MicroRNA-186-5p overexpression modulates colon cancer growth by repressing the expression of the FAM134B tumour inhibitor

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Abstract

Objectives: The role and underlying mechanism of miR-186-5p in colorectal cancer remain unknown. The present study aims to examine the various cellular effects of miR-186-5p in the carcinogenesis of colorectal cancer. Also, the interacting targets and association of clinicopathological factors with miR-186-5p expression in patients with colorectal cancer were analysed.

Methods: The miR-186-5p expression levels in colorectal cancer tissues (n=126) and colon cancer cell lines (n=3) were analysed by real-time PCR. Matched non-neoplastic colorectal tissues and a non-neoplastic colonic epithelial cell line were used as controls. Various in vitro assays such as cell proliferation, wound healing and colony formation assays were performed to examine the miR-186-5p specific cellular effects. Western blots and immunohistochemistry analysis were performed to examine the modulation of FAM134B, PARP9 and KLF7 proteins expression.

Results: Significant high expression of miR-186-5p was noted in cancer tissues (p< 0.001) and cell lines (p<0.05) when compared to control tissues and cells. The majority of the patients with colorectal cancer (88/126) had shown overexpression of miR-186-5p. This miR-186-5p overexpression was predominantly noted with in cancer with distant metastasis (p=0.001), lymphovascular permeation (p=0.037), microsatellite instability (MSI) stable (p=0.015), in distal colorectum (p=0.043) and with associated adenomas (p=0.047). Overexpression of miR-186-5p resulted in increased cell proliferation, colony formation, wound healing capacities and induced alteration of cell cycle kinetics in colon cancer cells. On the other hand, inhibition of endogenous miR-186-5p reduced the cancer growth properties. miR-186-5p overexpression reduced FAM134B expression significantly in the cancer cells (p<0.01). Also, FAM134B and miR-186-5p expressions are inversely correlated in colorectal cancer tissues and cells.
**Conclusion:** The miR-186-5p expression promotes colorectal cancer pathogenesis by regulating tumour suppressor *FAM134B*. Reduced cancer cells growth followed by inhibition of miR-186-5p highlights the potential of miR-186-5p inhibitor as a novel strategy for targeting colorectal cancer initiation and progression.

**Keywords:** Colorectal cancer, miR-186-5p, FAM134B, targeted therapy, oncogenic micro-RNA
**Introduction**

MicroRNAs (miRNAs) are a class of small (19-24 nucleotides), non-coding RNA molecules that play an important role in the pathogenesis of colorectal cancers [1-5]. Michael and co-workers first described altered expression of miRNAs in colorectal cancer in 2003 [6]. Subsequently, association of colorectal cancer pathogenesis and hundreds of miRNAs were demonstrated [1, 7]. These miRNAs play crucial roles in various cellular processes such as cell proliferation, cell cycle, apoptosis, maintenance of stemness, epithelial to mesenchymal transition (EMT), differentiation, angiogenesis and metastasis of colorectal cancer cells [8-10]. They modulate carcinogenesis of colorectal cancer by activating oncogenes and inactivating tumour suppressor genes, or DNA repair genes [1, 7].

miR-186-5p, located at 1p31.1, has been reported to have roles in the pathogenesis of cancers, Alzheimer’s disease, neuronal disorder and acute myocardial infarction [11-16]. Overexpression of plasma miR-186-5p was noted in patients with acute myocardial infarction, which have the potential to be used as a non-invasive biomarker for early detection of myocardial infarction [16]. A recent study reported that miR-186-5p controlled the expression of C-X-C motif chemokine 13 tightly. This in turn modulates the astrocyte signalling pathway, thereby regulates the neuropathic pain [14]. Another web-based miRNA data analysis study described that upregulation of a panel of miRNAs comprising miR-186-5p was associated with the incidence of Alzheimer’s disease and could be used as candidate miRNAs biomarkers for Alzheimer’s disease [13].

In cancer, miR-186-5p was significantly upregulated in head & neck and skin carcinomas [11, 15]. Importantly, circulating plasma miR-186-5p differentiated the patient with head & neck squamous cell carcinoma from healthy individuals with high specificity and sensitivity [11]. Also, high expression of miR-186-5p in neck squamous cell carcinoma correlated with worse patients’ prognosis [11]. Significant differential expression of
circulating plasma miR-186 has been noted in patients with colorectal adenomas [17]. The direct biological effects on cancer cells and the interacting targets of miR-186-5p in cancer have not been reported in the literatures. Thus, the present study, for the first time aims to examine the expression profiling as well as clinicopathological and prognostic implications of miR-186-5p in a large series of patients with colorectal cancer. *In vitro* miR-186-5p mediated cellular effects and target proteins expressions in colon cancer were also examined.
**Materials and Methods**

**Selection of patients**

Cancer tissues and matched non-cancer tissues (near the surgical resection margin) from the same patient who underwent resection of colorectal carcinomas by a colorectal surgeon (CTL) were prospectively collected from hospitals in Queensland, Australia. The collected tissues were snap frozen in liquid nitrogen and stored at -80°C. The rest of the cancer specimens were sampled for pathological examination and embedded in paraffin. Tissues samples were excluded from the study if they are lacking adequate cancer cells or the patients did not come back for clinical follow-up. Ethical approval for this work has been obtained from the Griffith University Human Research Ethics Committee (GU Ref No: MSC/17/10/HREC).

**Clinicopathological parameters**

The size (maximum dimension) and site of the cancers as well as associated polyp (s) were recorded on macroscopic examination. Proximal cancers were defined as cancer located in the caecum, ascending colon and transverse colon whereas distal cancers were defined as the cancers found in the region of descending colon, sigmoid colon and rectum.

