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이학석사학위논문

**ATR 의존적 인산화에 의한  
CHFR Ub-ligase의 활성 조절 연구**

**ATR-dependent phosphorylation and activation of  
human CHFR in response to DNA damage**

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서울대학교 대학원

생명과학부

김재은

## **ABSTRACT**

# **ATR –dependent phosphorylation and activation of human CHFR in response to DNA damage**

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CHFR is an E3 ubiquitin ligase and checkpoint protein that regulates cell cycle progression and tumorigenesis. Recently, it has been reported that CHFR is involved in cellular responses to DNA damage and plays a role in maintaining genome integrity. Functions of CHFR as a cell cycle checkpoint have been revealed by studies of its downstream targets such as PLK1, Aurora A and HDAC1. However, it still remains elusive how CHFR is regulated.

In this study, I showed that CHFR is phosphorylated by ATR in response to DNA damage. ATR is a new CHFR-interacting protein and CHFR is phosphorylated by

ATR *in vitro*. ATR is the master regulator of DNA damage response including cell cycle arrest, DNA repair and apoptosis. ATR activates the signaling pathway in response to DNA damage and replication stress. Therefore, I examined whether CHFR is phosphorylated upon DNA damage. In response to UV irradiation and doxorubicin treatment, CHFR is phosphorylated *in vivo* and it is ATR-dependent. There are five ATR consensus motifs (SQ or TQ) on CHFR. In order to narrow down the phosphorylation site, *in vitro* kinase assay was performed using CHFR deletion mutants. It was shown that the N-terminal region of CHFR is responsible for the phosphorylation. In addition, I found that CHFR is phosphorylated at threonine 130 *in vivo* upon UV irradiation. Furthermore, ATR-mediated phosphorylation of CHFR affects its checkpoint function. The phosphorylation-deficient mutant of CHFR does not exert anti-proliferative activity upon UV irradiation. Taken together, these results indicate that ATR-dependent phosphorylation is a new regulatory mechanism of CHFR for its checkpoint activity in response to DNA damage.

Keywords: CHFR, ATR, phosphorylation, DNA damage, cell cycle checkpoint, E3

ligase

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# Introduction

All cells have the control system for maintenance of genome integrity and cellular viability during cell divisions. Cells are confronted with various endogenous and exogenous damages during continuous cell divisions. To preserve the fidelity of genome transmission from genotoxic stress, each phase of cell cycle has checkpoints. Checkpoints include numerous proteins that control cell cycle progression and they are coordinated for elaborate and accurate cell division. G1 checkpoint, intra-S checkpoint, G2 checkpoint and M checkpoint delay entry into the next cell cycle phase and determine the cells' fates (Smith *et al.*, 2010).

CHFR (checkpoint with FHA and RING-finger domains) is a RING-finger type E3 ubiquitin ligase. CHFR was first identified as a checkpoint protein that delays cell cycle transition to metaphase in response to mitotic stress such as microtubule poisons (Scolnick *et al.*, 2000). CHFR contains an N-terminal FHA (forkhead-associated) domain, a RING-finger domain and a C-terminal CR (cysteine-rich) domain (Scolnick *et al.*, 2000). FHA domain plays a part in interactions with

phosphoproteins (Stavridi *et al.*, 2002). RING-finger domain is responsible for E3 ubiquitin ligase activity. CR domain functions in interaction with target proteins (Chaturvedi *et al.*, 2002; Oh *et al.*, 2009) and is known to be required for auto-ubiquitination. CHFR ubiquitinates targets for degradation or signal transduction. Downstream targets of CHFR are proteins that control cell cycle progression such as PLK1, Aurora A (Kang *et al.*, 2000; Yu *et al.*, 2005). In addition to mitosis, CHFR is involved in p21-dependent cell cycle checkpoint in G1 through downregulation of HDAC1 (Oh *et al.*, 2009). These findings imply that CHFR has a general checkpoint function not only in mitosis but also in interphase.

Several studies suggest that CHFR functions as a tumor suppressor. CHFR is epigenetically inactivated by hypermethylation in various cancer cells (Mizuno *et al.*, 2002; Corn *et al.*, 2003). CHFR-deficient mice promote tumor and chromosome instability (Yu *et al.*, 2005). In addition, CHFR regulates tumor metastasis through downregulation of HDAC1, resulting in the increase of metastasis suppressors (Oh *et al.*, 2009; Toyota *et al.*, 2003). Since CHFR plays a part in cell cycle progression and tumorigenesis, the importance of CHFR and its downstream targets are well

understood. However, the regulatory mechanism of CHFR is unclear. Recently, it has been known that the activity and stability of CHFR are regulated by ubiquitination and SUMOylation (Kwon *et al.*, 2013; Bae *et al.*, 2013). Nevertheless, how CHFR is regulated upon specific signals is still elusive.

