MiR-142-5p act as an oncogenic microRNA in colorectal cancer: clinicopathological and functional insights

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Abstract

Objectives: *miR-142-5p* was noted aberrantly expressed and plays important roles in different pathophysiological conditions in human. The present study aims to examine the expression of *miR-142-5p* and its association with clinicopathological factors in a large cohort of patients with colorectal cancer. In addition, the cellular effects of *miR-142-5p* and its interacting targets in colon cancer cells were investigated.

Methods: Expression of *miR-142-5p* in colorectal cancer tissues (n=125) and colon cancer cell lines were analysed using real-time polymerase chain reaction. *In vitro* assays (cell proliferation, wound healing and colony formation) were used to study the *miR-142-5p* induced cellular effects. Western blots were used to examine the modulation of FAM134B, KRAS, EPAS1 and KLF6 proteins expression followed by *miR-142-5p* expression-manipulation.

Results: Significant high expression of *miR-142-5p* was noted in cancer tissues and cells when compared to the controls ($p<0.001$). Overexpression of *miR-142-5p* in patients with colorectal cancer was common (72%; 90/125). *miR-142-5p* overexpression was associated with cancer in the proximal colorectum and with *B-raf* positive patients ($p=0.05$). Exogenous overexpression of *miR-142-5p* resulted in significantly increased cell proliferation, colony formation, and wound healing capacities, whereas inhibition of endogenous *miR-142-5p* led reduced cancer growth properties. The cellular effects of *miR-142-5p* were mediated by the modulation of tumour suppressor KLF6 expression, as the expression of *miR-142-5p* and KLF6 protein are inversely correlated in colon cancer cells.

Conclusion: High *miR-142-5p* expression was associated with the biological aggressiveness of cancer. Thus, suppression of *miR-142-5p* could be a therapeutic strategy for patients with colorectal cancers.

Keywords: Colorectal cancer, *miR-142-5p,* KLF6, targeted therapy, onco-miRNA.

1. Introduction

The *microRNA-142* (*miR-142*) hairpin, located at 17q22, give rise to the guide-strand (*miR-142-3p*) and the sister passenger-strand (*miR-142-5p*). They play a key role in many physiological processes including embryogenesis, haematopoiesis and immune responses [Shrestha et al., 2017]. Recent studies reported that *miR-142-5p* aberrantly expressed in different pathological conditions including cancers, inflammation, immune disorders, renal fibrosis, adaptive hypertrophy, cerebral ischemic/reperfusion injury [Zhang et al., 2011; Zarjou et al., 2011; Ben-Dov et al., 2012; Ding et al., 2012; Danger et al., 2013]. Also, *miR-142* differentially expressed in mouse embryonic stem cells [Sladitschek and Neveu, 2015]. Deletion of *miR-142* in mouse embryonic stem cells leads to negative expression of stem cell marker Oct4 that converts the undifferentiated stem cells to differentiated cells. On the other hand, overexpression of *miR-142* prevented the differentiation of stem cells [Sladitschek and Neveu, 2015]. *miR-142* is critical for the formation of different lineages of haematopoietic stem/progenitor cells and loss of *miR-142* caused abnormal haematopoiesis (*e. g.* hypoglobulinemia and megakaryocyte) and leads serious immunodeficiency in mice [Lu et al., 2013; Chapnik et al., 2014; Kramer et al., 2015]. In addition, *miR-142-5p* regulates neuronal cells survival, auto-immune diseases and bone repair [Zhu et al., 2016; Tu et al., 2017; Wang et al., 2017].

The expression and cellular implications of *miR-142-5p* in different types of cancers is paradoxical [Shrestha et al., 2017]. In cancers such as renal cell carcinoma, retinoblastoma, and gastric carcinomas, *miR-142-5p* exhibits oncogenic properties [Zhang et al., 2011; Liu et al., 2017; Jia et al., 2017]. In contrast, in pancreatic, lung, breast, liver (hepatocellular carcinoma) carcinomas, it behave as a tumour suppressor miRNA [Venkatesan et al., 2015; Ma et al., 2016;; Lou et al., 2017]. In colorectal carcinomas, growth promoting or oncogenic features of *miR-142-5p* been reported predominantly in recent

literatures [Ma et al., 2016; Vychytilova-Faltejskova et al., 2016; Yin et al., 2016; Liu et al., 2016]. A recent study by Shi et al. (2017) reported reduced expression in tumour tissue samples and *in vitro* cancer inhibitory properties (*e. g.* induction of apoptosis of cancer cell) of *miR-142-5p* in colorectal carcinomas [Shi et al., 2017].

MiR-142-5p gene located at the breakpoint junction of17q22- t(8;17) translocation, which is the cancer-associated genomic region or fragile site linked to aggressive B cell leukaemia [Calin et al., 2004]. This translocation brings the *miR-142-5p* under the promoter of highly expressed immunoglobulin heavy chain gene, consequently aberrant expression of *miR-142-5p* was noted in different cancers [Calin et al., 2004; Zhang et al., 2011; Venkatesan et al., 2015; Ma et al., 2016; Liu et al., 2017; Jia et al., 2017; Lou et al., 2017].

The underlying mechanism and association of *miR-142-5p* expression with clinical and pathological factors of patients with colorectal cancer have never been studied in depth. In addition, the survival of patients with colorectal cancer of *miR-142-5p* expression has never been reported. Therefore, the present study aims to examine the expression of *miR-142-5p* and its correlation with clinicopathological factors in a large cohort of patients with colorectal cancer. Furthermore, the effects of *miR-142-5p* on cellular behaviours and interacting targets of *miR-142-5p* in colon cancer cells were investigated in this study.

2. Materials and Methods

2.1 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. Ethical approval for this work has been obtained from the Griffith University Human Research Ethics Committee (GU Ref No: MSC/17/10/HREC).

