Downregulation of microRNA-498 in colorectal cancers and its cellular effects

Vinod Gopalan¹, Robert A Smith¹, Alfred K-Y Lam^{1*}

¹Cancer Molecular Pathology, School of Medicine and Griffith Health Institute,
Griffith University, Gold Coast, Queensland, Australia

***Correspondence to: Professor Alfred Lam**, Head of Pathology, Griffith Medical
School, Gold Coast Campus, Gold Coast QLD 4222, Australia.

E-mail: a.lam@griffith.edu.au

Telephone +61 7 56780718 Fax +61 7 56780303

Abstract

miR-498 is a non-coding RNA located intergenically in 19q13.41. Due to its predicted targeting of several genes involved in control of cellular growth, we examined the expression of miR-498 in colon cancer cell lines and a large cohort of patients with colorectal adenocarcinoma. Two colon cancer cell lines (SW480 and SW48) and one normal colonic epithelial cell line (FHC) were recruited. The expression of miR-498 was tested in these cell lines by using quantitative real-time polymerase chain reaction (qRT-PCR). Tissues from 80 patients with surgical resection of colorectum (60 adenocarcinomas and 20 non-neoplastic tissues) were tested for miR-498 expression by qRT-PCR. In addition, an exogenous miR-498 (mimic) was used to detect the miRNA's effects on cell proliferation and cell cycle events in SW480 using MTT calorimetric assay and flow cytometry respectively. The colon cancer cell lines showed reduced expression of miR-498 compared to a normal colonic epithelial cell line. Mimic driven over expression of miR-498 in the SW480 cell line resulted in reduced cell proliferation and increased proportions of G2-M phase cells. In tissues, miR-498 expression was too low to be detected in all colorectal adenocarcinoma compared to non-neoplastic tissues. This suggests that the down regulation of miR-498 in colorectal cancer tissues and the direct suppressive cellular effect noted in cancer cell lines implies that miR-498 has some direct or indirect role in the pathogenesis of colorectal adenocarcinomas.

Keywords: micro-RNA; colon; adenocarcinoma; miR-498

Introduction

Micro-RNAs have been shown to be important in the pathogenesis of cancers [1,2]. miR-498 is a non coding RNA located intergenically in 19q13.41. Schepeler *et al* reported the down regulation of miR-498 in stage II colon cancers and its role in cancer prognosis [3]. Patients with Stage II colon cancer having high miR-498 expressions had longer survival time than patients with low expression [1].

Down regulation of miR-498 has been seen in acute myeloid leukemia, while high expression has been reported in retinoblastoma [4,5]. Down regulation of miR-498 was also reported in CD4+T cells of rheumatoid arthritis patients [6]. In addition, Yan *et al* reported the role of miR-498 in herpes simplex virus-1 mediated Kaposi sarcoma associated herpesvirus replication [7]. Despite these studies, miR-498 has not been studied in isolation from other miRNAs in cancer, nor have its cellular effects, clinical and pathological implications been investigated. Due to its predicted targeting of several genes involved in control of cellular growth (Figure 1), we examined the expression of miR-498 in colon cancer cell lines and a large cohort of patients with colorectal cancer.

Materials and methods

Cell culture

Two human colon cancer cell lines (SW480 & SW48) and one normal colon epithelial cell line (FHC) were obtained from American Type Culture Collection (ATCC). These cell lines were cultured using the methods published previously [1,8-9].

Cell transfection

SW480 cells were passaged at $2-8 \times 10^4$ cells/cm² into 24 well plates in the recommended media, and were transfected by an miR-498 mimic at different concentrations (5nM, 10nM, 15nM). Each concentration of miRNA was treated with different volumes of HiperFect reagent (1.5ul, 3ul, 4.5ul) for making complexes. These complexes were transfected over the cells after 24 hours of seeding. Transfection efficiency was calculated by measuring the amount of miRNA in the transfected cells. A trypan blue exclusion test was performed for checking cell viability. The cells were also treated with scrambled miRNA and transfection reagent alone as negative controls.