It has been shown that various E3 ligases are involved in DNA damage response. Ubiquitination and SUMOylation are connected with recruitment and removal of protein for DNA damage signaling (Dantuma *et al.*, 2016). These modifications by E3 ligase such as EDD, RNF168 and COP1 initiate cellular responses in appropriate time and order for DNA damage repair (Munoz *et al.*, 2007; Doil *et al.*, 2009; Dornan *et al.*, 2006). Likewise, it has been studied that CHFR plays a role in ubiquitination of histones with RNF8 and is recruited to DNA damaged sites upon IR-induced DNA damage (Wu *et al.*, 2011; Liu *et al.*, 2013).

DNA damage response (DDR) is a signaling network for the maintenance of genome integrity. This pathway includes cell cycle arrest, DNA repair and apoptosis. Abrogation of DNA damage response leads to genomic instability. ATR (ataxia-telangiectasia and RAD3-related) is the master regulator of DNA damage response.

ATR is a member of PIKK (phosphatidylinositol 3 kinase (PI3K)-related protein kinases) family along with ATM (ataxia-telangiectasia mutated) and DNA-PKcs (DNA-dependent protein kinases). Generally, it is known that ATR is activated by single-stranded DNA breaks and ATM is activated by double-stranded breaks (DSB). Although replication stress is a main cause of ATR activation, ATR also responds to various DNA damage including DSB. In response to DSB, ATM mediates DNA strand resection and generates single-strand DNA that activates ATR. Accordingly, there is a cross-talk between ATM and ATR and it is possible that they are activated and coordinated in all cell cycle phases in response to diverse DNA damages (Smith *et al.*, 2010; Awasthi *et al.*, 2015; Cimprich *et al.*, 2008).

When ATR is activated, it phosphorylates proteins that have SQ or TQ motifs (serine or threonine residue followed by glutamine). ATR-dependent phosphorylation initiates DNA damage signaling cascade. Downstream targets of ATR are various effector proteins of DDR such as CHK1 (checkpoint kinases 1), BRCA1 (breast cancer 1), and p53 (Liu *et al.*, 2000; Tibbetts *et al.*, 2000; Tibbetts *et al.*, 1999). Recently, ATR is considered as a tumor suppressor. ATR-mediated signaling acts as a

barrier that delays tumor progression in early phase (Bartkova *et al.*, 2005). Moreover, ATR is a crucial regulator for cell survival because disruption of ATR leads to embryonic lethality (de Klein *et al.*, 2000; Cortez *et al.*, 2001).

In DNA damage response, a number of kinases including ATR participate as a part of signaling cascade and phosphorylation is a common post-translational modification of proteins that regulates their stability and activity. Since CHFR is involved in DNA damage response, it has the possibility that CHFR is modified and regulated by phosphorylation upon DNA damage.

In this study, ATR is identified as an interacting protein of CHFR. CHFR is phosphorylated upon DNA damage such as UV irradiation and doxorubicin in ATR-dependent manner. Among five ATR consensus motifs (SQ or TQ) on CHFR, I identified that the phosphorylation site of CHFR is threonine 130. ATR-mediated phosphorylation of CHFR affects its checkpoint function. The phosphorylation-deficient mutant of CHFR does not exert anti-proliferative activity in DNA damage condition. Taken together, ATR-dependent phosphorylation of CHFR is an important regulatory mechanism for its checkpoint activity in response to DNA damage.

# Materials and Methods

## 1. Cell culture, plasmids, and transfection

MCF7 cell, HEK293T cell, HeLa cell were cultured at 37°C in DMEM (Dulbecco's modified Eagle's medium, Hyclone) supplemented with 10% FBS and 100 units/ml penicillin, 100 µg/ml streptomycin. Transfection of p3XFLAG-CMV10-CHFR, pcDNA3-FLAG-ATR was carried out using polyethylenimine (Sigma). Alanine mutants of CHFR were generated using the QuickChange site-directed mutagenesis Kit (Stratagene).

## 2. Antibodies and reagents

Antibodies used for experiments were as follows: anti-FLAG (Sigma); anti-GAPDH and anti-ATR (Santa Cruz); anti-CHK1, anti-pCHK1 S317 and anti-pSQ/TQ (Cell signaling Technology); rabbit polyclonal anti-CHFR antiserum (raised against recombinant His-CHFR); peroxidase-conjugated AffiniPure goat anti-mouse and anti-rabbit IgGs (Jackson ImmunoResearch).

For UV irradiation, cells on culture dishes were washed twice with DPBS and subjected to UV-C 254 nm (UVP crosslinker). Doxorubicin (Sigma) 0.5  $\mu$ M and caffeine (Sigma) 5 mM were added to cell cultures with medium. Lambda phosphatase (New England Biolabs) was treated to resins before immunoprecipitates elution. The resin and lambda phosphatase were incubated in NEB buffer with  $MnCl_2$  at 37°C for 15 min.

