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## **Clinical and biological significance of miR-193a-3p targeted KRAS in colorectal cancer pathogenesis**

Running head: miR-193a-3p in colorectal cancer

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**Abstract**

This study was to investigate the expression pattern, mechanisms and clinicopathological implications of miR-193a-3p in colorectal cancer. Fresh frozen tissues from 70 matched colorectal adenocarcinomas and the adjacent non-neoplastic mucosae were prospectively collected. Two colorectal cancer cell lines (SW480 and SW48) and a non-neoplastic colon cell line (FHC) were also used. The expression levels of miR-193a-3p in the cells and tissues were measured by quantitative real-time polymerase chain reaction. The expression of KRAS protein as a predicted downstream target for miR-193a was studied by immunohistochemistry. Restoration of the miR-193a level in the cell lines by permanent transfection was achieved and multiple functional and immunological assays were performed to analyse the functions of miR-193a in vitro. Down-regulation of miR-193a-3p was noted in 70% of the colorectal cancer tissues when compared to non-neoplastic colorectal tissues. In addition, down regulation of miR-193a was significantly correlated with carcinoma of early stages ( $p < 0.05$ ). Significant inverse correlation between miR-193a-3p and its target KRAS protein was determined ( $p < 0.05$ ). Overexpression of miR-193a in colon cancer cells resulted in reduced cell proliferation, increased apoptosis, induced significant changes in cell cycle events and decreased the expression of epithelial mesenchymal transition marker TWIST. This study confirms the tumour suppressor roles of miR-193a-3p, its downstream target affinity to KRAS and clinical significance in patients with colorectal adenocarcinoma.

## 1. Introduction:

It is estimated that approximately 30% of protein coding for human genome is targeted by microRNAs [1]. Many of these miRNAs act either as oncogenes or tumour suppressor genes. They regulate multiple cellular and pathophysiological events in carcinogenesis [2-5]. The miR-193a is a member of the miR-193 family that consists of miR-193b and miR-193c. miR-193a is located on chromosome 17 at 17q11.2 of the human genome (Gene ID: 406968) [6]. Based on arm selection, miR-193a has two members: miR-193a-3p and miR-193a-5p. Previous studies have proposed that miR-193a primarily functions as a tumour suppressor miRNA in many carcinomas including non-small cell lung carcinoma [7] and breast carcinomas [8]. miR-193a can be aberrantly silenced by DNA hyper methylation which can leads to inhibition of its tumour suppressor properties and results in the development of no-small cell lung carcinomas and acute myeloid leukaemia [7, 9]. On the other hand, miR-193a has oncogenic roles in prostatic [10] and colorectal adenocarcinomas [11].

Previous studies have confirmed that miR-193a exhibits its pathogenic effects in carcinogenesis especially in the colorectal adenocarcinoma via targeting KRAS [12, 13]. *KRAS* mutations occur in approximately 40% of colorectal adenocarcinoma that lead to activation of downstream targets genes of this disease [14]. Until now, the significance of miR-193a in colorectal adenocarcinoma is not well understood in patient-derived tissue samples. The aim of this study is to investigate the expression pattern, target affinity, cellular changes and clinicopathological significance of miR-193a-3p in colon cancer cells and colorectal cancer tissues.

## 2. Materials and Methods

### 2.1 Recruitment of the tissues and sample selection

We prospectively collected fresh frozen colorectal cancer tissue with matched non-cancer mucosal tissues from patients who have undergone resection and stored at -80°C. In each case, formalin fixed paraffin tissue block from the cancer was also prospectively obtained at the same time. This study was approved by the Human Research Ethics Committee of Griffith University (GU Ref No: MSC/17/10/HREC).

### 2.2. Clinical and pathological data of the patients

After the collection of frozen tissues, the surgical specimens of colorectal cancer were processed for pathological examination by the author (AKL). As a result, 70 patients (35 men and 35 women) with colorectal adenocarcinoma were selected for this study. The cancer was graded according to the World Health Organization (WHO) [15]. In addition, the carcinoma was staged according to the 8<sup>th</sup> edition of Cancer Staging Manual of American Joint Committee on Cancer (AJCC) [16]. The pathological stages were also confirmed in a multidisciplinary team meeting of the pathologist and the surgeon (AKL & CTL). The other pathological parameters including peri-neural cancer invasion, lymphovascular permeation, cancer perforation and the presence of associated polyps (adenoma/hyperplastic polyp/serrated polyp/serrated adenoma) are also noted. Microsatellite instability (MSI) was tested in these cases by immunohistochemistry on four proteins (MLH1, PMS2, MSH2 and MSH6) as described previously [17].

