

ELUCIDATING THE ROLES OF miR-372 IN CELL PROLIFERATION AND APOPTOSIS OF NASOPHARYNGEAL CARCINOMA TW01 CELLS

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Aim: Deregulation of microRNA has been associated with cancer progression and the modification of cancer phenotypes could be achieved by targeting microRNA expression. This study aimed to determine the effects of miR-372 on cell progression and gene expression in nasopharyngeal carcinoma cell line, TW01. **Materials and Methods:** NPC TW01 cells were transfected with the miR-372 precursor molecules. Gene expression studies were conducted using RT-PCR assays for nine cancer related genes. The effects of miR-372 on cell proliferation, cell cycle arrest and apoptosis were also investigated. **Results:** Expression of miR-372 caused cell cycle arrest at the S phase that was accompanied by an overall decrease of cells entering the G2/M phase. miR-372 did not have any significant effect on apoptosis. Of the nine genes studied, four were up-regulated, namely CDKN1A, INCA1, LATS2 and BIRC5. The other five genes — CDK2, CCNA1, TP53, BAX and BCL2 were down-regulated by miR-372. **Conclusion:** This preliminary study indicated the tumor suppressing roles of miR-372 in cell cycle progression of TW01 cells, possibly via the down-regulation of CDK2 and CCNA1 as well as the up-regulation of CDKN1A and INCA1.

Key Words: apoptosis, microRNA, nasopharyngeal carcinoma, miR-372, CDK2, CCNA1.

Nasopharyngeal carcinoma (NPC) is common in areas such as Southern China and South East Asia. Populations originating from Hong Kong, Guangdong, Hainan, Guangxi, Hunan and Fujian Province have shown especially high incidence rates of NPC [1, 2]. In year 2007, NPC was recorded as the fourth most common cancers in Malaysia with a total of 900 cases registered with the National Cancer Registry. The Age-standardised Incidence Rate (ASR) for male was 6.4 and 2.3 per 100.000 population for males and females, respectively [3].

MicroRNAs (miRNAs) are 21 to 25 nucleotides small RNAs which function as gene regulators by pairing to the target mRNAs to execute post-transcriptional repression by either degradation of target mRNA or by preventing protein translation [4]. Although many human miRNAs have been discovered [5], the roles of most miRNAs remain unknown. Dysregulation of miRNAs could lead to great regulatory upheavals in the cell, perhaps leading to carcinogenesis [6]. For example, down-regulation of let-7 and miR-26a in NPC cells resulted in cell proliferation through up-regulation of c-Myc [7] and EZH2 [8], respectively. Down-regulation of miR-29c caused up-regulation of genes encoding extracellular matrix proteins which are associated with increased invasiveness and metastasis of NPC [9]. In addition, expression of miR-155 caused down-regulation of JMJD1A, which leads to poor prognosis of NPC patients [10].

miR-372 showed dual roles on cancer cells, acting as a tumor promoter as well as a tumor suppressor. Together with the oncogenic RAS, miR-372 allowed cell proliferation and stimulated neoplastic transformation phenotype although wild type p53 was activated with induction of p21cip1 [11]. Presence of miR-372 caused cells to be in-

sensitive to the cell cycle inhibitor and the CDK activity was not inhibited [11]. In AGS human gastric adenocarcinoma cell line, miR-372 played an oncogenic role in regulating cell proliferation, cell cycle and apoptosis via down-regulation of its target gene, LATS2 [12]. Expression of LATS2 resulted in the CDK inhibition and suppression of cell growth by blocking the G1/S transition [13]. On the contrary, miR-372 was found down-regulated in cervical carcinoma tissues and its expression down-regulated CDK2 and Cyclin A1 (CCNA1) resulted in the growth inhibition of HeLa cells and cell arrest in the S/G₂ phase [14]. In NPC TW01 cells, it was reported that miR-372 was down-regulated more than ten-fold in the presence of transforming growth factor beta 1 (TGF-β1) [15]. However, the roles of miR-372 in NPC have not been documented. Therefore, the present study aims to elucidate the role of miR-372 in NPC cell line, TW01.

