Tumour suppressor properties of miR-15a and its regulatory effects on BCL2 and SOX2 proteins in colorectal carcinomas

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Abstract:

Objectives: In this study, we aimed to investigate the expression pattern, clinicopathological significance and tumour suppressive properties of miR-15a in patients with colorectal carcinomas.

Methods: Tissue samples from 87 patients with primary colorectal carcinomas, 50 matched metastatic lymph node and 37 non-neoplastic colon (control) were prospectively recruited. The expression level of miR-15a was measured by quantitative real-time polymerase chain reaction. Restoration/overexpression of the miR-15a was achieved by exogenous transfection. Four colon cancer cell lines (SW480, CaCO2, SW48 and HCT116) and a non-cancer colon cell line (FHC) were also used for examining the miR-15a induced tumour suppression properties using various in-vitro and immunological assays.

Results: Downregulation of miR-15a was noted in ~ 62% of the colorectal carcinoma tissues and it was positively correlated with the presence of cancer recurrence in patients with colorectal carcinomas ($p = 0.05$). Also, these patients with low miR-15a expression showed relatively shorter survival time when compared to those with miR-15a overexpression. Following miR-15a exogenous overexpression, colon cancer cells showed reduced cell proliferation, low colony formation, less cell invasion properties and mitochondrial respiration when compared to control cells. In addition, BCL2 and SOX2 proteins showed a significant downregulation following miR-15a overexpression suggesting its regulatory role in cancer growth, apoptosis and stemness.

Conclusion: This study has confirmed the tumour suppressor properties of miR-15a in colorectal cancers. Therefore, its modulation has potential implications in controlling various biological and pathogenic processes in colon carcinogenesis via targeting its downstream proteins such as BCL2 and SOX2.

Keywords: Colorectal cancer; miR-15a; tumour suppression; BCL2; SOX2; carcinogenesis.

Introduction

MicroRNA (miRNA)s play a key role in the regulation of gene expression via pairing with the complementary sequence in the 3'-untranslated region (UTR) of a target mRNA and thereby regulating their protein expression [1]. Recent studies have confirmed that miRNAs expressions are critical in various cellular functions such as cell differentiation, cell cycle progression, stress response and apoptosis [2]. It has been postulated that approximately 30% of the human genome is regulated by miRNA [3]. It is worth noting that miRNAs can function either as a tumour suppressor or as an oncogene and are frequently interchangeable in its expression patterns in human cancers [4-7]. MiR-15a is one of the first group of miRNAs identified exhibiting significant role in carcinogenesis and deletion of miR-15a was first reported in patients with chronic lymphocytic leukaemia [8]

MiR-15a act as a tumour suppressor in carcinogenesis via inhibiting cell proliferation and promoting apoptosis both in vitro and in vivo [9]. Also, miR-15a exhibits its tumour suppressor properties via targeting multiple oncogenes, including *BCL2*, *MCL1*, *CCND1*, and *WNT3A* [10, 11]. Downregulation of miR-15a has recently been reported in patients with colorectal carcinoma and modified miR-15a has therapeutic potential for improving treatment of colorectal carcinomas in advanced stages via regulating its target proteins [12, 13]. Furthermore, miR-15 downregulation correlates with poor patient prognosis and advanced pathological stages in patients with colorectal carcinoma [12]. However, there are no reports on the biological impacts of miR-15a expression in colon cancer cells. In addition, clinicopathological correlations with cellular changes in vitro have never been studied in patients with colorectal carcinoma. Thus, in this study, we aimed to analyse the expression profiles and clinicopathological significance of miR-15a a large cohort of patients with matched primary and metastatic colorectal tissues. Also, the cellular ramifications of miR-15a in colon cancer cells were investigated in-vitro.

Materials and methods

Selection of patients

The patients were selected for this study had surgical resection of colorectal adenocarcinomas between January 2006 and December 2013 in Queensland, Australia. Ethical approval for this study was obtained from the Griffith University Human Research Ethics Committee (GU Ref No: MSC/17/10/HREC). After histopathological analysis, tissue samples from 124 patients (37 non-neoplastic; 87 primary adenocarcinomas together with 50 matched lymph nodes with metastatic adenocarcinoma) were used for this study. The non-neoplastic tissues (control) used in this study were recruited from patients diagnosed with hyperplastic polyps, diverticular diseases and intestinal volvulus. We select one tissue sample from each surgically resected specimen for miRNA extraction.