All the patients were followed-up regularly for any deterioration of their medical conditions, recurrence of cancer and metastasis by the same management protocol. The overall survival rate of the patients was calculated from the date of surgical resection of the colorectal carcinomas to the date of death due to cancer or at last follow-up of the patient.

### 2.3. Cell culture

Two human colon carcinoma cell lines, SW-480 (Stage II colon cancer, CCL-228™) and SW-48 (Stage III colon cancer, CCL-231™), were used. These cell lines were obtained from the American Type Culture Collection (ATCC). The control was a non-neoplastic colonic epithelial cell line (FHC). Each cell line was cultured in its required media and tested routinely to prevent mycoplasma contamination.

### 2.4. MicroRNA extraction and cDNA conversion

Total RNA including miRNA was extracted from the fresh frozen human tissues after cryotomy and from cell lines after culture. In brief, tissues were sectioned in thin slices (5µm) and followed by extraction using Qiagen miRNeasy mini kit which is specific for purification the total RNA including miRNA from the tissues and the cells (Qiagen, Hilden, North Rhine-Westphalia, Germany). RNA quality was assessed by using a Nano Drop ND.1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, U.S.A.). The purity of RNA was obtained by checking the optical density (OD) 260/280 ratio and concentration of RNA was noted in ng/µl.

Reverse transcription reactions were performed using 1 µg total RNA in a final reaction volume of 20 µl. The extracted miRNA was converted to cDNA using miScript reverse transcription kit (Qiagen) according to the manufacturer's instructions. Each cDNA sample was diluted to 1.5 ng/µl to provide uniformly concentrated sample for miRNA quantitative real-time PCR (polymerase chain reaction). The sample was stored at -20°C until the PCR analysis.

## 2.5. Quantitative real-time PCR

An IQ5 Multicolour Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA) was used to analyse the miR-193a-3p expression changes. PCR was performed in a total volume of 20  $\mu$ l reaction mixture containing 10  $\mu$ l 2x QuantiTect SYBR Green PCR Master Mix, 2  $\mu$ l 10x miScript Universal Primer, 2  $\mu$ l 10x miScript primer assay (Hs\_miR-193a-3P) (Qiagen) and 4  $\mu$ l of cDNA template at 1.5 ng/  $\mu$ l concentration. RNU6B RNA (Hs\_RNU6B\_2 miScript Primer Assay, Qiagen) was used for normalising the amount of target miRNA. All the samples were run in triplicates. The fold change in the target gene for the results of quantitative amplification was calculated for each sample using  $2^{-\Delta\Delta CT}$  (fold change) method as previously reported [4]. A fold change of more than 2 was considered as high miR-193a-3p expression, and a fold change of less than 1 was regarded as low expression for miR-193a-3p. In addition, the inverse ratio of miR-193a-3p versus RNU6B (control miRNA) [expression ratio] was used to represent the miRNA expression in relative to control samples [4, 17].

## 2.6. Immunohistochemical study of KRAS in colorectal cancer tissues:

A high PH ENVision™ FLEX Mini Kit (Dako, Glostrup, Hovedstaden, Denmark) was used for the immunohistochemical analysis of KRAS protein according to our previous published protocol [5]. Tissues were incubated with Anti –KRAS antibody, Rabbit polyclonal to KRAS (ab 137739, 1:150 dilution; Abcam, Cambridge, England) for 60 minutes at room temperature. A colon adenocarcinoma tissue that showed strong cytoplasmic staining was used as a positive control in each run of the experiment. A negative control sample was prepared by the same procedure, except that tissue was not incubated with the primary antibody. A grading scale ranging from “0” to “3” was used for this assessment, where “0” represented a negative staining, “1” represented weak staining (1-

30%), “2” represented moderate staining (31 to 70%), and “3” represented strong staining (>70%).

## 2.7. Cell transfection

The over expression of miR-193a in colon cancer cells was permanently achieved by using an OriGene pCMV/miR 193a plasmid vector (OriGene Technologies, Rockville, MD, USA). Transfection was performed according to the manufacturer’s instructions and optimisation was achieved by doubling the concentration of miR-193a-3p plasmid DNA. Approximately 0.5 µg of DNA was used to optimise the transfection in 96 well plates. Neomycin in starting concentration of 500µg/ml was used as a selective agent for permanent transfection and the dose of neomycin then adjusted to 375µg/ml. Cells transfected with empty vector were used as a control and non-transfected cells were used as mock.

