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Reduced miR-100 expression in cervical cancer and precursors and its carcinogenic effect through targeting PLK1 protein

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ABSTRACT

Aim: Although aberrant miRNAs expression has been documented, altered miR-100 expression in cervical cancer and precursor tissues and its carcinogenic effect and mechanism remain unexplored. The aim of our study was to investigate the role of miR-100 alteration in cervical carcinogenesis.

Methods: The expression of miR-100 was examined by quantitative real-time reverse transcriptase PCR (qRT-PCR) in 125 cervical tissues including normal cervical epithelium, cervical intraepithelial neoplasia (CIN), and cervical cancer, as well as in five cervical cell lines. Through modulating miR-100 expression using miR-100 inhibitor or mimic *in vitro*, cell growth, cycle and apoptosis were tested separately by MTT or flow cytometry and meanwhile Polo-like kinase1 (PLK1) mRNA and protein expressions were detected by qRT-PCR and immunoblotting. The expression of PLK1 in 125 cervical tissues was also examined by immunohistochemical staining and the correlation between miR-100 and PLK1 expression in the same tissues was analysed. Finally, HPV-16 E6/E7 expression was modulated by gene transfection and subsequently the level of miR-100 was examined by qRT-PCR.

Results: The miR-100 expression showed a significantly and gradually reduced tendency from low-grade CIN, high-grade CIN to cervical cancer tissues and a significant decrease in HPV positive cervical cancer cell lines. The modulation of miR-100 expression remarkably influenced cell proliferation, cycle and apoptosis, as well as the level of PLK1 protein, but not mRNA, *in vitro* experiments. PLK1 expression was negatively correlated with miR-100 expression in CIN3 and cervical cancer tissues. The modulation of HR-HPV E6/E7 expression did not change miR-100 level.

Conclusions: The reduced miR-100 expression participates in the development of cervical cancer at least partly through loss of inhibition to target gene PLK1, which probably occurs in a relative late phase of carcinogenesis. HR-HPV E6/E7 may not directly regulate miR-100 expression in cervical cells.

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1. Introduction

Cervical cancer (CC) is the second most common cancer following breast cancer in women worldwide, especially in developing countries. Persistent infection of high-risk human papillomavirus (HR-HPV) has been recognised as a necessary cause of cervical carcinogenesis. The development of cervical cancer induced by HR-HPV is the consequence of the multistep factors involving diverse activation of proto-oncogenes, inactivation of tumour suppressor genes, epigenetic alterations, genomic instability, increase of telomerase activity, immune evasion and others.[1,2] HR-HPV firstly infects the basal layer cells of epithelia in microlesions. Following host cell proliferation and differentiation, viruses initiate replication and spread into other cells. However, for some occasions that are still unclear, HR-HPV genome integrates into the host DNA, subsequently leading to the activation of viral oncogenes E6/E7 and eventually undergoing malignant transformation of host cells. It has been known that cervical carcinogenesis is a progressive process from normal tissues throughout CIN to cervical caner. However, the mechanisms by which viral oncogenes of HR-HPV induce the process of cervical carcinogenesis are not yet fully uncovered up to date.

MicroRNAs (miRNAs) are a sizable class of 19–25nt longs RNA molecules, most of which can negatively regulate target mRNAs in diverse biological processes, including cell proliferation, differentiation, development, metabolism and death. Accumulated evidences have demonstrated that miRNAs are often aberrantly expressed in a number of human malignancies and play key roles in tumour initiation and development. As a potential tumour suppressive miRNA, the reduction of miR-100 expression has been currently found in some tumours, such as low-grade bladder cancer,[3] oral cancer,[4] ovarian cancer[5] and hepatocellular carcinomas.[6] However, miR-100 expression and role in cervical lesions remain uncertain. To investigate the association between abnormal miRNA expression and cervical carcinogenesis, we recently identified miRNA expression profiles by microarray in normal cervical epithelium, HPV-16 infected CIN 2–3 and HPV-16 infected cervical cancer. The Chip result, validated by qRT-PCR, showed several differentially expressed miRNAs in three groups of cervical tissues. In a set of down-regulated expression of miRNAs, we found that miR-100 expression was remarkably decreased.[7] Considering aberrant expression of miR-100 is intimately associated with several virus infections,[8] we, therefore, presumed that reduction of miR-100 expression was involved in progressive process of cervical carcinogenesis induced by HR-HPV infection.

To elucidate our hypothesis, we examined the level of miR-100 expression by qRT-PCR in 125 cases of cervical tissues, including normal cervical epithelium, CIN, and cervical cancer and evaluated the correlation between miR-100 expression and cervical carcinogenesis. We further detected and modulated the expression of miR-100 in human normal epithelial cell line and cervical cancer cell lines to investigate miR-100 effect on the carcinogenetic processes in the cells. In addition, we validated a predicted target gene PLK1 for miR-100 by *in vitro* experiments and cervical tissues and finally investigated possible link between miR-100 and HR-HPV E6/E7. The aim of our study was to explore the role and mechanism of miR-100 during the processes of cervical carcinogenesis induced by HR-HPV infection.

