MiR-498 in oesophageal squamous cell carcinoma: clinicopathological impacts and functional interactions

Running title: MicroRNA-498 in oesophageal cancer

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Abstract

MicroRNA-498 plays a crucial role in progression of many carcinomas. The signalling pathways that miR-498 modulates carcinogenesis are still unknown. Also, miR-498 associated molecular pathogenesis has never been studied in the oesophageal squamous cell carcinoma (ESCC). Herein, we aimed to examine the expression and functional roles of miR-498 in the ESCC as well as its influences on the clinicopathological features in patients with ESCC. Expression of miR-498 was investigated in 93 ESCC tissues and five ESCC cell lines using quantitative real-time polymerase chain reaction. *In vitro* effects of miR-498 on cellular process were studied followed by overexpression of miR-498. Western blot and immunofluorescence techniques were used to identify the interacting targets for *miR-498* in ESCC. miR-498 expression was significantly reduced in the ESCC when compared to the non-neoplastic oesophageal tissues ($p < 0.05$). Patients with low miR-498 expression showed different histological grading of cancer and survival rates when compared to the patients with high miR-498 expression. Overexpression of miR-498 in ESCC cell lines induced remarkable reduction of cell proliferation, barrier penetration and colony formation when compared to control and wild type counterparts. Also, miR-498 activated the FOXO1/KLF6 transcriptional axis in ESCC. In addition, miR-498 overexpression increased p21 protein expression and led to reduced cancer cell growth. To conclude, reduced expression of miR-498 in ESCC as well as *in-vitro* analysis have confirmed the tumour suppressor properties of miR-498 by modulating the FOXO1/KLF6 signaling pathway. The changes in miR-498 expression may have impacts on the clinical pathological parameters of ESCC as well as in the management of the patients with ESCC.

Keywords: miR-498; grade; survival; oesophageal; squamous cell carcinoma

1. Introduction

Oesophageal squamous cell carcinoma (ESCC) is one of the most common malignancy worldwide and is the most common subtype of oesophageal cancer in Asian countries [1-2]. miRNAs (19-25 nucleotides non-coding single stranded RNA) could play a critical role in the regulation of pathogenesis of the oesophageal cancer by regulating the expression of growth related target genes [3-6]. MicroRNA-498 (miR-498) is located intergenically in 19q13.42 and play critical roles in the pathogenesis of different carcinomas including colorectal, breast, lung, ovarian and thyroid as well as in acute myeloid leukaemia and Kaposi's sarcoma [7-15]. In addition, *FOXO1* (Forkhead box O1)/*KLF-6* (Kruppel-like factor 6) signalling pathway has been reported to play critical roles in regulating cellular growth, development, differentiation, apoptosis and proliferation as well in different cancers [16-22].

The functions of *FOXO1/KLF-6* transcriptional axis and the miR-498 mediated regulation of this transcriptional network in the pathogenesis of ESCC have been not been studied. Also, the clinical and functional roles of miR-498 in ESCC have not been reported previously. Thus, the present study aimed to examine the roles of expression of miR-498 in ESCC as well as its clinical pathological impacts.

2. Materials and Methods

2.1 Selection of patients

Cancer tissue and non-neoplastic tissues were obtained prospectively from the patients who underwent resection of primary ESCC (AKL and SL). Patients were recruited with no selection bias. Ethic approval was obtained from Griffith University (MED/19/08/HREC).

2.2 Clinicopathological characteristics

The ESCCs were graded and staged according to the World Health Organization criteria [23]. The carcinomas were staged as per the TNM (tumour, lymph node and metastases) classification. For each patient, clinical and pathological parameters including gender and age of patients as well as the sites, grades, pathological stages of the ESCC were recorded. The follow-up period was defined as the interval between the date of surgery for ESCC and the date of death or closing date of the study. Only cancer-related death was counted as the end point in the statistical analysis.

2.3 Cell lines

Five oesophageal squamous cell carcinoma cell lines - KYSE-520, KYSE-450, KYSE-150, KYSE-70 and HKESC-1 were used in this study. Also, a non-neoplastic squamous cell line (HACAT) was recruited as control. All the cell lines used were maintained according to the manufacturer's guidelines.