## 2.8. Cell proliferation assay

The role of miR-193a-3p in colon cancer cell proliferation was detected by using 3-(4,5-dimethylthiazol-2-yl) - 5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS). Both transfected and non-transfected cells were seeded in flat-bottom 96-well plates with  $1 \times 10^4$  cells/well in quadruplicate and were incubated for 0 hours, 48 hours, 72 hours, and 96 hours. Approximately 10 ml CCK8 /100 ml complete media (Sigma–Aldrich, Australia) was added to each well. The cells were incubated at 37°C for 1 hour. Absorbance was determined by using a fluorescence polarisation microplate reader POLARstar Omega (BMG TABTECH, Ortenberg, Germany) at a wavelength of 450 nm

## 2.9. Wound healing assay

In vitro wound healing assay was performed to examine the migration of miR-193a transfected and control colon cancer cells. Briefly, the cells were grown in 6-well plates with their respective culture media. After reaching 90% confluence, a single scratch was made on the monolayer using a yellow pipette (200ul) tip, and the wounded layers were washed with phosphate buffered saline (PBS) to remove cell debris. Measurement of the closure or filling of the wounds at days 0, 1, 2 and 3 was done under a contrast microscope. All the experiments were performed in triplicate

## 2.10. Clonogenic assay

To assess miR-193a effect on clonogenic ability of colon cancer cell lines, all the treated/control cells were seeded in 6-well plates (200 cells/well). After two weeks, the cells were fixed with 70% cold ethanol for 10 minutes. Following fixation, 5% crystal violet solution was added and incubated at room temperature for two hours. Then, 10% fetal bovine serum (FBS) containing media was added and pipetted properly to wash off the cells. Plates were then allowed to dry, photos were taken and clones were counted under a microscope.

## 2.11. Western blot analysis

The cells were harvested and lysed with NP40 (ThermoFisher Scientific) buffer enriched with complete protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). They were then separated in 15% TGX-polyacrylamide gel according to the previously published protocol [2]. After that, the blots were incubated with primary antibody (anti-KRAS antibody, rabbit polyclonal to KRAS (ab 137739, Abcam, Cambridge, England) at the appropriate final concentration (after multiple optimisation steps). Other antibodies used are

OCT4 polyclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA), SOX-2 polyclonal antibody (Sc-17320, Santa Cruz Biotechnology), SNAIL1 polyclonal antibody (Santa Cruz Biotechnology) and TWIST polyclonal antibody (Santa Cruz Biotechnology). They are used to study the effects of miR-193a on the expressions of stem cell markers and epithelial-mesenchymal transition in the cancer respectively. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as a control. Each sample was then incubated with the appropriate secondary antibody. Finally, Western blot images were developed using VersaDoc-MP imaging system (Bio-Rad).

### **2.12. Cell cycle analysis**

To determine the effect of miR-193a on cell cycle regulation, cells were harvested by trypsinization and were fixed in phosphate buffered saline (PBS) with 70% ethanol. After 1 hour of incubation with RNase A, 400  $\mu$ l of propidium iodide was added directly to cells containing RNase A solution. Cell cycle analysis was performed with a flow cytometer (BD FACSCalibur™ flow cytometer- BD Bioscience, Franklin Lakes, NJ, USA) after incubation in a dark room for 5-10 minutes.

### **2.13. Annexin-V staining**

Annexin V-FITC conjugated apoptosis detection kit (Invitrogen, Carlsbad, CA, USA) was used to assess miR-193a induced apoptosis in colon cancer cell lines. The experiment was done according to the manufacturer's instructions. Briefly, all the treated/control cells were washed with PBS and resuspended in binding buffer containing Annexin V and propidium iodide (PI). Fluorescence intensity was measured using flow cytometry with a BD FACS Calibur™ flow cytometer (BD Bioscience).

#### **2.14. Apoptotic morphology by DAPI staining**

All the treated and control cells were used for assessing the miR-193a directed apoptotic changes. The cells were stained with 4, 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). Cells with fragmented or condensed nuclei under the fluorescence microscope were recorded as apoptotic cells.