MATERIAL AND METHODS

Transfection of miRNA-372 precursor into TW01 cells. NPC cell line, TW01 cells were cultured with DMEM/F12 (Invitrogen, USA) supplemented with 10% (v/v) fetal bovine serum (FBS). Transfection of Pre-miR™ hsa-miR-372 miRNA precursor molecules (Ambion, USA) into TW01 cells was carried out as described by the manufacturer's instruction using siPORT™ NeoFX™ Transfection Agent (Invitrogen, USA). The transfected cells were incubated in 5% CO₂ tissue culture incubator at 37 °C for 72 h. The same procedure was repeated for control (or untransfected TW01) where by miR-372 precursor in the transfection complexes was replaced with nuclease-free water.

MicroRNA isolation and MicroRNA assay. In order to determine the level of miR-372 in the transfected cells, miRNA was isolated from the transfected and untransfected TW01 cell using MirVana™ miRNA Isolation Kit (Ambion, USA). The isolated miRNA was converted to cDNA using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, USA) via MyCy-

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Abbreviations used: miRNA – microRNA; NPC – nasopharyngeal carcinoma.

cler Thermal Cycler (Bio-Rad, USA). The level of miR-372 in transfected cells was determined by conducting quantitative real-time PCR in the iQ5-Cycle (Bio-Rad, USA) using TaqMan® MicroRNA Assay and TaqMan® Fast Advanced Master Mix from Applied Biosystems, USA. The fold change was calculated based on $\Delta\Delta CT$ and SNORD44 was used for normalisation.

Cell progression assays. Transfected and untransfected (control) TW01 cells were subjected to cell progression assays, namely cell proliferation, cell cycle analysis and apoptosis assay. Cell proliferation assay was conducted using CellTiter 96® Aqueous One Solution Cell Proliferation Reagent (Promega, USA) and absorbance was measured at a wavelength of 490 nm using a microplate reader (Opsys, Dynex Technologies, USA).

For cell cycle analysis, the cells were fixed with 70% ethanol (v/v) (Merck, USA) for a week at 4 °C and rinsed twice with PBS. A solution containing RNase A (Sigma-Aldrich, USA) and propidium iodide (Sigma-Aldrich, USA) was added and the mixture was left in the dark for 30 min at room temperature. Cell cycle analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, USA) which measured the cell fluorescence at 536 nm excitation and 617 nm emissions.

In order to determine the apoptotic effect of miR-372 on TW01 cells, transfected and untransfected cells were induced to undergo apoptosis by adding Staurosporine (Sigma-Aldrich, USA). The cells were incubated at 37 °C for 3 h and subjected to the Caspase 3 Assay (Sigma-Aldrich, USA). Absorbance was measured at 405 nm using a microplate reader (Tecan, Switzerland). The final concentration of the pNA released was calculated based on the p-Nitroaniline standard curve. The apoptotic index of the transfected and untransfected TW01 cells was calculated as follows: apoptosis index = pNA induced/ pNA non-induced.

Gene expression assays. Gene expression assays were conducted for nine genes with primers purchased from Applied Biosystems, USA (Table). RNA was extracted from transfected and untransfected TW01 cells using RNeasy Mini Kit (Qiagen, Germany). RNA quantification was performed using NanoQuant™ (Tecan, Switzerland). Gene expression assay was performed using TaqMan® RNA-to-CT™ 1-Step Kit (Applied Biosystems, USA) via the iQ5-Cycle (Bio-Rad, USA). The fold change was calculated based on $\Delta\Delta CT$ and GAPDH was used for normalisation.