Clinicopathological characteristics

The demographic data and macroscopic features: age, gender, size and location of the tumours were documented for each patient in the study. A pathologist reviewed all the pathological characteristics of the carcinomas (AKL). Only adenocarcinomas were included in the study. The colorectal adenocarcinomas were classified, graded, and according to the World Health Organisation (WHO) criteria [14]. The carcinomas (66 from colon and 21 from rectum) were staged according to TNM (a tumour, lymph node and metastases) classification adopted in American Joint Committee on Cancer (AJCC) [15]. Both conventional (n=78) and mucinous (n=9) adenocarcinomas (>50% mucin distribution in conventional adenocarcinomas) were included in the study [16]. In addition, lymphovascular permeation were also recorded for all patients with colorectal adenocarcinoma. Microsatellite instability (MSI) was performed in 33 primary adenocarcinomas. The test was done by immunohistochemistry on four proteins (MLH1, PMS2, MSH2 and MSH6).

Colorectal carcinoma with the absence of any of these protein markers was considered as MSI high cases.

The management of all the patients was discussed in the weekly multidisciplinary team meeting and followed up by the same team of clinicians. The 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.

Cell culture

Four human colon cancer cell lines derived from different pathological stages (CaCo2, HCT116, SW480 and SW48) were used in this study and were purchased from American Type Culture Collection (ATCC). A non-neoplastic colon epithelial cell line, FHC (also obtained from ATCC) was used as a control. These cell lines were cultured and maintained according to the previously published protocol [6, 7].

miRNA extraction and quantitative real-time polymerase chain reaction (PCR)

The tissue samples were fixed in formalin and later embedded in paraffin for sectioning. Each tissue block was sliced into 7 microns and transferred into an Eppendorf tube for purification of miRNA. Isolation of miRNAs from both tissues and cell lines were performed as previously reported [6, 7]. After purification, complementary DNA (cDNA)s from these miRNAs were prepared using a commercial reverse transcription kit, miScript II RT Kit (Qiagen, Hilden, Germany). The cDNAs were diluted using RNAase free water to 1.5ng/µl for quantitative real-time PCR (qRT-PCR).

Each PCR reaction for miR-15a, RNU6B (internal control miRNA) was performed in triplicates and the mean cycle threshold (Ct) value of these PCR reactions was documented. Data analysis for determining miR-15a expression was performed using the $2^{-[delta][delta]Ct}$ (fold

change) method [6, 17]. Fold change greater than 2 was denoted as high miR-15a expression, while low miR-15a expression was regarded as a fold change of less than one [6, 17]. The Ct ratio of RNU6B versus miR-15a [expression ratio] was used to represent the miRNA expression in relative to control samples.

Cell transfection

Colon cancer cells (SW480 and HCT116) were seeded roughly at 3 x 10^5 cells/ cm^2 into 12-well plate in their respective growth media. Cells were transfected with miR-15a mimic ($SW480^{+miR\text{-}15a}$ and $HCT116^{+miR\text{-}15a}$) at 24 hours post seeding. For this, the diluted miRNA mimic in 100µl of serum-free media was treated with 6µl of HiperFect reagent (Qiagen) and incubated for 10 minutes for complex formation at room temperature. Subsequently, cells were transfected with these complexes and their effectiveness was examined at 24 hours and 48 hours after transfection by measuring the level of miR-15a expression. Cells treated with a scrambled miRNA ($SW480^{+miR\text{-}Scr}$ and $HCT116^{+miR\text{-}Scr}$) and transfection reagent (Hiperfect) alone ($SW480^{wildtype}$ and $HCT116^{wildtype}$) were used as controls.

Cell proliferation assay

The impact of miR-15a overexpression on colon cancer cell proliferation was analysed using a cell counting kit-8 (CCK-8) (Sigma-Aldrich, St Louis, USA). Colon cells were cultured in 96-well plates at $1x10^4$cells/well in quadruplets. After 24 hours of initial seeding, cells were treated with *miR-15a* mimic, a scrambled miRNA and transfection reagent alone as previously reported [6]. Cell proliferation rate was determined on days 0 to 4 with CCK-8 following manufacturer guidelines.