#### **2.15. Statistical analysis**

Statistical analysis was performed using the Statistical Package for Social Sciences for Windows (version 24.0, IBM SPSS Inc., New York, NY, USA). Chi-square test or likelihood ratio was used for categorical variables. Pearson correlation test was used for continuous variables. Independent t-test and ANOVA was performed for the analysis of continuous variables in categories. Kaplan-Meier survival analysis was used to study the impact of factors affecting survival of patients with colorectal carcinoma. A significance level of the tests was taken at  $p < 0.05$ .

### 3. Results

#### 3.1 Downregulation of miR-193a-3P and its clinicopathological significance

The miR-193a-3p expression was predominantly downregulated in the tissues from colorectal adenocarcinoma. Compared to matched non-neoplastic mucosa, approximately 70% (49/70) of the colorectal carcinomas showed reduced expression levels of miR-193a-3p. Upregulation/overexpression was noted in only 30% (21/70) of the colorectal adenocarcinomas. Down regulation of miR-193a-3p was also noted in the colon cancer cells (SW480 and SW48) when compared to the non-neoplastic colon epithelial (FHC) cells (Figure 1A).

During the analysis, miR-193a-3p expression was noted to be downregulated in the majority of patients with colorectal adenocarcinoma of early stages (I/II). From the 41 (58.6%) patients with colorectal adenocarcinoma of stages 1 or 2, 33 (80.5%) showed downregulation of miR-193a-3p whereas from 29 (41.4%) of patients with colorectal adenocarcinoma of stage 3 or 4, only 16 (55.2%) showed downregulation of miR-193a-3p ( $p < 0.05$ ). In addition, correlation between downregulation of miR-193a-3p and the presence of multiple polyps ( $>10$ ) in the colorectum was noted in these patients ( $p = 0.034$ ). Furthermore, there is a near significant value between downregulation of miR-193a-3p and perforation of colorectal cancer ( $p = 0.05$ ). Apart from the above results, there is no significant correlation between miR-193a-3p expression and other clinicopathological parameters (Table 1). Survival analysis is performed on the patients with stage IV colorectal cancer ( $n = 16$ ). Of these, 13 patients died due to cancer. In this group of patients, those with high levels of miR-193a-3p had better survival rate than those with low level of miR-193a-3p (33 months versus 20 months). However, this difference did not reach statistical significance ( $p > 0.05$ ) (Figure 1 B).

### **3.2 Correlation between miR-193a-3p and KRAS protein expression in colorectal cell lines and cancer tissues**

An inverse correlation was noted between miR-193a-3p and KRAS protein in colon cancer cells (Figure 1 C&D). Overexpression of miR-193a (miR-193a<sup>+</sup>) in vitro resulted in downregulation of KRAS protein expression (Figure 1E). In colorectal cancer tissue, KRAS protein showed cytoplasmic expression. Of the 70 cases, 37 showed no or low expression (score = “0” or “1”) of KRAS protein whereas 33 showed high expression (score = “2” or “3”) of KRAS protein. Overall, 55.1% of the miR-193a-3p downregulated colorectal cancer showed overexpression of KRAS protein (score = “2” or “3”) and 71.4% of the miR-193a-3p upregulated colorectal cancer showed downregulation of KRAS protein (score = “0” or “1”). These data showed a significant inverse correlation between miR-193a-3p and its target KRAS in the cancer tissues (Figure 2). On the other hand, there is no significant correlation between KRAS protein expression and the clinical pathological parameters.

### **3.3. miR-193a-3p reduces the proliferation, migration and colony formation**

Overexpression of miR-193a (miR-193a<sup>+</sup>) in colon cancer cells was achieved after stable transfection (Figure 1A). The cell proliferation was significantly reduced in miR-193a<sup>+</sup> transfected colon cancer cells when compared to the control cells after 48 and 72 hours of incubation ( $p < 0.05$ ) (Figure 3A). The cancer cell migration was significantly decreased after stable transfection with miR193a. The wound area showed a significant difference in these cells from both control and mock cells on day 2 and day 3 ( $p < 0.05$ ) (Figure 4). In addition, miR-193a<sup>+</sup> apparently inhibited the clonogenic activity of cancer cells (SW480 and SW48). There was a significant difference from both control and mock cancer cells ( $p < 0.01$ ) (Figure 3B).

