control group. Real-time quantitative PCR assays and immunohistochemistry method were performed to detect the expression of miRNA-135a and Bach1 in 60 colorectal carcinomas and adjacent normal tissues, and the clinical and pathological classifications had also been investigated. The SPSS 19.00 software was used. All data represented mean±SD of three independent experiments. P<0.05 was considered statistically significant.

Results
miRNA-135a expression levels increased significantly in the colon cancer tissues compared with the non-tumor control tissues (P<0.01). miRNA-135a expression levels were higher in stage III/IV than in stage I/II colon cancer patients. The expression level of Bach1 in colorectal cancer was significantly lower (P<0.01). Bach1 and miRNA-135a were negatively correlated.

Conclusions: The levels of miRNA-135a and Bach1 were opposite, the over-expression of miRNA-135a might downregulated the expression of Bach1, which might be involved in the pathogenesis of colorectal cancer.
The expression and Clinical Significance of miRNA-135a and Bach1 in colorectal cancer

Running title: miRNA-135a and Bach1 in colorectal cancer

Zhiyang Jiang, MD
Guoqing Tao, MD
Yanfei Zhu, MD PhD

Zhiyang Jiang, Guoqing Tao, Yanfei Zhu. Department of General Surgery, Wuxi People’s Hospital of Nanjing Medical University, wuxi 214023, China

Corresponding author: Yan-Fei Zhu, MD, Department of General Surgery, Wuxi People’s Hospital of Nanjing Medical University, No. 299, Qingyang Road, Wuxi 214023, Jiangsu Province, China. zhuyanfei_2002@163.com

Telephone: +86-510-85350091
Fax: +86-510-82828435

Authors’ contributions

Zhu YF and Tao GQ were responsible for overall study concept and design of experiments. Zhu YF analyzed and interpreted the patient data. Jiang ZY performed the IHC analysis and PCR, and Zhu YF was a major contributor in writing the manuscript. All authors read and approved the final manuscript.
Figure 1

Expression of miR-135a in CC tissues

![Bar chart showing expression levels of miR-135a in CC tissues compared to normal tissues. The chart indicates a statistically significant difference with P<0.01.](http://meridian.allenpress.com/international-surgery/article-pdf/doi/10.9738/INTSURG-D-20-00026.1/2790466/intsurg-d-20-00026.pdf)
Figure 2  Expression of miR-135a in different tumor stage

![Graph showing expression of miR-135a in different tumor stages I/II, III, and IV with statistical significance levels (P<0.01, P=0.04).]
Figure 3

Expression of BACH1 in CC tissues
Figure 4  BACH1 expression evaluated by immunohistochemistry (×200)

Compare to normal group, the expression of BACH1 in CC issues was much smaller.
### Table 1. Correlations between clinicopathological parameters and miRNA-135a expression in CC tissues