### **3. Immunoprecipitation and Western blot**

Cells were lysed in buffer A (20 mM Tris-HCl, pH 7.8, 150 mM NaCl, 0.5% Triton X-100) containing protease inhibitors and phosphatase inhibitors. Cell lysates were incubated with anti-FLAG M2 resin (Sigma) for 2 hour at 4°C. The resins were washed three times with buffer A and twice with buffer B (20 mM Tris-HCl, pH 7.8, 150 mM NaCl). Proteins were eluted by SDS sampling buffer. Samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed with appropriate antibodies.

#### **4. *In vitro* kinase assay**

Partially purified FLAG-ATR protein from HEK293T cells was incubated with purified GST-CHFR, GST-p53 protein from *E.coli* in kinase buffer (10 mM HEPES pH7.5, 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 10 mM MnCl<sub>2</sub>, 1 mM DTT, 10 μM ATP, 0.5 mM NaF, 0.5 mM Na<sub>3</sub>VO<sub>4</sub>) with 10 μCi [ $\gamma$ -<sup>32</sup>P] ATP at 30°C for 30 min. The reaction was stopped by SDS sampling buffer. The samples were resolved by SDS-PAGE and visualized by autoradiography and Coomassie brilliant blue staining.

#### **5. Cell proliferation assay and colony formation assay**

HeLa stable cells with p3XFLAG-CMV10, CHFR-WT, CHFR-T2A were plated into 35 mm culture dishes (1x10<sup>5</sup> cells). Cells were irradiated with UV 10 J/m<sup>2</sup>. The number of cells was counted for 3 days after UV treatment. For colony formation assay, HeLa stable cells were plated into 60 mm culture dishes and grown in G418 (250 μg/ml)-containing DMEM for 7 days. G418-resistant colonies were fixed with 10% formaldehyde and stained with 0.5% crystal violet in 20% methanol.

# Results

## 1. CHFR interacts with ATR

To identify proteins that interact with CHFR, immunoaffinity purification combined with mass spectrometry was performed using HEK293T cells transfected with FLAG-CHFR I306A and pCMV-3X FLAG as a control. CHFR I306A mutant lacks E3 ubiquitin ligase activity by substitution of isoleucine to alanine. Mass spectrometry of cells transfected with CHFR identified ATR as an interacting protein.

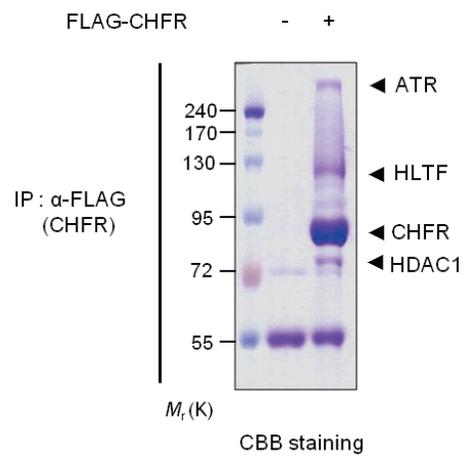
To validate the interaction between CHFR and ATR, HEK293T cells were transfected with FLAG-CHFR and pCMV-3X FLAG as a control. Cell lysates were immunoprecipitated with anti-FLAG resin and immunoblotted with anti-ATR antibody. ATR was immunoprecipitated from cells transfected with FLAG-CHFR but not from cells with pCMV-3X FLAG. These results indicate that CHFR interacts with endogenous ATR *in vivo* and raise the possibility that CHFR correlates with ATR-mediated pathway.

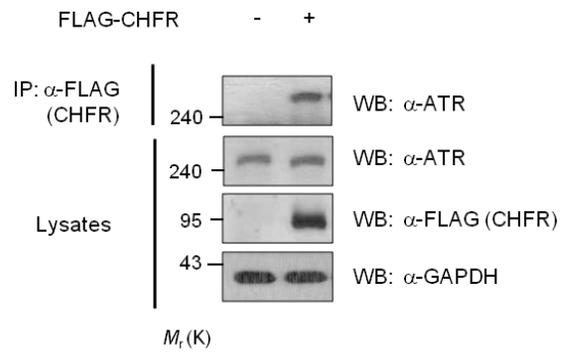
**Figure 1. CHFR interacts with ATR**

(A) HEK293T cells were transfected with FLAG-CHFR I306A mutant and pCMV-3X FLAG as a control. Lysates were immunoprecipitated with anti-FLAG resin. Sample was separated by SDS-PAGE and visualized with Coomassie Brilliant Blue staining. ATR was identified as an interacting protein by mass-spectrometry.

(B) HEK293T cells were transfected with FLAG-CHFR and immunoprecipitated to confirm the interaction between ATR and CHFR. Immunoprecipitates were resolved by SDS-PAGE and immunoblotted by anti-ATR antibody.