2.2 Clinicopathological parameters

The size (maximum dimension) and site of the cancers 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. The pathological features of patients with colorectal adenocarcinoma were analysed by examining the histological sections and were reviewed and graded by a pathologist (AKL). The adenocarcinoma is graded into grade 1 (well differentiated), grade 2 (moderately-differentiated) and grade 3 (poorly-differentiated) accoring to the WHO classification (Hamilton, et al, 2010). The cancer tissues were investigated for microsatellite instability (MLH1, PMS2, MSH2 and MSH6 by immunohistochemistry) according to the clinical guidelines. Braf muation was performed in patients with high microsatellite instability (Pakneshan et al., 2013; Smith et al., 2011). The

carcinoma was staged according to the 8th edtion of Cancer staging Manual of AJCC (American Joint Committee on Cancer) (Milburn Jessup et al, 2017) based on T, N and M staging. The pathological stages were obtained in multidisciplinary team meeting of the pathologist and the surgeon (AKL & CTL).

After reviewing, 125 patients (60 women; 65 men) with colorectal adenocarcinomas were included in the current study. The mean age of the patients used in this study was 66 years (range, 24-91). Forty four per cent (n = 55) of cancer was located in proximal colon while 56 % (n = 70) was located in the distal colorectum. Also, 23 % (n =29) of the patients had distant metastasis, whereas 77 % (n = 96) of patients had no distant metastasis. Overall, there were 9.6 % (n = 12) stage I, 41.6 % (n = 52) stage II, 24.8 % (n = 31) stage III and 24 % (n = 30) stage IV carcinomas.

2.3 Clinical Management

Clinical management was performed by a pre-agreed standardised multidisciplinary protocol. The use of postoperative adjuvant therapy was based on the pathological stages of cancer and the clinical status of the patients. 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.

2.4 Cell Culture

Colon cancer cell lines (SW480, SW48 and HCT116) and non-neoplastic colon epithelial cell line (FHC) were used in this study. SW480 (Duke's Type B; stage II) and

SW48 (Duke's Type C; stage III) cell lines were used in the expression-manipulation experiments as the representative cell model for colon cancer. All of these cells were purchased from ATCC (American type culture collection) and maintained as previously described [Islam et al., 2017a].

2.5 Extraction of RNA, miRNA and cDNA conversion

A cryostat (Leica Biosystems, Mt Waverley, VIC, Australia) was used to section tissues obtained from patients with colorectal carcinomas 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 [Islam et al., 2017b].

2.6 Quantitative Real-Time Polymerase Reaction (qRT-PCR)

The expression changes of *miR-142-5p* in colorectal cancer tissues and cell lines were examined using a QuantStudio ™6 Flex Real-time PCR system (Applied Biosystems, Foster city, CA, USA) as previously described [Gopalan et al., 2016]. Amplification efficiencies of *miR-142-5p* were normalised to that of multiple internal control genes including RNU6b, RNU44 and RNU48. RNU6b was selected on the basis of consistent results as internal control. Results were presented as relative expression ratio (expression of *miR-142-5p* normalised by internal control RNU6b expression). Fold changes were calculated according to previously published protocol [Gopalan et al., 2016]. A fold change of more than 2 was considered as high *miR-142-5p* expression and a fold change of 2 or less was considered as a low *miR-142-5p* expression.

2.7 Transfection of colon cancer cells with *miR-142-5p* mimic and anti-*miR-142-5p*

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-142-5p* mimic (Qiagen, Hilden, NRW, Germany) ($SW480^{+miR\text{-}142\text{-}5p}$ and $SW48^{+miR\text{-}142\text{-}5p}$) at 10 nM concentration and with anti-*miR-142-5p* (Qiagen) ($SW480^{+anti\text{-}miR\text{-}142\text{-}5p}$ and $SW48^{+anti\text{-}miR\text{-}142\text{-}5p}$) at 10 nM concentration according to the protocol published earlier [Islam et al., 2017c].

2.8 Cell proliferation assay

To examine the effect of *miR-142-5p* on the proliferation of colon cancer cell lines, cell proliferation assay using cell counting kit-8 (CCK-8) (Sigma-Aldrich, Sydney, VIC, Australia) was used. Cells (SW480 and SW48) 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-142-5p* mimic, anti-*miR-142-5p* and scramble miRNA as previously described [Islam et al., 2017d].

2.9 Colony formation assay

To determine the effect of *miR-142-5p* manipulation on clonogenic capacity, equal numbers of cells were seeded in 6-well plates with complete medium. Cells (SW480 and SW48) were then transfected with *miR-142-5p* mimic, anti-*miR-142-5p* 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 and the clone formation rates and surviving fractions were calculated according to the published protocol [Islam et al., 2017a,].

2.10 Would healing assay

Scratch assay was performed to determine the effect of *miR-142-5p* on the capacity of cells to migrate for repairing the scratch according to the previously published protocol [Islam et al., 2017a]. Wound areas on different days of all cell types were measured and compared with Image J 1.48 software.

2.11 Cell cycle analysis

Cells treated with *miR-142-5p* mimic, anti-*miR-142-5p* and scramble control miRNA were fixed with cold 70% ethanol for one hour as previously described [Islam et al., 2017a]. After washing with cold phophate buffered saline (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, Franklin Lakes, NJ, USA).