Patient selection

The patients who were chosen for this study had resection for primary colorectal adenocarcinomas and non-neoplastic colorectal tissues between January 2004 and December 2009 in Queensland, Australia. The patients were consecutively chosen and with no selection bias. Patients with non-neoplastic lesions were used as control population and these tissues were mainly recruited from patients with diverticular disease, polyps and volvulus to act as control tissue. Ethical approval of

this study has been obtained from the Griffith University Human Research Ethics Committee (GU Ref No: MED/05/06/HREC).

Tissue preparation and sampling

The resected colorectal tissues were fixed in 10 percent formalin and embedded in paraffin wax. Histological sections were cut and stained for haematoxylin and eosin for light microscopic examination. The pathological features of patients with colorectal adenocarcinomas and adenomas were analysed. The colorectal adenocarcinomas were classified, graded and staged according to the World Health Organization criteria [10]. After review, 82 patients (49 men; 33 women) with colorectal adenocarcinomas were selected for the analysis. The mean age of the patients with adenocarcinomas was 67 years (range, 32 to 88). One tissue block from each of the cancer samples were chosen for miRNA extraction. The tissue block was checked to ensure that it contained a representative cancer area. In addition, a tissue block with representative features was selected from each of the 20 patients (14 men; 6 women) with non-neoplastic colorectal lesions.

Extraction of miRNA

Tissue blocks from the selected samples were sectioned into 7 micron slices for miRNA extraction. H&E stained slides were used to distinguish the adenocarcinoma from surrounding morphologically non-neoplastic tissue. Paraffin was then removed with xylene, followed by centrifugation to move cellular contents to the bottom of the tube and allow removal of the xylene and dissolved paraffin. The miRNA was purified with Qiagen miRNeasy FFPE Kits (Qiagen Pty. Ltd., Hilden, NRW, Germany), which were specially designed for purifying total RNA and miRNA

from formalin-fixed, paraffin-embedded tissue sections. The quality and purity of the extracted miRNA and the cDNA preparations were determined according to the methods published previously [11].

Primers

Primers for detection of expression of miR-498 (Hs_miR-498_1 miScript Primer Assay) were purchased from Qiagen. RNU6B RNA (Hs_RNU6B_2 miScript Primer Assay, Qiagen) was chosen to be used as a ubiquitous control gene

Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed for the detection of miR-498 expression. An IQ5 Multicolour Real-Time PCR Detection system (Bio-Rad) was used to run the real-time quantification PCR. PCR was performed in a total volume of 20 μ L reaction mixture containing 10 μ L QuantiTect SYBR Green PCR Master Mix (Qiagen), 1 μ L of miScript Universal Primer, 1 μ L miScript Primer Assay as mentioned before (Qiagen), and 4 μ L of cDNA template at 1.5 ng/ μ L concentration and 4 μ L of RNase-free water. In the last tube, 4 μ L of deionized, diethylpyrocarbonate (DEPC) treated water was added as a non-template control. Thermal cycling conditions are mentioned in Table 1. For each tissue sample, the PCR reactions were performed in triplicate and accompanied by a non template control to increase the reliability of the results. Expression levels of miR-498 in different colorectal tissues were analysed as in our previous study [9].

Cell proliferation assay

An MTT calorimetric assay was performed for detecting cell proliferation. Both control and miRNA treated cells were used for this experiment. The cells were seeded in flat-bottom 96-well plates at 1×10^4 cells/well. At 12, 24 and 48 hrs after miRNA transfection, 0.5 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Invitrogen, CA, USA) was added to each well. After cells were incubated at 37 °C for another 4 hrs, the medium was removed and 150 µl dimethyl sulfoxide (DMSO, Sigma–Aldrich Co.) was added, and the cells were agitated for 10 min with protection from light. Absorbance was determined using a fluorescence polarization microplate reader- POLARstar Omega (BMG TABTECH, Ortenberg, Germany) using a wavelength of 570 nm with 630 nm as a reference.