**A**



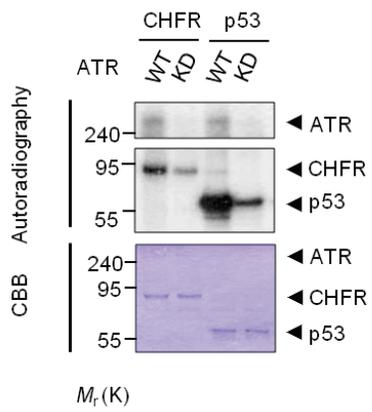
**B**

## **2. CHFR is phosphorylated by ATR *in vitro***

ATR is the master regulator of DNA damage response and phosphorylates effector proteins in the downstream pathway. To examine whether ATR phosphorylates CHFR, *in vitro* kinase assay was performed using partially purified FLAG-ATR WT (wild-type) and KD (kinase-dead) protein from HEK293T cell and GST-CHFR, GST-p53 protein from *E.coli*. Since it is known that ATR phosphorylates p53, GST-p53 was used as a positive control of ATR activity (Tibbetts *et al.*, 1999). The phosphorylated CHFR and p53 were revealed by autoradiography. Like p53, the phosphorylation of CHFR was detected in the presence of ATR WT. This result demonstrates that ATR phosphorylates CHFR *in vitro*.

**Figure 2. CHFR is phosphorylated by ATR *in vitro***

Partially purified FLAG-ATR protein from HEK293T cells was incubated with purified GST-CHFR, GST-p53 protein from *E.coli* at 30°C for 30 min. The samples were resolved by SDS-PAGE and visualized by autoradiography and Coomassie brilliant blue staining.



### 3. DNA damage induces ATR-dependent phosphorylation of CHFR

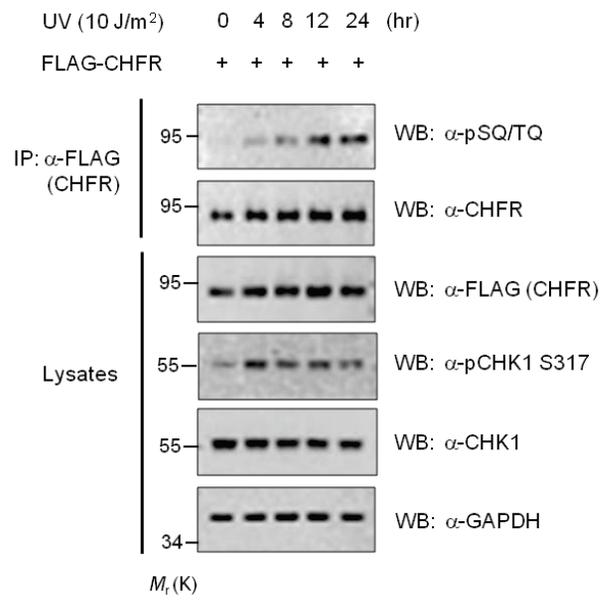
Given that DNA damaging agents induce ATR activation, I investigated whether CHFR is phosphorylated in response to DNA damage *in vivo*. MCF7 cells (human breast carcinoma) which lack endogenous CHFR were transfected with FLAG-CHFR and were treated with UV 10 J/m<sup>2</sup> or doxorubicin 0.5 μM. Lysates were immunoprecipitated by anti-FLAG resin and probed with phospho-SQ/TQ antibody. This antibody detects specific phosphorylation motif of ATR/ATM. In addition, the activation of ATR in response to DNA damage was checked by detecting the phosphorylation of endogenous CHK1. Phosphorylation of CHFR was maximized at 12 hours after UV irradiation. Similar result was obtained when treatment with doxorubicin. These data suggest that CHFR is ATR-dependent phosphorylated *in vivo*. Moreover, DNA damage and replication stress induced by UV and doxorubicin are upstream signals for modification of CHFR.

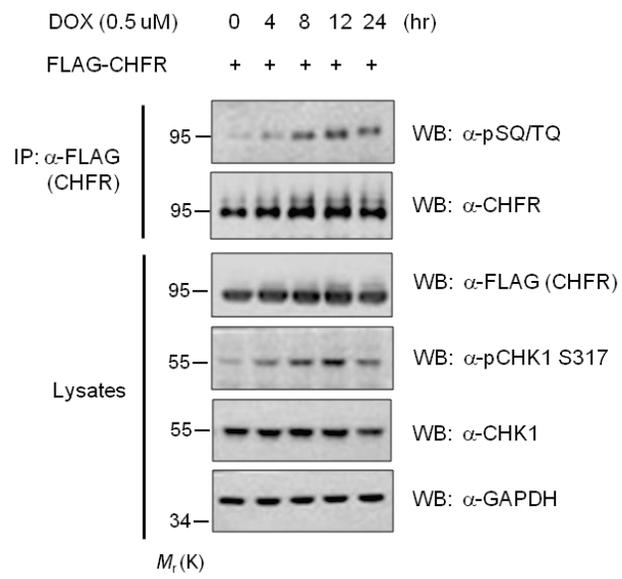
**Figure 3. DNA damage induces ATR-dependent phosphorylation of CHFR**

(A) MCF7 cells were transfected with FLAG-CHFR and were irradiated with UV 10 J/m<sup>2</sup>. Cells were harvested at indicated times. Cell lysates were immunoprecipitated with anti-FLAG resin and precipitates were analyzed by SDS-PAGE, followed by immunoblotting with phospho-SQ/TQ antibody for phosphorylated CHFR.