2.4 Extraction of miRNA and cDNA conversion

Prospectively collected ESCC tissues were sectioned. Haematoxylin and eosin stained sections were reviewed by the author (AKL). In cancers, blocks with > 70% cancer

were selected whereas in non-neoplastic mucosae, blocks with minimal stromal element were chosen. miRNA extraction from the tissues and the cultured cells were performed as previous reported [7, 24].

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

Alteration of miR-498 expression in different ESCC tissues and cell lines were examined as previously reported in colon cancer using qRT-PCR [7]. miR-498 expression was presented as expression ratio normalized by internal control miRNAs (RNU6B and RNU48) in tissues samples and in different ESCC and non-neoplastic cell lines. A fold change of >2 was considered as high miR-498 expression and <1 was considered as low miR-498 expression.

2.6 Transfection of oesophageal cancer cells with *miR-498* mimic

Oesophageal squamous cell carcinoma cells (KYSE-520 and KYSE-70) were cultured approximately at 2×10^4 cells/cm² into 24 wells plate. After 24 hours of seeding, cells were transfected with miR-498 mimic (KYSE-520^{+miR-498} and KYSE-70^{+miR-498}) at 10 nM concentrations according to the protocol published earlier [7].

2.7 Cell proliferation assay

Cell proliferation assay was performed to examine the effect of miR-498 mimic transfection on ESCC cell (KYSE-520 and KYSE-70) growth. For this experiment, a cell counting kit-8 (CCK-8) (Sigma-Aldrich, St Louis, MO, USA) was used. Cells were first seeded in a flat-bottom 96-well plate at 1×10^4 cells/well. After 24 hours of seeding, these cells were transfected with miR-498 mimic and scramble miRNA as previously described

[25]. Finally, proliferation of cells was determined on days 0 to 3 with CCK-8 following the manufacturer instructions.

2.8 Invasion assay

To investigate the ectopic expression effect of miR-498 mimic on *in vitro* cell penetration/invasion to a barrier, CultreCoat[®] 96 well basement membrane extract (BME)-coated cell invasion assay (Trevigen Inc., Gaithersburg, MD, USA) kit, consisting of basement membrane components was used as previously published [24].

2.9 Colony formation assay

To determine the effect of miR-498 overexpression on clonogenic capacity, equal numbers of cells were seeded in a 6-well plates with complete growth medium. The cancer cells (KYSE-520 and KYSE-70) were then transfected with the miR-498 mimic and scramble control miRNA and incubated at 37°C in 5% carbon dioxide and saturation humidity. After 17 days, when microscopic clones were noted in every single plate, growing of the cells was stopped. The colony formation capacity of cells was calculated as previously described [24].

2.10 Western blot analysis

To identify the target proteins that interact with miR-498, Western blot analysis was performed. Total proteins were extracted, quantified and separated by 15% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) [24]. Then, proteins were transferred to nitrocellulose membranes using Trans-Blot[®] Turbo transfer system (Bio-Rad, Hercules, CA, USA). After blocking, membranes were incubated with rabbit FOXO1 and p21 monoclonal antibodies (Cell signalling Technology Inc., Danvers, MA, USA) at 1:1000, rabbit polyclonal BCL2 antibody (Santa Cruz, Dallas, TX, USA) at 1:500, rabbit polyclonal

JHDM1D antibody (Santa Cruz) at 1:500, mouse monoclonal KLF6 antibody (Santa Cruz) at 1:500, mouse monoclonal KRAS antibody (Santa Cruz) at 1:500 and monoclonal GAPDH antibody (Santa Cruz) at 1:2000 overnight at 4°C. Membranes were then incubated with the anti-rabbit and anti-mouse secondary antibodies respectively (Santa Cruz) (1:5000) at room temperature for 2 hours. The proteins bands were developed, detected and analysed as previously published [24].