Cell invasion assays

Invasive ability of $HCT116^{+miR\text{-}15a}$, $HCT116^{+Scr,}$ and $HCT116^{+wildtype}$ cell lines were assayed using Cultrex® 96-well basement membrane extract (BME)-coated cell invasion kit (Trevigen, Gaithersburg, Maryland, USA) as per the recommendations of the manufacturer. Invasion chambers were first incubated overnight with 50 µl of BME-coated solution. After 24 hours of incubation in serum-free medium, HCT116 cells were collected and 50 µl (1 x 10^6 cells/ml) of cell suspension was added to each well of 96 wells top chamber. All the treated cells were added to the top chambers and incubated for 48 hours. Following incubation, 100µl of cell dissociation solution/Calcein AM was added to the bottom chamber and after a set of incubation, the number of invasive cells was quantitated using a plate reader (BMG Labtech, Ortenberg, Germany).

Colony formation

The clonogenic capacity of miR-15a transfected HCT116 cells was examined by seeding an equal number of cells in 6-well plates with 2 ml of complete medium at 37°C in 5% CO_2. On day 16, microscopic colonies were detected in the plate and cell growth was ceased. Using 70% cold ethanol for 15 minutes the cells were fixed in the plate. Next, the

cell colonies were stained with 0.5% crystal violet for 2 hours at room temperature, followed by a wash under running tap water. As a final step, plates containing stained cell colonies were air-dried, images were taken and the number of colonies was counted.

Western blot

Western blot analysis was undertaken to investigate the down-stream protein interaction of miR-15a. Whole cell proteins from all cell groups were extracted with a lysis buffer (Bio-Rad, California, USA). After quantification with an absorbance spectrometry, ~30 μg of whole cell protein was separated by 15% SDS-PAGE and transferred to nitrocellulose membranes using Trans-Blot® Turbo transfer system (Bio-Rad). After blocking with 5% non-fat milk powder for 2 hours at room temperature, the membranes were incubated with antibodies overnight at 4°C. The antibodies are anti-rabbit BCL2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz California, USA) (at 1:200), anti-rabbit TAB3 monoclonal antibody (Abcam, Melbourne, Victoria, Australia) (at 1:1000), anti-rabbit FAM134B (JK1) polyclonal antibody (Santa Cruz Biotechnology) (at 1:150), anti-goat polyclonal SOX2 (Santa Cruz Biotechnology) (at 1:50) and anti-mouse monoclonal GAPDH antibody (Santa Cruz Biotechnology) (1:2000). Finally, membranes were incubated with a secondary antibody (Santa Cruz Biotechnology) (1:5000) at room temperature for 2 hours. The bands for BCL2, TAB3, JK1 and SOX2 proteins were developed with a chemiluminescence HRP detection kit (Bio-Rad, Hercules, California, USA).

Immunofluorescence

Immunofluorescence analysis was achieved to visualise the localisation of target proteins regulated by miR-15a. The colon cancer cell (HCT116) were seeded, transfected, and prepared for incubation with primary antibodies according to the published protocol [18].

Cells were then blocked with 5% bovine serum albumin in phosphate buffered saline for 1 hour at room temperature. Subsequently, the HCT116$^{miR\text{-}15a}$ cells were washed with phosphate-buffered saline and incubated with rabbit monoclonal BCL-2 antibody (1:50) at 4°C overnight. This was followed by 2 hours of incubation with anti-rabbit secondary antibodies labelled with fluorescein isothiocyanate fluorophore (Sigma-Aldrich) at room temperature. Finally, coverslips were mounted on glass slides with mounting media containing 4′,6-diamidino-2-phenylindole (a fluorescent nuclear stain). The glass slides were observed under a confocal microscope (Nikon A1R+; Nikon Inc., Tokyo, Japan).

Seahorse XFp Cell Mito Stress Test

To measure the oxygen consumption rate (OCR) following miR-15a overexpression, an XFp analyser (Agilent Technologies, Santa Clara, California, USA) was used. Briefly, cells ($1.6x10^4$ cells per well) with DMEM (Dulbecco's modified Eagle's media) were seeded on Seahorse XFp cell culture mini plates (Seahorse Bioscience, North Billerica, Massachusetts, USA). After incubation for 24 hours at 37ºC, cells were transfected with miR-15a for 24 hours before analysis. Mitochondrial Stress Test assay was conducted by incubating the miR-15a transfected and control cells in XF Base Medium (Agilent Technologies) supplemented with 2mM Glutamine, 1mM sodium pyruvate and 10mM glucose (pH 7.4) for 45 minutes at 37°C in a non-CO_2 incubator. Mitochondrial function was evaluated using Mito Stress Test kit (by sequential injections of 1µM oligomycin, 0.5µM carbonyl cyanide-ptrifluoromethoxyphenylhydrazone (FCCP), and a mixture of 0.5µM antimycin-A and 0.5µM rotenone. After lysing these cells using an NP40 buffer (Thermo Fisher, Waltham, Massachusetts, USA), a BCA protein assay kit (Thermo Fisher) was used to quantify the protein levels, allowing relative expression of protein normalisation. Data

analysis was performed using the Seahorse XFe Wave software, including the Seahorse XF cell energy phenotype and MitoStress test report generators.

