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 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 up regulated 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 2×10^4 cells/cm^2 into 24 wells plate in the recommended media. After 24 hours of initial seeding, cells were transfected with *miR-186-5p* mimic (Qiagen) ($FHC^{+miR\text{-}186\text{-}5p}$, $SW480^{+miR\text{-}186\text{-}5p}$ and $SW48^{+miR\text{-}186\text{-}5p}$) at 10 nM concentrations and with anti-*miR-186-5p* (Qiagen) ($FHC^{+anti\text{-}miR\text{-}186\text{-}5p}$, $SW480^{+anti\text{-}miR\text{-}186\text{-}5p}$ and $SW48^{+anti\text{-}miR\text{-}186\text{-}5p}$) 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 ($FHC^{wildtype}$, $SW480^{wildtype}$ and $SW48^{wildtype}$) 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 1×10^4 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-*miR-186-5p* and scramble control miRNA. The cells were continued to grow at 37°C in 5% CO_2 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).

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

$SW480^{+miR\text{-}186\text{-}5p}$, $SW48^{+miR\text{-}186\text{-}5p}$ and $FHC^{+miR\text{-}186\text{-}5p}$ cells showed remarkably increased colony formation properties in comparison to the $SW480^{miRScr}$, $SW48^{miRScr}$ & $FHC^{+miRScr}$, and $SW480^{wildtype}$, $SW48^{wildtype}$ and $FHC^{wildtype}$ cells (Figure 2G-2I). The numbers of colonies in $SW480^{+miR\text{-}186\text{-}5p}$, $SW48^{+miR\text{-}186\text{-}5p}$ and $FHC^{+miR\text{-}186\text{-}5p}$ 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 $SW480^{+anti\text{-}}$

$^{miR\text{-}186\text{-}5p}$ and SW48$^{+anti\text{-}miR\text{-}186\text{-}5p}$ cells, respectively. Comparably, reduced surviving fractions (24%) were noted in FHC$^{+miR\text{-}186\text{-}5p}$ cells. On the contrary, 92 % and 94% surviving fractions were observed in SW480$^{+miR\text{-}186\text{-}5p}$ and SW48$^{+miR\text{-}186\text{-}5p}$ cells, respectively.

SW480$^{+miR\text{-}186\text{-}5p}$ cells had a higher cell migration potential than SW480^{miRScr} and SW480^{wildtype} cells as they healed the created scratch faster when compared to their counterpart (Figure 3A & 3B). SW480$^{+miR\text{-}186\text{-}5p}$ cancer cells healed the created wound completely on the 3rd day of initial scratch, whereas SW480^{wildtype} and SW480^{miRScr} cells took more time to heal the wounds. Anti-miR-186-5p treated cells (SW480$^{+anti\text{-}miR\text{-}186\text{-}5p}$) 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$^{+miR\text{-}186\text{-}5p}$ and FHC$^{+miR\text{-}186\text{-}5p}$ cells, wound healing was completed on day 3 whereas in SW48$^{+anti\text{-}miR\text{-}186\text{-}5p}$ and FHC$^{+anti\text{-}miR\text{-}186\text{-}5p}$ 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_0/G_1 phase of the cycle in SW480$^{+miR\text{-}186\text{-}5p}$ and SW48$^{+miR\text{-}186\text{-}5p}$ when compared to scramble control cells (Figure 4). In the case of SW480^{miRScr} cells, the percentage of S phase cells was 53.8 ± 4.5 %, while in SW480$^{+miR\text{-}186\text{-}5p}$ cells, the S phase population increased remarkably to 65.4 ± 3.2 %% ($p<0.05$). Proportion of G_0/G_1 phase in SW480$^{+miR\text{-}186\text{-}5p}$ cells was noted 4.4 ± 1.01% versus 25.2 ± 2.6 % in SW480^{miRScr} cells (Figure 4A-4B). On the other hand, treatment of cells with anti-miR-186-5p had shown opposite cell cycle distributions. In SW480$^{+anti\text{-}miR\text{-}186\text{-}5p}$, G_0/G_1 cells population increased to 32.1 ± 2.3 % and S phase cells reduced to 46.6 ± 3.1 %. Similar results were noted in SW48

cells (Figure 4C-4D). These results indicated that regulation of G_0/G_1 and synthesis phase of the cell cycle could be altered by the manipulation of miR-186-5p.

***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$^{+miR\text{-}186\text{-}5p}$ and SW48$^{+miR\text{-}186\text{-}5p}$ cells compared to that of scramble control and wild-type cells (Figures 5A and 5B). While anti-miR-186-5p treatment (SW480$^{+anti\text{-}miR\text{-}186\text{-}5p}$ and SW48$^{+anti\text{-}miR\text{-}186\text{-}5p}$) 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$^{+miR\text{-}186\text{-}5p}$ and SW48$^{+miR\text{-}186\text{-}5p}$ cells, whereas inhibition of miR-186-5p by the treatment of anti-miR-186-5p increased FAM134B protein expression in SW480$^{+anti\text{-}miR\text{-}186\text{-}5p}$ and SW48$^{+anti\text{-}miR\text{-}186\text{-}5p}$ 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, *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 $SW480^{+miR\text{-}186\text{-}5p}$ and $SW48^{+miR\text{-}186\text{-}5p}$ 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 $SW480^{+miR\text{-}186\text{-}5p}$ and $SW48^{+miR\text{-}186\text{-}5p}$ 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 ($SW480^{+anti\text{-}miR\text{-}186\text{-}5p}$ and $SW48^{+anti\text{-}miR\text{-}186\text{-}5p}$) minimized the oncogenic activities of miR-186-5p. Furthermore, in $SW480^{+anti\text{-}miR\text{-}186\text{-}5p}$ and $SW48^{+anti\text{-}miR\text{-}186\text{-}5p}$ 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 ($FHC^{+anti\text{-}miR\text{-}186\text{-}5p}$) 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