### 3.4 Altered cell cycle events and apoptotic changes by miR-193a-3p

Flow cytometry analysis of miR-193a<sup>+</sup> colon cancer cells showed a reduction of cells in the G<sub>0</sub>/G<sub>1</sub> phase when compared to both the control and mock cells. In addition, a significant increase of cells in the G<sub>2</sub>-M phase has been noted in the miR-193a<sup>+</sup> colon cancer cells when compared to the control cells (Figure 5). The majority of the miR-193a<sup>+</sup> colon cancer cells displayed apoptotic cell phenotypes by showing either shrinkage or fragmentation of the nucleus or/and disturbance of cell membrane (late stage apoptotic changes) under a fluorescence microscope. The control and mock cells showed less or no changes in its nuclear or cell membrane morphology (Figure 6 B). Flow cytometry using Annexin-V staining confirmed these findings by demonstrating a significant shift of miR-193a<sup>+</sup> cells to both late and early apoptosis when compared to the control and mock cells ( $p < 0.05$ ) (Figure 6A).

### 3.5. miR-193a-3p induced changes in colon cancer stemness

Immuno-markers specific to stemness and epithelial mesenchymal transition (EMT) were tested to examine the regulatory roles of miR-193a-3p in colon cancer stemness. Both stem cell markers (SOX-2 and OCT-4) did not show a significant change in their expression pattern following miR-193a<sup>+</sup> transfection. Among the EMT markers (SNAIL1 and TWIST), the TWIST protein showed a significant reduction in its expression in miR-193a<sup>+</sup> colon cancer cells (Figure7).

#### 4. Discussion

Changes in miR-193a expression have been reported in colon cancer. Iliopoulos *et al.* reported miR-193a as a tumour suppressor gene in both colorectal cancer tissues and colorectal cancer cell lines [12]. Similar to the findings of Iliopoulos *et al.*, this study noted tumour suppressor properties in colon cancer cells and patients' derived tissue samples. However, Yong and colleagues noted that miR-193a-3P was upregulated in both tissue and blood samples of colorectal carcinoma [11].

Tumour suppressor roles and oncogenic roles of miR-193a were reported in different types of cancer and the change in pathological behavioural of this miRNA was also reported in different cancer cell lines. For example, significant upregulation of miR-193a-3p was noted in H-bc (bladder cancer cell line) and SMMC-7721 (hepatocellular carcinoma cell line) while significant downregulation was reported in 5637 (bladder cancer cell line) and QGY-7703 (hepatocellular carcinoma cell line) [18, 19]. The difference in the methylation status of this miRNA was reported to have this significant effect on its biological behaviour in these cancers [18, 19]. It is also worth noting that, approximately 30% (21/70) of the patients with colorectal carcinomas in this study showed miR-193a overexpression. These findings imply that miR-193a expression and its deregulation will vary in different populations of the same cancer subtype.

In this study, there was a correlation between miR-193a-3p downregulation and patients with colorectal carcinomas of early stages (stage I or stage II). In addition, at the cellular levels, cells from a stage II cancer (SW480) exhibited a more reduction in miR-193a-3p expression when compared to cells from a stage III cancer (SW48). These findings were in agreement with those of Williams *et al.* who reported miR-193a-3p downregulation in the early phase of pathogenesis of mesothelioma [20]. On the other hand, Zhang *et al.* reported a significant correlation between miR-193a-5p downregulation and presence of lymph node

metastases in patients with colorectal carcinomas [21]. Furthermore, downregulation of miR-193a-3p was noted in non-small cell lung carcinoma of advanced stages [22]. The difference in these findings may attribute to the difference in the cancer subtypes, sample size and the modulation of different molecular pathways that miR-193a interacts with.

This research showed a correlations between the downregulation of miR-193a-3p and the presence of multiple polyps associated with the colorectal cancer. Overall, there were six patients with colorectal cancer having multiple polyps. None of them had familial adenomatous polyposis (FAP) or microsatellite instabilities. The finding implied that miR-193a inhibition promotes the presence of multiple colorectal epithelial lesions independent from above known genetic pathways. Further investigations are valid to confirm the role of and miR-193a in this setting.

Perforation of colorectal cancer could be a life threatening complication with serious consequence [23]. In our study, all the cases with cancer perforation occur in low expression of miR-193a-3p patients ( $p=0.05$ ). The association between miRNAs expression and perforation of colorectal cancer have been reported previously [24]. Thus, low miR-193a-3p expression could predict the risk of perforation in colorectal cancer.