Statistical analysis

Alterations in clinical, pathological, follow up and miRNA expression was recorded into a database. Statistical analysis was performed using the Statistical Package for Social Science for Windows (version 25.0, IBM SPSS, Inc., New York, USA). Normality test using skewness and kurtosis was performed prior to analysis. 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. A significance level of the tests was taken at $p < 0.05$. Survival analysis was checked via Kaplan-Meier method.

Results

Altered expression and clinicopathological significance of miR-15a

All four colon carcinoma cells exhibited a significant reduction of miR-15a when compared to FHC cells (Figure 1A). Compared to non-neoplastic colorectal tissues, down-regulation of miR-15a expression was noted in ~62% (n=54/87) of the colorectal adenocarcinoma tissues while remaining tissue samples (~38%) showed miR-15a overexpression (Figure 1B). Table 1 summarizes the associations between expressions of miR-15a with the clinicopathological features of the colorectal adenocarcinoma. Patients with colorectal adenocarcinoma with high miR-15a expression showed more cancer recurrence when compared to those with low miR-15a expression levels (33.3 % vs 18.4%, p=0.056).

The median overall follow-up of patients with colorectal cancer was 78 months and the pathological stages of cancer were significantly correlated with patient survival ($p < 0.05$) (Figure 2A). Patients with colorectal adenocarcinoma expressing low miR-15a expression had relatively shorter survival time when compared to those with high miR-15a expression (75 months versus 77 months) (Figure 2B (p=0.321). Similarly, patients with adenocarcinoma having low miR-15a expression levels in their metastatic lymph node tissues showed poor survival rate when compared to those without any metastatic lymph nodes (61 months versus 76 months) (Figure 2C). However, both these associations did not reach the statistical significance despite a good schematic difference.

Associations between primary and metastatic tumours

Among the patients with high levels of miR-15a expressions (n=26/52) in their primary colorectal adenocarcinoma, approximately 73% (n=19) patients presented with low miR-15a expressions in their metastatic lymph node tissues. Meanwhile, majority (77%,

20/26) of primary colorectal cancers with miR-15a low expression showed a consistent downregulation of its expression levels in the matched metastatic lymph node tissues.

miR-15a induced tumour suppression in-vitro

Colon cancer cells overexpressed with miR-15a ($HCT116^{+miR\text{-}15a}$) (Figure 3A) showed a reduction in cell proliferation levels when compared to control cells (Figure 3B). The decreased proliferation of cells was noted from day 1 until day 4; while the control cells ($HCT116^{+miRScr}$ and $HCT116^{wildtype}$) constantly proliferated until day 4 (Figure 3B). Correspondingly, colony formation assay indicated a significant decrease in the clonogenic capability of $HCT116^{+miR\text{-}15a}$ cells in contrast to control cells (Figure 3C). Moreover, it was observed that miR-15a mimic induced HCT116 cells to invade less profusely differing from $HCT116^{+miRScr}$ and $HCT116^{wild\text{-}type}$ cells (Figure 3D).

Downstream regulation on BCL2 and SOX2 protein expressions

Compared to control cells, protein expression of $SW480^{+miR\text{-}15a}$ cells showed a significant reduction of expression levels for BCL2 and SOX2 proteins, suggesting its regulatory effects via controlling cell apoptosis and stemness (Figure 4 A&B). Similar to SW480 cells; $HCT116^{+miR\text{-}15a}$ cells have also shown a reduction of BCL2 protein expression (Supplementary figure 1). The miR-15a induced downregulation of BCL2 protein was also confirmed by an immunofluorescence assay where the cytoplasmic expression of the BCL2 protein was significantly reduced in $HCT116^{+miR\text{-}15a}$ cells (Figure 4F, and 5). On the other hand, TAB3 and FAM134B proteins did not show any significant difference in its expression patterns following miR-15a overexpression.