2.11 Immunofluorescence

To visualize localisation and the miR-498 mediated regulation of targets proteins, immunofluorescence analysis was performed. The ESCC cells (KYSE-520 and KYSE-70) were seeded, transfected and prepared for incubation with primary antibodies according to the published protocol [24]. Cells were then blocked with 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for one hour at room temperature. After that, the cells were washed with PBS and incubated with rabbit monoclonal FOXO1 and p21 antibodies (1:100), rabbit polyclonal JHDM1D antibody (1:100), mouse monoclonal KLF6 antibody (1:50), rabbit monoclonal BCL2 antibody (1:50) and mouse monoclonal KRAS antibody (1:50) at 4°C overnight. This was followed by 2 hours of incubation with anti-rabbit and anti-mouse secondary antibodies labelled with fluorescein isothiocyanate (FITC) fluorophore (Sigma-Aldrich) at room temperature. Finally, cover slips were mounted on glass slides with mounting media containing DAPI (4', 6-diamidino-2-phenylindole, a fluorescent nuclear stain). The sections were observed under a confocal microscope (Nikon A1R+, Nikon Inc., Tokyo, Japan).

2.12 Statistical analysis

All clinical, pathological, follow-up information and miRNA expression changes were computerized. Statistical analysis was performed using the Statistical Package for Social Sciences for Windows (version 24.0, IBM SPSS Inc., New York, USA). Significance level of the tests was taken at $p < 0.05$.

3. Results

3.1 Reduced expression levels of miR-498 in ESCC tissues and cells

The mean expression ratio of miR-498 was significantly lower in ESCC tissues when compared to control tissues ($p < 0.05$) (Figure 1A). The expression levels for miR-498 was noted to be significantly ($p < 0.01$) decreased in all the five oesophageal cancer cell lines when compared to non-neoplastic squamous cells (Figure 1B). The relative expression (ratio of expression) of miR-498 in non-neoplastic squamous cells was 1.873 ± 0.04 whereas the ratios of expressions were 0.77 ± 0.09 , 0.75 ± 0.11 , $.48 \pm 0.07$, 0.63 ± 0.09 and 0.63 ± 0.1 in the cancer cells (KYSE-520, KYSE-450, KYSE-150, KYSE-70 and HKESC-1 cells) respectively (Figure 1B).

3.2 Clinicopathological correlation and impact of patient survival

The mean follow-up period for the patients with ESCC was 38 months (range from 1 to 214 months). During the follow-up period, 28 patients survived, 65 died of cancer. The survival time of the patients with ESCC was correlated with the pathological staging ($p = 0.017$) (Figure 2A). In stage 3 cancers, the survival rates appeared to be correlated with the expression level of miR-498 (Figure 2B). The mean survival time of patients with low miR-498 expression was 70 months whereas that high expression of miR-498 was 95 months. In stage 4 cancers, similar pattern of survival rates and expression of miR-498 was noted. However, there were only 9 stage IV cancers which was too small to reach any conclusion. Thus, patients with advanced stages ESCC having lower *miR-498* expression had poorer survival rates when compared to that of high miR-498 expression.

A trend of miR-498 expression changes was noted in histological grades of patients with ESCC (Supplementary table 1). Majority of the low grade (well or moderate

differentiated) ESCCs showed lower expression of miR-498 when compared with high grade (poorly differentiated cancer) ESCCs (57% versus 35%) (Figure 2C & 2D). However, these associations couldn't reach statistical significance (Supplementary table 1).

3.3 Induced overexpression of *miR-498* in oesophageal cancer cells

Cells treated with the miR-498 mimic (KYSE-520^{+miR498} and KYSE-70^{+miR-498}) showed remarkable overexpression of miR-498 levels (shown as ratio of expression) when compared to the scramble control miRNA (KYSE-520^{miRScr} and KYSE-70^{miRScr}) and the non-transfected wild type (KYSE-520^{wildtype} and KYSE-70^{wildtype}) cells (Figure 3A & 3B). Approximately four-thousands fold overexpression was noted in the KYSE-70^{+miR-498} when compared to that of non-neoplastic squamous cell line after 24 and 48 hours of transfection (Supplementary figure S1). Similar trend of expression changes was also noted with the KYSE-520^{+miR-498} (Supplementary figure S1).