2.12 Western blot analysis

Total proteins extraction, SDS_PAGE separation and transferred to nitrocellulose membranes were performed as previously described [Islam et al., 2017a, b]. Then, the membrane was incubated with the anti-rabbit family with sequence similarity 134, member B (FAM134B) polyclonal antibody (Santa Cruz, Starr County, TX, USA) (at 1:200), anti-mouse monoclonal Kirsten rat sarcoma (KRAS) and Kruppel-like factor 6 (KLF6) antibodies (Santa Cruz) (1: 500), anti-rabbit polyclonal Endothelial PAS domain-containing protein 1 (EPAS1) (Sigma) (at 1:500) and monoclonal β-actin 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, KRAS, KLF6 and β-actin protein bands were developed and detected with a chemiluminescence HRP detection kit (Bio-Rad,

Hercules, CA, USA). Images were taken with the ChemiDoc MP Imaging system (Bio-Rad). Expression of proteins was quantified and normalised to β-actin with Image J 1.48 software.

2.13 Statistical analysis

All clinical, pathological, follow-up 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, NY, USA). Chi-square test 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 $p < 0.05$.

3. Results

3.1 Overexpression of *miR-142-5p* in colorectal cancer

Expression of *miR-142-5p* in cancer tissue samples was noted significantly higher in comparison to that of the non-cancer samples (1.1518±0.203 versus 0.819±0.068) (Figure 1A). Overexpression of *miR-142-5p* was also noted in SW480, SW48 and HCT116 colon cancer cells in comparison to that of non-neoplastic colonic epithelial (FHC) cells (Figure 1B). Among the colorectal cancers, 72% (90/125) had shown high expression of *miR-142-5p* whereas 28% (35/125) exhibited low expression (Table 1).

Overexpression of *miR-142-5p* was predominantly noted in patients with cancers located in the proximal colon when compared to that of those in the distal colorectum (80 % versus 65.7%, p=0.05) (Table 1). In addition, higher expression of *miR-142-5p* was noted in patients with colorectal cancer recurrence when compared to those without any recurrence of cancer (79.6% versus 67.1%) (Table 1). In addition, significant (p=0.05) higher expression of *miR-142-5p* was noted in *B-raf* mutated cancer samples when compared to that of non *B-raf* mutated cancer samples (Table 1).

The overall median follow-up of patients with colorectal cancer was 50 months and a significant correlation was noted between poor patient survival and advanced pathological stages of colorectal carcinomas (p=0.0001) (Figure 2A). With *miR-142-5p*, patients with high *miR-142-5p* levels in their colorectal cancer tissues had a shorter survival time when compared to those with low *miR-142-5p* expression (97 months versus 127 months) (p=0.09) (Figure 2B). In addition, patients with higher level of *miR-142-5p* showed lower survival free time (103 months) whereas patients with lower *miR-142-5p* expression had comparatively higher disease free survival time (125 months) (Figure 2C). However, these differences did not reach to statistical significance despite showing a good schematic difference (p=0.14).

3.2 *miR-142-5p* stimulate colon cancer growth *in vitro*

Cancer cells treated with *miR-142-5p* mimic ($SW480^{+miR\text{-}142\text{-}5p}$ and $SW48^{+miR\text{-}142\text{-}5p}$) showed increased cell proliferation when compared to scramble control ($SW480^{miRScr}$ and $SW48^{miRScr}$) and non-transfected cells ($SW480^{wildtype}$ and $SW48^{wildtype}$). In $SW480^{+miR\text{-}142\text{-}5p}$ cells, 24, 18 and 13 % increase of cell proliferation was observed on day 1, day 2 and day 3, respectively, in comparison to that of $SW480^{miRScr}$ cells (Figure 3A). Whereas 52, 26 and 20 % enhancement of cell proliferation was noted on day 1, day 2 and day 3, respectively, in $SW48^{+miR\text{-}142\text{-}5p}$ when compared to that of $SW48^{miRScr}$ cells (Figure 3B). On the other hand, treatment of cancer cells with anti-*miR-142-5p* ($SW480^{+anti\text{-}miR\text{-}142\text{-}5p}$ and $SW48^{+anti\text{-}miR\text{-}142\text{-}5p}$) significantly inhibited cells proliferations in comparison to that of $SW480^{miRScr}$ and $SW48^{miRScr}$ and $SW480^{wildtype}$ and $SW48^{wildtype}$ cells. $SW480^{+anti\text{-}miR\text{-}142\text{-}5p}$ cells had shown 34, 30 and 29 % reduction of cells proliferation on day 1, day 2 and day 3, respectively. Similarly, $SW48^{+anti\text{-}miR\text{-}142\text{-}5p}$ cells exhibited 22, 20 and 18 % reduction of cells growth on day 1, day 2 and day 3, respectively, in comparison to that of control cells (Figure 3A-3B). Figure 3C and 3D presents the normalised expression ratio of *miR-142-5p* in different cell types used in this study at various time intervals followed by expression-manipulation treatments.

$SW480^{+miR\text{-}142\text{-}5p}$ cells showed higher migration potential when compared to that of $SW480^{miRScr}$ and $SW480^{wildtype}$ cells as they healed the created scratch in advance (Figure 4A & 4B). Compard to control cells, $SW480^{+miR\text{-}142\text{-}5p}$ cells healed the wound completely on 3rd day of initial scratch. On the other hand, opposite findings were observed in anti-*miR-142-5p* treated cells ($SW480^{+anti\text{-}miR\text{-}142\text{-}5p}$). Anti-*miR-142-5p* reduced the migratory capacity of cells; thereby the wound remained unhealed until day 3 (Figure 4A & 4B). Similar to SW480 cells, the wounds in $SW48^{+miR\text{-}142\text{-}5p}$ cells were healed completely on day 3 whereas in $SW48^{+anti\text{-}miR\text{-}142\text{-}5p}$ cells the scratch remained unhealed until day 3 (Figure 4C & 4D).

