

## ORIGINAL ARTICLE

# CHAF1A overexpression in human retinoblastoma promotes cell proliferation and suppresses apoptosis

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## Summary

**Purpose:** Retinoblastoma causes significant human mortality especially in children. Although retinoblastoma may be treated if detected at early stage, however, it becomes destructive at advanced stages. The treatment involves surgery and chemotherapy. However, the chemotherapeutic agents have severe adverse effects. Therefore, development of viable drugs and identification of novel molecular therapeutic targets may enable efficient management of retinoblastoma. This study was designed to examine the expression profile of Chromatin Assembly Factor-1 (CHAF1A) and explore its therapeutic implications in retinoblastoma.

**Methods:** The expression of CHAF1A was determined by qRT-PCR. MTT assay was used for the determination of the cell viability. Apoptosis was detected by acridine orange (AO)/ethidium bromide (EB) and annexin V/propidium iodide (PI) assay. Cell cycle analysis was determined by flow cytometry. Protein expression was determined by western blot analysis.

**Results:** The results showed that CHAF1A is significantly

upregulated in human retinoblastoma, with 7.3 folds up-regulation in retinoblastoma cells relative to normal cells. Knockdown of CHAF1A resulted in significant decline in the viability of the RB355 retinoblastoma cells. The flow cytometric analysis showed that knockdown of CHAF1A caused arrest of the RB355 cells at G0/G1 phase of the cell cycle. This was also linked with significant downregulation of cyclin D1 and cyclin E1. The AO/EB staining assay showed that CHAF1A knockdown promotes apoptosis which is associated with downregulation of Bcl-2 and upregulation of Bax.

**Conclusion:** Taken together, these results suggest that CHAF1A is upregulated in retinoblastoma and regulates its proliferation and apoptosis. As such CHAF1A may act as biomarker as well as therapeutic target for the management of retinoblastoma.

**Key words:** retinoblastoma, chromatin assembly factor-1, apoptosis, proliferation

## Introduction

In eukaryotes the genetic material is organised in the form of chromatin. There are different proteins which are involved the assembly of DNA into chromatin [1]. One of the proteins, the Chromatin Assembly Factor-1 (CHAF1A) plays vital role in the chromatin assembly by enabling the histone proteins, H3 and H4 to deposit on to the DNA [2]. Studies have shown that aberrant expression of CHAF1A is associated with development of cancers and development of drug resistance in different cancers such

as ovarian cancer [3]. In a recent study, CHAF1A has been reported to be involved in the promotion of metastasis of neuroblastoma [4]. The potential of CHAF1A as biomarker has also been explored as it has been reported to be considerably overexpressed in certain cancers such as colon cancer and cervical cancer to name a few [5]. However, the expression profile of CHAF1A in retinoblastoma is unknown and its therapeutic implications unexplored. This study was therefore designed to explore the role

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Received: 19/01/2020; Accepted: 11/02/2020

and therapeutic implications of CHAF1A in retinoblastoma. One of the rare forms of eye malignancies, retinoblastoma is generally seen in children below the age of 5 years. Retinoblastoma can be cured when diagnosed at early stages. Nonetheless, it may become aggressive and even cause death if untreated [6]. It may occur only in one eye (unilateral) or develop in both eyes (bilateral). Retinoblastoma causes significant mortality and the children who survive generally lose their eye vision. Factors such as papilloma virus and mutations in RB1 gene have been implicated in the development of retinoblastoma [7]. Surgery followed by chemotherapy is generally applied for the treatment of this disease. Herein, we report that CHAF1A is overexpressed in retinoblastoma and promotes its proliferation by suppressing apoptosis.

## Methods

### Cell lines and culture

The human retinoblastoma RB355, Y79 and WERI-Rb-1 cell lines as well as normal RPE cell line were purchased from the ATCC collection centre, USA. The cell lines were cultured using Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Scientific, Waltham, Mass, USA) and were maintained in a humidified incubator at 37°C with 5% CO<sub>2</sub>/95% air.

### Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

The total RNA was extracted from the retinoblastoma cell lines (Hs255.T, and normal cell line using RNeasy kit (Qiagen, Inc., Valencia, CA, USA). To reverse transcribe the cDNA, the Omniscript RT (Qiagen, Inc.) was employed using 1 µg of the extracted RNA. The cDNA was then used as a template for RT-qPCR analysis with the assistance of the Taq PCR Master Mix kit (Qiagen, Inc.) according to the manufacturer's protocol. The reaction mixture consisted of 20 µl containing 1.5 mM MgCl<sub>2</sub>, 2.5 units Taq DNA Polymerase, 200 µM dNTP, 0.2 µM of each primer and 0.5 µg DNA. The cycling conditions were as follows: 95°C for 20 sec, followed by 40 cycles of 95°C for 15 sec, and 58°C for 1 min. GAPDH was used as internal control and the relation was determined by the 2<sup>-ΔΔC<sub>q</sub></sup> method.