Histological sections were cut and stained with haematoxylin and eosin (H&E) for light microscopic examination [18-21]. The pathological features of patients with colorectal cancer and associated polyp (adenoma) were analysed by examining the histological sections and were graded by a pathologist (AKL). The cancer tissues were investigated for microsatellite instability (MLH1, PMS2, MSH2 and MSH6 by immunohistochemistry) according to the clinical guidelines. The stages were obtained in multidisciplinary team meeting of the pathologist and the surgeon (AKL & CTL).
After reviewing, 126 patients (67 women; 59 men) with colorectal adenocarcinomas were included in the present study. The mean age of the patients used in this study was 66 years (range, 24-91). The cancers were noted in proximal colon in 44.4 % (n = 56) of the cases whereas the rest were in distal colon and rectum (55.6 %; n = 70). Among the 126 cases studied, 24 % (n = 30) of the carcinomas had distant metastasis, whereas 76 % (n = 96) of cancer patients had no distant metastasis. There were 9.5 % (n = 12) stage I, 39.7 % (n = 50) stage II, 26.2 % (n = 33) stage III and 24.6 % (n = 31) stage IV carcinomas.

Clinical Management

Clinical management was performed by a pre-agreed standardised multidisciplinary protocol. The use of postoperative adjuvant therapy was based on the pathological stage of cancer. The follow-up period was defined as the interval between the date of surgery for colorectal carcinoma and the date of death or closing date of the study. The actuarial survival rate of the patients was calculated from the date of surgical resection of the colorectal carcinomas to the date of death or last follow-up. Only cancer-related death was counted as an end point in the statistical analysis. Persistence or recurrence of the disease was also recorded.

Cell Culture

Colon cancer cell lines (SW-480, SW-48 and HCT116) and a non-neoplastic colon epithelial cell line (FHC) were used in this study. The miR-186-5p induced cellular changes were examined using the cell models from FHC, SW480 (stage II) and SW48 (stage III) cell lines. All of these cells were purchased from ATCC (American type culture collection) and maintained as previously described [21].
Extraction of RNA, miRNA and cDNA conversion

A cryostat (Leica Biosystems, Mt Waverley, VIC, Australia) was used to section tissues from these patients which had >70% representation of cancer cells for RNA and miRNA extraction. Tissues from the selected samples were sectioned and RNA, miRNA extraction was performed as previous reported [21-22]. Briefly, the miRNA extraction from tissues and cell lines were performed with Qiagen miRNeasy FFPE Kits (Qiagen Pty. Ltd., Hilden, NRW, Germany) and miRNeasy Mini kits (Qiagen), respectively. The purity of RNA and miRNA was examined by checking the optical density (OD) method using a nanodrop spectrophotometer. The purified RNA and miRNAs were converted to cDNA using miScript Reverse Transcription kits (Qiagen) according to the manufacturer's instructions.

Quantitative Real-Time Polymerase Reaction (qRT-PCR)

The expression changes of miR-186-5p in colorectal cancer tissues and 3 cell lines were examined using a QuantStudio™ 6 Flex Real-time PCR system (Applied Biosystems, Foster city, CA, USA). qPCR was performed in a total volume of 20 µl reaction mixture containing 10µL of QuantiTech SYBR® Green PCR kits (Qiagen), 1.5 µL of each 5 µmol/L primer, 3 µl of cDNA at 50ng/µl, and 4 µL of 0.1% diethylpyrocarbonate (DEPC) treated water as previously described [21-22]. The amplification efficiencies of a family with sequence similarity 134, member B (FAM134B) mRNA were normalised to that of multiple housekeeping genes including beta actin, 18s and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH and β-actin were selected on the basis of consistent results. Similarly, amplification efficiencies of miR-186-5p were normalised to that of multiple internal control genes including RNU6b, RNU44 and RNU48. RNU6b was selected on the basis of consistent results. Sequences of the primers used in this study are detailed in supplementary
data 1. Results were presented as relative expression ratio (expression of miR-186-5p normalised by internal control RNU6b expression). Fold changes were calculated according to previously published protocol [22]. A fold change of more than two was considered as high miR-186-5p expression and a fold change of two or less was considered as a low miR-186-5p expression.

Transfection of colon cancer cells with miR-186-5p mimic and anti-miR-186-5p

FHC and colon cancer cells, SW480 and SW48, were cultured approximately at 2×10⁴ cells/cm² into 24 wells plate in the recommended media. After 24 hours of initial seeding, cells were transfected with miR-186-5p mimic (Qiagen) (FHC+miR-186-5p, SW480+miR-186-5p and SW48+miR-186-5p) at 10 nM concentrations and with anti-miR-186-5p (Qiagen) (FHC+anti-miR-186-5p, SW480+anti-miR-186-5p and SW48+anti-miR-186-5p) at 10 nM concentrations according to the protocol published earlier [3, 22-23]. Briefly, 3 μl of transfection reagent, Hiperfect (Qiagen), was added to the miR-186-5p mimic / anti-miR-186-5p and incubate for 5 minutes at room temperature to form the complexes for cell transfection. Cells treated with scrambled miRNA (FHC+miRScr, SW480+miRScr and SW48+miRScr) and transfection reagents (Hiperfect) alone (FHCwildtype, SW480wildtype and SW48wildtype) were used as controls in this study.