(B) MCF7 cells were transfected with FLAG-CHFR and treated with doxorubicin 0.5 μM. They were also harvested at indicated times and lysates were immunoprecipitated and immunoblotted with phospho-SQ/TQ antibody

**A**



**B**

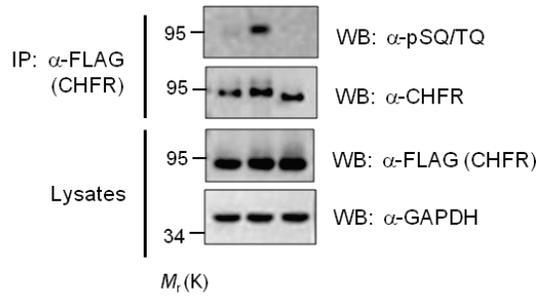
#### **4. The phosphorylation of CHFR is diminished by lambda phosphatase**

To further validate phosphorylated CHFR, I treated lambda phosphatase. MCF7 cells were transfected with FLAG-CHFR. Then cells were irradiated with UV 10 J/m<sup>2</sup> and incubated for 12 hours. Lysates were immunoprecipitated with anti-FLAG resin and the precipitated lysates with resin were incubated with lambda phosphatase at 37°C for 15 min. The reaction was stopped by adding SDS sampling buffer. The sample was resolved by SDS-PAGE and immunoblotting. The phosphorylation band was not detected in the sample which was treated with lambda phosphatase. Therefore, it is sure that the band detected by phospho-SQ/TQ antibody is phosphorylated protein and CHFR is modified by phosphorylation upon UV irradiation.

**Figure 4. The phosphorylation of CHFR is diminished by lambda phosphatase**

MCF7 cells were transfected with FLAG-CHFR, treated with UV 10 J/m<sup>2</sup> and incubated for 12 hours. Lysates were immunoprecipitated with anti-FLAG resin. The immunoprecipitates were incubated with lambda phosphatase at 37°C for 15 min and the reaction was stopped and eluted by SDS sampling buffer. The samples were subjected to SDS-PAGE and immunoblotting.

$\lambda$ Phosphatase	-	-	+
UV (10 J/m <sup>2</sup> )	-	+	+
FLAG-CHFR	+	+	+

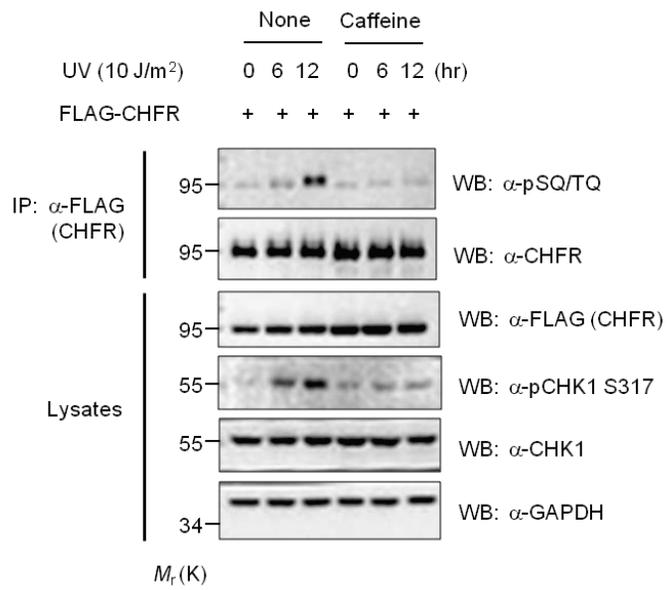


## **5. Caffeine inhibits the ATR-dependent phosphorylation of CHFR**

To verify additionally whether ATR is responsible for phosphorylation of CHFR *in vivo*, I treated caffeine, an ATR/ATM inhibitor. MCF7 cells were transfected with FLAG-CHFR and were incubated with caffeine immediately after exposure to UV 10 J/m<sup>2</sup>. Caffeine treatment inhibited phosphorylation of CHFR as well as CHK1. These results indicate that ATR mediates the phosphorylation of CHFR.

**Figure 5. Caffeine inhibits the ATR-dependent phosphorylation of CHFR**

MCF7 cells were transfected with FLAG-CHFR. After exposure to UV 10 J/m<sup>2</sup>, cells were incubated with caffeine and harvested at indicated times. Lysates were immunoprecipitated with anti-FLAG resin and immunoblotted by phospho-SQ/TQ antibody.