1. Thomas J, Ohtsuka M, Pichler M, Ling H. MicroRNAs: Clinical Relevance in Colorectal Cancer. Int J Mol Sci. 2015; 16: 28063-28076.
2. Ebrahimi F, Gopalan V, Wahab R, Lu CT, Smith RA, Lam AK. Deregulation of miR-126 expression in colorectal cancer pathogenesis and its clinical significance. Exp Cell Res. 2015; 339: 333-341
3. Gopalan V, Smith RA, Lam AK. Downregulation of microRNA-498 in colorectal cancers and its cellular effects. Exp Cell Res. 2015; 330: 423-428.
4. Ebrahimi F, Gopalan V, Smith RA, Lam AK. miR-126 in human cancers: clinical roles and current perspectives. Exp Mol Pathol. 2014; 96: 98-107.
5. Gopalan V, Pillai S, Ebrahimi F, Salajegheh A, Lam TC, Le TK, Langsford N, Ho YH, Smith RA, Lam AK. Regulation of microRNA-1288 in colorectal cancer: altered expression and its clinicopathological significance. Mol Carcinog. 2014; 53: E36-44.
6. Michael MZ, O' Connor SM, van Holst Pellekaan NG, Young GP, James RJ. Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res. 2003; 1: 882-891.
7. Stiegelbauer V, Perakis S, Deutsch A, Ling H, Gerger A, Pichler M. MicroRNAs as novel predictive biomarkers and therapeutic targets in colorectal cancer. World J Gastroenterol. 2014; 20: 11727-11735.
8. Yu Y, Kanwar SS, Patel BB, Oh PS, Nautiyal J, Sarkar FH, Majumdar AP. MicroRNA-21 induces stemness by downregulating transforming growth factor beta receptor 2 (TGFβR2) in colon cancer cells. Carcinogenesis. 2012; 33:68-76.
9. Xiong B, Cheng Y, Ma L, Zhang C. MiR-21 regulates biological behavior through the PTEN/PI-3 K/Akt signaling pathway in human colorectal cancer cells. Int J Oncol. 2013; 42: 219-228.

10. Ling H, Pickard K, Ivan C, Isella C, Ikuo M, Mitter R, Spizzo R, Bullock MD,Braicu C, Pileczki V, Vincent K, Pichler M, Stiegelbauer V, Hoefler G, Almeida MI, Hsiao A, Zhang X, Primrose JN, Packham GK, Liu K, Bojja K, Gafà R, Xiao L, Rossi S, Song JH, Vannini I, Fanini F, Kopetz S, Zweidler-McKay P, Wang X, Ionescu C, Irimie A, Fabbri M, Lanza G, Hamilton SR, Berindan-Neagoe I, Medico E, Mirnezami AH, Calin GA, Nicoloso MS. The clinical and biological significance of MIR-224 expression in colorectal cancer metastasis. Gut. 2016; 65: 977-989.
11. Summerer I, Unger K, Braselmann H, Schuettrumpf L, Maihoefer C, Baumeister P, Kirchner T, Niyazi M, Sage E, Specht HM, Multhoff G, Moertl S, Belka C, Zitzelsberger H. Circulating microRNAs as prognostic therapy biomarkers in head and neck cancer patients. Br J Cancer. 2015; 113: 76-82.
12. Wu D, Tang R, Qi Q, Zhou X, Zhou H, Mao Y, Li R, Liu C, Wang W, Hua D, Zhang X. Five functional polymorphisms of B7/CD28 co-signaling molecules alter susceptibility to colorectal cancer. Cell Immunol. 2015; 293: 41-48.
13. Satoh J, Kino Y, Niida S. MicroRNA-Seq Data Analysis Pipeline to Identify Blood Biomarkers for Alzheimer's Disease from Public Data. Biomark Insights. 2015; 10: 21-31.
14. Jiang BC, Cao DL, Zhang X, Zhang ZJ, He LN, Li CH, Zhang WW, Wu XB, Berta T, Ji RR, Gao YJ. CXCL13 drives spinal astrocyte activation and neuropathic pain via CXCR5. J Clin Invest. 2016; 126: 745-761.
15. Balci S, Ayaz L, Gorur A, Yildirim Yaroglu H, Akbayir S, Dogruer Unal N, Bulut B, Tursen U, Tamer L. microRNA profiling for early detection of nonmelanoma skin cancer. Clin Exp Dermatol. 2016; 41: 346-351.
16. Wang KJ, Zhao X, Liu YZ, Zeng QT, Mao XB, Li SN, Zhang M, Jiang C, Zhou Y, Qian C, Feng KG, Guan HQ, Tang TT, Cheng X, Chen ZJ. Circulating MiR-19b-3p,

MiR-134-5p and MiR-186-5p are promising novel biomarkers for early diagnosis of acute myocardial infarction. Cell Physiol Biochem. 2016; 38: 1015-1029.

17. Verma AM, Patel M, Aslam MI, Jameson J, Pringle JH, Wurm P, Singh B.Circulating plasma microRNAs as a screening method for detection of colorectal adenomas. Lancet. 2015; 385: S100.

18. Gopalan V, Smith RA, Nassiri MR, Yasuda K, Salajegheh A, Kim SY, Ho YH, Weinstein S, Tang JC, Lam AK. GAEC1 and colorectal cancer: a study of the relationships between a novel oncogene and clinicopathologic features. Human pathology. 2010; 41: 1009-10015.
19. Gopalan V, Yasuda K, Pillai S, Tiang T, Leung M, Lu CT, Tang JC, Smith RA, Lam AK. Gene amplified in oesophageal cancer 1 (GAEC1) amplification in colorectal cancers and its impact on patient's survival. Journal of clinical pathology. 2013; 66:721-3.
20. Lam AK, Gopalan V, Nassiri MR, Kasim K, Dissanayake J, Tang JC, Smith RA. Altered JS-2 expression in colorectal cancers and its clinical pathological relevance. Molecular oncology. 2011; 5:475-81.
21. Islam F, Gopalan V, Wahab R, Smith RA, Qiao B, Lam AK. Stage dependent expression and tumor suppressive function of FAM134B (JK1) in colon cancer. Mol Carcinog. 2017; 56: 238-249.
22. Gopalan V, Islam F, Pillai S, Tang JC, Tong DK, Law S, Chan KW, Lam AK. Overexpression of microRNA-1288 in oesophageal squamous cell carcinoma. Exp Cell Res. 2016; 348: 146-154.

23. Ebrahimi F, Gopalan V, Wahab R, Lu CT, Smith RA, Lam AK. Deregulation of miR-126 expression in colorectal cancer pathogenesis and its clinical significance. Exp Cell Res. 2015; 339: 333-341.

24. Kasem K, Gopalan V, Salajegheh A, Lu CT, Smith RA, Lam AK. The roles of JK-1 (FAM134B) expressions in colorectal cancer. Exp Cell Res. 2014; 326:166-173.

25. Hamilton SR, Bosman FT, Boffetta P, Ilas M, Morreau H, Nakamura SI, Quirke P, Riboli E, Sobin LH. Carcinoma of the colon and rectum. In: Bosman FT, Carneiro F, Hruban RH, Theise ND (eds) WHO classification of tumors of the digestive system. IARC Press, Lyon, 2010, pp 134–146.