SW480$^{+miR\text{-}142\text{-}5p}$ and SW48$^{+miR\text{-}142\text{-}5p}$ cells showed significantly higher colony formation properties in comparison to control cell groups (Figure 5A & 5B). On the contrary, the cancer cells with anti-*miR-142-5p* displayed a significant reduction in the number of colonis (Figure 5A & 5B). Significant reductions in surviving fractions were also noted in SW480$^{+anti\text{-}miR\text{-}142\text{-}5p}$ (33 versus 95%) and SW48$^{+anti\text{-}miR\text{-}142\text{-}5p}$ (31 versus 91%) cells, in comparison to SW480$^{+miR\text{-}142\text{-}5p}$ and SW48$^{+miR\text{-}142\text{-}5p}$ cells, respectively (Figure 5C). On the contrary, 71 % and 65% surviving fractions were observed in SW480^{miRScr} & SW48^{miRScr} cells (Figure 5C).

3.3 *miR-142-5p* altered the cell cycle kinetics in colon cancer

Analysis of cell cycle distribution showed accumulation of cells in the S phase and reduction of the G_2/M phase of the cycle in SW480$^{+miR\text{-}142\text{-}5p}$ when compared to scramble control cells (Figure 6A). In SW48$^{+miR\text{-}142\text{-}5p}$ cells, accumulation of cells in the S phase and reduction of G_0/G_1 was observed (Figure 6B). In the case of SW48^{miRScr} cells, the percentage of S phase cells was 54.1 ± 5.4 %, while in SW480$^{+miR\text{-}142\text{-}5p}$ cells, the S phase population increased remarkably to 50.2 ± 4.7 %%. Proportion of G_0/G_1 phase in SW48$^{+miR\text{-}142\text{-}5p}$ cells was noted 25.2 ± 5.1% versus 31.5 ± 3.2 % in SW48^{miRScr} cells (Figure 6A-6B). On the other hand, treatment of cells with anti-*miR-142-5p* had shown opposite results in cell cycle distributions. These results indicated that regulation of G_0/G_1, synthesis phase and G_2/M of the cell cycle could be altered by *miR-142-5p* in colon cancer cells.

3.4 *miR-142-5p* regulate KLF6 protein expression in colon cancer cells

Bioinformatics tools miRDB (http://mirdb.org/miRDB/), miRbase (http://www.mirbase.org/index.shtml) were used to identify the interacting targets for *miR-142-5p*. The expression of KLF6, KRAS, EPAS1 and FAM134B proteins among the various

targets followed by exogenous manipulation of *miR-142-5p* expression were analysed in this study. Notable down-regulation of KLF6 expression was observed in SW480$^{+miR\text{-}142\text{-}5p}$ and SW48$^{+miR\text{-}142\text{-}5p}$ cells compared to that of scramble control cells (Figure 7). While anti-*miR-142-5p* treatment (SW480$^{+anti\text{-}miR\text{-}142\text{-}5p}$ and SW48$^{+anti\text{-}miR\text{-}142\text{-}5p}$) increased KLF6 expression significantly in both cells (Figure 7). The interaction of *miR-142-5p* with the 3′ untranslated region of *KLF6* mRNA may mediate reduced expression of KLF6. In SW480$^{+anti\text{-}miR\text{-}142\text{-}5p}$ cells, EPAS1 expression was increased while in SW480$^{+miR\text{-}142\text{-}5p}$ and SW48 cells it did not show any significant changes (Figure 7). Similarly, in SW48$^{+anti\text{-}miR\text{-}142\text{-}5p}$ cells, KRAS protein showed overexpression but in SW48$^{+miR\text{-}142\text{-}5p}$ cells, the KRAS expressions remain unchanged (Figure 7).

4. Discussion

The present study revealed that *miR-142-5p* promotes colorectal cancer cells growth by repressing tumour suppressor *Kruppel like factor 6* (*KLF6*). This study has also observed significant ($p<0.001$) overexpression of *miR-142-5p* in cancer cell lines and in a significant number of colorectal cancer tissues when compared to that of the non-neoplastic cell and tissue samples, respectively. Also, patients with colorectal cancer expressing high *miR-142-5p* had poor prognosis in comparison to those of expressing low *miR-142-5p*.

Studies reported the aberrant expression of *miR-34b-5p* in colorectal cancer cells and tissue samples [Vychytilova-Faltejskova et al., 2016; Yin et al., 2016; Liu et al., 2016; Shi et al., 2017]. For example, Vychytilova-Faltejskova and co-workers noted the significant elevated expression of *miR-142-5p* with some other miRNAs in serum samples (n=427) from patients with colorectal cancer when compared to those with healthy individuals [Vychytilova-Faltejskova et al., 2016]. Liu and colleagues demonstrated tumour promoting properties and overexpression of *miR-142-5p* in colorectal samples (n=12) and cells [Liu et al., 2016]. Yin et al. (2016) noted overexpression of *miR-142-5p* in colorectal cancer samples (n=15) in comparison to those of adenoma tissues samples (n=7), whereas Shi et al. (2017) noted downregulation of *miR-142-5p* in tissue samples (n=80) from patients with stage III colorectal cancer when compared to that of adjacent non-neoplastic tissues [Yin et al., 2016; Shi et al., 2017]. In the present study, we noted 72% patient with colorectal cancer had shown overexpression of *miR-142-5p*. Up-regulation of *miR-142-5p* associated with clinicopathological factors including sites of the cancers and *B-raf* mutation. We have found that cancers arising from proximal and distal colorectum had shown a significant difference in overexpressing *miR-142-5p* (80 % versus 65 %). As cancers from proximal and distal colorectum have different physiological and biochemical make-up, differential genes expression pattern is common in these sites [Islam et al., 2017c]. The differential expression

of *miR-142-5p* in the proximal and distal sites of the colorectum implied its role in colorectal cancer pathogenesis could be tissue specific and may target genes in these locations differently.