### Cell transfection

The negative control (NC) and pcDNA-CHAF1A (Tajin Saier Biotechnology, China) were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., USA) as per the manufacturer's protocol.

### MTT cell viability assay

The seeding of the RB355 cells was done in 96-well plates with 4500 cells per well. The RB355 cells were then transfected with si-NC or CHAF1A and cultured for 24 h. After replacing the Dulbecco's Modified Eagle medium (DMEM), the cells were treated with 10 µL of 5

mg/mL MTT solution at 37°C for 4 h. Finally, 100 µL of DMSO were added and the absorbance was taken at 570 nm by spectrophotometer at different time intervals to determine its cell viability.

### AO/EB dual staining

The si-NC and CHAF1A-siRNA transfected RB355 cancer cells were cultured for 24 h. The cells were collected by centrifugation and cell pellets were washed with PBS and then fixed using 70% ethanol. The cells were then stained with acridine orange and ethidium bromide (AO/EB) dual staining mix and analyzed for morphological appearances under fluorescent microscope.

### Annexin V-FITC/PI fluorescent staining

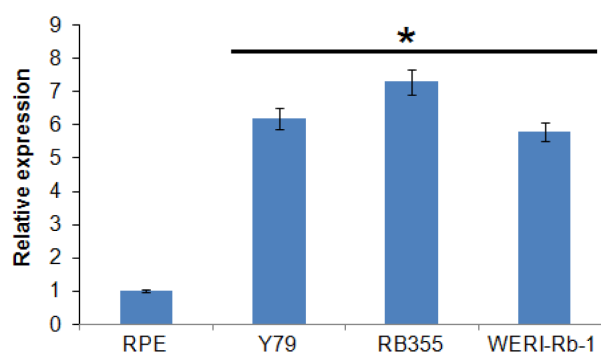
The annexin V-FITC/PI staining assay was performed to assess the level of cell apoptosis in transfected RB355 cells. The RB355 cancer cells were fixed using methanol and stained with dual annexin V-FITC/PI staining solution. Then, the cells were examined for nuclear morphology under fluorescent microscope and the percentage of apoptotic cells was determined.

### Cell cycle analysis

For cell cycle analysis, the miR-NC and CHAF1A-siRNA transfected RB355 retinoblastoma cells were cultured for 24 h. Centrifugation was used to harvest the treated cells which were then washed with PBS three times, fixed using 4% formaldehyde and stained with PI solution. The cells were then examined using flow cytometer for the analysis of mitosis.

### Western blot analysis

To extract the total proteins the RB355 cells were lysed with RIPA lysis buffer (Thermo Scientific). The Bradford method was used to determine the total protein concentrations of the cell lysates. Equal protein concentrations were loaded on 8% SDS-PAGE gel. The page gel was then blotted to transfer the contents to nylon membranes which were exposed to primary and secondary antibodies designed for respective proteins of interest which were then visualized and their expression levels were assessed by the chemiluminescence method.



**Figure 1.** Expression of CHAF1A in normal RPE and retinoblastoma cell lines as determined by qRT-PCR. The experiments were performed in triplicate and expressed as mean ± SD (\*p<0.05).

### Statistics

The experiments were performed in triplicate. The values are shown as mean  $\pm$  SD. Student's t-test and one-way ANOVA were used for statistical analyses.  $P < 0.05$  was considered statistically significant.

## Results

### CHAF1A is overexpressed in retinoblastoma

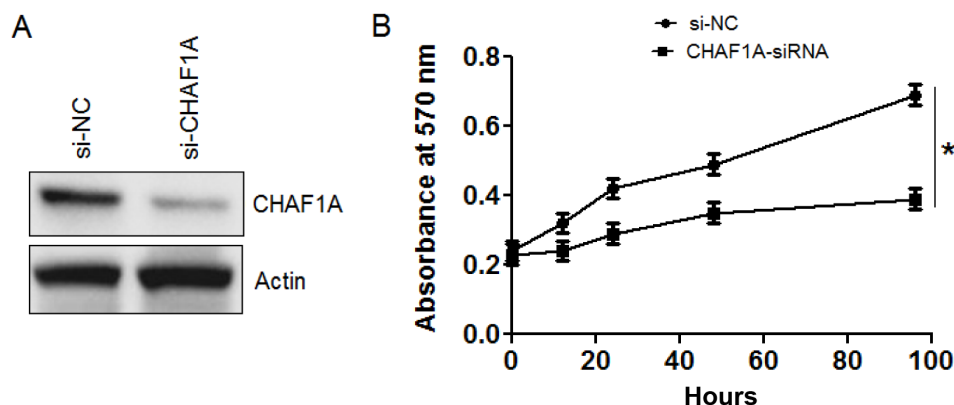
The expression of CHAF1A was examined in normal RPE cells and the Y79, RB355 and WERI-Rb-1 retinoblastoma cell lines. The results showed that CHAF1A was significantly ( $p < 0.05$ ) overexpressed in all the retinoblastoma cell lines relative to the normal RPE cells (Figure 1). The upregulation was upto 7.3 folds with highest expression of CHAF1A in RB355 cells. Hence, these cells were used in further experiments.