*UPA*, *MCL-1*, *JNK1* and *ERBB4* have been suggested as the downstream targets of miR-193a in different cancers [25-28]. In colorectal carcinoma, KRAS protein has been reported as a downstream target of miR-193a [12]. Iliopoulos *et al.* demonstrated an inverse correlation between miR-193a and KRAS mRNA in colorectal carcinoma [12]. In this study, an inverse correlation was noted between miR-193a-3p and KRAS protein expression in both tissue samples and cell lines.

*KRAS* gene plays a crucial role in colorectal cancer carcinogenesis [29]. Mutation of *KRAS* is responsible for the refractory of anti-epidermal growth factor receptor treatments which are widely used in the treatment of metastatic colorectal carcinoma [30]. Moreover,

*KRAS* exerts its oncogenic effects in colorectal cancer by targeting two major signalling pathways, RAF-MEK-ERK and P13K-AKT pathways [31]. Thus, miR-193a downregulation and its association with colorectal carcinomas of early stages could be attributed to its activation of highly oncogenic *KRAS* protein for the initiation and progression of colorectal carcinomas, especially in its early pathological stages. These findings have potential therapeutic use to target *KRAS* associated pathogenesis in colorectal carcinomas.

The present study has confirmed the growth inhibitory properties of colon cancer cells *in-vitro* following the overexpression of miR-193a-3p indicating that miR-193a-3p is a tumour suppressor. Current literature has very limited evidence on the cellular roles of miR-193a-3p in the colon [12, 32]. The miR-193a induced inhibition of cell proliferation, colony formation and cell migration in this study are in consensus with previous. This also indicates the growth inhibitory function of miR-193a in colon cells by regulating *KRAS* and by other growth associated molecular pathways.

MicroRNA could affect the pathogenesis of colorectal cancer by regulating the cell cycle phases [3, 4]. Recently, Khoo *et al.* reported that miR-193a could stop the cell cycle of endothelial colony forming cells [33]. In addition, Teng *et al.* stated that miR-193a-3p could arrest the G1 phase of colon cancer cell cycle in a mouse [34]. In our study, the arrest of cells upon their passing from the S phase to the G2/M phase and the accumulation of these cells in G2/M phase following overexpression of miR-193a could make the colon cells vulnerable to the apoptotic changes. This in turn leads to the inhibition of their cell growth and migration properties. These results are in concur with the findings of Lam *et al* in 2010 who demonstrated that miR-193a could sensitise the colon cancer cells (HCT-116) to BCL2 family inhibitor (navitoclax) by 50% via enhancing apoptosis [32]. This study also reported that miR-193a could increase the apoptosis in colon cancer cells treated with navitoclax via targeting *MCL-1* gene (myeloid cell leukaemia 1) [32]. The findings of our study imply that

miR-193a could induce cell apoptosis in colon cancer cells without sensitized by BCL2 inhibitors. Such difference in apoptotic properties of miR-193a could be due to the differences in pathological stages and genetic composition of cell lines. Importantly, these findings support our hypothesis that miR-193a plays a role in the pathogenesis of colorectal carcinomas of early stages.

The roles of miR-193a in the cancer stem cells have been reported in colon cancers [12, 35]. The current study has shown no significant change in the expression of stem cell markers (SOX-2 and OCT-4) following stable transfection of miR-193a in colon cancer cells. Future study is needed to explore the role of miR-193a in stem cell regulation, as other stem cell markers could be targeted directly or indirectly by this miRNA. For example, beside its role in the regulation of apoptosis, MCL1 (a direct target of miR-193a) was noted to play important roles in self-renewal of cancer stem cells [36].

Epithelial-mesenchymal transition (EMT) is a process by which epithelial cells lose its polarity and cell to cell adhesion leading to increase cell invasion, metastasis and chemotherapeutic resistance in cancer cells. This process also plays a key role in the maintenance and differentiation of cancer stem cells [37]. SNAIL1 and TWIST are two prominent EMT markers having a strong association with colorectal cancer metastasis and poor prognosis of patients with the cancer [38]. In addition, TWIST protein can directly regulate cancer stem cells by increasing the expression of CD44, a key stem cell regulator [39]. The current study has noted significant downregulation of TWIST protein following miR-193a transfection in cancer cells in both cell lines, suggesting its potential roles in EMT and cancer stemness. It is worth noting that, *RAS* family genes could have essential roles in the regulating of EMT [40]. Furthermore, recent studies have confirmed a synergic association between miR-193a and EMT in cancers including colon cancers [41-42]. Taken together, it can be hypothesised that miR-193a exhibits its tumour suppressor properties by

regulating the EMT process. This could be through modulating the expressions of its downstream targets such as KRAS, TWIST and other molecular signalling pathways.