Inactivation of tumour suppressor genes *e.g.* adenomatous polyposis coli (APC), loss of 18q and mutational activation of oncogenes such as *K-ras, B-raf* are involved in the pathogenesis of colorectal carcinoma [Leslie et al., 2002]. The association with high *miR-142-5p* expression with *B-raf* mutation positive patients with colorectal cancer in comparison to those of *B-raf* mutation negative patients imply that *miR-142-5p* could either directly or indirectly involve in the carcinogenesis by modulating the expression of *B-raf* in the adenoma-carcinoma sequence of colorectal cancer pathogenesis.

miR-142-5p induced colon cancer cells growth and proliferation *in vitro*, which is consistent with the overexpression of *miR-142-5p* in cancer tissue samples in the present study. Ectopic overexpression of *miR-142-5p* ($SW480^{+miR\text{-}142\text{-}5p}$ and $SW48^{+miR\text{-}142\text{-}5p}$) cells had shown high tumour promoting features such as increased cell proliferation, high scratch healing and colony formation capacities when compared to that of controls cells (Figure 3 to Figure 5). Accumulation of cells in S phase and reduction in G_0/G1 and G_2/M phases of $SW480^{+miR\text{-}142\text{-}5p}$ and $SW48^{+miR\text{-}142\text{-}5p}$ cells further strengthen the tumorigenic potential of *miR-142-5p* in colon cancer via regulating cell cycle checkpoints. On the other hand, inhibition of *miR-142-5p* through anti-*miR-142-5p* (miR-142-5p inhibitor) treatment had exhibited opposite results by minimising the oncogenic properties of endogenous *miR-142-5p* in the present study. Significant reduction of cell proliferation, wound healing and colony formation features of $SW480^{+anti\text{-}miR\text{-}142\text{-}5p}$ and $SW48^{+anti\text{-}miR\text{-}142\text{-}5p}$ cells in comparison to that of control cells. It was reported that ectopic overexpression of *miR-142*-5p stimulates growth and proliferation of cells in pancreatic, renal, lung, blood and breast cancer by regulating different target genes [Liu et al., 2017; Ma et al., 2016; Jia et al., 2017]. In contrast, in

hepatocellular carcinoma, overexpression of *miR-142-5p* led inhibition of cancer cells growth and proliferation [Lou et al., 2017]. Thus, the modulation of cellular behaviours of cancer cells by *miR-142-5p* could be tissues and cells type specific. The oncogenic features of *miR-142-5p* noted in the present study demonstrated using both knocks in and knock down models could be directly attributed to the effects of *miR-142-5p* or by its interacting target proteins. Therefore, *miR-142-5p* may act as a tumour promoting miRNA in colon cancer pathogenesis via modulating different physiological processes in cells by regulating interacting targets.

As a single miRNA can regulate a few hundred of genes, previous studies demonstrated that *miR-142-5p* modulates growth and proliferation of various type of cancer cells by regulating different targets *e. g* SDHB, EPAS1, SMAD3, FOXO, PD-L1, BTG3 in various cancers [Nawrocki et al., 2015; Ma et al., 2016; Shi et al., 2017; Liu et al., 2017a, b; Jia et al., 2017; Lou et al., 2017]. Liu et al. (2017b) illustrated that *miR-142-5p* promote colon cancer growth by repressing SDHB, whereas Shi et al., (2017) found that *miR-142-5* inhibited colon cancer growth by inducing apoptosis via repressing the expression of EPAS1 [Shi et al., 2017]. In the present study, using bioinformatics tools miRDB (http://mirdb.org/miRDB/) and miRbase (http://www.mirbase.org/index.shtml), modulation of several predictive targets of *miR-142-5p* including FAM134B, KLF6, EPAS1 and KRAS were examined [Wong et al., 2015]. We have noted the significant reduction of KLF6 in *miR-142-5p* overexpressing ($SW480^{+miR\text{-}142}$ and $SW48^{+miR\text{-}142}$) cells when compared to that of control cells. On the other hand, inhibition of endogenous *miR-142-5p* by anti-*miR-142-5p* treatment caused significant upregulation of KLF6 expression (Figure 7). *KLF 6*, a tumour suppressor involved in cancer cells growth, proliferation, apoptosis and colony formation [Ito et al., 2004].

Expression-manipulation of *miR-142-5p* in colon cancer cells caused a substantial enhancement of a large panel of cancer type activities including higher cellular proliferation,

increased colony formation, cell migration was followed by repression of KLF6 expression. Thus, *miR-142-5p* attributed increment in cancer activity might be mediated by reduced expression of KLF6 in colon cancer cells. Also, the anti-*miR-142-5p* induced growth suppression properties in-vitro imply that regulation of *miR-142-5p* expressions in turn can regulate biological behaviours of cancer cells. Inhibitions of oncogenic miRNAs have the potential to develop miRNAs interference therapy for efficient management of cancer [Wen et al., 2015; Monroig Pdel et al., 2015; Li et al., 2013]. Therefore, overexpression of KLF6 by targeting the miRNAs such as *miR-142-5p* could be useful to develop effective therapeutics target for cancer cells. However, further research is needed to understand the precise mechanisms involved in *miR-142-5p* mediated modulation of colorectal cancer pathogenesis.

5. Conclusion

In conclusion, *miR-142-5p* is often expressed high in patients with colorectal carcinomas and exerts its oncogenic propertis *in-vitro* by targetting KLF6 protein, which plays a key role in regulating the growth properties in colon carcinogenesis. Also, the correlations of high *miR-142-5p* expression with poor patients' survival rates with colorectal cancer, *B-raf* mutation and different location of tumours signify the importance of *miR-142-5p* expressions in predicting molecular progression, tumour growth and patient prognosis in colorectal carcinoams. Thus, *miR-142-5p* related carcinogenesis would raise the possibility of anti-*miRNAs* agents as a potential therapeutic strategy for patients with colorectal cancers.