26. Lim SB, Yu CS, Jang SJ, Kim TW, Kim JH, Kim JC. Prognostic significance of lymphovascular invasion in sporadic colorectal cancer. Dis Colon Rectum. 2010; 53: 377-384.

27. Yang MH, Yu J, Chen N, Wang XY, Liu XY, Wang S, Ding YQ. Elevated microRNA-31 expression regulates colorectal cancer progression by repressing its target gene SATB2. PLoS One. 2013; 8:e85353.

28. Zhou T, Zhang G, Liu Z, Xia S, Tian H. Overexpression of miR-92a correlates with tumor metastasis and poor prognosis in patients with colorectal cancer. Int J Colorectal Dis. 2013; 28: 19-24.

29. Tang W, Zhu Y, Gao J, Fu J, Liu C, Liu Y, Song C, Zhu S, Leng Y, Wang G, Chen W, Du P, Huang S, Zhou X, Kang J, Cui L. MicroRNA-29a promotes colorectal cancer metastasis by regulating matrix metalloproteinase 2 and E-cadherin via KLF4. Br J Cancer. 2014; 110: 450-458.

30. Wu W, Wang Z, Yang P, Yang J, Liang J, Chen Y, Wang H, Wei G, Ye S, Zhou Y. MicroRNA-135b regulates metastasis suppressor 1 expression and promotes migration and invasion in colorectal cancer. Mol Cell Biochem. 2014; 388: 249-259.

31. Pritchard CC, Grady WM. Colorectal cancer molecular biology moves into clinical practice. Gut 2011; 60:116-129.
32. Lam AK, Ong K, Giv MJ, Ho YH. p16 expression in colorectal adenocarcinoma: marker of aggressiveness and morphological types. Pathology. 2008; 40: 580-585.
33. Saleh S, Lam AK, Ho YH. Real-time PCR quantification of human telomerase reverse transcriptase (hTERT) in colorectal cancer. Pathology. 2008; 40: 25-30.
34. Lam AK, Ong K, Ho YH. Aurora kinase expression in colorectal adenocarcinoma: correlations with clinicopathological features, p16 expression, and telomerase activity. Hum Pathol. 2008; 39: 599-604.
35. Leslie A, Carey FA, Pratt NR, Steele RJ. The colorectal adenoma-carcinoma sequence. Br J Surg. 2002; 89: 845-860.
36. Strum WB. Colorectal Adenomas. N Engl J Med. 2016; 374: 1065-75.
37. Atkin WS, Morson BC, Cuzick J. Long-term risk of colorectal cancer after excision of rectosigmoid adenomas. N Engl J Med. 1992; 326:658-662.
38. Müller AD, Sonnenberg A. Protection by endoscopy against death from colorectal cancer. A case-control study among veterans. Arch Intern Med. 1995; 155: 1741-1748.
39. Kasem K, Sullivan E, Gopalan V, Salajegheh A, Smith RA, Lam AK. FAM134B (FAM134B) represses cell migration in colon cancer: a functional study of a novel gene. Exp Mol Pathol 2014; 97:99-04.
40. Kasem K, Gopalan V, Salajegheh A, Lu CT, Smith RA, Lam AK. FAM134B (FAM134B) gene and colorectal cancer: a pilot study on the gene copy number alterations and correlations with clinicopathological parameters. Exp Mol Pathol 2014; 97:31-36.

41. Wong N, Wang X. miRDB: an online resource for microRNA target prediction and functional annotations. Nucleic Acids Res. 2015; 43: D146-52.

42. Wang X. Improving microRNA target prediction by modeling with unambiguously identified microRNA-target pairs from CLIP-ligation studies. Bioinformatics. 2016; 32: 1316-22.

43. Wen D, Danquah M, Chaudhary AK, Mahato RI. Small molecules targeting microRNA for cancer therapy: Promises and obstacles. J Control Release. 2015; 219: 237-247.

44. Monroig Pdel C, Chen L, Zhang S, Calin GA. Small molecule compounds targeting miRNAs for cancer therapy. Adv Drug Deliv Rev. 2015; 81: 104-116.

45. Li Y, Ahmad A, Kong D, Bao B, Sarkar FH. Targeting MicroRNAs for personalized cancer therapy. Med Princ Pract. 2013; 22: 415-417.

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

Characteristics	Number	High Expression	Low Expression	p value
Sex				
Male	67 (53.2%)	46 (68.7%)	21 (32.3%)	0.455
Female	59 (46.8%)	42 (71.2%)	17 (28.8%)	
Age				
< 60	35 (27.8%)	23 (65.7%)	12 (34.3%)	0.337
≥ 60	91 (72.2%)	65 (71.4%)	26 (28.6%)	
Site				
Proximal	56 (44.4%)	36 (64.3%)	20 (35.7%)	**0.043**
Distal	70 (55.6%)	52 (74.3%)	18 (25.7%)	
Size (mm)				
≤40	61 (48.4%)	42 (68.9%)	19 (31.1%)	0.055
>40	65 (51.6%)	46 (70.8%)	19 (29.2%)	
MSI*				
Positive	20 (18.9%)	10 (50%)	10 (50%)	**0.015**
Negative	86 (81.1%)	67 (77.9%)	19 (22.1%)	
Histological grade				
Grade 1 or grade 2	107 (84.9%)	74 (69.2%)	33 (30.2%)	0.461
Grade 3	19 (15.1%)	14 (73.7%)	5 (26.3%)	
Polyposis				
Positive	9 (7.1%)	4 (44.4%)	5 (55.6%)	0.093
Negative	117 (92.9%)	84 (71.8%)	33 (28.2%)	
Associated adenoma				
Positive	59 (46.8%)	46 (78%)	13 (22%)	**0.047**
Negative	67 (53.2%)	42 (62.7%)	25 (37.3%)	
Lymphovascular permeation				
Present	35 (27.8%)	24 (68.6%)	11 (31.4%)	**0.037**
Absent	91 (72.2%)	64 (70.3%)	27 (29.7%)	
Distant metastasis				
Present	30 (23.8%)	21 (70%)	9 (30%)	**0.001**
Absent	96 (76.2%)	67 (69.8%)	29 (30.2%)	
Pathological staging				
I &II	62 (49.2%)	41 (66.1%)	21 (33.9%)	0.242
III & IV	64 (50.8%)	47 (73.4%)	17 (26.4%)	

* 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.