Effects of miR-15a on mitochondrial respiration

A Seahorse XFp Cell Mito stress test was performed to measure the impact of miR-15a overexpression in mitochondrial function and glycolysis of colon carcinoma cells. After 24 hours of transfection, we observed that the maximal oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were reduced in $SW480^{+miR\text{-}15a}$ cells when compared to controls (Figure 6A&C). Also, compared to control cell groups, Seahorse XF report generator derived values demonstrated a reduction of basal respiration, ATP production, proton leak, maximal respiration, coupling effect, PPR, and glycolytic capacity in $SW480^{+miR\text{-}15a}$ (Figure 6B). Analogous findings were also noted with miR-15a overexpression on mitochondrial function and glycolysis after 36 hours of transfection (Supplementary figure 1).

Discussion

The current study has confirmed the tumour suppressor properties of miR-15a in-vitro and ~ 62% of the primary colorectal adenocarcinoma tissues showed low levels of miR-15a expression. These study findings are in consensus with the previous reports on the tumour suppressive properties of miR-15a in other human cancers including breast, lung, liver, ovary, prostate and colorectal carcinomas [19-24]. In addition, the expression levels of miR-15a were significantly reduced among colorectal cancer patients with metastasis to either lymph node or distant organs. Analogues findings were also noted with Bonci et al., were miR-15a was frequently downregulated in pathologically advanced prostate [25] and colorectal adenocarcinomas [12]. Though not statistically significant, among metastatic colorectal cancers, patients with low miR-15a expression levels showed poor patient prognosis when compared to those with high/normal levels of miR-15a. In addition, miR-15a low expression was associated with patients with colorectal cancer recurrence during their post-surgical follow up. Taken together, these findings are in consensus with the previous studies suggesting the potential use of miR-15a as a molecular target in predicting aggressive tumour progression in cancer patients.

Several miRNAs have recently come to light as potential prognostic biomarkers in colorectal carcinomas. There is limited evidence on the miR-15a induced biological and growth changes in cancer cells. A study by Luo et al. have noted that miR-15a overexpression in breast cancer cells led to inhibition of cancer cell proliferation [19]. Similar growth inhibition properties of miR-15a have also been noted in lung and gastric carcinomas [20, 26, and 27]. A most recent study by Fesler et al. have found that miR-15a overexpression in colon cancer cells induced cell cycle arrest and cell proliferation [13]. They also noted that miR-15a expression enhances the effectiveness of chemotherapy (5-FU, fluorouracil) in colon cancers both in-vitro and in-vivo suggesting the therapeutic potential of this miR-15a

[13]. The present study has observed similar findings where miR-15a overexpression has led to reduced cell proliferation, colony formation and cell invasion properties in colon cancer cells (Figure 3). These results in conjunction with previous findings suggest that miR-15a modification has a significant impact on targeting colon cancer cell growth and has potential therapeutic potential in patients with colorectal carcinomas.

Previous studies have confirmed that miR-15a exhibits its tumour suppressive properties by targeting some key growth-related genes in vitro and in vivo [28, 20, and 19]. Low expression levels of miR-15a in human cancers have been attributed to the overall deletion or allele loss at the chromosome level (ch13q) [29]. In this study, we have identified BCL2 and SOX2 as important targets of miR-15a in colon cancer. Both proteins have shown a significant down regulation following miR-15a overexpression. BCL2 is an inhibitor of apoptosis in colon cancer, and high expression of BCL2 has been shown to have oncogenic potential as well as chemotherapy resistance in colon cancer cells [30, 13]. Similar to the current study, miR-15a induced regulatory effects on BCL2 have been previously reported in colon cancers and chronic lymphocytic leukaemia [13, 31]. On the other hand, SOX2 is a well-known transcription factor and its expression induces stemness in colon cancers and is associated with poor prognosis of the patients with cancer [32]. The downstream target effects of miR-15a overexpression on SOX2 were not reported previously. These findings imply that the growth inhibition properties of miR-15a in colon cancers are mainly exhibited by regulating cancer cell apoptosis and its stemness via directly targeting BCL2 and SOX2 proteins.