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Conflict of Interest: Authors have no conflict of interest.

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Figure legends

Figure 1. Altered *miR-142-5p* expression in colorectal cancer and cells. A) Box plot of *miR-142-5p* expression levels in colorectal cancer and matched non-neoplastic colorectal tissue samples. Paired T-test showed a significant difference in expression of *miR-142-5p* (ratio of miR-142-5p normalised by RNU6B) in cancer and matched non-neoplastic colorectal tissues (p=0.00047). **B)** Expression of *miR-142-5p* in colon cancer (SW480, SW48 and HCT116) and non-neoplastic colon epithelial cell (FHC). Significant high expression of *miR-142-5p* was noted in cancer cells when compared to those non-cancer cells. Results are shown as mean ±SD (standard deviation). Level of significance **p<0.01 and ***p<0.001 when compared with that of control FHC cell.

Figure 2. Survival distribution of patients with colorectal carcinomas. **A)** Survival rates of patients with colorectal cancer of different stages (p=0.0001). **B)** Overall survival time of patients expressing high and low *miR-142-5p*. Patients overexpressing *miR-142-5p* had poor survival time when compared to those with low levels of *miR-142-5p* expression (97 months versus 127 months) (p=0.09). **C)** Disease free survival of patients with colorectal cancer expressing high and low levels of *miR-142-5p*. Patients which had high level of *miR-142-5p* showed lower (103 months) survival rates, whereas patients with low *miR-142-5p* expression had higher (125 months) disease free survival rates (p=0.14).

Figure 3. *miR-142-5p* promoted colon cancer cell proliferation *in vitro*. Exogenous manipulation of *miR-142-5p* in SW-480 **(A)** and SW-48 **(B)** cells induce altered proliferation in comparison to control and non-transfected cells on different days after transfection. Ectopic overexpression of *miR-142-5p* increased cells proliferation in $SW480^{+miR\text{-}142\text{-}5p}$ and $SW48^{+miR\text{-}142\text{-}5p}$ whereas downregulation of *miR-142-5p* in $SW480^{+anti\text{-}miR\text{-}142\text{-}5p}$ and $SW48^{+anti\text{-}}$

$^{miR\text{-}142\text{-}5p}$ cells caused inhibition of cells proliferation significantly. Expression of *miR-142-5p* in SW480 **(C)** and SW48 **(D)** on day 1, day 2 and day 3 in different cells groups followed by transfection with *miR-142-5p* mimic and *miR-142-5p* inhibitor. Results are shown as mean ± standard deviation (SD). Level of significance *p<0.05, and **p<0.01 when compared with that of scramble control and untransfected wild type cells.

Figure 4. *miR-142-5p* stimulate wound healing of colon cancer cells. Exogenous overexpression of *miR-142-5p* in SW480$^{+miR\text{-}142\text{-}5p}$ cells showed more migration capacity, healing the wound faster than the control (SW480^{miRScr}) and non-transfected (SW480^{wildtype}) cells **(A-B)**. Anti-*miR-142-5p* 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)**. Similar results were noted in SW48$^{+miR\text{-}142\text{-}5p}$ cells **(C-D)**. Results are shown as mean ± standard deviation (SD). Level of significance *p<0.05, **p<0.01 and ***p<0.001 when compared with that of scramble control and untransfected wild type cells.

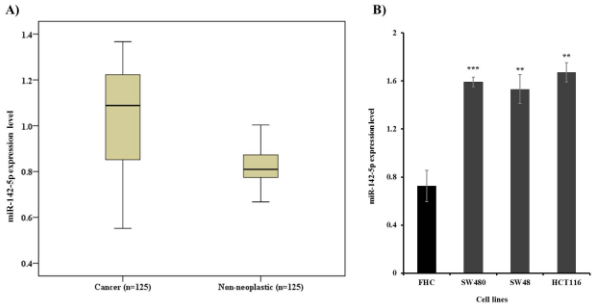
Figure 5. *miR-142-5p* increased the clonogenic potential of colon cancer cells. A) Overexpression of *miR-142-5p* increased the colony formation capacity of SW480$^{+miR\text{-}142\text{-}5p}$ and SW48$^{+miR\text{-}142\text{-}5p}$ cells. Suppression of *miR-142-5p* by anti- *miR-142-5p* treatment reduced clonogenic properties of SW480$^{+anti\text{-}miR\text{-}142\text{-}5p}$ and SW48$^{+anti\text{-}miR\text{-}142\text{-}5p}$. **B)** Bar graphs presented the number of colonies generated in different experimental groups. **C)** Surviving fraction of cells followed in different groups followed by manipulation of *miR-142-5p* expression. Anti-*miR-142-5p* significantly reduced the surviving fraction of cells when compared to the controls. Results are shown as mean ± standard deviation (SD). Level of significance

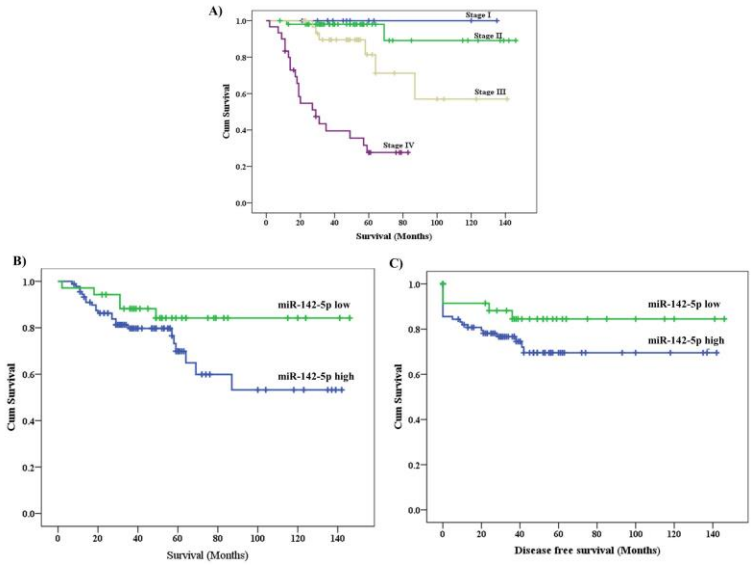
*p<0.05, **p<0.01 and ***p<0.001 when compared with that of scramble control and untransfected wild type cells.