Cell proliferation assay

To examine the effect of miR-186-5p on the proliferation of colon cancer cell lines, cell proliferation assay using cell counting kit-8 (CCK-8) (Sigma-Aldrich, St Louis, MO, USA) was used. SW480, SW48 and FHC cells were first seeded in a flat-bottom 96-well plate at 1 × 10⁴ cells/well. After 24 hours of initial seeding, cells were treated with miR-186-5p mimic, anti-miR-186-5p and scramble miRNA as previously described [21]. Cell
proliferation rate was determined on days 0 to 3 with CCK-8 following manufacturer guidelines.

**Colony formation assay**

To determine the effect of miR-186-5p manipulation on clonogenic capacity, equal numbers of cells were seeded in 6-well plates with complete medium. The cells (SW480, SW48 and FHC) were then transfected with miR-186-5p mimic, anti-\textit{miR-186-5p} and scramble control miRNA. The cells were continued to grow at 37°C in 5% CO₂ and saturation humidity. After two weeks, when microscopic clones were noted in the plate, growing of the cells was stopped. Then, the media was discarded and cells were washed with phosphate buffered saline (PBS). Afterwards, cells were fixed with 70% cold ethanol for 15 minutes at room temperature. Subsequently, the clones were stained with crystal violet (0.5%) for 2 hours at room temperature and washed with tap water. Finally, after being air-dried, images of the plates were taken. The clone formation rates and surviving fractions were calculated (Supplementary data 2).

**Wound healing assay**

A wound healing assay was performed to determine the effect of miR-186-5p on the capacity of cells to migrate for repairing the scratch. Cells were cultured in complete medium until 70-80% confluence as a monolayer. Then scratches were made with a 200μl pipette tip across the centre of culture plates. Cells were then treated with miR-186-5p mimic, anti-miR-186-5p and control miRNA and incubated for three days of the investigation. Images were taken to monitor the changes among the cells type on day 0 to day 3. Wound areas on different days of all cell types were measured and compared with Image J 1.48 software.
Cell cycle analysis

Cells treated with miR-186-5p mimic, anti-miR-186-5p and control miRNA were fixed with cold 70% ethanol for one hour as previously described [21]. After washing with cold PBS, 5μl of RNase A (10 mg/ml) was added to the cells and incubated for one hour at 37 °C. Finally, 10 μl of propidium iodide solution (1 mg/ml) was added to the cell suspension. Analysis was performed by flow cytometry (BD FACSCalibur, BD Biosciences).

Western blot analysis

Total proteins from all cell groups were extracted with a lysis buffer (Bio-Rad, Gladesville, NSW, Australia). Approximately 30 μg of total protein was separated by 15% SDS-PAGE (Bio-Rad) and transferred to nitrocellulose membranes using Trans-Blot® Turbo transfer system (Bio-Rad). The membrane was blocked with 5% non-fat milk powder for 2 hours at room temperature. Then the membrane was incubated with the anti-rabbit FAM134B polyclonal antibody (Santa Cruz, Texas, USA) (at 1:200), anti-mouse monoclonal PARP9 and KLF7 antibodies (Santa Cruz) (1: 500) and anti-rabbit monoclonal GAPDH antibody (Santa Cruz) (1:1000) overnight at 4°C. Membranes were then incubated with secondary antibody (Santa Cruz) (1:5000) at room temperature for 2 hours. FAM134B, PARP9, KLF7 and GAPDH protein bands were developed and detected with a chemiluminescence HRP detection kit (Bio-Rad). Images were taken with the ChemiDoc MP Imaging system (Bio-rad). Expression of proteins was quantified and normalised to GAPDH with Image J 1.48 software.

Immunohistochemistry

Immunohistochemical study was performed on the selected tissues paraffin blocks (n = 42 colorectal adenocarcinomas) to investigate the relationship between miR-186-5p and
FAM134B protein expression in cancer tissues samples. After histological review, the selected tissues were cut at 5 to 6 μm thickness. Following this, they were allowed to dry overnight at 37°C in an incubator. The sections were then deparaffinised with xylene and hydrated in 100% and 70% ethanol. They were then heated in a pressure vessel at 120°C for 7 min in a citrate buffer (at pH 6) solution and were allowed to cool down for 30 min and were rinsed with phosphate buffered saline (PBS). Immunohistochemical staining was performed using previously published protocol [24].

**Statistical analysis**

All clinical, pathological, follow-up, mRNA and miRNA expression changes were computerised. Statistical analysis was performed using the Statistical Package for Social Sciences for Windows (version 24.0, IBM SPSS Inc., New York, USA). Chi-square test or likelihood ratio was used for categorical variables. Independent t-test and ANOVA was performed for the analysis of continuous variables in categories. Survival analysis was tested using Kaplan-Meier method with the log-rank test and multivariate Cox regression. A significance level of the tests was taken at p <0.05.
Results

Overexpression of miR-186-5p in colorectal cancer

miR-186-5p expression in cancer tissue samples was noted significantly higher in comparison to that of the non-cancer samples (0.98±0.01 versus 0.84±0.07) (Figure 1A). Also, increased expression of miR-186-5p was noted in SW480, SW48 and HCT116 colon cancer cells in comparison to that of non-neoplastic colonic epithelial cells (FHC) (Figure 1B). Among the colorectal cancers, 70% (88/126) had shown high expression of miR-186-5p whereas 30% (38/126) exhibited low expression (Table 1).