3.4 MiR-498 overexpression decreases the proliferation of oesophageal cancer cells

Cells growth from all the treated cell groups were measured at different time interval (day 0 to 3). The KYSE-520^{+miR-498} and KYSE-70^{+miR-498} cells showed a remarkable decrease in cell proliferation when compared to the other cells (KYSE-520^{miRScr}, KYSE-70^{miRScr}, KYSE-520^{wildtype} and KYSE-70^{wildtype}) (Figure 4A & 4B). The reduced proliferation of cells was noted in all three days of the experiment with a similar trend in all cancer cell lines. However, on day 1, the growth rate of miR-498 treated groups was significantly ($p < 0.01$) lower when compared to that of control (Figure 4A & 4B).

3.5 Reduced invasion of BME-barrier of cancer cells with miR-498 mimic transfection

The KYSE-520^{+miR-498} and KYSE-70^{+miR-498} cells showed a mild reduction in cell invasion properties. The relative fluorescence unit (RFU; which is proportional to the BME-barrier invading cells) from KYSE-520^{+miR-498} and KYSE-70^{+miR-498} cells was noticeably less when compared to that of the control cell groups (Figure 4C). In the KYSE-520 cells, RFU from the miR-498 mimic treated cells was 171±11 whereas RFU from scramble miRNA was 244±17. Similar results (183±16 versus 267±19) were also noted in the KYSE-70 cells (Figure 4C).

3.6 MiR-498 induced inhibition of the clonogenic capacity of oesophageal cancer cells

Following transfection, the oesophageal cancer cells (KYSE-520^{+miR-498} and KYSE-70^{+miR-498}) caused remarkably reduction of colony formation capacity in comparison to the control and non-transfected wild type oesophageal cancer cells (Figure 5A & 5B). The number of colonies in cells with exogenous miR-498 treatment groups were significantly ($p<0.01$) lower in comparison to the scramble miRNA treated (KYSE-520^{miRScr} and KYSE-70^{miRScr}) and un-transfected KYSE-520^{wildtype} and KYSE-70^{wildtype} cells (Figure 5 C & 5D).

3.7 MiR-498 modulates FOXO1/KLF6/p21 transcriptional axis

The target for miR-498 were predicted using miRDB target prediction tools (<http://mirdb.org/miRDB/>). Among the various targets, FOXO1, KLF6, JHDM1D, BCL2, p21 and KRAS protein expressions were examined in this study followed by miR-498 mimic transfection. In KYSE-70^{+miR-498} cells, FOXO1, KLF6 and p21 proteins expression were remarkably upregulated when compared to KYSE-70^{miRScr} and KYSE-70^{wildtype} cells (Figure 6A). The relative protein band intensity of FOXO1 and KLF6 expressions in the KYSE-

KYSE-70^{+miR-498} cells were 1.0 ± 0.07 , 1.13 ± 0.1 and 1.34 ± 0.05 , respectively (Figure 6B). On the other hand, the intensities of expression of the FOXO1, KLF6 and p21 proteins in the KYSE-70^{miRScr} cells were 0.58 ± 0.2 , 0.45 ± 0.04 and 0.43 ± 0.06 , respectively (Figure 6B). Almost similar results were noted in KYSE-520 cells (Figure 6A, 6C). The relative expressions for FOXO1, KLF6 and p21 proteins in KYSE-520^{+miR-498} when compared to KYSE-520^{miRScr} cells were 1.2 ± 0.08 versus 0.415 ± 0.14 and 1.94 ± 0.4 versus 1.24 ± 0.17 and 1.1 ± 0.04 versus 0.42 ± 0.1 , respectively (Figure 6C). There was no notable up or down regulation were observed in JHDM1D, BCL2 and KRAS expression in the KYSE-520^{+miR-498} and KYSE-70^{+miR-498} cells when compared to the control cells (Supplementary figure S2).

Alteration of FOXO1, KLF6, and p21 expressions upon miR-498 mimic transfection was further confirmed by immunofluorescence microscopy. Remarkable increases in the expressions (strong green signal) of cytoplasmic FOXO1, KLF6 and p21 protein were noted in KYSE-70^{+miR-498} cells when compared to that of control cells (Figure 7A). Similarly, higher FOXO1, KLF6 and p21 expressions were noted in the KYSE-520^{+miR-498} cells when compared to both controls (Figure 7B). There were no notable changes in the expression of JHDM1D, BCL2 and KRAS in the KYSE-70^{+miR-498} and KYSE-520^{+miR-498} cells when compared to the control cells (Supplementary figure S3).