Table. List of genes studied for gene expression in TW01 cells using Taqman primers from Applied Biosystems (USA)

Genes	Description (Taqman primer code)
<i>CDK2</i>	Cyclin-dependent kinase 2 (Hs01548894_m1)
<i>CDKN1A</i>	Cyclin-dependent kinase inhibitor 1A (Hs00355782_m1)
<i>CCNA1</i>	Cyclin A1 (Hs01652223_m1)
<i>INCA1</i>	Inhibitor of CDK, cyclin A1 interacting protein 1 (Hs00171105_m1)
<i>TP53</i>	Tumour protein p53 (Hs01034249_m1)
<i>LATS2</i>	Large tumour suppressor, homologue 2 (Hs00324396_m1)
<i>BAX</i>	B-cell CLL/lymphoma 2 – associated X protein (Hs00180269_m1)
<i>BCL2</i>	B-cell CLL/lymphoma 2 (Hs00608023_m1)
<i>BIRC5</i>	Baculoviral IAP repeat containing 5 (Hs00153353_m1)

Statistical analysis. All statistical analyses were performed using the software SPSS 17.0 (SPSS Inc., USA). Student t-test was used to analyze data of cell proliferation and apoptosis assays whereas Mann – Whitney U test was used to analyze data of cell cycle analysis.

RESULTS AND DISCUSSION

Based on the results obtained from the TaqMan® MicroRNA Assay, the level of miR-372 in transfected TW01 cells was determined to be more than 100.000 folds compared to untransfected cells. The high level of miR-372 caused significant decrease in cell proliferation of TW01 cells ($p < 0.001$, Fig. 1). As shown in Fig. 2, high level of miR-372 resulted in significant increase of cells at the S phase (1.74%, $p \leq 0.05$) and a reduction of cells in both the G2/M phase (1.97%, $p \leq 0.05$) and the G0/G1 phase (0.75%, $p > 0.05$). A slight decrease in apoptotic index was observed in TW01 cells transfected with miR-372, however, it was not statistically significant ($p > 0.05$, Fig. 3).

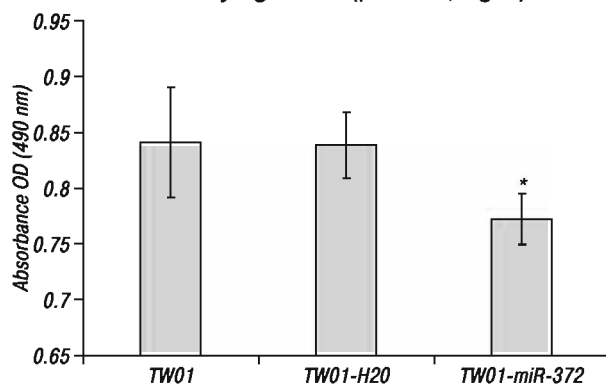


Fig. 1. Effects of miR-372 on cell proliferation of NPC TW01 cells. Cells were transfected with miR-372 precursor. Control consisted of TW01 cells exposed to transfection agent without the presence of miR-372 precursor. Cell proliferation assay was conducted using CellTiter 96® Aqueous One Solution Cell Proliferation Reagent (Promega, USA) as described in the Methodology. Statistical analysis was conducted using student t-test. * $p \leq 0.001$ (n = 9)

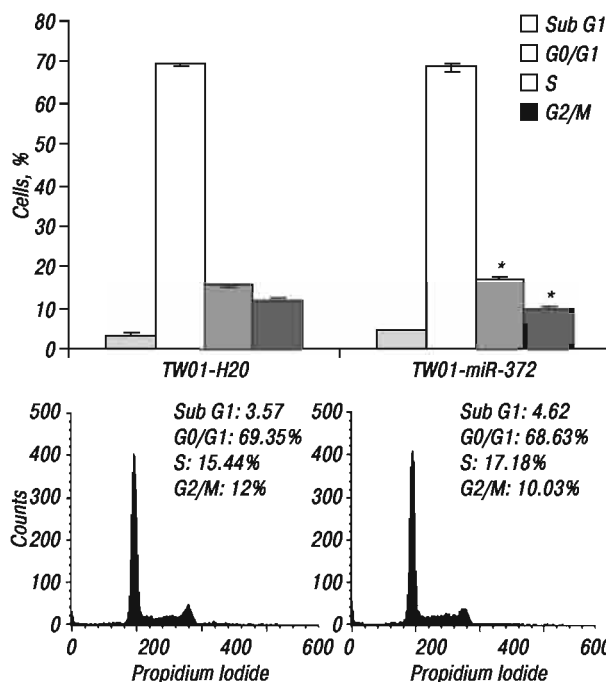


Fig. 2. Effects of miR-372 on cell cycle progression of TW01 cells. Cell cycle analysis was done using propidium iodide staining coupled with flow cytometry as described in Methodology. Statistical analysis was conducted using the Mann – Whitney U test. * $p \leq 0.05$ (n = 3)