## 6. ATR phosphorylates CHFR at the N-terminal region *in vitro*

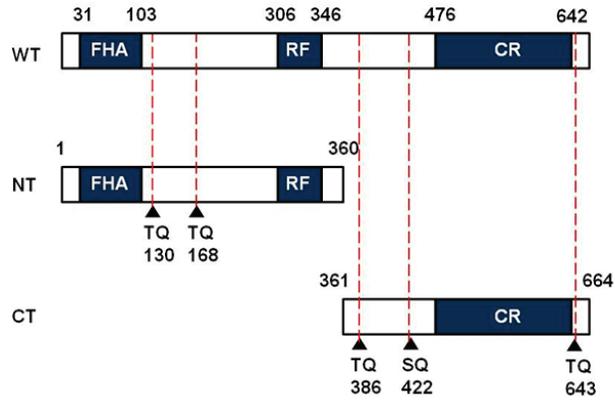
ATR consensus motif for target proteins is well known. ATR phosphorylates serine or threonine residue followed by glutamine of substrates. There are five ATR consensus motifs on CHFR. (Thr<sup>130</sup>, Thr<sup>168</sup>, Thr<sup>386</sup>, Ser<sup>422</sup>, Thr<sup>643</sup>). In order to narrow down the phosphorylation sites, CHFR deletion mutants were used for *in vitro* kinase assay. The C-terminal or N-terminal region of CHFR was truncated for each mutant. The CHFR deletion mutant proteins purified from *E.coli* were incubated with partially purified ATR protein from HEK293T cells. The samples were analyzed by autoradiography. The N-terminal region of CHFR (a.a 1-360) was phosphorylated by ATR, whereas the C-terminal region of CHFR (a.a 361-664) was not. This data indicates that the N-terminal region of CHFR is indispensable for its phosphorylation.

**Figure 6. ATR phosphorylates CHFR at the N-terminal region *in vitro***

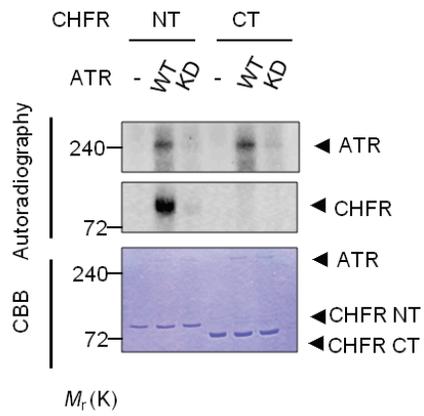
(A) Schematic representation of CHFR and CHFR deletion mutants. ATR consensus motifs on CHFR are shown. (FHA, forkhead associated domain; RF, RING finger domain; CR, cysteine- rich domain)

(B) Partially purified FLAG-ATR protein from HEK293T cells was incubated with purified GST-CHFR NT, CT mutants from *E.coli* at 30°C for 30 min. The samples were resolved by SDS-PAGE and visualized by autoradiography and Coomassie brilliant blue staining

**A**



**B**

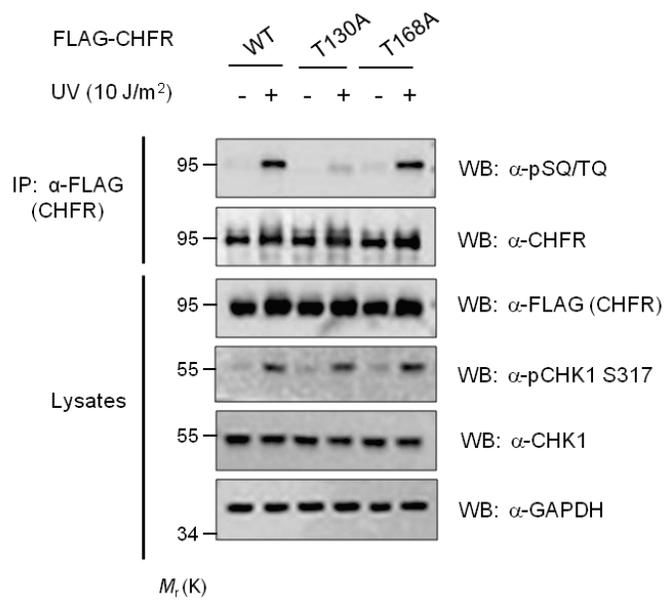


## **7. Threonine 130 on CHFR is the major phosphorylation site**

Given that the N-terminal region of CHFR is responsible for phosphorylation, I examined the phosphorylation of CHFR *in vivo* to further clarify the site. Since the N-terminal region contains two threonine residues (Thr 130, Thr 168), I used CHFR mutants in which threonine is replaced with alanine. (T130A, T168A). Phosphorylation of these mutants was analyzed by phospho-SQ/TQ antibody. MCF7 cells were transfected with FLAG-CHFR WT (wild-type), T130A and T168A and cells were irradiated with UV 10 J/m<sup>2</sup>. After incubation for 12 hours, lysates were immunoprecipitated with anti-FLAG resin and immunoblotted by phospho-SQ/TQ antibody. While WT and T168A mutant of CHFR were phosphorylated, T130A mutant was not. Taken together, I concluded that the major phosphorylation site on CHFR is threonine 130.