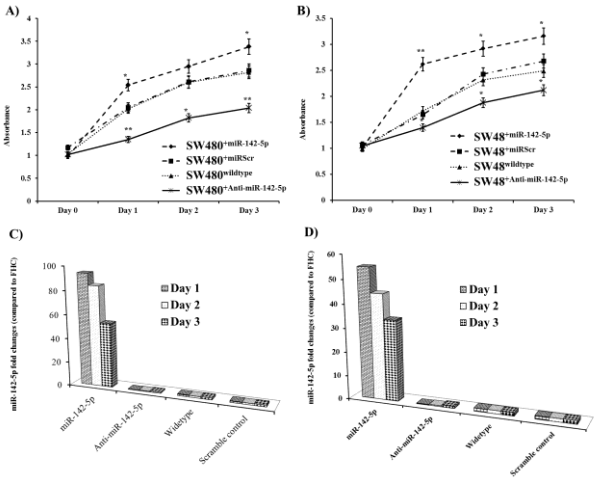
Figure 6. *miR-142-5p* altered cell cycle distribution of colon cancer cells. Flow cytometry analysis showed that *miR-142-5p* overexpression increased S phase population and a parallel reduction of G_0/G_1 and G_2/M of colon cancer cell. On the other hand, inhibition of endogenous *miR-142-5p* by the treatment with anti-*miR-142-5p* exhibited opposite results. **A)** Presented the representative histograms of cell cycle analysis of $SW480^{+miR\text{-}142\text{-}5p}$, $SW480^{+anti\text{-}miR\text{-}142\text{-}5p}$ and $SW480^{miRScr}$ cells. **B)** Representative histograms of cell cycle followed by *miR-142-5p* manipulation in SW-48 cells. Bar graphs presented the percentage of cells in different phases after *miR-142-5p* manipulation **(C-D)**. The results represented as mean ± standard deviation (SD) from three independent experiments.

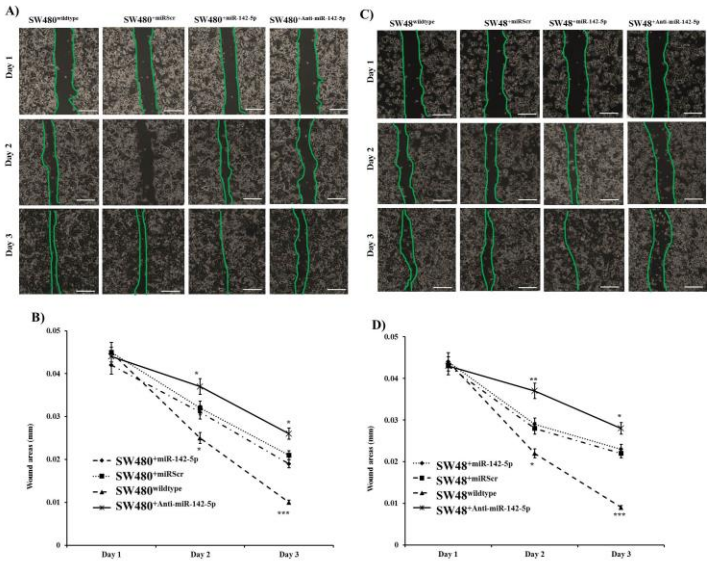
Figure 7. *miR-142-5p* modulate colon cancer cell growth by repressing tumour suppressor KLF6. KLF6 protein expression inversely correlated with the expression of *miR-142-5p* in colon cancer cell lines (SW480 and SW48). Overexpression of *miR-142-5p* caused reduces expression of KLF6 protein in SW480 **(A)** and SW48 **(B)** colon cancer cells. Inhibition of *miR-142-5p* expression by anti- *miR-142-5p* treatment minimized *miR-142-5p* mediated repression of KLF6 and increased its expression in $SW480^{+anti\text{-}miR\text{-}142\text{-}5p}$ and $SW48^{+anti\text{-}miR\text{-}142\text{-}5p}$ cells **(A)** and **(B)**, respectively. The bar graphs for relative expression of proteins in SW480 cells **(C)**. The proteins band intensity of KLF6 in $SW480^{+miR\text{-}142\text{-}5p}$ cells was noted to be decreased significantly when compared to the controls ($SW480^{miRScr}$ and $SW480^{wildtype}$) cells. Significant enhanced KLF6 protein band intensity was noted in $SW480^{+anti\text{-}miR\text{-}142\text{-}5p}$ cells **(C)**. Similar results were noted in SW48 cells **(D)**. The results are shown as mean ± standard deviation (SD) from three independent tests. Level of significance

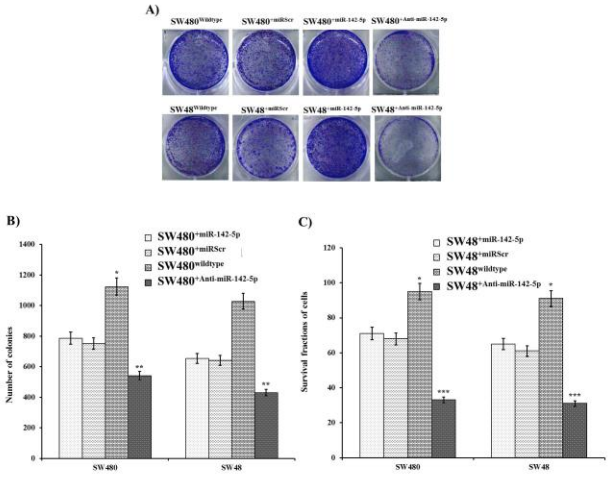
*p<0.05, and **p<0.01 when compared with that of scramble control and untransfected wild type cells.