Fig. 4 shows that four genes, namely *CDKN1A*, *INCA1*, *LATS2* and *BIRC5* were up-regulated in the transfected TW01 cells as compared to untransfected cells. However,

five genes including *CDK2*, *CCNA1*, *TP53*, *BAX* and *BCL2* were down-regulated by miR-372. miR-372 was reported to target both *CDK2* and *CCNA1* by direct binding to their 3'UTR regions [14]. Down-regulation of both genes might lead to the significant decrease in TW01 cell proliferation as well as S phase cell arrest, observed in the present study. *CCNA1* and *CDK2* were reported to function as activators of transcription factor E2F1, which plays a critical role in the expression of genes required for cell progression [16]. E2F-mediated transcription could be repressed by retinoblastoma protein (pRB), a tumor suppressor protein which inhibitory effect could be abolished by its phosphorylation with *CCNA1/CDK2* [17]. Moreover, our observations were consistent with the report by Tian *et al.* [14] which stated that the expression of miR-372 in HeLa cells caused cell arrest at S phase and a decreased entrance of cells into G2/M phase via the down-regulation of *CDK2* and *CCNA1*. Hence, this indicating that both *CCNA1* and *CDK2* were targets of miR-372 in NPC TW01 cells.

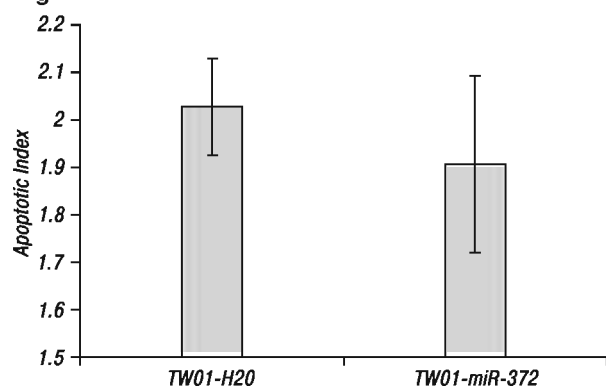


Fig. 3. Effects of miRNA-372 on apoptosis of TW01 cells. Apoptosis was induced by adding staurosporine. Cells were subjected to the caspase 3 assay and the apoptotic index of the transfected group as well as the untransfected TW01 cells was calculated as described in Methodology. Statistical analysis was conducted using student t-test ($n = 9$)

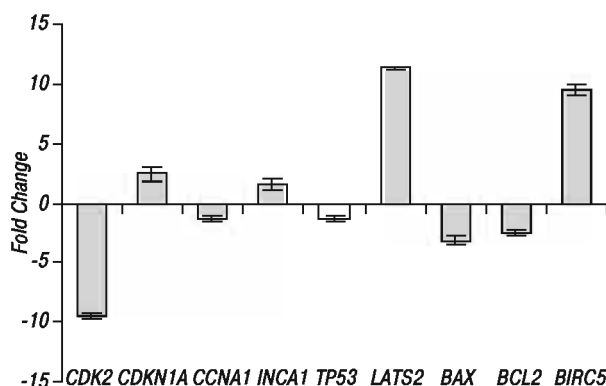


Fig. 4. Effects of miR-372 on the expression of *CDK2*, *CDKN1A*, *CCNA1*, *INCA1*, *TP53*, *LATS2*, *BAX*, *BCL2* and *BIRC5* in TW01 cells. The CT values of each genes obtained from qRT-PCR were normalised against housekeeping *GAPDH*. Fold change value greater than zero indicates up-regulation of gene whilst value lesser than zero indicates down-regulation of gene