Mitochondrial respiration is essential for the generation of cellular energy under normal conditions. This study showed miR-15a induced a reduction in oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) as well as fall in basal respiration, ATP production and glycolytic capacity in colon cancer cells (Figure 5). According to

"Warburg effect", defects in mitochondrial respiration can lead the cancer cells to ferment glucose in the presence of oxygen, suggesting its central role in malignant tumour progression [33]. However, further studies have confirmed that most cancers still retain mitochondrial function, including respiration and the oncogenic mutations in these cancer cells, are responsible for the aerobic glycolysis [34, 35]. Previously Gao et al. have reported that by targeting *BCL2* oncogene, miR-15a in a cluster with miR-16 expression has induced apoptosis via regulating mitochondrial function [11]. Consistent with these findings, this miRNA cluster has reported causing mitochondria-mediated apoptosis via targeting *BMI1* oncogene [36]. Another study by Liu et al. have also noted a similar miR-15a induced apoptosis in cardiomyocytes via affecting the mitochondrial apoptotic pathway [37]. Thus, it can be hypothesized that miR-15a helps in regulating mitochondrial functions in normal colon cells and during carcinogenesis, it enhances mitochondria directed apoptotic pathways via directly targeting BCL2 or/and other downstream targets.

To conclude, exogenous over expression of miR-15a has shown downstream suppression of BCL2 and SOX2 proteins and led to lowered colon cancer cell proliferation, invasion and colony formation ability. This study has also noted regulatory effects of miR-15a mitochondrial respiration potential via controlling the BCL2 induced mitochondria directed apoptosis. Taken together, these findings further emphasize a tumour suppressive role of miR-15a, wherein its inhibition or absence, epithelial cells in the intestine can proliferate faster and can lead to a rapidly growing tumour in vivo. Downregulation of miR-15a in patients with metastatic colorectal adenocarcinomas and pathologically advanced colon carcinoma cells in vitro, infer the tumour dissemination capacity of miR-15a by its own or via regulating its key downstream gene targets.

Figure legends

Figure 1: Expression levels of miR-15a colorectal cancers. A- Colon cancer cells with advanced pathological stage, HCT116 (stage IV), SW48 (stage III), SW480, and CaCo2 (stage II) showed reduced expression of miR-15a compared to normal epithelial cell line (FHC). Inverse ratio of miR-15a versus RNU6B (control miRNA) (expression ratio) was used to illustrate the miRNA expression levels in various cell lines. **B**- Expression levels of miR-15a were altered in primary colorectal cancer (CRC) tissues, CRC lymph node tissues and distant metastasis tissues. Higher expression levels of miR-15a were noted in primary CRC tissues while lower miR-15a expression was found in lymph node and distant metastasis tissues

Figure 2: Expression levels of miR-15a and its correlation with patient survival. A- Patient survival was significantly reduced with advanced pathological stages ($P < 0.05$). A gradual decline in survival period (cum survival) was noted as the pathological stage in patients with colorectal carcinoma was advanced from stage I to stage IV. **B-** A marginal improvement of survival was noted among colorectal cancer patients (n=87) with high levels of miR-15 expression. **C**- Patients with low expression levels of miR-15a in stage III colorectal adenocarcinomas (n=52) showed a reduced survival compared to those with high miR-15a levels ($P > 0.05$).

Figure 3: Tumour suppressor properties of miR-15a in vitro. A- Compared to control cells (scramble and wild-type), exogenous transfection has resulted in a significantly higher expression of miR-15a in Colon cancer cells ($HCT116^{+miR\text{-}15a}$) and **B-** overexpressed $HCT116^{+miR\text{-}15a}$ showed a reduction in cell proliferation levels when compared to control cells ($P = 0.046$). **C-** Colony formation ability of $HCT116^{+miR\text{-}15a}$ were reduced compared to control groups ($P = 0.005$), **D-** Overexpression of miR-15a also reduced the invasiveness of HCT116 cells ($P > 0.05$).

Figure 4: miR-15a target protein quantification. A, B- Target proteins BCL-2 (*** $P = 0.002$) and SOX-2 (** $P = 0.019$) expression was notably reduced in $SW480^{+miR\text{-}15a}$ compared to control and wild-type. **C**- $HCT116^{+miR\text{-}15a}$ cells also showed reduced expression levels of BCL-2 in comparison to control groups (*** $P = 0.005$). **D, E**- Expression levels of TAB3 and FAM134B showed no change in $SW480^{+miR\text{-}15a}$ cells when compared to control and wild-type. GAPDH antibody was used as an immunoblot loading control.

Figure 5: Intracellular localisation and expression of BCL-2. Immunofluorescence analysis indicated reduced expression levels of BCL-2 in $HCT116^{miR\text{-}15a}$ cells compared to control groups. Green colour stands for BCL-2 staining while blue colour represents nuclear staining by DAPI. All images are in their original magnifications at 40x; scale bar is at 50 um.