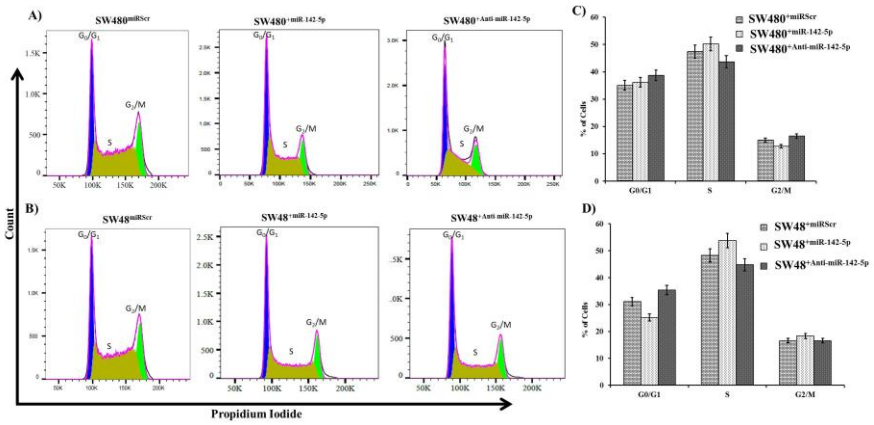












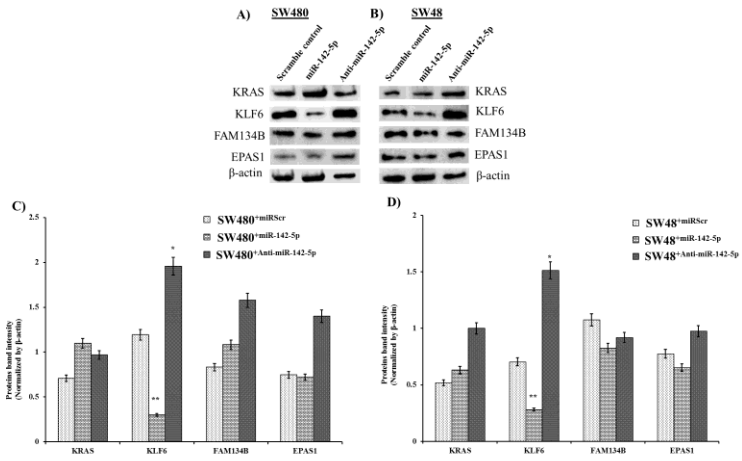


Table 1. Association of miR-142-5p expression with clinicopathological factors of patients with colorectal cancers

Features	Number	High Expression	Low Expression	p-values
Sex				
Female	60 (48%)	43 (71.7%)	17 (28.3%)	0.54
Male	65 (52%)	47 (72.3%)	18 (27.7%)	
Age				
≥60	35 (28%)	27 (77.1%)	8 (22.9%)	0.28
<61	90 (72%)	63 (70%)	27 (30%)	
Site				
Proximal	55 (44%)	44 (80%)	11 (20%)	**0.05**
Distal	70 (56%)	46 (65.7%)	24 (34.3%)	
Size (mm)				
≤40	61 (48.8%)	42 (68.9%)	19 (31.1%)	0.28
>40	64 (51.2%)	48 (75%)	16 (25%)	
Grade				
Well or moderate	107 (85.6%)	76 (71%)	31 (29%)	0.39
Poor	18 (14.4%)	14 (77.8%)	4 (22.2%)	
LVI				
Presence	35 (28%)	24 (68.6%)	11 (31.4%)	0.37
Absence	90 (72%)	66 (73.3%)	24 (26.7%)	
T-stage				
I & II	13 (10.4%)	8 (61.5%)	5 (38.5%)	0.27
III & IV	112 (89.4%)	82 (73.2%)	30 (26.8%)	
N-stage				
Absence	67 (53.6%)	48 (71.6%)	19 (28.4%)	0.54
Presence	58 (46.4%)	42 (72.4%)	16 (27.6%)	
M-stage				
Presence	29 (23.2%)	22 (75.9%)	7 (24.1%)	0.39
Absence	96 (76.8%)	68 (70.8%)	28 (29.2%)	
Overall stage				
I & II	64 (51.2%)	45 (70.3%)	19 (29.7%)	0.18
III & IV	61 (48.8%)	45 (73.8%)	16 (26.2%)	
B-raf*				
Absence	15 (50%)	10 (66.7%)	5 (33.3%)	**0.05**
Presence	15 (50%)	14 (93.3%)	1 (6.7%)	
Recurrence				
Positive	49 (39.2%)	39 (79.6%)	10 (20.4%)	0.09
Negative	76 (60.8%)	51 (67.1%)	25 (32.9%)	

* B-raf screening was carried out in 30 cases; LVI: lymphovascular permeation by carcinoma

Highlights

- Significant high expression of miR-142-5p was noted in cancer tissues and cells
- Patients had higher miR-142-5p expression had poor prognosis
- Overexpression of miR-142-5p associated with biological aggressiveness of cancer cells
- miR-142-5p overexpression reduced KLF6 expression significantly in cancer cells
- Anti-miR-142-5p treatment inhibited colon cancer cells growth remarkably

MiR-142-5p act as an oncogenic microRNA in colorectal cancer: Clinicopathological and functional insights

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