Cell Cycle analysis

To determine the miR-498 effect on cell cycle regulation, control and miR-498 (mimic) treated cells were harvested by trypsinization and fixed in phosphate buffered saline (PBS) with 70% ethanol for 1 hour at 4⁰C. Then, the cells were resuspended in PBS and treated with 5ul of 10mg/ml Rnase A. After 1 hour of incubation at 37⁰C, 10ul of 1mg/ml propidium iodide was added and kept it in dark until the analysis was performed. Analysis was performed with a flow cytometer (Millipor-Guava, Billerica, MA, USA) by reading on a cytometer at 488 nm. To determine the difference between controls and treatments, the analysis was performed with triplicate samples for each treatment.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences for Windows (version 22.0, 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. Significance level of the tests was taken at $p < 0.05$.

Results

Expression of miR-498 in colon cancer cell lines

Inverse ratio of miR-498 versus RNU6B (control miRNA) cycle threshold values [expression ratio] was used to represent the miRNA expression levels in different cell lines. Cancer cell lines (SW480 & SW48) showed lower expression of miR-498 compared to non-neoplastic epithelial cell line (FHC) (Figure 2).

Over expression induced by miR-498 mimic in SW480 cell line

miR-498 over expression was noticed in the cancer cells after transfection of 10nM miR-498 mimic with 3ul of transfection reagent (Figure 3A). miR-498 expression was also analysed over a time period of 12 hours, 24 hours and 48 hours after transfection (Figure 3B). Expression levels were altered and the highest expression was noted at 48 hours of transfection with miR-498.

Reduced cell proliferation in miR-498 transfected cells

To assess the role of miR-498 in terms of cell growth, SW480 cells were treated with 10nM miR-498 mimic at various time points. Cell proliferation assay was performed on transfected SW480 cells with miR-498 mimic, scrambled siRNA and Hi-Perfect reagent (control) respectively (Figure 3C). The miR-498 transfected cancer cells showed reduced proliferation from day 2 of the experiment, compared to control cells and cells transfected with scrambled siRNA (not shown here).

Alteration of cell cycle events

Readings for G0-G1, S and G2-M phases were obtained after flow cytometry analysis. Cells treated with miR-498 showed accumulation of cells in the G2-M phase

compared to the controls (Figure 4). Also, cells transfected with miR-498 showed a reduced number of cells at the S phase compared with the control cells (treated with Hi-Perfect reagent only).

Down regulation of miR-498 in colorectal cancer tissues

The mean expression ratio (inverse ratio) of miR-498 was much lower in colorectal adenocarcinomas compared to non-neoplastic colon tissue (Figure 5). miR-498 expression was too low to be detected via qRT-PCR in all colorectal adenocarcinoma samples.

Discussion

In this study, the expression levels of miR-498 in colon cancer tissues and cell lines were demonstrated for the first time. Reduced expression of miR-498 in colon cancer cells (SW480 and SW48) compared to controls coincides with miR-498 expression in colorectal cancer tissues.

Recent studies proved that miRNAs can alter many signalling pathways and cellular process such as cell proliferation and differentiation by controlling the expression of target transcripts [1,2, 12-14]. As miR-498 is naturally low expressing in the majority of solid tumours and cancer cell lines, we aimed to over express the miR-498 in colon cancer cell lines to identify its downstream effects. In the present study, we determined that upregulation of miR-498 inhibits cell proliferation in colon cancer cell lines. This inhibitory effect of miR-498 on cell proliferation can be attributed to the tumour suppressor properties of this microRNA. In addition, the inhibitory effect of miR-498 on cell growth are analogous with the previous work by Yan et al [7]. These results indicated that miR-498 plays a role in cancer development by modulating the expression of target oncoproteins involved in carcinogenesis of colorectal cancer.

Accumulation of cells in G2-M phase was noted high in cells transfected with exogenous miR-498. Also, the number of cells in the S phase was reduced in cells over expressed with miR-498. This reduction in cell proliferation and the cell cycle changes suggested that miR-498 regulates cell growth and division by controlling the DNA replication at the S phase. Additionally, in response to miR-498 activation, the targeted cells accumulated in the G2-M phase, indicating that they were prevented from completing mitosis and entering into the G0-G1 phase for the next set of cell division.