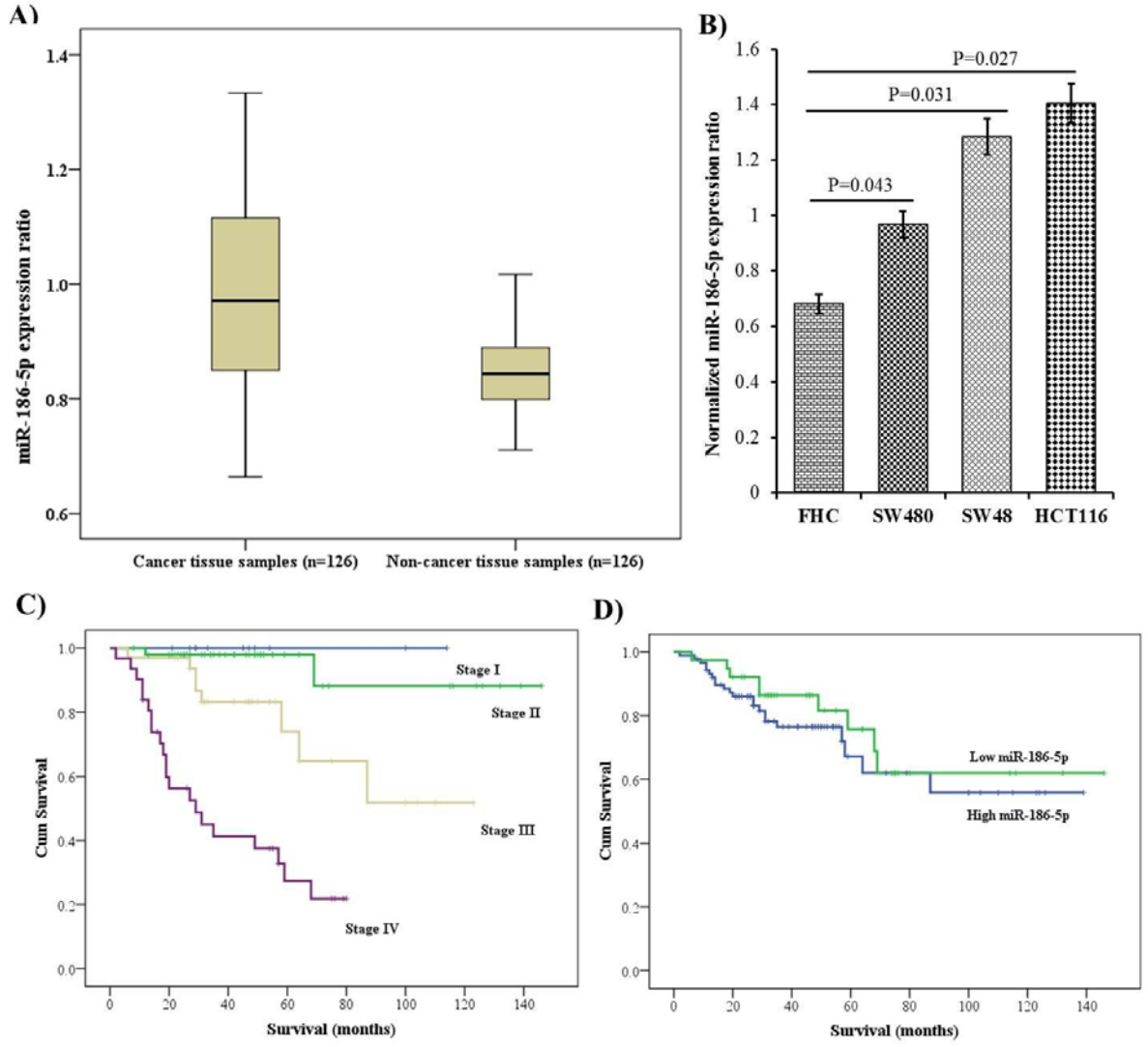


Figure 2. miR-186-5p promoted in vitro colon cancer growth. Exogenous manipulation of miR-186-5p in SW-480 **(A),** SW-48 **(B)** and FHC **(C)** cells induce altered proliferation in comparison to control and non-transfected cells on different days after transfection. Overexpression of miR-186-5p increased cells proliferation in $SW480^{+miR\text{-}186\text{-}5p}$ and $SW48^{+miR\text{-}186\text{-}5p}$ whereas downregulation of miR-186-5p in $SW480^{+anti\text{-}miR\text{-}186\text{-}5p}$ and $SW48^{+anti\text{-}miR\text{-}186\text{-}5p}$ 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 $SW480^{+miR\text{-}186\text{-}5p}$ **(G),** $SW48^{+miR\text{-}186\text{-}5p}$ **(H)** and $FHC^{+miR\text{-}186\text{-}5p}$ **(I)** cells. Suppression of miR-186-5p by anti-miR-186-5p treatment reduced clonogenic properties of $SW480^{+anti\text{-}miR\text{-}186\text{-}5p}$ **(D),** $SW48^{+anti\text{-}miR\text{-}186\text{-}5p}$ and $FHC^{+miR\text{-}186\text{-}5p}$ **(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.