| Parameters          | Patient | colorectal cancers miRNA-135a | P-value | Adjacent normal tissues miRNA-135a | P-value |
|---------------------|---------|-------------------------------|---------|-----------------------------------|---------|
| **Age**             |         |                               |         |                                   |         |
| <60                 | 26      | 0.086±0.050                   | 0.086   | 0.041±0.003                       | 0.041   |
| ≥60                 | 34      | 0.079±0.019                   | 0.56    | 0.038±0.006                       | 0.038   |
| **Gender**          |         |                               |         |                                   |         |
| Male                | 33      | 0.085±0.042                   | 0.079   | 0.040±0.006                       | 0.040   |
| Female              | 27      | 0.076±0.011                   | 0.47    | 0.039±0.005                       | 0.039   |
| **Tumor size**      |         |                               |         |                                   |         |
| <5 cm               | 31      | 0.086±0.044                   | 0.066   | 0.042±0.005                       | 0.042   |
| ≥5 cm               | 29      | 0.077±0.012                   | 0.46    | 0.037±0.003                       | 0.037   |
| **Tumor location**  |         |                               |         |                                   |         |
| Ascending colon     | 8       | 0.085±0.049                   | 0.085   | 0.040±0.005                       | 0.040   |
| Transverse colon    | 3       | 0.084±0.043                   | 0.034   | 0.038±0.002                       | 0.038   |
| Descending colon    | 6       | 0.082±0.027                   | 0.072   | 0.041±0.003                       | 0.041   |
| Sigmoid colon       | 9       | 0.078±0.015                   | 0.078   | 0.040±0.004                       | 0.040   |
| Rectum              | 4       | 0.083±0.033                   | 0.74    | 0.040±0.002                       | 0.040   |
| **Differentiation** |         |                               |         |                                   |         |
| Well and moderately | 29      | 0.085±0.041                   | 0.085   | 0.041±0.005                       | 0.041   |
| Poorly              | 31      | 0.077±0.011                   | 0.54    | 0.038±0.003                       | 0.038   |
| **Depth of invasion** |       |                               |         |                                   |         |
| T1~T2               | 22      | 0.087±0.049                   | 0.09    | 0.039±0.004                       | 0.039   |
| T3~T4               | 38      | 0.078±0.017                   | 0.078   | 0.040±0.006                       | 0.040   |
| **Lymph node**      |         |                               |         |                                   |         |
| Negative            | 21      | 0.054±0.015                   | 0.054   | 0.041±0.002                       | 0.041   |
| Positive            | 39      | 0.100±0.028                   | 0.100   | 0.039±0.005                       | 0.039   |
| **Tumor stage**     |         |                               |         |                                   |         |
| I-II                | 26      | 0.062±0.021                   | <0.01   | 0.040±0.005                       | 0.040   |
| III-IV              | 34      | 0.103±0.032                   | <0.01   | 0.039±0.006                       | 0.039   |
Table 2  Expression of BACH1 in different tumor stage

| stage | n  | Bach1 (+) | Bach1 (-) | P 值 |
|-------|----|-----------|-----------|------|
| I/II  | 26 | 10        | 16        |      |
| III/IV | 34 | 3         | 31        | <0.05|

The low expression of BACH1 in tumor III-IV stages presented the potential correlation with tumor stage.
| miRNA-135a | Bach1  | R     | P   |
|-----------|--------|-------|-----|
| low       | high   | 0.375 | 0.04|
|           | low    | 8     | 5   |
|           | high   | 41    | 6   |

Bach1 and mirna-135a are negatively correlated. (P < 0.05)
The expression and Clinical Significance of miRNA-135a and Bach1 in colorectal cancer
Abstract

BACKGROUND

AIM

To explore the correlation between the expression of miRNA-135a and Bach1 in colorectal cancer tissue and the patient's clinical information.

Methods

60 patients with colorectal carcinoma were treated as a control group. Real-time quantitative PCR assays and immunohistochemistry method were performed to detect the expression of miRNA-135a and Bach1 in 60 colorectal carcinomas and adjacent normal tissues, and the clinical and pathological classifications had also been investigated. The SPSS 19.00 software was used. All data represented mean±SD of three independent experiments. P<0.05 was considered statistically significant.

Results

miRNA-135a expression levels increased significantly in the colon cancer tissues compared with the non-tumor control tissues(P<0.01). miRNA-135a expression levels were higher in stage III/IV than in stage I/II colon cancer patients. The expression level of Bach1 in colorectal cancer was significantly lower(P<0.01). Bach1 and miRNA-135a were negatively correlated.

Conclusions: The levels of miRNA-135a and Bach1 were opposite, the over-expression of miRNA-135a might downregulated the expression of Bach1, which might be involved in the pathogenesis of colorectal cancer.