2. Materials and methods

2.1. Patients and tissue specimens

The use of all tissue specimens was approved by the Hospital Research Ethics Committee. A total of 105 cervical tissues were collected, which were derived from the patients undergoing colposcopy and biopsy in cervical lesion clinic of Women's Hospital, Zhejiang University School of Medicine from January 2009 to March 2010. The informed consent was obtained from the patients. All tissues were histologically diagnosed by two senior pathologists in a double-blind manner and divided into three groups, including HR-HPV infected normal cervical epithelium ($n = 20$), CIN ($n = 43$) and cervical caner ($n = 42$). In 42 cervical cancer tissues, 35 were Stage I and 7 Stage II (International Federation of Obstetrics and Gynaecology, 2000). Again, cervical tissues ($n = 20$) with normal histology and negative HR-HPV DNA were obtained as controls, which were from patients undergoing hysterectomy due to benign gynaecologic diseases. All collected tissues were immediately snap frozen in liquid nitrogen after surgical removal and stored at −70 °C until used.

2.2. Cell culture

The HPV negative human normal epithelial cell line Hacat was cultured in Dulbecco's modified Eagle medium (Gibco) with 10% foetal bovine serum and 1% penicillin/streptomycin (Invitrogen). The HPV-16 positive cervical cancer cell line Siha and Caski, the HPV-18 positive cervical cancer cell line Hela and the HPV negative cervical cancer cell line C33A were cultured separately in RPMI 1640 medium (Gibco) with 10% foetal bovine serum and 1% penicillin/streptomycin. All cells were grown at 37 °C in a humidified incubator with 5% CO_2. All the cell lines were purchased from the American Type Culture Collection (ATCC).

2.3. MiR-100 inhibitor and mimic

MiR-100 hairpin inhibitor (single-stranded chemically modified oligonucleotides) and miR-100 mimic (double-stranded oligonucleotides) were purchased from Thermo Scientific Dharmacon. Hacat, Siha, and Caski cells were cultured separately in plates overnight, then transfected with miR-100 inhibitor or miR-100 mimic to under-express or over-express the miR-100 level using DharmaFECT 1 reagent (Thermo Scientific Dharmacon) at a final concentration of 50 nM based on manufacturer's instruction. The relative level of miR-100 in transfected cells was examined by qRT-PCR (Supplementary Fig. 1). At the indicated times after transfection, cells were harvested and used to further study. Mock-transfected cells served as blank control and negative control inhibitor

or mimic (Thermo Scientific Dharmacon) was transfected as negative control.

2.4. qRT-PCR for mature miR-100

SYBR Green-based real-time quantification of miRNAs was used to determine the matur-miR-100 expression as previously described.[9,10] Briefly, the RNAs were reverse-transcribed to cDNA with a miR-100-specific stem-loop-like RT prime following the manufacturer's protocol. The RT prime for miR-100 is 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCG-CACTGGATACGACCACAAG-3′. Then, qRT-PCR was performed using SYBR Green (Takara) with primers specific to miR-100. The forward sequence was 5′-GAGCCAACCCGTAGATCCGA-3′. The reverse primer was common to all miRNAs and the sequence was 5′-GTGCAGGGTCCGAGGT-3′. The accuracy of mature-miR-100 sequence was identified by cloning and sequencing. Small nucleolar RNA RNU6 was used as endogenous control. Relative quantification of the miRNA expression was calculated with the $2^{-\Delta\Delta CT}$ method.

2.5. qRT-PCR for mRNA

The cDNAs were synthesised using an RNA reverse transcription amplification kit (Takara). Then, qRT-PCR for mRNA was performed using SYBR Green (Takara) detection. All primers used for qRT-PCR were listed: for PLK1, forward 5′-GGCAACCTTTTCCTGAATGA-3′, antisense 5′-AATGGACCAC ACA TCC ACCT-3′; for HPV-16 E6, forward 5′-CTGCAAGCAACA GTTACTGC-3′, antisense 5′-GGCTTTTGACAGTTAATACACC-3′; for HPV-16 E7, forward 5′-CATGGAGATACACCTACATTGC-3′, antisense, 5′-CACAACCGAAGCGTAG AGTC-3′. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as endogenous control. Relative quantification of the mRNA expression was calculated with the $2^{-\Delta\Delta CT}$ method.

2.6. Cell proliferation analysis

Hacat (3×10^3 cells/well), Siha (6×10^3 cells/well) and Caski cells (4×10^3 cells/well) were separately cultured in 96-well plates overnight, then transfected with miR-100 inhibitor or mimic as described above. At 24, 48 and 72 h after transfection, cell growth activities were examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. The absorbance of samples was recorded at 490 nm using a microplate spectrophotometer (ELx800, Bio-Tek).