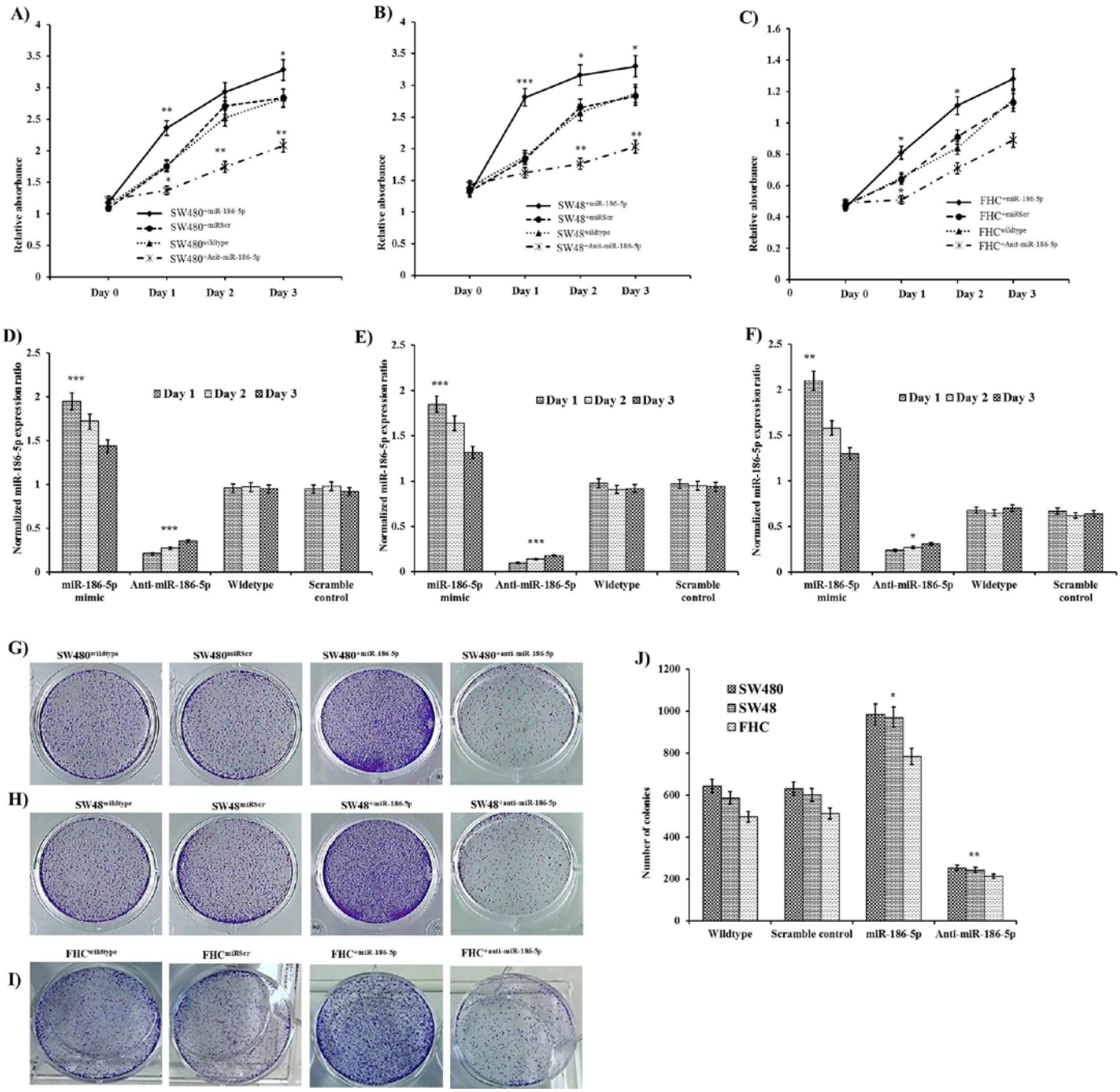


Figure 3. miR-186-5p stimulate wound healing capacity in colon cancer cells.

Overexpression of miR-186-5p in SW480$^{+miR\text{-}186\text{-}5p}$ cells showed more migration capacity, healing the wound faster than the control (SW480^{miRScr}) and non-transfected (SW480^{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$^{+miR\text{-}186\text{-}5p}$ and FHC$^{+miR\text{-}186\text{-}5p}$ cells, similar results were noted **(C-D & E-F)**. Scale bars (A, C & E) 200 μm. 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.

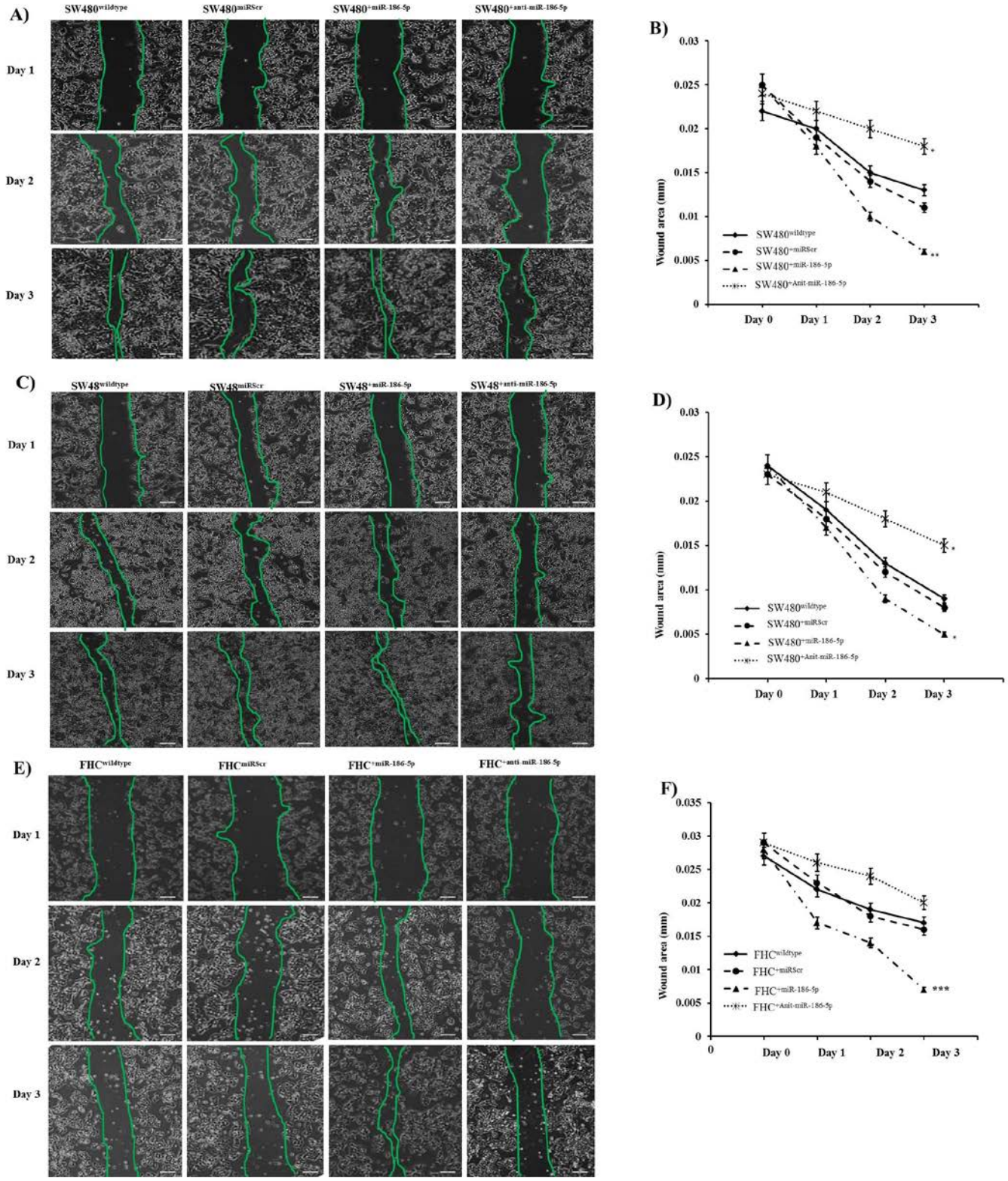


Figure 4. miR-186-5p altered cell cycle kinetics of colon cancer cells. Cell cycle analysis by flow cytometry revealed that miR-186-5p overexpression caused S phase accumulation and a parallel reduction of G_0/G_1 of colon cancer cell. Inhibition of endogenous miR-186-5p by the treatment with anti-miR-186-5p exhibited accumulation of cells in G_0/G_1 phase and reduction of S phase cells. **A)** Presented the representative histograms of cell cycle analysis of $SW480^{+miR\text{-}186\text{-}5p}$, $SW480^{+anti\text{-}miR\text{-}186\text{-}5p}$ 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.