Colorectal cancers that were located in the distal colorectum showed high prevalence of miR-186-5p over expression when compared to cancers in the proximal colon (74 % versus 64.3%, p=0.043) (Table 1). Also, miR-186-5p over expression was significantly higher in cancers with adenomas in their colorectum (63% versus 78%, p=0.047). Also, high expression of miR-186-5p was noted more often in colorectal cancers with lympho-vascular permeation. Nearly 69% (n=24/35) of lymph-vascular permeation positive cancer samples had shown miR-186-5p high expression and only 31% (n=11) of these patients had low expression of miR-186-5p (p=0.037). Furthermore, 70% of patients with distant metastasis had shown high expression of miR-186-5p (p=0.001). Furthermore, there was a significant increase in miR-186-5p expression levels were noted in cancers that were MSI stable when compared to those with MSI (78% versus 50%, p=0.015).

The median overall follow-up of patients with colorectal cancer was 42 months and the survival rates correlated with the pathological stages of cancer (p=0.0001) (Figure 1C). Patients with colorectal cancer expressing high miR-186-5p had shorter survival time when compared to those with low miR-186-5p expression (96 months versus 108 months). However, the difference in survival time between the groups did not reach statistical significance despite a good schematic difference (Figure 1D) (p=0.28).
**miR-186-5p stimulate colon cancer growth in vitro**

Cancer cells treated with miR-186-5p mimic (SW480\(^{\text{miR-186-5p}}\) and SW48\(^{\text{miR-186-5p}}\)) showed increased cell proliferation when compared to scramble control (SW480\(^{\text{miRScr}}\) and SW48\(^{\text{miRScr}}\)) and non-transfected cells (SW480\(^{\text{wildtype}}\) and SW48\(^{\text{wildtype}}\)). In SW480\(^{\text{miR-186-5p}}\) cells, 18% to 35% increased cell proliferation was observed at different days in comparison to that of SW480\(^{\text{miRScr}}\) cells (Figure 2A). Whereas 17% to 53% enhancement of cell proliferation was noted in SW48\(^{\text{miR-186-5p}}\) when compared to that of SW48\(^{\text{miRScr}}\) cells (Figure 2B). The normal colon epithelial FHC\(^{\text{miR-186-5p}}\) cells also showed similar results (Figure 2C). On the other hand, treatment of cancer cells with anti-miR-186-5p (SW480\(^{\text{anti-miR-186-5p}}\) and SW48\(^{\text{anti-miR-186-5p}}\)) significantly inhibited cells proliferations in comparison to that of SW480\(^{\text{miRScr}}\) and SW48\(^{\text{miRScr}}\) and SW480\(^{\text{wildtype}}\) and SW48\(^{\text{wildtype}}\) cells. SW480\(^{\text{anti-miR-186-5p}}\) cells had shown 21% to 36% reduced cells proliferation and SW48\(^{\text{anti-miR-186-5p}}\) cells exhibited 14% to 41% cells growth inhibition in comparison to that of control cells (Figure 2A-2B). Similarly, FHC\(^{\text{anti-miR-186-5p}}\) showed reduced cell proliferation when compared with FHC\(^{\text{wildtype}}\) or FHC\(^{\text{miRScr}}\) cells (Figure 2C). Figure 2D-2F presents the normalised expression ratio of miR-186-5p on day 1, day 2 and day 3 in different cell types used in this study.

SW480\(^{\text{miR-186-5p}}\), SW48\(^{\text{miR-186-5p}}\) and FHC\(^{\text{miR-186-5p}}\) cells showed remarkably increased colony formation properties in comparison to the SW480\(^{\text{miRScr}}\), SW48\(^{\text{miRScr}}\) & FHC\(^{\text{miRScr}}\), and SW480\(^{\text{wildtype}}\), SW48\(^{\text{wildtype}}\) and FHC\(^{\text{wildtype}}\) cells (Figure 2G-2I). The numbers of colonies in SW480\(^{\text{miR-186-5p}}\), SW48\(^{\text{miR-186-5p}}\) and FHC\(^{\text{miR-186-5p}}\) cells were noted significantly higher in comparison to that of control cells (Figure 2J). Treatment of the cancer cells with anti-miR-186-5p reduced the colony formation capacity of cells remarkably (Figure 2G-2I). Approximately, 14% and 16% surviving fractions were noted in SW480\(^{\text{anti-}}\)
miR-186-5p and SW48\textsuperscript{+anti-miR-186-5p} cells, respectively. Comparably, reduced surviving fractions (24\%) were noted in FHC\textsuperscript{+miR-186-5p} cells. On the contrary, 92\% and 94\% surviving fractions were observed in SW480\textsuperscript{+miR-186-5p} and SW48\textsuperscript{+miR-186-5p} cells, respectively.

SW480\textsuperscript{+miR-186-5p} cells had a higher cell migration potential than SW480\textsuperscript{miRS\textsubscript{cr}} and SW480\textsuperscript{\text{wildtype}} cells as they healed the created scratch faster when compared to their counterpart (Figure 3A & 3B). SW480\textsuperscript{+miR-186-5p} cancer cells healed the created wound completely on the 3rd day of initial scratch, whereas SW480\textsuperscript{\text{wildtype}} and SW480\textsuperscript{miRS\textsubscript{cr}} cells took more time to heal the wounds. Anti-miR-186-5p treated cells (SW480\textsuperscript{+anti-miR-186-5p}) had shown opposite results. Anti-miR-186-5p reduced the migratory capacity of cells, thereby the wound remained unhealed until day 3 (Figure 3A & 3B). Also, in SW48\textsuperscript{+miR-186-5p} and FHC\textsuperscript{+miR-186-5p} cells, wound healing was completed on day 3 whereas in SW48\textsuperscript{+anti-miR-186-5p} and FHC\textsuperscript{+anti-miR-186-5p} cells, the scratch remained unhealed until day 3 (Figure 3C-3D & 3E-3F).