It was interesting to note that *LATS2* was up-regulated (11.29-fold) in TW01 cells when transfected with miR-372 precursors, although *LATS2* was reported as a target of miR-372 in human testicular germ cell tumors [11], gastric adenocarcinoma [12], and colorectal carcinoma [18]. In addition, *LATS2* was also reported to be associated with

pRB-induced silencing of E2F target genes [19]. Even though *LATS2* has been reported to have tumor suppressive roles [13, 20]; it was reported to induce tumorigenesis of NPC [21]. Down-regulation of *LATS2* in 5–8F and CNE2 cells caused the inhibition of growth, induction of apoptosis and S phase cell arrest, while expression of *LATS2* in NP69 cells increased cell proliferation [21].

In addition, *CDKN1A* (or p21) and *INCA1* were noted to be up-regulated in transfected TW01 cells. *CDKN1A* and *INCA1* are known inhibitors to the Cyclin/CDKs complexes [22, 23]. *INCA1* binds to *CDK1*- and *CDK2*-bound cyclins via a cyclin interaction domain, which is important to regulate the CDK inhibitory and the proliferation inhibitory activities [23]. *CDKN1A* is known to bind to CDK and directly inhibiting the activity of CDK on pRB, hence, preventing the release of transcriptionally active E2F [24]. It was also reported that *CDKN1A* could affect E2F activity via a pRB-independent pathway [25]. Although *TP53* was reported to initiate the transcription of p21, it was interesting to note that *TP53* was down-regulated while p21 was up-regulated. This phenomenon was not unusual as the *TP53*-independent up-regulation of p21 has also been reported in the presence of tumor suppressor genes such as *Chk2* [26] and *PML* [27].

Down-regulation of the tumor suppressor *TP53* and pro-apoptotic *BAX* gene as well as up-regulation of anti-apoptotic gene (*Survivin*, *BIRC5*) were observed in the transfected TW01 cells. *TP53* exhibits anti-neoplastic activity through the induction of apoptosis. *TP53* could increase the permeability of *BAX* protein into mitochondria and thus, leading to the apoptotic program [28]. *BAX* has been reported to be a death effector and it is needed to induce mitochondrial dysfunction and death [29]. On the other hand, *BIRC5* which was up-regulated has been reported to be associated with distant metastasis and poor prognosis in NPC [30, 31]. It was noted that *BIRC5* was up-regulated in the background of reduce population of cells in G2/M phase although *BIRC5* has been reported to be expressed mostly at G2/M phase [32, 33]. At this point, we could not explain the mechanism that has resulted in this phenomenon, although it might be related to the CDK inhibition. It was reported that suppression of Thr34 phosphorylation of *BIRC5* would abolish its anti-apoptotic activity [34]. Loss of phosphorylation might result in dissociation of *survivin*-caspase 9 complexes, leading to apoptosis or death of cancer cells at mitosis [35]. Hence, in this study, high level of miR-372 did not cause any significant effect on apoptosis (see Fig. 3). This observation might also be due to the down-regulation of *BCL2* which is an anti-apoptotic gene. The death-protective activity of *BCL2* is associated with an inhibition of cell progression due to a prolong duration of G_1 phase. It was observed that cells which lacked *BCL2* expression would die from any stage of the cell cycle in the presence of apoptotic agents [36]. It was reported that in advanced staged NPC patients, those with negative *BCL2* expression had a better disease-free survival rate as compared to patients with *BCL2* positive tumors [37]. Therefore, it was suggested that the interaction between genes, namely *TP53*, *BAX*, *BIRC5* and *BCL2* could have antagonised and neutralised each other [38],

thus explaining the marginal change in apoptotic indices between the control and transfected TW01.

Finally, this study showed that expression of miR-372 decreased cell proliferation and induced S phase cell cycle arrest in TW01 cells. These effects could perhaps, be attributed to the down-regulation of CDK2 and CCNA1 by miR-372. There was no significant effect of miR-372 on the apoptosis of TW01 cells. The study provides a preliminary overview of the potential application of miR-372 in targeting CDK2 and CCNA1 in NPC.

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