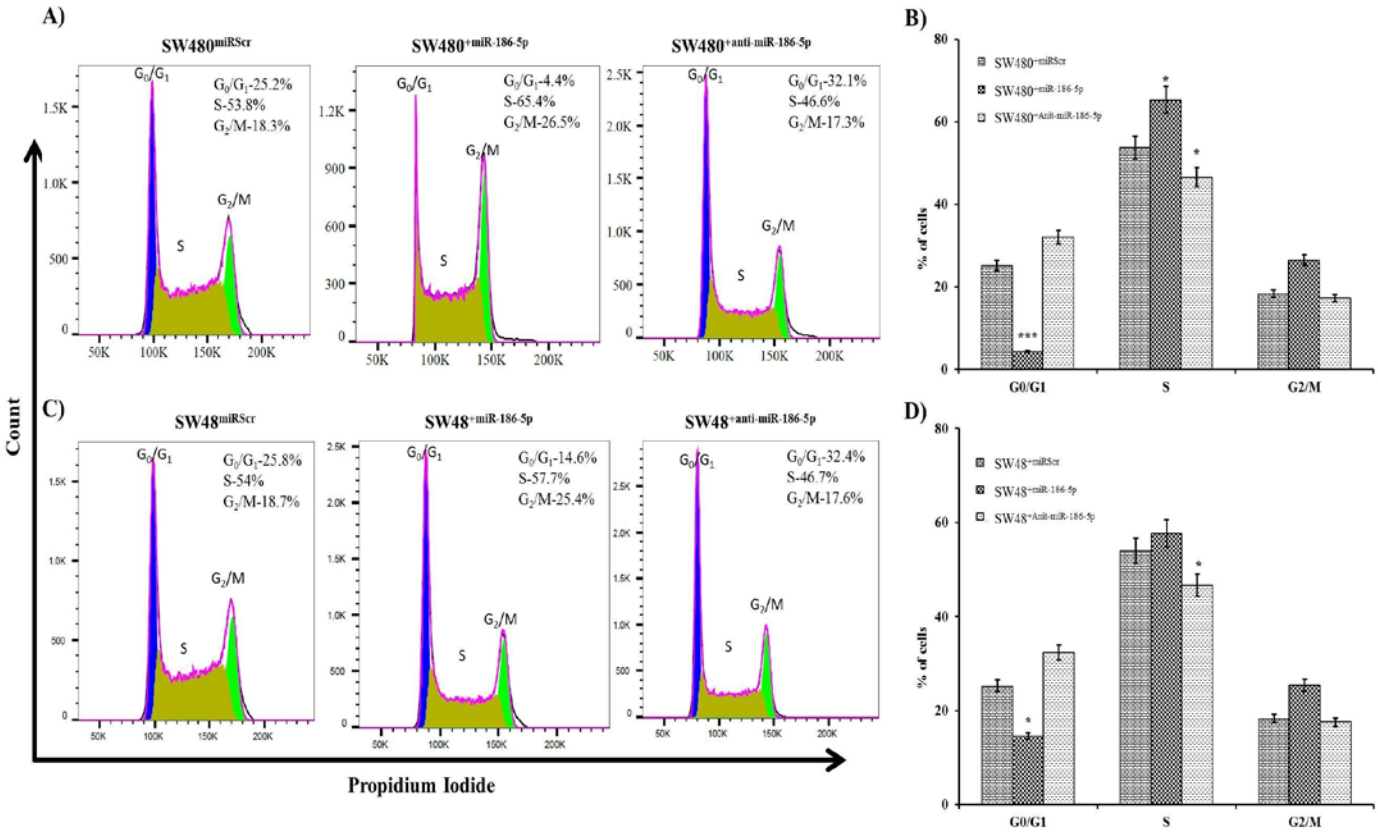
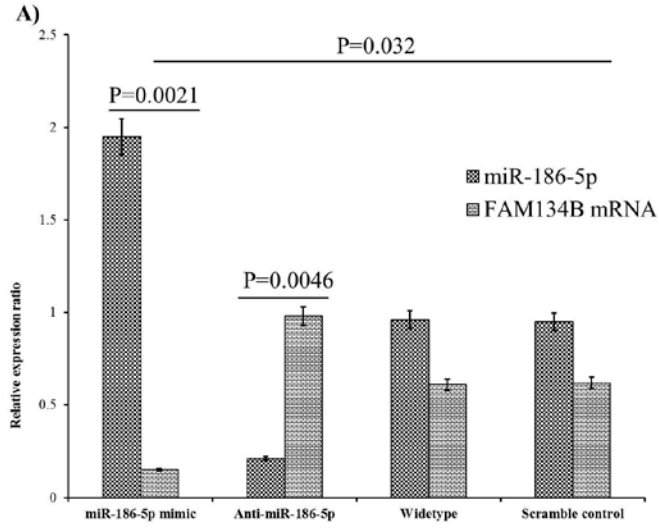
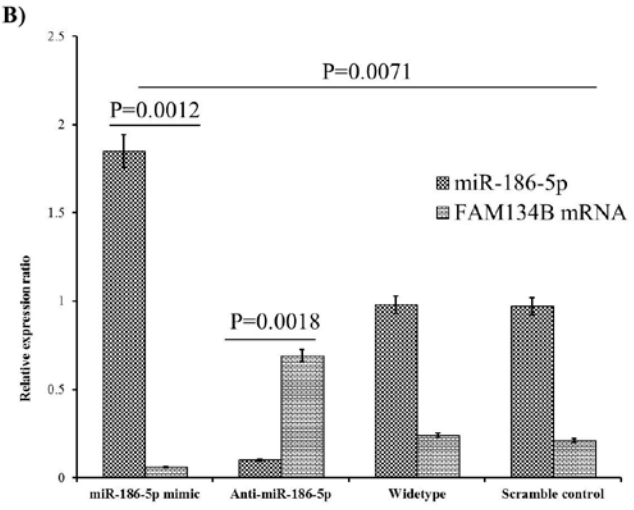
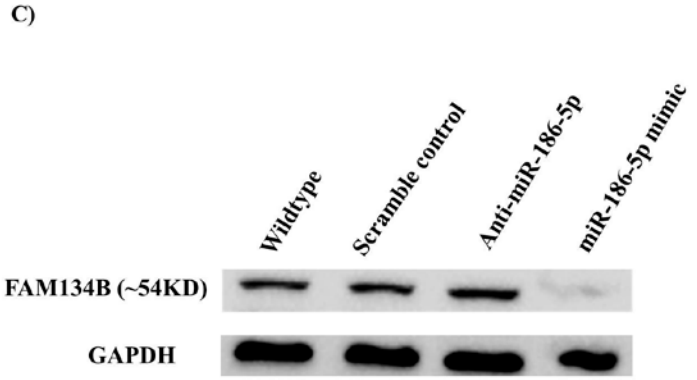
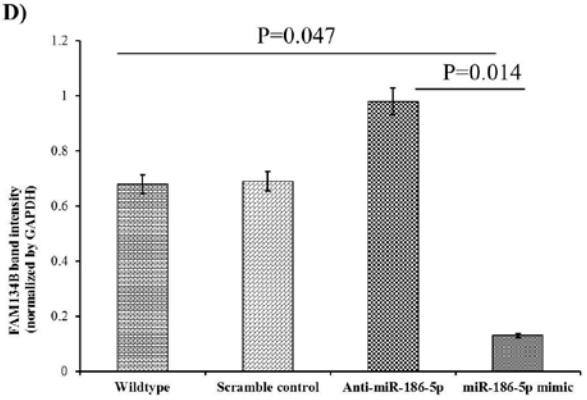