2.7. Cell cycle and apoptosis analysis

Hacat (5×10^4 cells/well), Siha (1×10^5 cells/well) and Caski cells (7×10^4 cells/well) were cultured separately in 12-well plates overnight, then transfected with miR-100 inhibitor or miR-100 mimic as described above. At 72 h following transfection, a flow cytometer was used to determine cell cycle distribution and detect cell apoptosis. Briefly, for cell cycle analysis, cells were fixed in 70% ethanol for 48 h at −20 °C. Then, cells were stained with a propidium iodide solution containing 100 ug/ml DNase-free RNase A, 0.2% Triton X-100 and 50 ug/ml propidium iodide (PI) in PBS for 1 h at 37 °C. For cell apoptosis, apoptosis cells were stained using annexin-V-FITC and PI according to the manufacturer's protocol.

2.8. Western-blots analysis

Transfected Hacat, Siha and Caski cells were harvested; then, cells were lysed in lysis buffer. Western-blots were done as described previously. The membranes were incubated with primary antibodies specific for PLK1 (diluted 1:600, ABGENT) and β-actin (diluted 1:5000, Sigma).

2.9. Immunohistochemistry for PLK1

The expression of PLK1 protein was detected by immunohistochemical staining in 125 cases of cervical tissues that were simultaneously used to examine the level of miR-100 expression. Briefly, all samples were incubated with antihuman PLK1 antibody (AP7937a, ABGENT) at 1:200 dilutions for 1 h at room temperature as described previously.[11] The immunohistochemical staining of each sample was scored based on staining intensity and percentage of the cells stained. In detail, the staining intensity was assigned a rating from 0 to 3: 0 means negative in cells; 1 weakly positive; 2 moderately positive; and 3 strongly positive. The percentage of positive cells was quantitated in four randomly selected fields and graded using following five categories: 0 = 0–9% of cells positively stained; 1 = 10–24% of cells positively stained; 2 = 25–49% of cells positively stained; 3 = 50–74% of cells positively stained and 4 = 75–100% of cells positively stained. Finally, the intensity score and percentage score were multiplied to yield an overall score. According to overall score, immunohistochemical reactivity for PLK1 expression in each sample was classified into 4 groups: 0 – was defined as 'negative'; 4 – defined as 'weak'; 6 – defined as 'moderate' and 8 – defined as 'strong'.

2.10. HPV-16 E6/E7 transfection and interference

On the basis of our previous study,[12] we constructed a pcDNA3.1/NT-GFP-TOPO-HPV-16E6/E7 expressed vector (Invitrogen). The expression of HPV-16 E6/E7 was confirmed by RT-PCR (Supplementary Fig. 5A). In brief, Hacat cells were plated at a density of 1×10^5 cells per well in 6-well plates and cultured overnight. Then, cells were transiently transfected with recombinant vector containing the HPV-16 E6/E7 gene or empty vector using GenJet™ reagent (SignaGen) according to manufacturer's protocol. At 24, 48 and 72 h after transfection, cells were harvested and used to further study. Mock-transfected cells (blank control) and cells transfected with pcDNA3.1/NT-GFP-TOPO empty vector (negative control) served as controls.

We also synthesised a HPV-16 E6/E7 promoter-targeting siRNA sequence based on our previous study.[13] A pLenti6/BLOCK-iT™-DEST system (Invitrogen) was used to transfect the promoter-targeting siRNA into HPV-16 positive Siha and Caski cells according to manufacturer's instruction. The inhibition rates of HPV-16 E6/E7 were identified by qRT-PCR (Supplementary Fig. 5B). Mock-transfected cells served as blank control and a scrambled oligonucleotides siRNA was used as a negative control.

2.11. Statistical analysis

All statistical analyses were performed using SPSS 16.0 for windows software. Data were presented as the mean ± SE. P-values less than 0.05 were considered to be statistically significant (* means <0.05, ** represents <0.01).

3. Results

3.1. The miR-100 expression was reduced in CIN and cervical cancer, as well as cervical cancer-derived cell lines

In three groups of cervical tissues, a gradually reduced expression of miR-100 was observed from normal cervical epithelium throughout CIN ($P = 0.015$) to cervical cancer ($P = 6.13E-5$) and cervical cancer also exhibited significantly decreased expression of miR-100 compared to CIN ($P = 0.014$), as shown in Fig. 1A. In different grades of CIN, including CIN 1 ($n = 14$), CIN 2 ($n = 13$) and CIN 3 ($n = 16$), the level of miR-100 in CIN 3 was significantly lower than that in CIN 1 ($P = 0.001$) and CIN 2 ($P = 0.044$), but there was no difference ($P = 0.662$) between CIN 1 and CIN 2, as shown in Fig. 1B. Our findings showed that the tendency of reduced miR-100 expression was consistent with that of cervical lesion progression, suggesting that miR-100 probably contributes to cervical carcinogenesis.