4. Discussion

The current study has for the first time provided insights on the functional roles of the miR-498 and its clinical pathological impacts in the patients with ESCCs. We noted significant reduction ($p < 0.001$) of miR-498 expression in ESCC tissues and cell lines (Figure 1). Exogenous overexpression of miR-498 in ESCC induced tumour inhibitory properties *in vitro* by upregulating tumour suppressors *-FOXO1*, *KLF6* and *p21*. This suggests that the regulatory roles of the miR-498 in the pathogenesis of oesophageal squamous cell carcinoma by modulating FOXO1/KLF6 transcriptional axis.

Downregulation of miR-498 have been reported in different carcinomas [11, 14]. In this study, we observed that patients with advanced stages ESCC having low expression of miR-498 had shorter survival time when compared to those with high miR-498 expression. Thus, the reduced expression of miR-498 in ESCC tissues and cell lines and miR-498⁺ induced tumour inhibitor properties implies its regulatory effects in pathogenesis of ESCC. In addition, the expression of miR-498 correlates with the grade of ESCC which may indicate that there are 2 distinctive groups of ESCC in term of biological behaviour. It may imply that with identical pathological stage, lower histological grade (well or moderately-differentiated) squamous cell carcinomas are biological more aggressive than higher histological grade squamous cell carcinomas.

The inhibition of cancer cell proliferation following miR-498 over expression has been noted in colon carcinoma [7]. In addition, tumour suppressive cellular behavior of miR-498 was observed in ovarian cancer [15]. Thus, the miR-498 induced inhibition of ESCC cell proliferation in this study is consistent with the earlier findings. These results indicate that the regulatory roles of miR-498 expression are not cancer specific. In addition to reduced cell proliferation, other tumour suppressive *in vitro* properties such as reduced cell invasion and migration, reduced clonogenic capacity were noted in ESCC cells following miR-498

ectopic activation [Figures 3 to 5]. Significant reduction ($p < 0.01$) of colony number, cell proliferation in miR-498 treated ESCC cells implies that miR-498 plays important role in cancer development via modulating various check points in cell growth and development. Therefore, miR-498 acts as tumour suppressor in ESCC and plays a vital role in the pathogenesis of ESCCs by interacting and regulating various physiological and pathological processes in this cancer.

FOXO1, a member of FOXO family, acts as a tumor inhibitor in various cancers by inducing apoptosis, cell cycle arrest and oxidative and cellular stress [26]. It was demonstrated that FOXO1 mRNA stability was regulated by the binding of RNA-binding protein tristetraprolin (TTP) to its adenylate-uridylate-rich elements in fibroblast-like synoviocytes [27]. Also, the 3' UTR of FOXO1 mRNA contains miRNAs binding sites and it can be regulated by several microRNAs [26-28]. Using miRDB target prediction tool (<http://mirdb.org/miRDB/>), we noted that there is a putative binding site in the 3' UTR of FOXO1 mRNA to which miR-498 seed sequence can bind [29]. Thus, it is predicted that the increased expression of FOXO1 protein followed by miR-498 transfection in ESCC cells may be due to the increased stability of FOXO1 mRNA. The exogenous miR-498 can interact with FOXO1 mRNA and prevent from RNA-binding protein mediated degradation. This in turns increased FOXO1 expression.

KLF 6, a tumour suppressor and a downstream regulator of FOXO1, is involved in cancer cells growth, proliferation, apoptosis and colony formation [16-19]. It is predicted that miR-498 can interact with *KLF6* mRNA at the 3' UTR (<http://mirdb.org/miRDB/>). Thus, up-regulation of KLF6 could be due to the increasing the stability of KLF6 mRNA, which in turn, may lead to increase protein expression [30]. The increased expression of KLF6 protein in our present study may be associated with the direct interaction of miR-498 with its mRNA, or due to the downstream effects of increased expression of FOXO1 (upstream regulator of