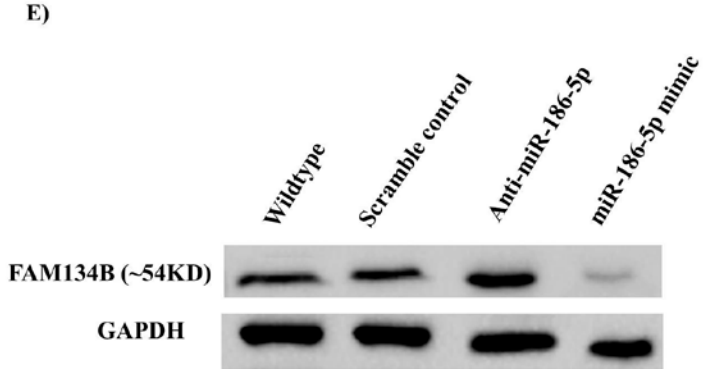
Figure 5. miR-186-5p repress FAM134B expression in colon cancer cells. FAM34B mRNA expression inversely correlated with the expression of miR-186-5p in colon cancer cell lines (SW480 and SW48). Overexpression of miR-186-5p caused reduces expression of FAM134B mRNA in SW480 **(A)** and SW48 **(B)** colon 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 $SW480^{+anti\text{-}miR\text{-}186\text{-}5p}$ and $SW48^{+anti\text{-}miR\text{-}186\text{-}5p}$ cells **(A)** and **(B)**, respectively. Also, altered FAM134B protein expression was noted upon miR-186-5p manipulation in $SW480^{+miR\text{-}186\text{-}5p}$, $SW480^{+anti\text{-}miR\text{-}186\text{-}5p}$ compared to that of $SW480^{miRScr}$ and $SW480^{wildtype}$ cells **(C)**. In $SW480^{+miR\text{-}186\text{-}5p}$ cells, FAM134B proteins expression was downregulated significantly in comparison to the $SW480^{miRScr}$ and $SW480^{wildtype}$ cells, whereas in $SW480^{+anti\text{-}miR\text{-}186\text{-}5p}$ 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 $SW480^{+miR\text{-}186\text{-}5p}$ 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.


A)
P=0.032
P=0.0021
P=0.0046
miR-186-5p
FAM134B mRNA
Relative expression ratio
2.5
2
1.5
1
0.5
0
miR-186-5p mimic
Anti-miR-186-5p
Widetype
Scramble control


B)
P=0.0071
P=0.0012
P=0.0018
miR-186-5p
FAM134B mRNA
Relative expression ratio
2.5
2
1.5
1
0.5
0
miR-186-5p mimic
Anti-miR-186-5p
Widetype
Scramble control


C)
Wildtype
Scramble control
Anti-miR-186-5p
miR-186-5p mimic
FAM134B (~54KD)
GAPDH


D)
P=0.047
P=0.014
FAM134B band intensity
(normalized by GAPDH)
1.2
1
0.8
0.6
0.4
0.2
0
Wildtype
Scramble control
Anti-miR-186-5p
miR-186-5p mimic


E)
Wildtype
Scramble control
Anti-miR-186-5p
miR-186-5p mimic
FAM134B (~54KD)
GAPDH