Keywords: miRNA-135a, Bach1, colorectal cancer
Background

The colorectal cancer (CC) mortality rate has been decreasing in Western advanced countries, while it is still growing in China. Recently, CC has become the third-ranking cause of cancer death in China. During the early stages of CC, some patients could be treated effectively with radical surgery and chemotherapy. Due to the high rates of postsurgical recurrence and metastasis, the prognosis remains disappointing for patients with advanced-stage.[1-3].

MicroRNAs (miRs), a category of non-protein-coding RNAs, have been recognized as critical participants in many pathways, especially proliferation and apoptosis. Besides, more and more researches have displayed their carcinogenic or cancer suppressive functions in many solid tumors[4-6]. Furthermore, it is a challenge to distinguish invasive carcinomas from high-grade intraepithelial neoplasms in colonoscopy biopsy tissues. One recent research has found a microRNA panel that accurately differentiates carcinomas from high-grade intraepithelial neoplasms in colonoscopy biopsy tissues, which has potential clinical application in the early diagnosis and optimal surgical decision-making of colorectal cancer[7]. Recently, miR-135a has been explored widely and deeply because of its controversial role in cancers[8-10]. For example, the expression of miR-135a increases in hepatocellular carcinoma and human bladder cancer, which is implicated in the development of them. By contrast, some studies show that it decreases and plays a suppressive role during the development of malignant glioma, such as epithelial ovarian cancer and renal cell carcinoma[11-14]. These controversial results may reflect the various roles of miR-135a
in different types of cancer. Furthermore, miR-135a has been found to be up-regulated in CC cells\textsuperscript{[9,15]}. As one of potential target genes, Bach1(BTB and CNC homology 1), plays a vital role in adjustment of oxidative stress and ascribed as a repressor of its main target hemoxygenas-1(HO-1). The expression of HO-1 increases significantly in various types of cancer, which might promote tumor growth and metastasis\textsuperscript{[16-18]}. In this study, we examined the expression levels of miR-135a and Bach1 in CC tissues by quantitative PCR and immunohistochemistry respectively, and investigated the association between them to evaluate the possible role in the development of CC.

Methods

Tissue samples and clinical data

Sixty patients diagnosed with CC at Wuxi People’s Hospital of Nanjing Medical University between 2016 and 2017 were recruited in our study. These patients were treated by colorectectomy with lymphadenectomy. The clinical stage of postoperative patients was evaluated. All patients should not received any chemotherapy, radiotherapy or other treatment prior to surgery. Human tissues including sixty colorectal cancer tissues and sixty matched adjacent normal tissues were immediately collected after surgical resection. The clinicopathologic characteristics of these patients were collected from electronic medical records. The study was approved by the Research Ethics Board of Wuxi People’s Hospital of Nanjing Medical University. All patients signed an informed consent form for this investigation.

quantitative PCR

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, California) and
then measured by spectrophotometer (BioPhotometer, Eppendorf, Hamburg, German).

The transcriptions of miRNA-135a and Bach1 were detected with the primers. The transcription of β-actin was used for normalization. The PCR products were detected by ethidium bromide staining. Images were obtained and the gray values of all the products were measured by ImageJ.

**Immunohistochemistry**

Immunohistochemical study was performed using the EnVision method (Dako, Glostrup, Danmark) on 2-mm formalinfixed, paraffin-embedded sections. The staining intensity was scored semiquantitatively as described by two independent observers without knowledge of the clinical status of the samples. All the images were captured using a digital camera mounted on a light microscope (Axioscprop, Zeiss, Gottingen, Germany).

**Statistical analyses**

Data presented as the mean ± SD and analyzed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Statistical analyses were performed using an independent samples t-test and one-way AVOVA. Spearman correlation analysis was performed. P<0.05 was considered significant.