KLF6) protein. *p21* (*CIP1/waf1*) is a cyclin-dependent kinase inhibitor (CKI), which inhibits the activity of cyclin-CDK2, CDK1 and CDK4/6 complexes as well as proliferating cell nuclear antigen (PCNA) [31-32]. Studies have demonstrated that p21 protein negatively regulated the growth and development oesophageal carcinomas [33-34]. In this study, we noted significant increment of p21 protein expression in miR-498 up regulated ESCC cells when compared to that of control cells. Such transfection of miR-498 in ESCC cells was followed by reduction of cancer enhancing activities including cellular proliferation, low colony formation, cell invasion and migration. This miR-498 attributed reduction in cancer enhancing activities might be mediated by the increase expression of p21 in these cells.

FOXO1 acts as a transcriptional regulator and suppresses tumour cell growth via regulation of the expressions of *KLF6*, *p21*, *p27* and *CCND1* genes [19]. In the current study, we noted miR-498 overexpression significantly increased expression of FOXO1, which in turn upregulates the expression of its downstream effectors - KLF6 and p21 proteins in the ESCC cells (Figure 6 & 7). It was reported that KLF6 inhibits cell growth and proliferation. This is due to the effect of upregulating p21/waf1 and repressing CCND1, thus disrupting the formation of CCND1/cyclin-dependent kinase 4 complex [35]. Thus, miR-498 induced inhibition of cell growth in ESCC might be due to the modulation of the levels of FOXO1, KLF6 and p21 (Figure 8).

5. Conclusions

In summary, we identified a transcriptional network regulated by miR-498 in ESCC. This involves *FOXO1*, *KLF6* and *p21* tumour suppressors which in-turn negatively regulates the cellular response in ESCC (Figure 8). MiR-498 was downregulated in ESCC and its overexpression triggered various downstream effects such as inactivation of ESCC cell proliferation, colony formation and cell invasion *in vitro*. These anti-oncogenic effects of miR-498 might be due to the upregulation of the tumor suppressor proteins such as FOXO1, KLF6 and p21 in ESCC. The expression of miR-498 was related to grade of the cancer as well as have implications on patients' survival rate in patients with ESCC. These novel findings imply that miR-498 expression plays a key role in the molecular carcinogenesis of ESCCs.

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Figure 1. Expression of miR-498 in ESCC and relationship of patient's survival with miR-498 expression.

A: In ESCC, miR-498 expression was significantly downregulated when compared with non-cancer oesophageal mucosa ($p < 0.05$).

B: ESCC cell lines (KYSE-520, KYSE-450, KYSE-150, KYSE-70 and KHEC-1) showed lower expression of miR-498 when compared to non-neoplastic HACAT cells. The results are shown as mean $\pm$ SD (Standard Deviation) from three independent tests. Level of significance ** $p < 0.001$ and * $p < 0.01$ was noted when compared with that of HACAT cells.

Figure 2. Clinical pathological correlations with expression of miR-498 in ESCC

A: The survival rates of patients with ESCC depend on the pathological stages of the cancer. Patients with lower staged cancers (stage 1 or stage 2) have better survival rates when compared to the patients with advanced staged cancers (stage 3 or stage 4).

B: Patients with Stage 3 ESCC having low miR-498 expression level had poorer survival rates than with high miR-498 expression levels.

C: Well-differentiated squamous cell carcinoma often shows low expression of miR-498 (haematoxylin & eosin, x 15)

D: Poorly-differentiated squamous cell carcinoma less often shows low expression of miR-498 (haematoxylin & eosin, x 15)

Figure3. Overexpression of *miR-498* in ESCC cell lines following exogenous transfection of *miR-498* mimic.

Expression level of *miR-498* in KYSE-520^{+miR-498} (A) and KYSE-70^{+miR-498} cell lines (B). Significant difference in miRNA expression was noted in post transfect cells with *miR-498* mimic. Expression levels were analysed in expression ratio. These were compared to the control cell lines (HACAT). A highest level of *miR-498* expression was noted after 24 hours of transfection and this duration was considered as the optimum for the downstream in vitro experiments. The results were shown as mean $\pm$ SD from three independent tests. Level of significance ^a $p < 0.01$ was noted when compared to that of HACAT cells.