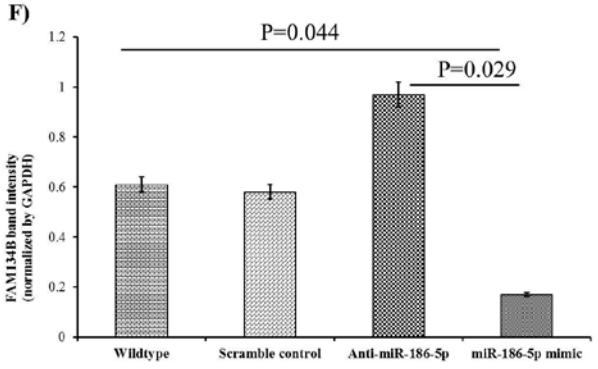
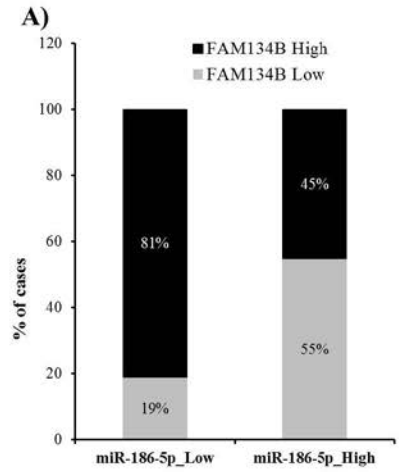
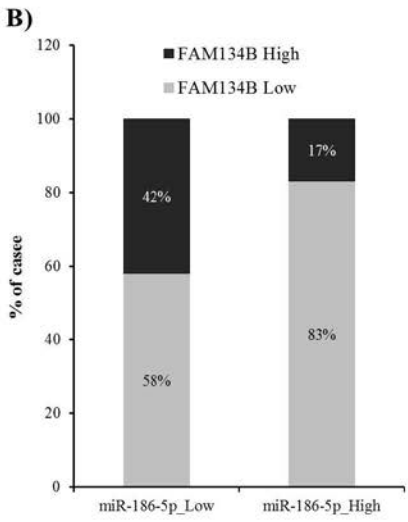
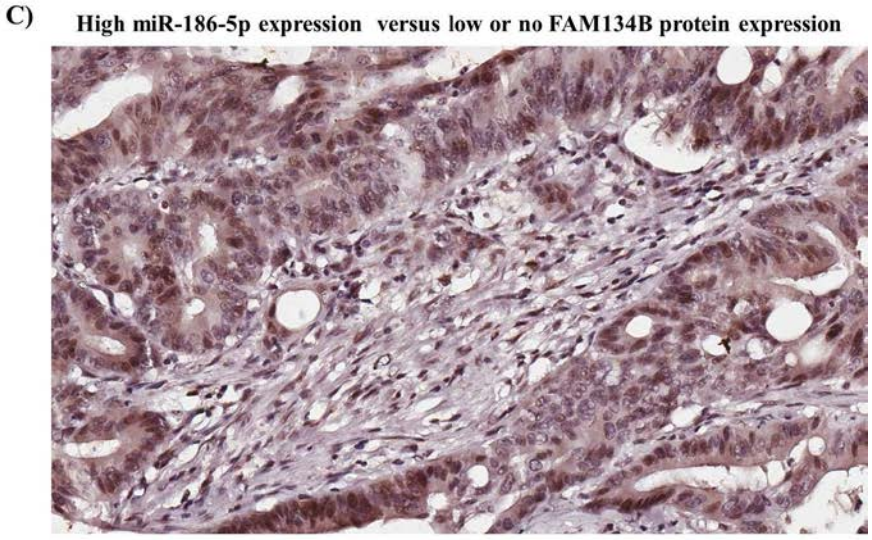
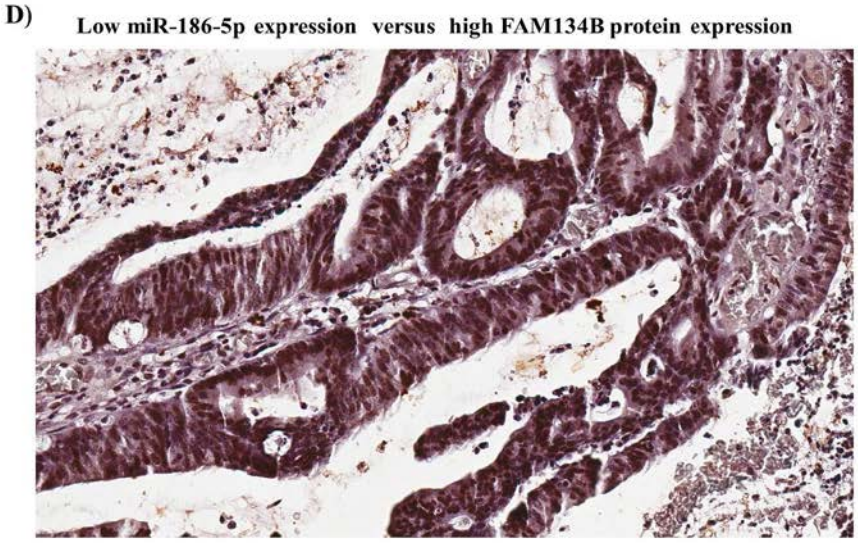

F)
P=0.044
P=0.029
FAM134B band intensity
(normalized by GAPDH)
1.2
1
0.8
0.6
0.4
0.2
0
Wildtype
Scramble control
Anti-miR-186-5p
miR-186-5p mimic

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

Name of Genes	Primer sequence (Forward and Reverse)
FAM134B	5′-TGACCGACCCAGTGAGGA-3′ 5′-GGGCAAACCAAGGCTTAA-3′
GAPDH	5′- TGCACCACCAACTGCTTAGC-3′ 5′- GGCATGGACTGTGGTCATGAG-3′
β-actin	5′-ACCCACACTGTGCCCATCTACGA-3′ 5′-CAGGAGGAGCAATGATCTTGATCTTC-3′
has-miR-186-5p	5′CAAAGAAUUCUCCUUUUGGGCU

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-

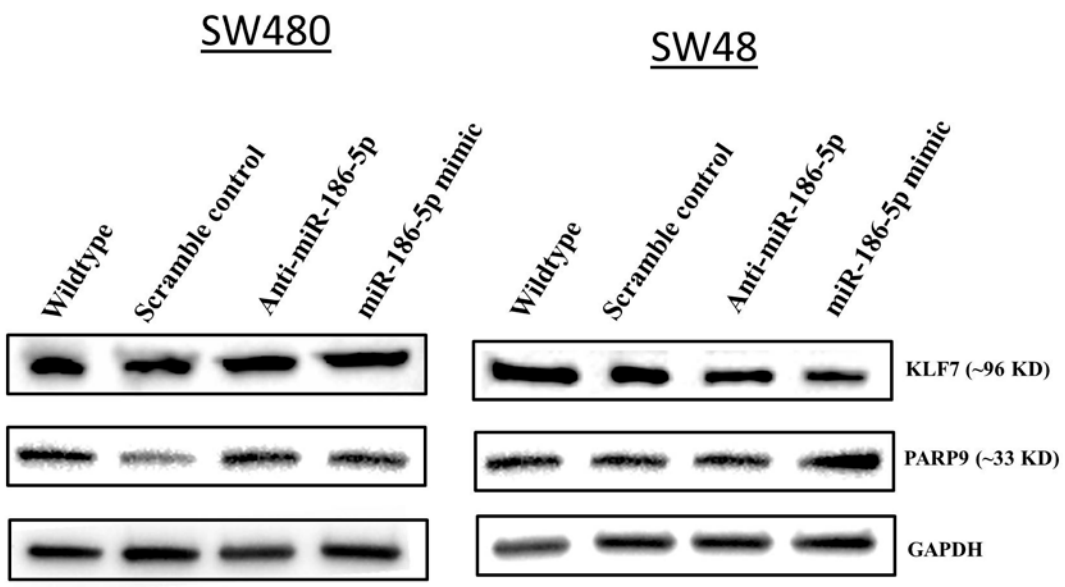
$$\text{Clone formation rate or plating efficiency (PE)} = \frac{\text{No. of colony formation}}{\text{No. of cells seeded}} \times 100$$

$$\text{Surviving fractions} = \frac{\text{No. of colony formation}}{\text{No. of cells seeded}} \times \text{PE}$$

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′ atcctcctataaatATTCTTT 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).