**Results**

The clinicopathologic characteristics of these patients showed in Table 1. There was no difference between the expression of miRNA-135a with many aspects in CC tissues, including age, tumor size, location, differentiation and etc. It was different in
lymph node involvement group and tumor stage group. Compare to lymph node negative involvement group, miRNA-135a expression levels were higher in lymph node involvement group. Besides, miRNA-135a expression levels were higher in stage III/IV than in stage I/II colon cancer patients (P < 0.01) (Fig 1). The results suggested that the high expression of miRNA-135a in lymph node metastasis (LNM) group and tumor III-IV stages presented the potential correlation with LNM and tumor stage.

miRNA-135a had a statistical difference between the CC tissues and matched normal tissues (P < 0.01). The expression levels of miRNA-135a were significantly increased in CC tissues (figure 2).

The expression of Bach1 by RT-PCR result was 0.032±0.002 vs 0.073±0.004, P<0.05, in the CC tissues and matched normal tissues, respectively, suggesting a significant decrease of Bach1 during the development of CC (Fig 3).

Compared with the normal tissues group, a significant decrease of Bach1 in CC tissues was also observed (Fig 4).

The expression of Bach1 was different in tumor stage group (P < 0.01) (table 2). The results suggested that the low expression of Bach1 in tumor III-IV stages presented the potential correlation with tumor stage.

miRNA-135a expression level was higher in stage III/IV colon cancer patients, while the expression level of Bach1 was significantly lower in the same stage. The levels of miRNA-135a and Bach1 were opposite and negatively correlated (P < 0.05) (table 3).

**Discussion**
The morbidity incidence of CC has ranked no. 3 among all malignant tumor diseases in China. CC affects approximately 390,000 new patients in China annually, and the mortality has ranked no. 5 among all malignant tumors. Tumor metastasis is the major cause of death in CC patients. miRNAs are small non-coding RNAs, they induce their degradation or block the translation of the encoded protein via binding to specific complementary sequences in the 3′ UTR of target mRNAs. With the diverse abilities, reducing of their expression has might been involved with promoting or suppressing tumor metastasis, providing a new perspective on the metastatic process. As is well-known, miRNAs could promote or inhibit various traits related to tumor aggressiveness such as proliferation, cell migration and invasion in various cancer cell lines. As one member of them, miRNA-135a can produce an identical and active sequence through being encoded by two genes localized on different chromosomes. Current reports have shown that the effects of miRNA-135a on cancer progression are contradictory. Previous researches showed that the expression of miRNA-135a decreased in human gastric cancer, the proliferation of gastric cancer cells was repressed while the apoptosis was promoted \[^{[10]}\]. On the other hand, miRNA-135a showed a inhibitive role during the migration and invasion of lung cancer cells, due to targeting a transcription factor \[^{[19]}\]. However, the functions and mechanisms of miRNA-135a during tumors are largely unknown\[^{[20-23]}\]. Recent studies have demonstrated that miRNA-135a is up-regulated in CC cell lines SW480 and SW620, while in our study, the expression levels of miRNA-135a were significantly increased in CC tissues, which was in accordance with previous studies. With the analysis of
clinicopathologic characteristics, the expression levels of miRNA-135a were obviously different between different tumor stage groups, and it was different in lymph node involvement group. They were positively related. The data also showed no correlation between miRNA-135a and many aspects in CC tissues, including age, tumor size, location, differentiation and etc. These results supported the hypothesis that miRNA-135a was involved in CC progression, which might function as an oncogenic factor.