Figure 4. Overexpression of miR-498 induced reduced cell proliferation and invasion in ESCC cells.

A-B: Results were plotted on different time intervals (day 0 to 3) with mean value of absorbance from three independent experiments. Both ESCC cells treated with miR-498 mimic (KYSE-520^{+miR-498} and KYSE-70^{+miR-498}) showed notable reduced cell proliferation when compared to the controls (KYSE-520^{miRScr} and KYSE-70^{miRScr}) and non-transfected wild type (KYSE-520^{wildtype} and KYSE-70^{wildtype}) counterparts (A&B) respectively. The decreased cell proliferation was noted in all three days for both cell lines. Interestingly, on 24 hours after transfection, the difference of absorbance was statistically significant among the groups. Level of significance **p<0.01 was noted when compared to that of control (KYSE-520^{miRScr}) cells and *p<0.05 when compared to the corresponding control (KYSE-70^{miRScr}) cells.

C: KYSE-520^{+miR-498} and KYSE-70^{+miR-498} cells showed decreased penetration of basement membrane extract (BME)-barrier following *miR-498* transfection. The BME-barrier invasion of cells was noted as relative fluorescence unit (RFU) and was noted lower in cells groups with *miR-498* over expression. A RFU of 171±11 was noted in KYSE-520^{+miR-498} cells compared to 244±17 in scrambled control (KYSE-520^{miRScr}) cells. Similar trends (183±16 versus 267±19) were also noted in case of KYSE-70 cells. Results are shown as mean ±SD. This difference couldn't reach statistical significance (p<0.05).

Figure 5. Effect of miR-498 overexpression on colony formation in ESCC.

A: Overexpression of miR-498 resulted in decreased colony formation in KYSE-70^{+miR-498} compared to control scramble miRNA treated (KYSE-70^{miRScr}) and non-transfected cells (KYSE-70^{wildtype}).

B: KYSE-520^{+miR-498} cells showed significant reduction of clonogenic capacity compared to that of controls (KYSE-520^{miRScr} and KYSE-520^{wildtype}).

C: Bar charts indicates that the number of colonies with miR-498 mimic (KYSE-70^{+miR-498}) were significantly ($p < 0.01$) lower in comparison to control and un-transfected cells.

D: Numbers of colonies generated from cells treated with miR-498 mimic (KYSE-520^{+miR-498}) and controls (KYSE-520^{miRScr} and KYSE-520^{wildtype}).

Results are shown as mean $\pm$ SD. Level of significance ** $p < 0.01$ when compared with that of control and untransfected cells.

Figure 6. miR-498 alters FOXO1/KLF6 tumour suppressor.

A: FOXO1, KLF6, and p21 expressions were changed upon miR-498 mimic transfection in the KYSE-70 and KYSE-520 cells. FOXO1, KLF6 and p21 proteins expression were upregulated in KYSE-70^{+miR-498} and KYSE-520^{+miR-498} cells when compared to un-transfected (KYSE-70^{wildtype} and KYSE-70^{wildtype}) and scramble control miRNA (KYSE-70^{miRScr} and KYSE-70^{miRScr}) transfected cells.

B: The bar graphs for relative expression of targets proteins in the KYSE-70 cells. The proteins band intensity of FOXO1, KLF6 and p21 in the miR-498 treated cells was noted to be increased significantly ($p < 0.001$) when compared to the controls (KYSE-70^{wild type} and the KYSE-70^{miRScr} [scramble control]).

C: Bar graphs of targets proteins band intensity (relative protein expression) in the KYSE-520 cells. Increased FOXO1, KLF6 and p21 protein expressions were observed in the KYSE-520^{+miR-498} cells when compared to that of the KYSE-520^{miRScr} and KYSE-520^{wildtype} cells. The results are shown as mean $\pm$ SD from three independent tests. Level of significance, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared to the control group.

Figure 7. Confirmation of miR-498 mediated alteration of targets protein via immunofluorescence microscopy.