In this study, the down regulation of miR-498 in colorectal cancer tissues and the direct suppressive cellular effect noted in cancer cell lines implied that miR-498 has some direct or indirect role in the pathogenesis of colorectal cancer. Target prediction for miR-498 indicated a number of potential genes that have a role in cell growth and metabolism (Figure 1). Among the various targets predicted by miRDB target prediction tool (<http://mirdb.org/miRDB/>), the most likely interactors (target score = 95) was noted with cancer related genes such as JHDM1D and CD93 [15-16]. These target genes have specific cellular functions such as cell growth, angiogenesis and apoptosis [17-18]. This gives some clues for the reason behind the association of low miR-498 levels with cell growth and progression in colorectal cancers. Further research is needed to understand the precise mechanism of action of this miRNA and its association with its targets in the pathogenesis of colorectal cancers.

To conclude, over expression of miR-498 suppresses cell proliferation and alters cell cycle events. Also, reduced expression of miR-498 in colorectal cancer tissues indicates its role in cancer pathogenesis via activation of its target proteins such as JHDM1D and CD93.

Reference

1. Gopalan V, Pillai S, Ebrahimi F, Salajegheh A, Lam TC, Le TK, Langsford N, Ho YH, Smith RA, Lam AK. Regulation of microRNA-1288 in colorectal cancer: Altered expression and its clinicopathological significance. *Mol Carcinog* 53 (2014) E36-E44.
2. Ebrahimi F, Gopalan V, Smith RA, Lam AK. miR-126 in human cancers: Clinical roles and current perspectives. *Exp Mol Pathol* 96 (2014) 98-107.
3. Schepeler T, Reinert JT, Ostensfeld MS, Christensen LL, Silahtaroglu AN, Dyrskjot L, Wiuf C, Sørensen FJ, Kruhøffer M, Laurberg S, Kauppinen S, Ørntoft TF, Andersen CL. Diagnostic and prognostic microRNAs in stage II colon cancer. *Cancer Res* 68 (2008) 6416-6424.
4. Garzon R, Garofalo M, Martelli MP, Briesewitz R, Wang L, Fernandez-Cymering C, Volinia S, Liu CG, Schnittger S, Haferlach T, Liso A, Diverio D, Mancini M, Meloni G, Foa R, Martelli MF, Mecucci C, Croce CM, Falini B. Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. *Proc Natl Acad Sci U S A* 105 (2008) 3945-3950.
5. Zhao JJ, Yang J, Lin J, Yao N, Zhu Y, Zheng J, Xu J, Cheng JQ, Lin JY, Ma X. Identification of miRNAs associated with tumorigenesis of retinoblastoma by miRNA microarray analysis. *Childs Nerv Syst* (25) 2009 13-20.
6. Li J, Wan Y, Guo Q, Zou L, Zhang J, Fang Y, Zhang J, Zhang J, Fu X, Liu H, Lu L, Wu Y. Altered microRNA expression profile with miR-146a upregulation in CD4⁺ T cells from patients with rheumatoid arthritis. *Arthritis Res Ther* 12 (2010) R81.

7. Yan Q, Li W, Tang Q, Yao S, Lv Z, Feng N, Ma X, Bai Z, Zeng Y, Qin D, Lu C. Cellular microRNAs 498 and 320d regulate herpes simplex virus 1 induction of Kaposi's sarcoma-associated herpes virus lytic replication by targeting RTA. *PLoS One* 8 (2013) e55832.
8. Alqurashi N, Gopalan V, Smith RA, Lam AK. Clinical impacts of mammalian target of rapamycin expression in human colorectal cancers. *Hum Pathol* 44 (2013) 2089-2096.
9. Lam AK, Gopalan V, Nassiri MR, Kasim K, Dissanayake J, Tang JC, Smith RA. Altered JS-2 expression in colorectal cancers and its clinical pathological relevance. *Mol Oncol* 5 (2011) 475-481.
10. Hamilton SR, Bosman FT, Boffetta P, Ilyas M, Morreau H, Nakamura SI, Quriq P, Riboli E, Sobin LH. Carcinoma of the colon and rectum. In: WHO classification of tumours of the digestive system, Bosman FT, Carneiro F, Hruban RH, Theise ND (eds). IARC Press, Lyon, France (2010) 134-146.
11. Gopalan V, Smith RA, Nassiri MR, Yasuda K, Salajegheh A, Kim SY, Ho YH, Weinstein S, Tang JC, Lam AK. GAEC1 and colorectal cancer: a study of the relationships between a novel oncogene and clinicopathologic features. *Hum Pathol* 41 (2010) 1009-1015.
12. Inui M, Martello G, Piccolo S. MicroRNA control of signal transduction. *Nat Rev Mol Cell Biol* 11 (2010) 252-263.
13. Bueno MJ, Pérez de Castro I, Malumbres M. Control of cell proliferation pathways by microRNAs. *Cell Cycle* 7 (2008) 3143-3148.
14. Foshay KM, Gallicano GI. Small RNAs, big potential: the role of MicroRNAs in stem cell function. *Curr Stem Cell Res Ther* 2 (2007) 264-271.