Bach1, a member of the basic leucine zipper transcription factor family, is a critical participant in the process of oxidative stress\[^{24}\]. Recent researches demonstrate that Bach1 is a widely expressed transcriptional repressor, it takes part in many vital cell processes through the targeted genes, such as cell cycle progression, apoptosis, and the hypoxia response negatively\[^{25-28}\]. Heme-oxygenase-1 (HO-1), one of the target genes, might be significant in induction of the tumorigenic pathway. The significant increasing expression of HO-1 in various types of cancer is contributed to promote tumor growth and metastasis. Furthermore, Bach1 is recognized to inhibit growth and survival of acute myeloid leukemia (AML) cells by down-regulation of HO-1 expression. Bach1 functions as a repressor of HO-1 in human renal cancer cells, though the possible mechanism has not been established\[^{17,29}\]. In addition, the up-regulation of HO-1 might inhibit apoptosis of renal cancer cells via activation of the nuclear factor (erythroid-derived 2)-like 2(Nrf2) pathway\[^{29,30}\]. Our results show that the expression of Bach1 significantly decreased in CC tissues. With further analysis, there was no difference in tumor I-II stages while the majority decrease was
in tumor III-IV stages, which was similar to miRNA-135a. These findings indicated that Bach1 might play a inhibition role during the development of CC, especially in the advanced stages. Additionally, Bach1 and miRNA-135a were negatively correlated. Thus, it could be concluded that miRNA-135a played an oncogenic role in CC through down-regulation of Bach1 at least partially. Bach1 might be one of targets of miRNA-135a. However, our study indicated the potential role of miRNA-135a and Bach1, further research should be needed to explore the exact signal pathway between them during the development of CC.

Conclusions

In summary, the results presented here indicated that the expression of miRNA-135a was activated significantly, which likely decreased the expression of Bach1 and involved in CC progression. Thus, the expression of miRNA-135a might be useful as a prognostic biomarker and a possible therapeutic target for CC patients.
Abbreviations

CC: colorectal cancer; miRs: MicroRNAs; HO-1: hemoxygenase-1; AML: acute myeloid leukemia; BACH1: BTB and CNC homology 1; PCR: Polymerase Chain Reaction; LNM: lymph node metastasis; Nrf2: nuclear factor (erythroid-derived 2)-like 2.
REFERENCES

1. Brenner H, Kloor, M, Pox CP, et al. Colorectal cancer. Lancet 2014;383:1490-1502.

2. Saif MW, Chu E. Biology of colorectal cancer. Cancer J 2010;16:196-201.

3. Yiu AJ, Yiu CY. Biomarkers in Colorectal Cancer. Anticancer Res 2016; 36:1093-1102.

4. Cekaite L, Eide PW, Lind GE, et al. microRNAs as growth regulators, their function and biomarker status in colorectal cancer. Oncotarget 2016;7:6476-6505.

5. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. Nature 2005;435:834-838.

6. Hollis M, NairK, Vyas A, et al. MicroRNAs potential utility in colon cancer: Early detection, prognosis, and chemosensitivity. World J Gastroenterol 2015;21:8284-8292.

7. Wang S, Wang L, Bayaxi N, et al. A microRNA panel to discriminate carcinomas from high-grade intraepithelial neoplasms in colonoscopy biopsy tissue. Gut 2013;62:280-289.

8. Liang C, Sun W, He H, et al. Antitumor effect of a new nano-vector with miRNA-135a on malignant glioma. Int J Nanomedicine 2018;13:209-220.

9. Zhou W, Li X, Liu F, et al. MiR-135a promotes growth and invasion of colorectal cancer via metastasis suppressor 1 in vitro. Acta Biochim Biophys Sin (Shanghai) 2012;44:838-46.

10.Liu L, Ye JX, Qin YZ, et al. Evaluation of miR-29C, miR-124, miR-135a and
miR-148a in Predicting lymph node metastasis and tumor stage of Gastric Cancer. Int J Clin EXP Med 2015;8:22227-22236.

11.Mao XP, Zhang LS, Huang B, et al. Mir-135a enhances cellular proliferation through post-transcriptionally regulating PHLPP2 and FOXO1 in human bladder cancer. J Transl Med 2015;13:86.

12.Yamada Y, Hidaka H, Seki N, et al. Tumor-suppressive MicroRNA-135a inhibits cancer cell proliferation by targeting the c-MYC oncogene in renal cell carcinoma. Cancersci 2013;104:304-312.