**miR-186-5p altered the cell cycle kinetics in colon cancer**

Analysis of cell cycle kinetics revealed an accumulation of cells in the S phase and reduction of the G\textsubscript{0}/G\textsubscript{1} phase of the cycle in SW480\textsuperscript{+miR-186-5p} and SW48\textsuperscript{+miR-186-5p} when compared to scramble control cells (Figure 4). In the case of SW480\textsuperscript{miRS\textsubscript{cr}} cells, the percentage of S phase cells was 53.8 \pm 4.5 \%, while in SW480\textsuperscript{+miR-186-5p} cells, the S phase population increased remarkably to 65.4 \pm 3.2 \% (p<0.05). Proportion of G\textsubscript{0}/G\textsubscript{1} phase in SW480\textsuperscript{+miR-186-5p} cells was noted 4.4 \pm 1.01\% versus 25.2 \pm 2.6 \% in SW480\textsuperscript{miRS\textsubscript{cr}} cells (Figure 4A-4B). On the other hand, treatment of cells with anti-miR-186-5p had shown opposite cell cycle distributions. In SW480\textsuperscript{+anti-miR-186-5p}, G\textsubscript{0}/G\textsubscript{1} cells population increased to 32.1 \pm 2.3 \% and S phase cells reduced to 46.6 \pm 3.1 \%. Similar results were noted in SW48
miR-186-5p regulate tumour suppressor FAM134B expression in colorectal cancer

Interacting targets for miR-186-5p were predicted using miRDB target prediction tools (http://mirdb.org/miRDB/). The expression of KLF7, PARP9, and FAM134B proteins among the various targets followed by exogenous manipulation of miR-186-5p expression were analysed in this study. Notable reduction of FAM134B mRNA expression was observed in SW480^{+miR-186-5p} and SW48^{+miR-186-5p} cells compared to that of scramble control and wild-type cells (Figures 5A and 5B). While anti-miR-186-5p treatment (SW480^{anti-miR-186-5p} and SW48^{anti-miR-186-5p}) increased FAM134B mRNA expression significantly in both cells (Figures 5A and 5B). Western blot analyses revealed that cells treated with miR-186-5p mimic reduced FAM134B protein expression in SW480^{+miR-186-5p} and SW48^{+miR-186-5p} cells, whereas inhibition of miR-186-5p by the treatment of anti-miR-186-5p increased FAM134B protein expression in SW480^{anti-miR-186-5p} and SW48^{anti-miR-186-5p} cells (Figures 5C-5F). The target site of miR-186-5p on FAM134B mRNA is presented in supplementary data 3. The interaction of seed 325 ATTCTTT location of FAM134B mRNA with miR-186-5p mediated reduced expression of FAM134B. Expression of KLF7 and PARP9 did not show any significant difference in the present study (Supplementary data 4).

Among the high miR-186-5p expressed colorectal carcinomas, 81% (71/88) of tumours have shown low FAM134B mRNA expression (Figure 6A). On the other hand, colorectal carcinomas expressing low miR-186-5p, 55.3% (21/38) tissues samples exhibited high FAM134B mRNA expression (Figure 6A). These inverse correlations of miR-186-5p and FAM134B mRNA expressions have shown high statistical significance (p=0.0001). Analogues findings were also noted with FAM134B protein expressions.
Immunohistochemical analysis on 42 colorectal cancer tissues samples have also revealed that high expression of miR-186-5p down regulates FAM134B expression significantly \((p=0.013)\) in their corresponding tissues (Figure 6B). Interestingly, high miR-186-5p expression was associated with no or reduced FAM134B protein (19/23) expression, while low miR-186-5p expression cases correlated with high FAM134B protein (11/19) expression (Figures 6C & 6D). In addition, these associations were also reciprocated in colon cancer cell line models (Supplementary data 5).
Discussion

The present study has for the first time reported insights on the functional roles of miR-186-5p in colorectal cancer. The results have confirmed the association of a known tumour suppressor gene, \textit{FAM134B}, through which miR-186-5p exhibits modulatory roles in carcinogenesis. This study has noted significant overexpression of miR-186-5p in large number of colorectal cancer tissues samples (p<0.001) and cell lines (p<0.05) when compared to their control tissues and cells, respectively (Figure 1). In addition, patients with colorectal cancer having high expression of miR-186-5p had shorter survival time when compared to that of low miR-186-5p expressing patients.

Expression of miR-186-5p has only been reported in two studies, one on head & neck squamous cell carcinoma and another on non-melanoma skin cancer [11, 15]. Overexpression of miR-186-5p was associated with poor prognosis of patients with head and neck squamous cell carcinoma [15]. In this study, we noted that overexpression of miR-186-5p in colorectal cancer was correlated with distant metastasis and lymphovascular permeation.

The prognosis of patients with colorectal cancer depends on the TNM pathological staging, which is a collective assessment of cancer invasion, lymph node and distant metastasis [25]. Lympho-vascular permeation in patients with colorectal cancer is an unfavourable prognostic factor and associated with more aggressive of cancer [26]. In addition, previous studies have demonstrated that dysregulation of different oncogenic miRNAs promoted the metastatic phenotype of colorectal cancer by suppressing their target genes [27-30]. For example, overexpression of miR-29a associated with metastasis and poor prognosis of patients with colorectal cancer [29]. miR-29a promoted cell invasion by blocking the expression of KFL4, which in turn induced overexpression of matrix metalloproteinase 2, thereby stimulated the metastasis of cancer cells [29]. Lympho-vascular permeation could lead to distant metastases which in turn lead to the poor prognosis of patients with colorectal cancer [25]. Thus, the
association of high miR-186-5p expression with cancer having lympho-vascular permeation and distant metastatic cancer implied that miR-186-5p could play an important role in colorectal cancer metastasis by modulating the expression of key genes involved in cancer metastasis.