In addition, we examined the level of miR-100 expression in one HPV negative human normal epithelial cell line Hacat and four cervical cancer-derived cell lines, including HPV16 positive Siha and Caski, HPV18 positive Hela, and HPV negative C33A. The result showed that miR-100 expression was remarkably reduced in all HPV positive cervical cancer cell lines, but not in HPV negative C33A cells. In detail, miR-100 level was 89.66% reduction in Caski cells, 83.57% in Siha cells and 40.76% in Hela cells compared with that in Hacat cells (Fig. 2A), suggesting that reduction of miR-100 expression might be associated with HR-HPV infection.

3.2. Altered miR-100 expression influenced growth, cell cycle, and apoptosis of cervical cells

To investigate the role of miR-100 in cervical carcinogenesis, we modulated miR-100 expression and examined the influence of aberrantly expressed miR-100 on cell growth, cycle and apoptosis in cervical cell lines. Firstly, we measured cellular proliferation rate using the MTT assay after the cells were transfected with miR-100 inhibitor or mimic for 24, 48 and 72 h. No significant change in the proliferation rate was observed in Hacat, Siha and Caski cells at 24 h after transfection. However, Hacat cells treated by miR-100 inhibitor showed a 16% and 19% increased rate of growth compared with blank control ($P = 0.032$) and negative control ($P = 0.02$) at 48 h and a 15% and 16% increased compared with blank control ($P = 0.019$) and negative control ($P = 0.017$) at 72 h (Supplementary Fig. 2A). Conversely, at the same two time points after transfection with miR-100 mimic, the cell proliferation was significantly decreased in both Siha and Caski cells (Supplementary Fig. 2B and C). In detail, overexpression of miR-100 resulted in a 22% and 17% decrease in cellular proliferation rate of Siha compared with blank control ($P = 0.002$) and negative control ($P = 0.011$) at 48 h, a 24% and 22% decrease compared with blank control ($P = 0.005$) and negative control ($P = 0.009$) at 72 h. Similarly, Caski cells treated by miR-100 mimic showed a 24% and 19% decrease in cellular proliferation rate compared with blank control ($P = 0.014$) and negative control ($P = 0.048$) at 48 h and a 31% and 21% decrease compared with blank control ($P = 0.003$) and negative control ($P = 0.032$) at 72 h.

Furthermore, an accelerated cell cycle progression at the G2/M phase was observed by flow cytometer in miR-100 inhibitor transferred Hacat cells. In detail, flow cytometer showed that the G2/M phase population decreased by 65% and 71% compared with those of blank control ($P = 0.018$) and negative control ($P = 0.005$) at 72 h after transfection (Supplementary Fig. 3A). Contrarily, the cell population at the G2/M phase was increased by 68% and 102% in miR-100 mimic transferred Siha cells (Supplementary Fig. 3B) compared with those of blank control ($P = 0.03$) and negative control ($P = 0.009$), or was increased by 71% and 53% in miR-100 mimic transferred Caski cells (Supplementary Fig. 3C) compared with those of blank control ($P = 0.003$) and negative control ($P = 0.008$), at 72 h after transfection.

Finally, the early apoptosis rate of Hacat, Siha and Caski cells transfected with miR-100 inhibitor or mimic was examined using annexin-V-FITC and PI assay. Supplementary Fig. 4 and Supplementary Table 1 showed together that, at 72 h

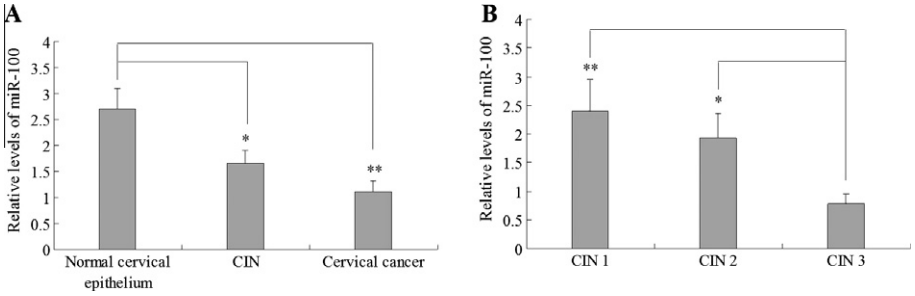

Fig. 1 – The miR-100 expression was reduced gradually in CIN and cervical cancer. (A) CIN (1.6529 ± 0.2477) and cervical cancer (1.1115 ± 0.2073) presented a significantly reduced miR-100 expression compared to normal cervical epithelium (2.7101 ± 0.3834). Moreover, the level of miR-100 was significantly lower in cervical cancer than that in CIN. (B) Among CIN tissues, the miR-100 level in CIN 3 (0.7814 ± 0.1698) was significantly reduced compared with that in CIN 1 (2.397 ± 0.5497) and CIN 2 (1.9242 ± 0.424), but no difference between CIN 1 and CIN 2. Each sample was performed in three independent experiments. (Mann–Whitney U test).