### CHAF1A promotes the growth of retinoblastoma cells

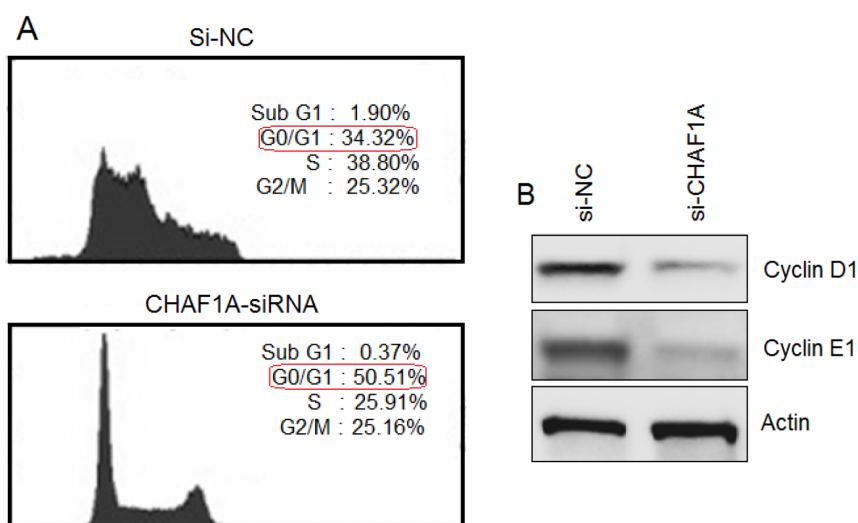
To gain insights about the effect of CHAF1A on the cell growth, the RB355 retinoblastoma cells were transfected with CHAF1A-siRNA. Next, western blot analysis was performed to authenticate the knockdown of CHAF1A (Figure 2A). The cells were then cultured for 24h and subjected to MTT assay. The results showed that knockdown of CHAF1A in RB355 cells caused significant ( $p < 0.05$ ) decline in the viability of the RB355 cells in a time-dependent manner (Figure 2B). Taken together, overexpression of CHAF1A was responsible for promotion of retinoblastoma cell proliferation.

### CHAF1A knockdown promotes RB355 cell cycle arrest

Next, the RB355 cells with CHAF1A knockdown were subjected to cell cycle analysis by flow cytometry. The results showed that CHAF1A knock-



**Figure 2. A:** Western blots showing the expression of CHAF1A in si-NC and CHAF1A-siRNA transfected RB355 cells. **B:** Cell viability of the si-NC and CHAF1A-siRNA transfected RB355 cells. The experiments were performed in triplicate and expressed as mean  $\pm$  SD ( $*p < 0.05$ ).



**Figure 3. A:** Cell cycle analysis of the si-NC and CHAF1A-siRNA transfected RB355 cells. **B:** Western blots showing the expression of Cyclin D1 and Cyclin E1 in si-NC and CHAF1A-siRNA transfected RB355 cells. The experiments were performed in triplicate and expressed as mean  $\pm$  SD ( $p < 0.05$ ).

down caused a significant increase of RB355 cells in G0/G1 phase of the cell cycle. The percentage of G0/G1 cells was 34.32% and 50.51% in si-NC and CHAF1A-siRNA transfected RB355 cells, suggestive of G0/G1 cycle arrest (Figure 3A). The expression of cyclin D1 and cyclin E1 were also significantly downregulated in RB355 cells (Figure 3B).