Figure 6: Effects of miR-15a on mitochondrial respiration post 24 hours. A, D- Oxygen consumption rate (OCR), **B, E-** extracellular acidification rate (ECAR) were reduced in $SW480^{+miR\text{-}15a}$ cells, **C-** maximal respiratory capacity, spare respiratory capacity, ATP coupled respiration, proton leak showed a reduction in $SW480^{+miR\text{-}15a}$ cells compared to control groups. **F-** the Metabolic potential of $SW480^{+miR\text{-}15a}$ cells were lower in stressed OCR than control groups while increased levels of $SW480^{+miR\text{-}15a}$ cells were noted in stressed ECAR. All samples were analysed after 24 hours or transfection using the Seahorse XF analyser. OCR and ECAR were measured, followed by treatment with oligomycin, FCCP, and rotenone/antimycin A pharmacological inhibitors of ETC.

Supplementary figure

Figure 1: Effects of miR-15a on mitochondrial respiration post 36 hours. A, D- Oxygen consumption rate (OCR), **B, E-** extracellular acidification rate (ECAR) were reduced in SW480+miR-15a cells, **C-** maximal respiratory capacity, spare respiratory capacity, ATP coupled respiration, proton leak showed a reduction in SW480+miR-15a cells compared to control groups. **F-** the Metabolic potential of SW480+miR-15a cells were lower in stressed OCR and stressed ECAR than control groups. All samples were analysed after 36 hours or transfection using the Seahorse XF analyser. OCR and ECAR were measured, followed by treatment with oligomycin, FCCP, and rotenone/antimycin A pharmacological inhibitors of ETC.

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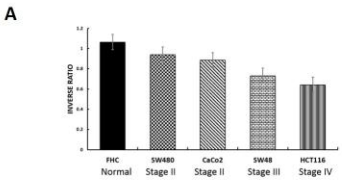
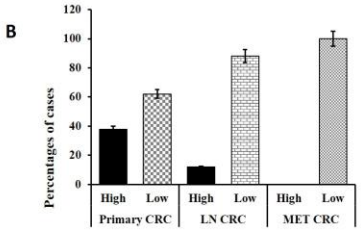
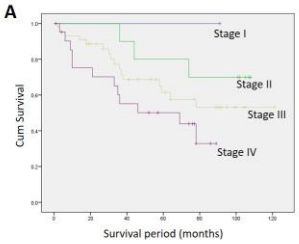
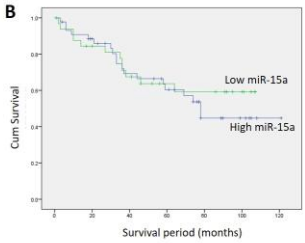
Table: miR-15a expression and correlation with clinicopathological features

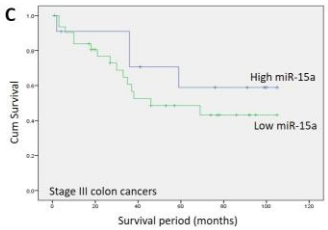
Type	Total no. (%)	High expression	Low expression	p-value
Age				
≤ 60	20 (23.0 %)	12 (60%)	8 (40%)	0.408
>60	67 (77.0%)	36 (53.7%)	32 (46.3%)	
Gender				
Male	46 (52.9%)	27 (58.7%)	19 (41.3%)	0.314
Female	41 (47.1%)	21 (51.2%)	20 (48.8%)	
MSI				
High	8 (24.2%)	3 (37.5%)	5 (62.5%)	0.091
Stable	25 (75.8%)	18 (72.0%)	7 (28.0%)	
Location				
Proximal colon	41 (47.1%)	22 (53.7%)	19 (46.3%)	0.479
Distal colorectum	46 (52.9%)	26 (56.5%)	20 (43.5%)	
Size				
≤ 50	64 (73.6%)	36 (56.3%)	28 (43.8%)	0.462
> 50	23 (26.4%)	12 (52.2%)	11 (47.8%)	
Grade				
Well	8 (10.1%)	4 (50%)	4 (50%)	0.550
Moderate	55 (69.6%)	30 (54.5%)	25 (45.5%)	
Poor	16 (20.3%)	11 (68.8%)	5 (31.3%)	
Subtypes				
Conventional	78 (89.7%)	42 (53.8%)	36 (46.2%)	0.651
Mucinous Adenocarcinoma	9 (10.3%)	6 (66.7%)	3 (33.3%)	
Lymphovascular permeation				
Presence	47 (54.0%)	28 (59.6%)	19 (40.4%)	0.249
Not detected	40 (46.0%)	20 (50%)	20 (50%)	
T stage				
II	3 (3.4%)	2 (66.7%)	1 (33.3%)	0.743
III	54 (62.1%)	31(57.4%)	23 (42.6%)	
IV	30 (34.5%)	15 (50%)	15 (50%)	
N stage (Distant metastasis)				
Positive	74 (85%)	43(58.1%)	31(41.9%)	0.156
Negative	13(15%)	5 (38.5%)	8 (61.5%)	
M stage (Distant metastasis)				
Positive	26 (29.9%)	15 (57.7%)	11 (42.3%)	0.472
Negative	61 (70.1%)	33 (54.1%)	28 (45.9%)	
Recurrence				
Presence	45 (51.7%)	29 (64.4%)	16 (35.6%)	**0.056**
Not detected	42 (48.1%)	19 (45.2%)	13 (54.8%)	