13.Tang W, Jiang Y, Mu X, et al. MiR-135a functions as a tumor suppressor in epithelial ovarian cancer and regulates HOXA10 expression. Cell Signal 2014;26:1420-6.

14.Leung CO, Deng W, Ye TM, et al. miR-135a leads to cervical cancer cell transformation through regulation of β-catenin via a SIAH1-dependent ubiquitin proteosomal pathway. Carcinogenesis 2014;35:1931-40.

15.Schlörmann W., Naumann S., Renner C, et al. Influence of miRNA-106b and miRNA-135a on butyrate-regulated expression of p21 and Cyclin D2 in human colon adenoma cells. Genes Nutr 2015;10:50.

16.Davudian S, Mansoori B, Shajari N, et al. BACH1, the master regulator gene: A novel candidate target for cancer therapy. Gene 2016;588:30-37.

17.Chen Y, Zhang J, Wang H, et al. miRNA-135a promotes breast cancer cell migration and invasion by targeting HOXA10. BMC Cancer 2012;12:111.

18.Kocanova S, Buytaert E, Matrouble JY, et al.
Induction of heme-oxygenase 1 requires the p38MAPK and PI3K pathways and suppresses apoptotic cell death following hypericin-mediated photodynamic therapy. Apoptosis 2007;12:731-41.

19. Shi H, Ji Y, Zhang D, et al. MiR-135a inhibits migration and invasion and regulates EMT-related marker Genes BY targeting KLF8 in lung Cancer Cells. Biochem Biophys Res Commun 2015;465:125-130

20. Schlörmann W, Naumann S, Renner C, et al. Influence of miRNA-106b and miRNA-135a on butyrate-regulated expression of p21 and Cyclin D2 in human colon adenoma cells. Genes Nutr 2015;10:50.

21. Ahmad A, Zhang W, Wu M, et al. Tumor-suppressive miRNA-135a inhibits breast cancer cell proliferation by targeting ELK1 and ELK3 oncogenes. Genes Genomics 2018;40:243-251.

22. Ren JW, Li ZJ, Tu C. MiR-135 post-transcriptionally regulates FOXO1 expression and promotes cell proliferation in human malignant melanoma cells. Int J Clin Exp Pathol 2015;8:6356-6366.

23. Wang Q, Zhang H, Shen X, Ju S. Serum microRNA-135a-5p as an auxiliary diagnostic biomarker for colorectal cancer. Ann Clin Biochem 2017;54:76-85.

24. Davudian S, Mansoori B, Shajari N, et al. BACH1, the master regulator gene: A novel candidate target for cancer therapy. Gene 2016;588:30-37.

25. Anderson NM, Simon MC. BACH1 Orchestrates Lung Cancer Metastasis. Cell 2019;178:265-267.

26. Lee J, Yesilkanal AE, Wynne JP, et al. Effective breast cancer combination
therapy targeting BACH1 and mitochondrial metabolism. Nature 2019;568:254-258.

27. Shajari N, Davudian S, Kazemi T, et al. Silencing of BACH1 inhibits invasion and migration of prostate cancer cells by altering metastasis-related gene expression. Artif Cells Nanomed Biotechnol 2018;46:1495-1504.

28. Davudian S, Shajari N, Kazemi T, et al. BACH1 silencing by siRNA inhibits migration of HT-29 colon cancer cells through reduction of metastasis-related genes. Biomed Pharmacother 2016;84:191-198.

29. Li S1, Chen T, Zhong Z, et al. microRNA-155 silencing inhibits proliferation and migration and induces apoptosis by upregulating BACH1 in renal cancer cells. Mol Med Rep 2012r;5:949-954.

30. Chang LC, Fan CW, Tseng WK, et al. Immunohistochemical Study of the Nrf2 Pathway in Colorectal Cancer: Nrf2 Expression is Closely Correlated to Keap1 in the Tumor and Bach1 in the Normal Tissue. Appl Immunohistochem Mol Morphol 2013;21:511-517.