To conclude, this study has confirmed the downregulation and tumour suppressor properties of miR-193a in colon cancer tissues and cell lines by regulating various cellular processes such as EMT. The miR-193<sup>+</sup> colon cancer cells showed a significant reduction in cell proliferation, colony formation, migration. Furthermore, miR-193a could arrest cell cycle progression and induction of apoptosis via targeting KRAS and EMT process.

### **Figure legends**

**Figure 1: Expression pattern miR-193a-3p in colorectal cancer cell lines, correlation to KRAS protein expression and survival analysis.** Downregulation of miR-193a-3p was noted in SW480 and SW48 colon cancer cells when compared to the non-neoplastic colon cells (FHC). There is more downregulation of the miRNA noted in SW480. Significant upregulation of miR-193a-3p was obtained in SW480 and SW48 transfected with pCMV/miR 193a-3p plasmid vector (fold change compared to FHC cells). These data were obtained by qRT-PCR (A). Patients with Stage IV colorectal carcinoma having low expression levels of miR-193a-3p had reduced survival when compared to patients with high miR-193a-3p expression levels (B). KRAS protein shows upregulation in cancer cells in SW480 and SW48 when compared to non-neoplastic cells (FHC) as detected by Western blot before transfection of these cells with pCMV/miR 193a plasmid vector (C). There is an inverse correlation between miR-193a-3p expression and its target protein KRAS in colon cancer cell lines (D). KRAS is significantly down regulated in SW480 and SW48 cells transfected with miR-193a-3p when compared to both control and mock cells (E).

**Figure 2: Correlation of miR-193a expression to its target KRAS protein by the immunohistochemical study**

The top figure shows an inverse correlation between miR-193a and KRAS protein expression in colorectal carcinoma. The bottom figure shows strong cytoplasmic expression of KRAS protein in colorectal carcinoma (x 25)

**Figure 3: Proliferation assay and colony formation of colon cancer cells.** The figure shows decrease proliferation of both SW48 and SW480 cells transfected with pCMV/miR 193a plasmid vector when compared to mock and control cells after 48 hours of the transfection ( $p < 0.05$ ) (A). SW480 and SW48 cells transfected with miR-193a-3p have less ability to form colonies when compared to non-transfected cells and cells transfected with empty vector ( $P < 0.01$ ) (B)

**Figure 4: The effect of miR-193a-3p on colon cancer cell migration was determined by wound healing assay in SW480 and SW48 cell lines.** The picture shows the migration of transfected and non-transfected cells towards the wound area. The cells transfected with miR-193a have less ability to migrate when compared to control and mock cells in both cell lines (A). The level of cells migration was detected by measuring the wound area after seeding the cells for 4 consecutive days. The results show that miR-193a-3p inhibits the cell migration in both cell lines (B).

**Figure 5: miR-193a-3p induces changes in cell cycle events in colon cancer cell cycle.** Flow cytometry analysis reveals decreased accumulation of SW480 and SW48 cells transfected with miR-193a-3p in the G0-G1 phase and increased accumulation in the G2-M phase when compared to control and non-transfected cells (A & B respectively).

**Figure 6: miR-193a-3p induces apoptosis in colon cancer cells:** SW480 and SW48 cells transfected with miR-193a-3p and stained by DAPI staining show apoptotic changes when compared to non-transfected cells and cells transfected with empty vector. Apoptotic changes include both pyknotic nuclei (head arrow) and fragmented nuclei (arrow) (B). Annexin-V staining shows that SW480 and SW48 cells transfected with miR-193a-3p have significant apoptotic changes when compared to both mock and control cells ( $p < 0.05$ ).

**Figure 7: The effect of miR-193a-3p on stem cell and epithelial mesenchymal transition factors (Western blot analysis).** miR-193a-3p significantly down regulates TWIST in cancer cells (SW480 and SW48) and does not affect SOX-2, OCT-4 and SNAIL1 expression (A&B). GAPDH was used as a control.