**Figure 7. Threonine 130 on CHFR is the major phosphorylation site**

MCF7 cells were transfected with FLAG-CHFR WT, CHFR T130A and T168A mutants. Cells were irradiated with UV 10 J/m<sup>2</sup> and incubated for 12 hours. Cell lysates were immunoprecipitated with anti-FLAG resin and precipitates were analyzed by immunoblotting with phospho-SQ/TQ antibody for phosphorylated CHFR.



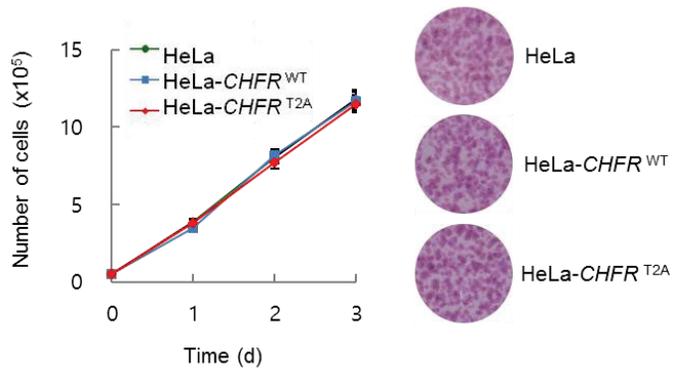
## **8. Phosphorylation of CHFR affects the checkpoint activity**

Post-translational modifications such as phosphorylation usually regulate the activity of target proteins. To investigate the effect and physiological meaning of phosphorylation of CHFR, cell proliferation assay and colony formation assay were performed using CHFR-WT and CHFR-T2A mutant. CHFR T2A is a double substitution mutant of both threonine 130 and threonine 168. It was used to clarify the effect of phosphorylation-deficiency. Since CHFR has been known to have anti-proliferative activity as a checkpoint protein, I examined the cell growth upon UV irradiation. HeLa cells stably expressing CHFR-WT or CHFR-T2A were exposed to UV 10 J/m<sup>2</sup> and then the number of cells was counted for a period of 3 days. Only HeLa CHFR-WT cells grew slowly and formed less G418 resistant colonies compared to HeLa CHFR-T2A cells upon UV irradiation. The slow proliferation of HeLa CHFR-WT cells is owing to the intact checkpoint function upon DNA damage while HeLa CHFR-T2A cells could not exert anti-proliferative activity. Accordingly, this result implies that phosphorylation of CHFR upon DNA damage is required for the checkpoint activity of CHFR.

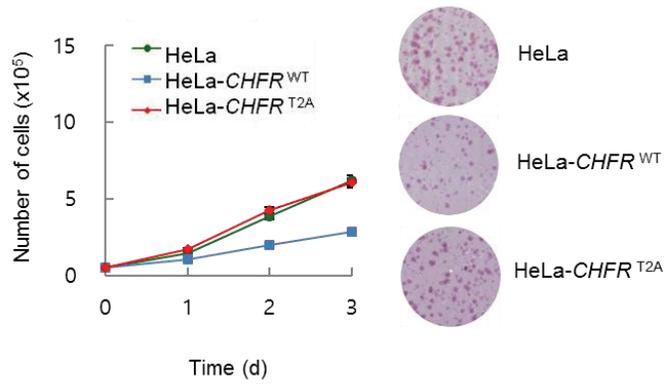
**Figure 8. Phosphorylation of CHFR affects the checkpoint activity**

HeLa stable cells with p3XFLAG-CMV10, CHFR-WT, CHFR-T2A were plated into 35 mm culture dishes ( $1 \times 10^5$  cells). Cells were irradiated with UV  $10 \text{ J/m}^2$ . The number of cells was counted for 3 days after UV treatment. Representative images were obtained by colony formation assay after 7 days of G418 selection using HeLa-CHFR stable cells. G418<sup>R</sup> colonies were fixed and stained with crystal violet.

### Untreated



### UV 10 J/m<sup>2</sup>



## Discussion

CHFR was originally identified as an early mitotic checkpoint. Upon microtubule poison, CHFR delays entry into metaphase. CHFR regulates mitotic progression through ubiquitination of PLK1 and Aurora A. In addition, CHFR is involved in p21-dependent G1 arrest by downregulation of HDAC1. Function of CHFR as a general cell cycle checkpoint has been revealed by studies of its downstream targets. On the other hand, how CHFR is regulated still remains elusive. Since CHFR acts as tumor suppressor, elucidating the regulatory mechanism is important to understand tumorigenesis as well as cell cycle checkpoint.

In this study, I identified ATR as an upstream regulator of CHFR. ATR was discovered as an interacting protein of CHFR and interaction between ATR and CHFR was analyzed *in vivo*. Thereby it raised the possibility that ATR phosphorylates CHFR. *In vitro* kinase assay demonstrated that CHFR is phosphorylated by ATR. Since ATR is the master regulator of DNA damage response, I investigated whether CHFR is ATR-dependent phosphorylated upon DNA damage. I examined phosphorylation of

CHFR after UV irradiation and doxorubicin treatment using ATR/ATM consensus motif antibody. Furthermore, lambda phosphatase treatment eliminated the phosphorylation and caffeine treatment inhibited this modification. These results verified that CHFR is phosphorylated by ATR in response to DNA damage. To investigate phosphorylation site of CHFR, I used CHFR mutants. *In vitro* kinase assay using CHFR deletion mutants indicated that the N-terminal region of CHFR is responsible for phosphorylation. Two threonine residues (T130, T168) in the N-terminal that are ATR consensus motifs were replaced with alanine to identify the exact site. Upon UV irradiation, T130A mutant was not phosphorylated by ATR. Hence, I concluded that threonine 130 of CHFR is the major phosphorylation site *in vivo*.