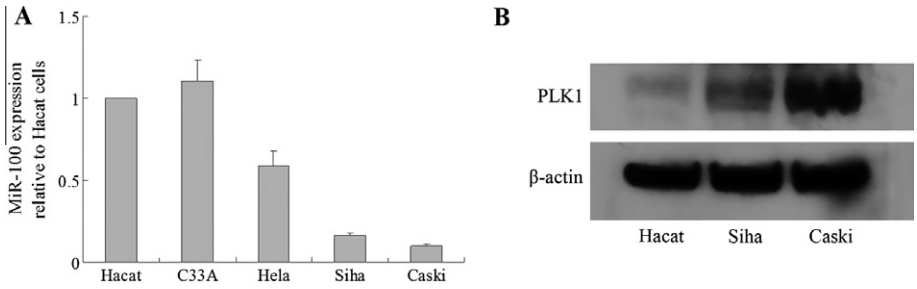

Fig. 2 – The relative expression of miR-100 and PLK1 protein in different cervical cell lines. (A) A substantial reduction of miR-100 was observed in all HPV positive cervical cancer cell lines, but not in HPV negative C33A cells, compared with Hacat cells. (B) The levels of PLK1 protein were significantly higher in Siha cells and Caski cells than those in Hacat cell by immunoblotting. Each sample was performed in three independent experiments. (One-way ANOVA followed by Least-significant difference).

after transfection, the early apoptosis rate was significantly decreased in Hacat cells transfected with miR-100 inhibitor and significantly increased in Siha and Caski cells transfected with miR-100 mimic, compared with blank control and negative control (all $P < 0.05$).

Our results together suggest that the restoration of miR-100 inhibits cell proliferation, induces G2/M phase arrest of cell cycle, and promotes cell apoptosis. The miR-100 may function as a tumour suppressive miRNA during cervical carcinogenesis.

3.3. *MiR-100 negatively regulated PLK1 expression at post-transcriptional level*

Considering PLK1 as a predictive target gene of miR-100 through TargetScan, PicTar and miRBase software, we examined the level of PLK1 protein by immunoblotting in Hacat, Siha and Caski cells, and found that Hacat cells, as normal epithelium, presented 6-fold and 10-fold higher miR-100 expression (Fig. 2A) and 3-fold and 5-fold lower PLK1 protein expression (Fig. 2B) than cervical cancer cell line Siha and Caski, respectively.

And then, we manipulated miR-100 expression using miR-100 inhibitor or mimic and observed the influence of altered miR-100 expression on PLK1 expression by qRT-PCR and immunoblotting analysis. We found that decreased miR-100 expression by miR-100 inhibitor caused an approximately 2-fold higher expression of PLK1 protein, but not PLK1 mRNA, in Hacat cells at 72 h post-transfection (Fig. 3A). Contrarily, increased miR-100 expression by miR-100 mimic significantly inhibited PLK1 protein, but not mRNA, in both Siha and Caski cells at 72 h post-transfection (Fig. 3B and C). Our findings suggest that PLK1 is negatively regulated by miR-100 at post-transcriptional level in cervical normal and cancer cells *in vitro*.

3.4. *The expression of miR-100 was negatively correlated with the expression of PLK1 protein in high-grade cervical lesion and cervical cancer*

To validate further the negative regulation of miR-100 on PLK1 protein *in vivo*, we examined the expression of PLK1 protein by immunohistochemical staining in the same cervical tissues in which miR-100 expression had been detected. As shown in Fig. 4, PLK1 immunoreactivity was majorly localised in the cytoplasm and nucleus of staining cells, while it was very faint or absent in the adjacent stromal cells. In 125 cases of cervical tissues, 40 cases showed negative, 43 weak, 32 moderate and 10 strong expression of PLK1 protein. Furthermore, a significantly negative correlation ($P = 1.45E-10$) between miR-100 and PLK1 expression was identified (Fig. 5A). But, after all cervical tissues were stratified according to grades of cervical lesion, significant correlation between miR-100 and PLK1 expression was merely presented in CIN 3 ($P = 0.005$) and cervical cancer ($P = 0.001$) group, but not in normal cervical epithelium ($P = 0.347$), CIN 1 ($P = 0.589$) and CIN 2 ($P = 0.315$), as shown in Fig. 5B and C. Thus, our results further supported by cervical tissues that PLK1, as a target gene, could be negatively regulated by miR-100, but this regulation merely occurred in high-grade cervical lesion, suggesting that it may be a relative late event during cervical carcinogenesis.

3.5. *HR-HPV E6/E7 may not directly regulate miR-100 expression in cervical tissues and cervical cell lines*

To determine whether viral proteins of HR-HPV regulate the expression of miR-100 prior to histological changes, we compared the miR-100 level in the normal cervical epithelium with ($n = 20$) and without ($n = 20$) HR-HPV infection, but did not find significant difference ($P = 0.424$) between two groups (Supplementary Fig. 5C). Furthermore, we observed miR-100 expression in gene transferred Hacat cells that expressed exogenous HPV-16 E6/E7 using pcDNA3.1/NT-GFP-TOPO-HPV-16E6/E7 expression vector. Following transfection for 24, 48 and 72 h, no significant alterations of miR-100 expression in Hacat cells were observed at each time point compared with those in blank control and negative control (Supplementary Fig. 5D). And then, we used a HPV-16 E6/E7 promoter-targeting siRNA to knock down simultaneously HPV-16 E6 and E7 expression in Siha and Caski cells that contain integrated HPV-16 genome. But, the inhibition of E6 and E7 expression in Siha and Caski cells did not increase the expression of miR-100 compared with those in blank control