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**Table 1:** The correlations of miR-193a expression level with different clinical and pathological parameters in patients having colorectal adenocarcinoma.

| Parameters                      | Total no. | High expression | Low expression | p-value |
|---------------------------------|-----------|-----------------|----------------|---------|
| <b>Age</b>                      |           |                 |                |         |
| ≤60                             | 14(20.0%) | 4(28.6%)        | 10(71.4%)      | 0.89    |
| >60                             | 56(80.0%) | 17(30.4%)       | 39(69.6%)      |         |
| <b>Gender</b>                   |           |                 |                |         |
| Male                            | 35(50.0%) | 11(31.5%)       | 24(68.6%)      | 0.79    |
| Female                          | 35(50.0%) | 10(28.6%)       | 25(71.4%)      |         |
| <b>Size</b>                     |           |                 |                |         |
| ≤ 40mm                          | 39(55.7%) | 12(30.8%)       | 27 (69.2%)     | 0.875   |
| > 40 mm                         | 31(44.3%) | 9(29.0%)        | 22(71.0%)      |         |
| <b>Location</b>                 |           |                 |                |         |
| Colon                           | 55(78.6%) | 19(34.5%)       | 36 (66.7%)     | 0.092   |
| Rectum                          | 15(21.4%) | 2(12.5%)        | 13 (81.3%)     |         |
| <b>Perforation</b>              |           |                 |                |         |
| Yes                             | 5(7.1%)   | 0(0.0%)         | 5(100.0%)      | 0.05*   |
| No                              | 65(92.9%) | 21(32.3%)       | 44(67.7%)      |         |
| <b>Grade</b>                    |           |                 |                |         |
| I                               | 7(10.0%)  | 2(28.6%)        | 5(71.4%)       | 0.251   |
| II                              | 55(78.6%) | 18(32.7%)       | 37(67.3%)      |         |
| III                             | 8(11.4%)  | 1(12.5%)        | 7(87.5%)       |         |
| <b>LVI</b>                      |           |                 |                |         |
| Yes                             | 15(21.4%) | 7(46.7%)        | 8(53.3%)       | 0.11    |
| No                              | 55(78.6%) | 14(25.5%)       | 41(74.5%)      |         |
| <b>Peri-neural infiltration</b> |           |                 |                |         |
| Yes                             | 6(8.6%)   | 3(50.0%)        | 3(50.0%)       | 0.264   |
| No                              | 64(91.4%) | 18(28.1%)       | 46(71.9%)      |         |
| <b>MSI status</b>               |           |                 |                |         |
| High                            | 12(17.1%) | 4(33.3%)        | 8(66.7%)       | 0.782   |
| Stable                          | 58(82.9%) | 17(29.3%)       | 41(70.7%)      |         |
| <b>Multiple polyps</b>          |           |                 |                |         |
| Presence                        | 6(8.6%)   | 0(0.0%)         | 6(100.0%)      | 0.034*  |
| Not detected                    | 64(91.4%) | 21(32.9%)       | 43(67.2%)      |         |
| <b>Stage</b>                    |           |                 |                |         |
| I/II                            | 41(58.6%) | 8(19.5%)        | 33(80.5%)      | 0.023*  |
| III/IV                          | 29(41.4%) | 13(44.8%)       | 16(55.2%)      |         |
| <b>Recurrence</b>               |           |                 |                |         |
| Yes                             | 25(35.7%) | 9(36.0%)        | 16 (64.0%)     | 0.432   |
| No                              | 45(64.3%) | 12(26.7%)       | 33 (73.3%)     |         |

\*: significant p value; MSI: microsatellite instability; Multiple polyps: >10 polyps associated with the colorectal cancer; LVI: lymphovascular permeation by carcinoma

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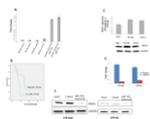


Figure 1

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Figure 2

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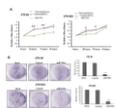


Figure 3

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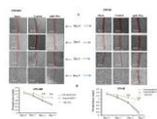


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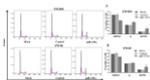


Figure 5

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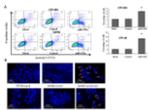


Figure 6

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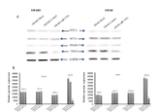


Figure 7

ACCEPTED MANUSCRIPT

- 1- miR-193a-3p plays a key role in regulating KRAS protein expression.
- 2- miR-193a-3p is downregulated in most cases of the colorectal cancer tissues.
- 3- miR-193a overexpression inhibits colorectal cancer via targeting KRAS and EMT.
- 4- miR-193a downregulation correlated with early stages colorectal cancer.