Microsatellite instability (MSI) occurs in 15% of colorectal cancer and results from the inactivation of DNA mismatch repair genes [31]. In this study, colorectal cancers that were MSI stable showed high miR-186-5p expressions indicating that miR-186-5p perhaps suppress the genes involved in adenoma-carcinoma sequence of colorectal cancer pathogenesis but not the genes involved in the maintenance of genetic integrity or DNA repair.

We also found that the levels of miR-186-5p expression in cancers arising from proximal or distal colorectum were significantly different. As cancers from proximal colon and distal colorectum have different physiological and trans-epithelial function in terms of biochemical composition and epithelial membrane transport, differential genes expression patterns in these sites of colorectum are not uncommon [5, 18-20, 25, 32-34]. The differential expression of miR-186-5p in the distal and proximal site of the colorectum implied that its role in colorectal cancer pathogenesis could be tissue specific and it may regulate its target genes in the proximal and distal colorectum differently.

The adenoma-carcinoma sequence, a process that illustrates the stepwise progression from normal to dysplastic epithelium to carcinoma linked with the accumulation of multiple clonally selected genetic alterations, is a widely accepted hypothesis responsible for the development of a significant proportion (85%) of colorectal carcinomas [35-36]. Several studies have demonstrated that appropriate management of precursor adenomatous polyps (adenoma) reduces the long-term risk of colorectal cancer [35, 37-38]. Abnormalities of tumour suppressor genes such as adenomatous polyposis coli (APC), loss of 18q etc. and
oncogenes *e. g.* *K-ras*, *B-raf* are involved in adenoma-carcinoma sequence [35]. As adenoma represents the early events of colorectal cancer pathogenesis, the association of high miR-186-5p expression with associated adenoma positive patients with cancer imply that miR-186-5p could either directly or indirectly induce carcinogenesis via modulating the expression of other genes in adenoma-carcinoma sequence.

miR-186-5p induced *in vitro* cancer cell growth in this study is consistent with overexpression of miR-186-5p in cancer tissue samples, which implied its cancer-promoting role in the pathogenesis and progression of colorectal carcinomas. In addition to increased cell proliferation, a high wound healing and colony formation capacities (tumour promoting properties) were noted in SW480^miR-186-5p^ and SW48^miR-186-5p^ cells [Figures 2 and 3]. Also, miR-186-5p notably increased proliferation, migration and colony formation in the non-neoplastic colon epithelial (FHC) cells which further imply its oncogenic potential in colon cells. Significant (p<0.01) cellular features such as enhancement of colony number, increased cell proliferation, faster wound healing and accumulation of cells in the synthetic phase of cell cycle in SW480^miR-186-5p^ and SW48^miR-186-5p^ cells further strengthens the carcinogenic potential of miR-186-5p via regulating various checkpoints in cell growth and development in colon cancers.

On the other hand, treatment of cancer cells with anti-miR-186-5p (SW480^anti-miR-186-5p^ and SW48^anti-miR-186-5p^) minimized the oncogenic activities of miR-186-5p. Furthermore, in SW480^anti-miR-186-5p^ and SW48^anti-miR-186-5p^ cells, the cancer growth properties reduced significantly in comparison to that of control cells. In addition, treatment of FHC cells with anti-miR-186-5p (FHC^anti-miR-186-5p^) has led to slow cell growth and proliferation. Thus, the oncogenic properties noted in the present study using both knock in and down models in colon cancer cells could be directly attributed by the effects of miR-186-5p or by its affiliated protein targets. Therefore, it can be hypothesised that miR-186-5p could act as an oncogenic
miRNA in colon cancer and might play a vital role in the pathogenesis of the diseases by interacting and regulating various physiological and pathological processes in cancer cells.

*FAM134B* is a tumour suppressor and its downregulation plays a key role in the initiation and progression of colorectal carcinomas [21, 24, 39-40]. Recent studies have demonstrated that *FAM134B* inhibits colon cancer cells proliferation, migration, wound healing, colony formation and tumour formation *in vivo* [21, 39]. In addition, the expression of *FAM134B* significantly correlated with cancer stages, grade and prognosis of patients with colorectal cancer [39-40]. FAM134B expressions (both mRNA and protein) were downregulated whereas the expression of miR-186-5p upregulated in colorectal cancer tissues and cells significantly. Thus, miR-186-5p induced cancer promoting activities observed in this study might be due to the repression of FAM134B expression followed by the interaction of 3′ untranslated region (UTR) of FAM134B mRNA and seed sequence of miR-186-5p [41-42].

As described earlier, anti-miR-186-5p treatment has resulted into reduced cell growth and low oncogenic features indicating that miR-186-5p expressions can be manipulated and regulates its effects in cancer cells. It has been reported that inhibitions of oncogenic miRNAs have the potential to develop miRNAs interference therapy for effective management of cancer [43-45]. Thus, overexpression of FAM134B by targeting the miRNAs such as miR-186-5p could be useful to therapeutically target cancer cells. However, further research is needed to understand the precise mechanisms involved in miR-186-5p mediated modulation of colorectal cancer pathogenesis.