15. Wang X, El Naqa IM. Prediction of both conserved and nonconserved microRNA targets in animals. *Bioinformatics* 24 (2008) 325-332.
16. Wang X. miRDB: a microRNA target prediction and functional annotation database with a wiki interface. *RNA* 14 (2008) 1012-1017.
17. Osawa T, Muramatsu M, Wang F, Tsuchida R, Kodama T, Minami T, Shibuya M. Increased expression of histone demethylase JHDM1D under nutrient starvation suppresses tumor growth via down-regulating angiogenesis. *Proc Natl Acad Sci U S A* 108 (2011) 20725-20729.
18. Ikewaki N, Tamauchi H, Inoko H. Decrease in CD93 (C1qRp) expression in a human monocyte-like cell line (U937) treated with various apoptosis-inducing chemical substances. *Microbiol Immunol* 51 (2007) 1189-1200.

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Disclosure Statement

There is nothing to disclose for this manuscript.

Figure captions

Figure 1: Target prediction for miR-498 using miRDB target prediction tool (<http://mirdb.org/miRDB/>). Various targets of miR-498 were predicted computationally. Strong candidates (target score = 95-87) were noted as JHDM1D, NAP1L3, CD93, DCTN4, CCPG1, PDK3 and KCNK10. Among these, high association scores (target score = 95) were noted with cancer related genes such as JHDM1D and CD93 which have specific cellular functions such as cell growth, angiogenesis and apoptosis [15-16].

Figure 2: Down regulation of miR-498 in different colon cancer cell lines. Inverse ratio of miR-498 versus RNU6B (control miRNA) [expression ratio] was used to represent the miRNA expression levels in different cell lines. Cancer cell lines (SW480 and SW48) showed lower expression of miR-1288 compared to a normal epithelial cell line (FHC).

Figure 3: miR-498 transfection induced changes in expression and cell proliferation in colon cancer cell line (SW480). (A) The miR-498 expression levels in transfected and control cells. Significant difference in miRNA expression was noted in post-transfected cells with the miR-498 mimic. Expression levels were analyzed by fold change and were compared with FHC. (B) Expression levels of miR-498 at different time intervals (12, 24, and 48 h) of transfection. Expression levels were altered and the highest expression was noticed at 48 h of transfection with miR-498. (C) MTT calorimetric assay for detecting miR-498 induced suppression cell growth after day 1. Results were plotted on different intervals (days 1, 2 and 3) with mean value of absorbance. Decreased rate of absorbance was noticed in miR-498 transfected cells compared to control cells after day 1 of transfection.

Figure 4: Exogenous miR-498 enhances changes in cell cycle events. G₀–G₁, S, and G₂–M phases were obtained after flow cytometry analysis. Debris/dead cells were highlighted as dark green. Following miR-498 transfection, there was more accumulation of cells in the M phase than the controls. Also cells entering into the G₀–G₁ phase was significantly lower in cancer cells overexpressed with miR-498.

Figure 5: Reduced expression levels of miR-498 in colorectal tissues. The mean expression inverse ratio showed low or no expression in majority of cancer tissues compared to that of non tumour tissues. A small proportion of outliers show expression near the normal range.