FOXO1 and KLF6 proteins were predominantly expressed in the cytoplasm of ESCC cells whereas p21 was noted in the nucleus as well as within the cytoplasm. Similar to the Western blot analysis, increased level of FOXO1, KLF6 and p21 proteins expressions were noted in KYSE-70^{+miR-498} and KYSE-520^{+miR-498} cells. The results were compared to that of un-transfected (KYSE-70^{wildtype}/ KYSE-520^{wildtype}) and scramble control miRNA (KYSE-70^{miRScr}/ KYSE-520^{miRScr}) transfected cells.

Figure 8. Proposed **miR-498 mediated inhibition of ESCC cells growth.**

miR-498 mimic in the KYSE-70^{+miR-498} and KYSE-520^{+miR-498} cells interact with FOXO1 mRNA at 3' UTR and increased the stability of *FOXO1* mRNA. This in turn increased the expression of FOXO1 protein. FOXO1 protein then translocates to the nucleus which binds to the DNA and activates the transcription of downstream targets such as KLF6. KLF6 in turn, upregulated the expression of cell cycle regulator p21/waf1 expression. Thus, the transcriptional activation of this axis (FOXO1/KLF6) disrupts the formation of cyclin D 1/cyclin-dependent kinase 4/6 complex and causes reduced cellular proliferation, migration and colony formation.

Table 1. Expression pattern of miR-498 and its correlation with clinicopathological parameters of ESCC

Characteristics	Number	High Expression	Low Expression	p value
Gender				
Male	79 (84.95%)	39 (49.36%)	40 (50.64%)	0.258
Female	14 (15.05%)	5 (35.71%)	9 (64.28%)	
Age				
< 60	33 (35.48%)	16 (48.48%)	17 (51.51%)	0.519
≥ 60	60 (64.51%)	28 (46.66%)	32 (53.33%)	
Site				
Upper	10 (10.75%)	6 (60%)	4 (40%)	0.109
Middle	52 (55.91%)	28 (53.84%)	24 (46.15%)	
Lower	31 (33.33%)	10 (32.25%)	21 (63.63%)	
Size				
< 6cm	62 (66.66%)	26 (41.93%)	36 (58.06%)	0.106
≥ 6cm	31 (33.33%)	18 (58.06%)	13 (41.93%)	
Histological grade				
Well or moderate	76 (81.72%)	33 (43.42%)	43 (56.57%)	0.093
Poor	17 (18.27%)	11 (64.70%)	6 (35.29%)	
T staging				
T1&T2	9 (9.67%)	3 (33.33%)	6 (66.66%)	0.478
T3&T4	84 (90.32%)	41 (48%)	43 (51.19%)	
Lymph node metastasis				
Present	71 (76.34%)	35 (49.29%)	36 (50.7%)	0.330
Absent	22 (23.65%)	9 (40.90%)	13 (59.09%)	
Distant metastasis				
Present	9 (9.67%)	4 (44.44%)	5 (55.55%)	0.569
Absent	84 (90.32%)	40 (47.61%)	44 (52.38%)	
Pathological staging				
I & II	26 (27.95%)	10 (38.46%)	16 (61.53%)	0.639
III & IV	67 (72.04%)	34 (50.74%)	33 (49.25%)	

Supplementary Information

For

***MiR-498* alters FOXO1/KLF6 signaling in oesophageal squamous cell carcinoma**

Farhadul Islam, Vinod Gopalan, Alfred King-Yin Lam

Supplementary data 1: Fold changes of miR-498 expression in ESCC cell lines followed by mimic transfection at 24 and 48 hours of treatment compared to the control microRNA. We noted highest (~4000) fold changes of miR-498 expression at 24 hours of transfection in both cell lines.

Supplementary data 2: Expression of KRAS, BCL2, JHDM1D in ESCC cell lines followed by miR-498 transfection. No notable upregulation or downregulation was observed of these expressions in Western blot analysis among the cells type tested (A). Bar graphs representing relative expression of proteins in KYSE-70 (B) and KYSE-520 (C) cell lines. Results are shown as mean $\pm$ standard deviation (SD) from three individual measurements.

Supplementary data 3: KRAS, BCL2, JHDM1D expression in ESCC cell lines followed by miR-498 transfection by immunofluorescence. Similar to the Western blot, there was no remarkable modulation of expressions was noted in both ESCC cell lines in immunofluorescence microscopic analysis.