In conclusion, miR-186-5p is frequently overexpressed in colorectal carcinomas and contributed to the poor survival of patients with colorectal cancer. Exogenous overexpression of miR-186-5p in colon cancer cells promoted cancer growth properties *in vitro* by suppressing of tumour suppressor *FAM134B*, whereas inhibition of miR-186-5p
induced cancer growth reduction by upregulating *FAM134B* expression. Thus, the cancer-promoting effects of miR-186-5p are possibly due to the repression of the tumour suppressor, *FAM134B*, by negative regulation at the post-transcriptional level. Also, the association of high miR-186-5p expression with clinical and pathological factors of patients with colorectal cancer implies that miR-186-5p plays a key role in the molecular carcinogenesis of colorectal cancer. Therefore, findings of the present study raise the possibility that anti-microRNA-186-5p agents may have potential therapeutic value for patients with colorectal cancer.
References


Table 1. Expression pattern of miR-186-5p and its correlation with clinicopathological parameters of colorectal cancer

<table>
<thead>
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<th>Characteristics</th>
<th>Number</th>
<th>High Expression</th>
<th>Low Expression</th>
<th>p value</th>
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<tr>
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<td>67 (53.2%)</td>
<td>46 (68.7%)</td>
<td>21 (32.3%)</td>
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<td>Female</td>
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<td>42 (71.2%)</td>
<td>17 (28.8%)</td>
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<tr>
<td>&lt; 60</td>
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<td>23 (65.7%)</td>
<td>12 (34.3%)</td>
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<tr>
<td>≥ 60</td>
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<td>65 (71.4%)</td>
<td>26 (28.6%)</td>
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<tr>
<td>Proximal</td>
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<td>36 (64.3%)</td>
<td>20 (35.7%)</td>
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<td>Distal</td>
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<td>52 (74.3%)</td>
<td>18 (25.7%)</td>
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<td><strong>Size (mm)</strong></td>
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<tr>
<td>≤40</td>
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<td>42 (68.9%)</td>
<td>19 (31.1%)</td>
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<tr>
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<tr>
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<td>10 (50%)</td>
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<td>67 (77.9%)</td>
<td>19 (22.1%)</td>
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<td>74 (69.2%)</td>
<td>33 (30.2%)</td>
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<td>Grade 3</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>21 (70%)</td>
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<tr>
<td>Absent</td>
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<tr>
<td><strong>Pathological staging</strong></td>
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<tr>
<td>I &amp; II</td>
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<td>III &amp; IV</td>
<td>64 (50.8%)</td>
<td>47 (73.4%)</td>
<td>17 (26.4%)</td>
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</tr>
</tbody>
</table>

* MSI screening was performed in 106 cases
Figure 1. Altered miR-186-5p expression and survival distribution in colorectal cancer.

A) Scatter plot of miR-186-5p expression levels in colorectal cancer and matched non-neoplastic colorectal tissues. Paired T-test showed a significant difference in expression of miR-186-5p (ratio of miR-186-5p normalised by RNU6B) in cancer and matched non-neoplastic colorectal tissues (p=0.00063).

B) Expression of miR-186-5p in colon cancer (SW480, SW48 and HCT116) and non-neoplastic colon epithelial cell (FHC). Significant high expression of miR-186-5p was noted in cancer cells when compared to those non-cancer cells.

C) Survival rates of patients with colorectal cancer of different stages.

D) Patients with high levels of miR-186-5p expression had shorter survival time when compared to those with low levels of miR-186-5p expression.
Figure 2. miR-186-5p promoted in vitro colon cancer growth. Exogenous manipulation of miR-186-5p in SW-480 (A), SW-48 (B) and FHC (C) cells induce altered proliferation in comparison to control and non-transfected cells on different days after transfection. Overexpression of miR-186-5p increased cells proliferation in SW480\(^{\text{miR-186-5p}}\) and SW48\(^{\text{miR-186-5p}}\) whereas downregulation of miR-186-5p in SW480\(^{\text{anti-miR-186-5p}}\) and SW48\(^{\text{anti-miR-186-5p}}\) cells caused inhibition of cells proliferation significantly. Expression of miR-186-5p in different cells groups in SW480 (D), SW48 (E) and FHC (F) on different days after transfections. Overexpression of miR-186-5p increased the colony formation capacity of SW480\(^{\text{miR-186-5p}}\) (G), SW48\(^{\text{miR-186-5p}}\) (H) and FHC\(^{\text{miR-186-5p}}\) (I) cells. Suppression of miR-186-5p by anti-miR-186-5p treatment reduced clonogenic properties of SW480\(^{\text{anti-miR-186-5p}}\) (D), SW48\(^{\text{anti-miR-186-5p}}\) and FHC\(^{\text{miR-186-5p}}\) (G-I) cells. Bar graphs presented the number of colonies generated in different experimental groups (J). Results are shown as mean ±SD (standard deviation). Level of significance *p<0.05, **p<0.01 and ***p<0.001 when compared with that of scramble control and untransfected wildtype cells.
Figure 3. miR-186-5p stimulate wound healing capacity in colon cancer cells.