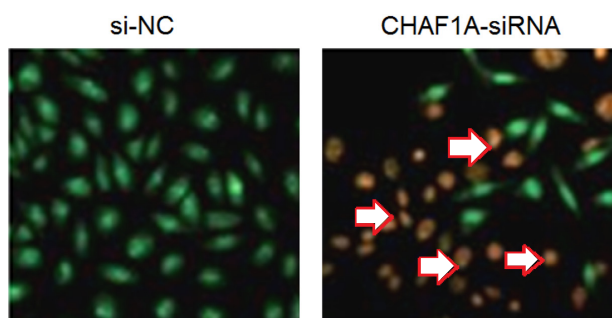
*CHAF1A suppresses apoptosis of RB355 cells*

The si-NC and CHAF1A-siRNA transfected RB355 cells were subjected AO/EB staining to gain

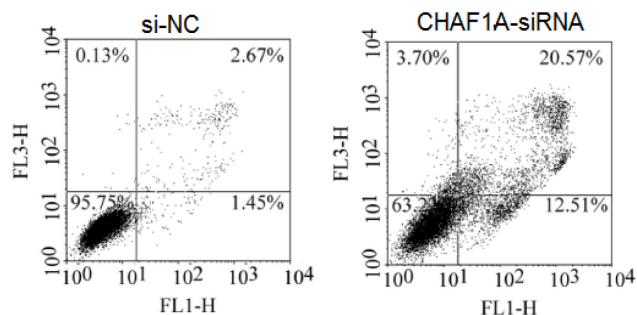
insights about the impact of CHAF1A knockdown on apoptosis. The results showed that knockdown of CHAF1A caused significant increase of red and orange colored cells, suggestive of apoptosis (Figure 4). The annexin V/PI staining showed 1.45% early and 2.67% late apoptosis and si-NC 12.51% early and 20.57% late apoptosis in CHAF1A-siRNA (Figure 5). The CHAF1A knockdown also caused suppression of Bcl-2 and upregulation of Bax, further confirming apoptosis in RB355 cells (Figure 6). Taken together, these results suggest that CHAF1A silencing promotes apoptosis and its over-expression in retinoblastoma cells suppresses its apoptosis.

**Discussion**

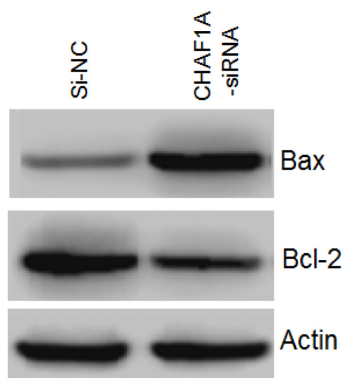
Retinoblastoma is a lethal and aggressive cancer of the eye, prevalently observed in children. The mortality rate of this disease is as high as 70% among the children of developing or under developed countries [8]. The aggressiveness of retinoblastoma is increased by its metastasis to neighbouring tissues along with increased intracranial pressure [9]. The treatment methods being applied against the retinoblastoma are unsatisfactory [10]. Therefore, there is urgent need to improve the efficacy of anticancer treatments against the human retinoblastoma by looking for more potent chemotherapeutic agents or alternatively identify efficient therapeutic targets that could be easily targeted by anticancer drugs [11]. Herein we explored the expression profile, role and therapeutic potential of CHAF1A in retinoblastoma. The results showed remarkable upregulation of CHAF1A in human retinoblastoma cells relative to the normal cells. These findings are in concordance with previous findings wherein CHAF1A has been shown to be considerably upregulated in breast cancer regulating its proliferation [12] as CHAF1A has been shown to promote the tumorigenesis of neuroblastoma [4]. Consistently herein we found that knockdown of CHAF1A suppresses the proliferation of the retinoblastoma cells and therefore it may act as an oncogene in retinoblastoma. CHAF1A has also been shown to act as an oncogene in glioblastoma, further confirming our results [13]. In this study we found that knockdown of CHAF1A triggered G0/G1 cell cycle arrest and also promoted apoptosis. These observations are in agreement with a previous study wherein CHAF1A has been reported to play similar roles in ovarian cancer [13]. Taken together, CHAF1A may act as an oncogene in human retinoblastoma and may be utilised as a therapeutic target for its management.



**Figure 4.** AO/EB staining of the si-NC and CHAF1A-siRNA transfected RB355 cells (Arrows depict apoptotic cells). The experiments were performed in triplicate and expressed as mean ± SD (p<0.05).



**Figure 5.** Annexin V/PI staining of the si-NC and CHAF1A-siRNA transfected RB355 cells. The experiments were performed in triplicate.



**Figure 6.** Western blot analysis showing the expression of Bax and Bcl-2 in si-NC and CHAF1A-siRNA transfected RB355 cells. The experiments were performed in triplicate.

## Conclusion

The findings of the present study revealed significant upregulation of CHAF1A in human retinoblastoma cells. The CHAF1A was found to promote proliferation and suppress apoptosis and therefore may act as oncogene in retinoblastoma.

Taken together, these findings point towards the therapeutic implications of CHAF1A in retinoblastoma treatment.

## Conflict of interests

The authors declare no conflict of interests.

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