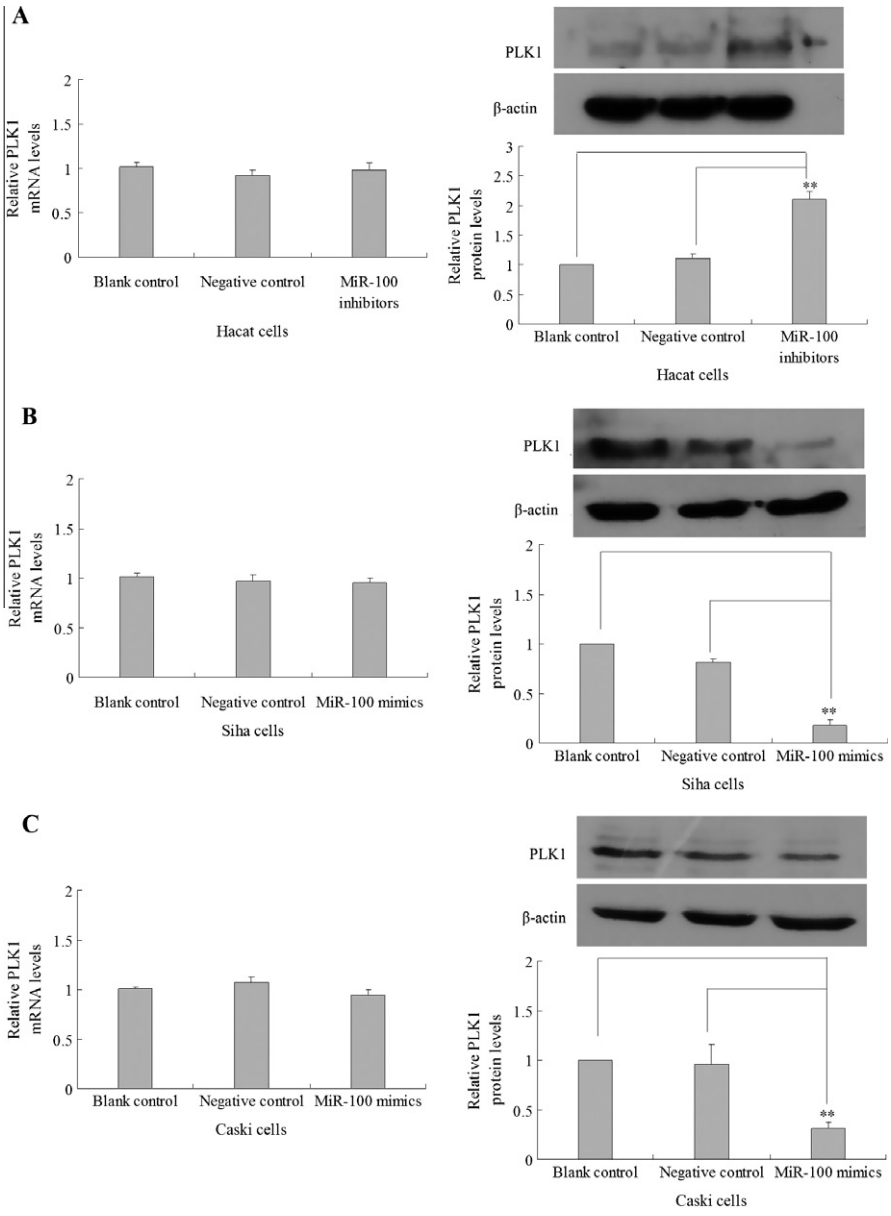

Fig. 3 – MiR-100 negatively regulated PLK1 expression *in vitro* at post-transcriptional level. Hacat cells were transfected with miR-100 inhibitor; meanwhile, Siha and Caski cells were transfected separately with miR-100 mimic. The expression of PLK1 mRNA and protein were examined by qRT-PCR and immunoblotting. At 72 h after transfection, PLK1 protein expression was significantly up-regulated in Hacat cells transfected with miR-100 inhibitor (A), significantly down-regulated in Siha (B) and Caski cells (C) with miR-100 mimic, but not PLK1 mRNA, compared with blank control and negative control. Each sample was performed in three independent experiments. (One-way ANOVA followed by Least-significant difference).

and negative control (Supplementary Fig. 5E and F). Collectively, these results suggest that HR-HPV E6/E7 may not directly regulate miR-100 expression in cervical cells.