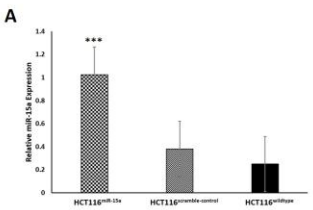


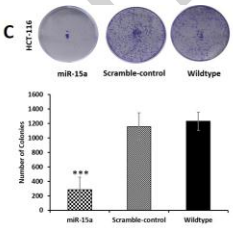


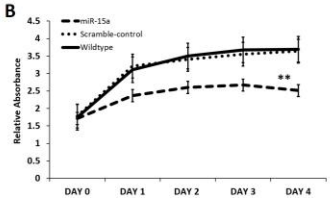


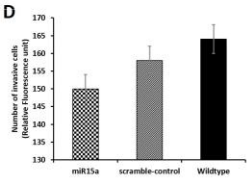


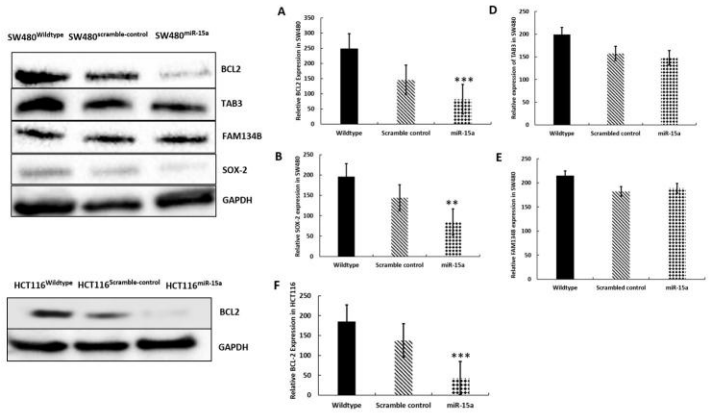


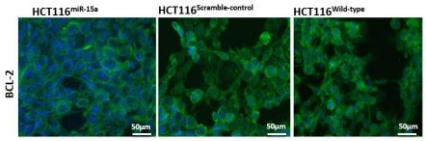


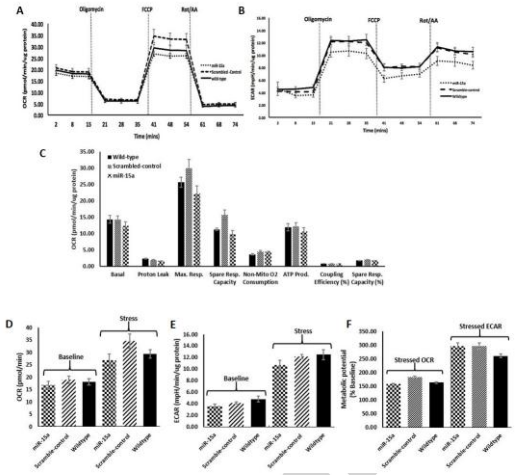












Highlights

1. Majority of patients with colorectal adenocarcinomas (62%) and colon cancer cell lines showed a significant downregulation of miR-15a

2. Expression levels of miR-15a was significantly altered with their matched metastatic lymph node tissues.

3. Downregulation and tumour suppressor properties of miR-15 has confirmed in-vitro via cell proliferation, colony formation, cell invasion and mitochondrial respiration assays

4. BCL2 and SOX2 proteins showed a significant downregulation following miR-15a overexpression suggesting its regulatory role in cancer growth, apoptosis and stemness.

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Tumour suppressor properties of miR-15a and its regulatory effects on BCL2 and SOX2 proteins in colorectal carcinomas

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