It has been known that ubiquitination and SUMOylation regulate stability or activity of CHFR (Kwon *et al.*, 2013; Bae *et al.*, 2013). Phosphorylation also has the possibility that it affects the activity of CHFR. As CHFR plays a part in cell cycle arrest upon mitotic poison, I examined checkpoint function of CHFR upon DNA damage. I performed cell proliferation assay to examine whether the anti-proliferative

activity of CHFR was dependent on phosphorylation. As expected, the phosphorylation-deficient mutant of CHFR did not exert checkpoint activity upon UV irradiation. Taken together, these results demonstrate that ATR-dependent phosphorylation is required for the checkpoint activity of CHFR.

It is important to elucidate specific signals or environments that trigger cellular response and modifications of CHFR. The signals for other known modifications of CHFR remain elusive. For this reason, ATR-dependent phosphorylation of CHFR in response to DNA damage is a new regulatory mechanism. Furthermore, phosphorylation is known to interplay with other post-translational modification. Hence, this finding contributes to investigating other modification of CHFR in response to various cellular stresses.

It needs to be further studied whether phosphorylation of CHFR alters the interaction with downstream targets and which specific cell cycle is affected by CHFR in this response. It has been reported that the domains of CHFR responsible for interaction with target proteins are different. Aurora A and HDAC1 interact with the CR domain while PLK1 interacts with FHA domain (Kang *et al.*, 2002; Yu *et al.*,

2005; Oh *et al.*, 2009). There is a chance that phosphorylation affects the property of CHFR and the interaction with substrates. Since CHFR needs to be precisely controlled as a checkpoint protein, it will be interesting to elucidate how the phosphorylation upon DNA damage modulates the downstream target and specific cell cycle transition.

In conclusion, this study presents not only the regulatory mechanism of CHFR but also the connection of CHFR with ATR-mediated pathway. Therefore, it implies that CHFR plays a role in DNA damage-induced checkpoint.

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## 국문 초록

CHFR은 E3 ubiquitin ligase로 세포 주기 진행과 암 형성을 조절하는 checkpoint protein이다. 또한 CHFR은 DNA 손상으로 인한 세포 반응에 관여한다고도 알려져 있다. CHFR이 genome integrity 유지에 중요한 역할을 함에 따라 CHFR의 기능과 그 하위 기질 단백질에 대한 연구의 중요성이 대두되고 있지만 이를 조절하는 그 상위 기작에 대한 연구는 미비한 편이다.

본 연구에서는 CHFR의 상위 조절 인자로 ATR을 밝히고, 이를 바탕으로 CHFR의 조절 기작을 연구하고자 하였다. CHFR과 상호작용하는 새로운 단백질로서 ATR을 동정하였고, CHFR을 인산화시킴을 확인하였다. ATR은 DNA damage response에서 역할을 하는 중요한 주 조절자로서, 세포에 DNA damage와 replications stress가 오면 ATR은 세포 주기 지연, DNA 손상 복구, 세포 사멸을 유도하는 신호 전달 경로를 활성화시킨다. 따라서 세포에 DNA 손상 자극이 가해질 때 CHFR이 인산화되는지 알아보려고 하였고, 세포에 UV irradiation과 doxorubicin 처리하여 DNA

손상 자극을 유도하였을 때 CHFR이 ATR에 의해 인산화됨을 확인하였다. CHFR 내 ATR consensus motif가 5개임을 바탕으로, CHFR mutant 를 이용하여 인산화 사이트를 알고자 하였다. 먼저 범위를 좁히기 위해 CHFR deletion mutant를 사용한 *in vitro* kinase assay로 CHFR의 N-말단이 인산화에 필요함을 확인하였다. 그리고 CHFR substitution mutant를 이용하여 세포 내에서 CHFR의 threonine 130이 ATR에 의해 인산화되는 사이트를 확인하였다. 또한 인산화가 되지 않는 CHFR substitution mutant는 DNA 손상 자극에도 불구하고 anti-proliferative 활성을 나타내지 않았다. 이를 통해 ATR 의존적 인산화가 CHFR의 checkpoint 기능을 조절함을 확인하였다. 이러한 결과들을 통해 CHFR의 새로운 조절 기작으로 DNA 손상 자극에 의한 ATR 의존적 인산화를 제시하였다.

주요어 : CHFR, ATR, DNA 손상 자극, 인산화, cell cycle checkpoint, E3 ligase

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