4. Discussion

Aberrantly expressed miRNAs in human cervical cancer have been well documented.[14–17] Those altered miRNAs can promote cell proliferation, reduce cell apoptosis, affect cell invasion and eventually contribute to the initiation and development of cervical cancer. MiR-100, as a potential tumour suppressive miRNA, has been reported to be involved in tumour occurrence, development and drug resistance,[18] but the association between miR-100 and cervical cancer is poorly understood. In the present study, we for the first time described the reduced expression of miR-100 in CIN and cervical cancer tissues, presenting a gradually decreased tendency following the progression from normal throughout precancerous lesions to cancer. Moreover, this altered tendency similarly occurred in different grades of CIN, in which miR-100 expression was more decreased in high-grade lesion than that in low-grade lesion. Our findings suggest that decreased miR-100 expression is probably involved in cervical carcinogenesis. To validate this possibility, we further modu-

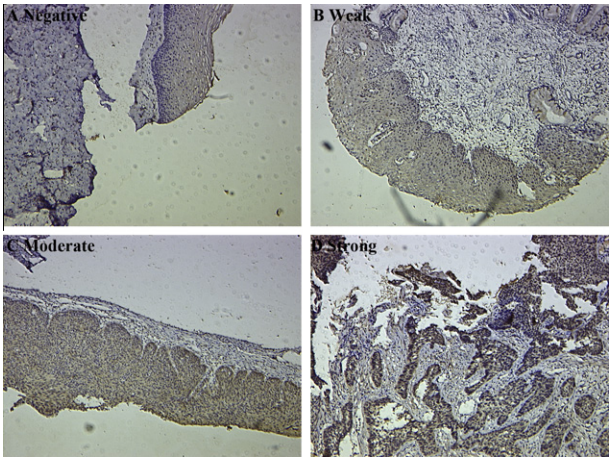

Fig. 4 – Representative immunohistochemical staining of PLK1 in cervical tissues. The positive staining was majorly localised in the cytoplasm and nucleus of cells. Immunohistochemical staining showed no detectable PLK1 expression in normal cervical epithelium (A), weak expression in CIN 2 (B), moderated expression in CIN 3 (C), and strong expression in cervical cancer tissues (D). Magnifications: ×100.

lated miR-100 expression in cervical cells *in vitro*. We found that the down-regulation of miR-100 expression by its specific inhibitor distinctly promoted cell growth, decreased cell apoptosis, and accelerated G2/M phase progression in Hacat cells that originally express high level of miR-100; conversely, the up-regulation of miR-100 expression by its specific mimic remarkably inhibited cell growth, increased cell apoptosis and induced G2/M phase arrests in Siha and Caski cells that originally express low level of miR-100. Thus, our results together suggest that reduced expression of miR-100 is involved in the process of cervical carcinogenesis by regulating cell growth, cycle and apoptosis.

MiRNAs can negatively regulate target gene expression by fully or partially sequence complementary binding to its 3′-UTR leading to mRNA cleavage or mRNA translation repression. Individual miRNA can potentially regulate many target genes, whereas individual target gene can be targeted by multiple miRNAs. Moreover, the target genes regulated by the single miRNA often have similar biological functions. Presently, the several identified targets for miR-100 are a subset of genes involved in cell proliferation and cell cycle regulation, including FGFR3,[3,4] mTOR,[8,19] and Smarca5.[20] The FGFR3 belongs to a family of fibroblast growth factor receptor and plays vital roles in cell proliferation, differentiation and angiogenesis through binding to its cognate ligands (FGFs).[21] The mTOR, namely mammalian target of rapamycin, is a serine/threonine kinase and affects cell size, cell cycle progression and cell death. Recently, it was also found that overexpression of miR-100 repressed mTOR mRNA and protein levels in clear cell ovarian cancer cells and enhanced sensitivity of OVSAYO cells to the rapamycin analogue RAD001.[19] The Smarca5 is a member of the SWI/SNF family of proteins and can form a chromatin remodelling complex with Rsf-1 responsible for a variety of growth signals, such as DNA replication and cell cycle progression.[22] Thus, reduced miR-100 level could up-regulate these targets expression so as to promote cell growth, regulate cell differentiation and induce tumourigenesis. However, the target genes for miR-100 that affect cellular processes during cervical carcinogenesis remain still unknown now. By a series of predicting miRNA targets software, we predicted PLK1 as a novel candidate target gene for miR-100 to participate in cervical carcinogenesis. PLK1 is a key mitotic checkpoint regulatory protein, especially G2/M phase checkpoint regulation, and its defects may cause chromosome instability or aneuploidy.[23] As a well-characterised oncoprotein, PLK1 is usually highly expressed in many tumours,[24–26] including cervical cancer.[27] A further study demonstrated that depletion of PLK1 by small interfering RNA (siRNA) resulted in inhibition of cell proliferation, increase of cell apoptosis and arrest of cell cycle progression at the G2/M phase in Hela cells.[28] However, the regulation mechanism of PLK1 expression is still less understood. A recent report identified that decreased expression of miR-100 led to PLK1 over-expression and in turn contributed to human nasopharyngeal cancer (NPC) progression.[29] But this report only used a luciferase reporter gene system to validate the association between miR-100 and PLK1 in pre-miR-100 transfected NPC cells. It has been also reported that PLK1 expression is regulated by HPV-16 E6 and consequently contributes to the generation of tetraploidy in human foreskin keratinocyte.[30] In this study, we firstly observed PLK1 mRNA and protein expression by modulating miR-100 expression *in vitro* and found that decreased or increased miR-100 expression could