Overexpression of miR-186-5p in SW480\textsuperscript{miR-186-5p} cells showed more migration capacity, healing the wound faster than the control (SW480\textsuperscript{miRScr}) and non-transfected (SW480\textsuperscript{wildtype}) cells (A-B). Anti-miR-186-5p had shown reduced migratory and wound healing capacity (A-B). Wound areas of all experimental cell types on different days (day-0 to day-3) were
recorded from three independent measurements \((B)\). In SW48\(^{+}\)miR-186-5p and FHC\(^{+}\)miR-186-5p cells, similar results were noted \((C-D \& E-F)\). Scale bars (A, C & E) 200 \(\mu\)m. Results are shown as mean ±SD (standard deviation). Level of significance *\(p<0.05\), **\(p<0.01\) and ***\(p<0.001\) when compared with that of scramble control and untransfected wildtype cells.
**Figure 4. miR-186-5p altered cell cycle kinetics of colon cancer cells.** Cell cycle analysis by flow cytometry revealed that miR-186-5p overexpression caused S phase accumulation and a parallel reduction of G0/G1 of colon cancer cell. Inhibition of endogenous miR-186-5p by the treatment with anti-miR-186-5p exhibited accumulation of cells in G0/G1 phase and reduction of S phase cells. **A)** Presented the representative histograms of cell cycle analysis of SW480^+miR-186-5p, SW480^+anti-miR-186-5p and SW480^miRScr cells. **B)** The bar graphs presented the percentage of cells in a different phase. **C)** Representative histograms of cell cycle followed by miR-186-5p manipulation in SW-48 cells. **D)** Bar graphs presented the percentage of cells in different phase after miR-186-5p manipulation. The results represented as mean±SD from three independent experiments. Level of significance *p<0.05 and ***p<0.001 when compared with that of scramble control cells.
Figure 5. miR-186-5p repress FAM134B expression in colon cancer cells. FAM34B mRNA expression inversely correlated with the expression of miR-186-5p in colon cancer cell lines (SW480 and SW48). Overexpression of miR-186-5p caused reduces expression of FAM134B mRNA in SW480 (A) and SW48 (B) colon cancer cells. Inhibition of miR-186-5p expression by anti-miR-186-5p treatment minimized miR-186-5p mediated repression of FAM134B mRNA and increased its expression in SW480 anti-miR-186-5p and SW48 anti-miR-186-5p cells (A) and (B), respectively. Also, altered FAM134B protein expression was noted upon miR-186-5p manipulation in SW480 miR-186-5p, SW480 anti-miR-186-5p compared to that of SW480 miRScr and SW480 wildtype cells (C). In SW480 miR-186-5p cells, FAM134B proteins expression was downregulated significantly in comparison to the SW480 miRScr and SW480 wildtype cells, whereas in SW480 anti-miR-186-5p cells elevated expression of the FAM134b protein was observed (C). D) The bar graphs for relative expression of FAM134B proteins in SW480 cells. The proteins band intensity of FAM134B in SW480 miR-186-5p cells was noted to be decreased significantly (p=0.014) when compared to the controls (SW480 miRScr and SW480 wildtype) cells. Similar results were noted in SW48 cells (E & F). The results are shown as mean±SD from three independent tests.
Figure 6. miR-186-5p regulates FAM134B expression in colorectal cancer tissue samples. RT-qPCR analysis of colorectal cancer tissue samples (n=126) revealed that high expression of miR-186-5p inversely associated (p=0.0001) with low expression of FAM134B mRNA expression (A). Immunohistochemistry analysis of colorectal cancer tissue samples (n=42) exhibited that high miR-186-5p expression induces low or no expression of the FAM134B protein (p=0.013) and low miR-186-5p expression associated with high FAM134B protein expression (B). Representative photographs presented FAM134B protein expression in high miR-186-5p versus low FAM134B protein (C) and low miR-186-5p versus high FAM134B protein (D) groups of colorectal cancer samples (magnification- 20x).
Supplementary Information

Supplementary data 1. Primers pairs used in the present study

<table>
<thead>
<tr>
<th>Name of Genes</th>
<th>Primer sequence (Forward and Reverse)</th>
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<tr>
<td>FAM134B</td>
<td>5’-TGACCGACCCAGTGAGGA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GGGCAAACCAAGGCTTTA-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-TGCACCACCAACTGCTTAGC-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GGCATGGACTGTGTCATGAG-3’</td>
</tr>
<tr>
<td>β-actin</td>
<td>5’-ACCCACACTGTGCCCATCTACGA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-CAGGAGGAGCAATGATCTTGATCTTC-3’</td>
</tr>
<tr>
<td>has-miR-186-5p</td>
<td>5’CAAAGAAUUCUCCUUUGGGCU</td>
</tr>
</tbody>
</table>

Hs_miR-186-5p_1 miScript Primer Assay and Hs_RNU6B_2 miScript Primer Assay (Qiagen) were used in this study.
**Supplementary data 2.** Calculation of clone formation and surviving fraction determination were carried out using the following formulae-

Clone formation rate or plating efficiency (PE) = \(\frac{\text{No. of colony formation}}{\text{No. of cells seeded}} \times 100\)

Surviving fractions = \(\frac{\text{No. of colony formation}}{\text{No. of cells seeded}} \times \text{PE}\)
Supplementary data 3. The target site of miR-186-5p on FAM134B mRNA. The interaction of seed 325 ATTCTTT location of FAM134B mRNA with miR-186-5p mediated FAM134B reduced expression.

FAM134B 3’ atcctctataaatATTCTTT 5’
    | | | | |
miR-186-5p 5’ ggguuuuccucuUAAGAAA 3’
Supplementary data 4. Expression of PRAP9 and KLF7 protein were examined followed by miR-186-5p exogenous manipulation in SW480 and SW48 colon cancer cells. No notable modulation of these proteins was noted.
**Supplementary data 5.** With the increment of miR-186-5p expression in cancer cells, reduced FAM134B mRNA (A) and protein (B) expressions were noted in colon cancer cell lines (SW480, SW48 and HCT116) when compared to that of nonneoplastic colon epithelial cells (FHC).

![Graph A) FAM134B mRNA expression ratio](image)

![Graph B) FAM134B and GAPDH protein expression](image)