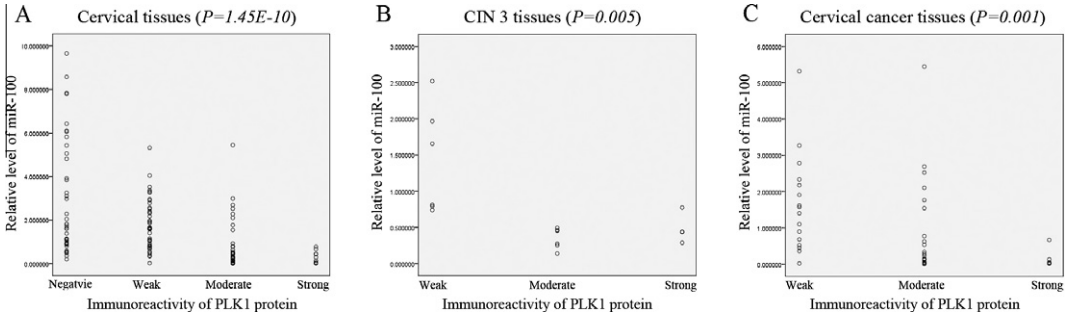

Fig. 5 – PLK1 protein expression was negatively correlated with miR-100 expression in cervical tissues. (A) Totally 125 cervical tissues ($r = -0.534$, $P = 1.45E-10$). (B) CIN 3 tissues ($r = -0.670$, $P = 0.005$). (C) Cervical cancer tissues ($r = -0.506$, $P = 0.001$). (Correlation).

up-regulate or down-regulate the level of PLK1 protein, but not that of PLK1 mRNA in cervical cell lines. To validate further the negative regulation of miR-100 on PLK1 protein *in vivo*, we also examined simultaneously miR-100 and PLK1 expression in the same cervical tissues and found that higher expression of PLK1 was significantly correlated with lower expression of miR-100. Interestingly, we found that negative correlation between miR-100 and PLK1 protein expression merely occurred in high-grade cervical lesions and cervical cancer. Thus, taken previous reports together, our results suggest that miR-100 participates in cervical carcinogenesis through loss of inhibition to PLK1 protein at post-transcriptional level, probably at a relative late stage.

Whether decreased miR-100 expression is directly regulated by HR-HPV is still unclear. Wang et al. demonstrated that human cytomegalovirus (HCMV) infection can down-regulate cellular miR-100 expression that affects its replication in the mTOR-dependent pathway.[8] Up to date, HPV has not been shown to express any virally encoded miRNAs,[31,32] but it indeed exerts diverse and profound effects through regulating host miRNAs expression.[12,14,33,34] In the study, although miR-100 expression in HPV positive cervical cancer cell lines was remarkably lower than that in HPV negative cervical cell line, such altered levels of miR-100 expression did not occur between in normal cervical epithelium with and without HR-HPV infection. Furthermore, we examined miR-100 level in HPV-16 E6/E7 transferred Hacat cells and HPV-16 E6/E7 inhibited Siha and Caski cells and also did not find any significant changes of miR-100 expression. Thus, HR-HPV E6/E7 seems to not directly regulate miR-100 expression in cervical normal and cancer cells, but the causation is not clear. Recently, the role of HR-HPV E5 as an oncogene in cervical carcinogenesis has been noticed.[35,36] It was also reported that HPV-16 L2 targeted and regulated miR-125b in NIH 3T3 cells.[37] Therefore, it is possible that viral genes other than E6/E7 of HR-HPV regulate miR-100 expression during cervical carcinogenesis. In addition, other factors might be involved in reduced miR-100 expression in cervical carcinogenesis. For example, miR-100 primary transcript is located on chromosome 11q24+1. It was found that the copy number loss of 11q contributes to the reduction of miR-100 expression in oral squamous cell carcinoma[4], while loss of 11q has also been identified as a highly prevalent event in cervical cancer tissues[38,39] and cell lines.[40] Accordingly, a further study is needed to identify exact causation of reduced miR-100 in cervical carcinogenesis.

In summary, we for the first time showed a gradually reduced tendency of miR-100 expression from cervical normal epithelium throughout precancerous lesions to cancer tissues. The decrease of miR-100 expression promoted cell proliferation, induced G2/M phase progression in cell cycle and inhibited cell apoptosis. The carcinogenetic procedure induced by miR-100 probably occurred mainly in late phase of cervical carcinogenesis and was at least partly through loss of inhibitive effect on PLK1 protein at the post-transcriptional level. The reduced miR-100 expression in cervical cancer and its precursors may be not directly regulated by HR-HPV E6/E7. Our findings may provide insight into a novel mechanism of cervical carcinogenesis.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.04.037.

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