이학석사학위논문

O-linked *N*-acetylglucosamine modification에 의한 CHFR의 활성 조절에 관한 연구

Modification of CHFR by *O*-linked *N*-acetylglucosamine regulates its stability

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ABSTRACTS

O-linked β-N-acetylglucosamine (*O*-GlcNAc) modification (*O*-GlcNAcylation) of proteins is important for modulating many cellular processes such as signal transduction, transcription, translation and proteasomal degradation. Only two enzymes are known to regulate *O*-GlcNAcylation in mammals: *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA).

CHFR tumor suppressor is a checkpoint protein that delays cell cycle progression upon DNA damage. Moreover, CHFR ubiquitinates key mitotic kinases such as Aurora A and PLK1 leading to their degradation by proteasomes. Althrough the significance role in the control of cell growth, little is known on how CHFR activity is regulated.

Here, i showed that tumor suppressor CHFR was modified with *O*-GlcNAc. Moreover, the levels of *O*-GlcNAcylated CHFR increased when overexpressed OGT and/or OGA was blocked by Thiamet G, an *O*-GlcNAcase inhibitor. And CHFR was modified with *O*-GlcNAc at Ser164 and Ser165, analysed by mass

spectrometry. I confirmed that *O*-GlcNAcylation of CHFR positively regulates its stability. Thus, *O*-GlcNAcylation may contribute a new regulatory mechanism of CHFR.

Keywords : CHFR, Tumor suppressor, *O*-GlcNAcylation, OGT, OGA

Student Number : 2011-23247

INTRODUCTION

The tumor suppressor CHFR (Checkpoint with fork head-associated and RING finger domains) is identified as a mitotic checkpoint protein that delays entry into metaphase in response to mitotic stress such as interference with microtubule polymerization or stability (Scolnick and Halazonetics, 2000). It plays an important role in the control of cell growth and in the maintenance of genome integrity. CHFR gene has been linked to cancer. CHFR is expressed ubiquitously in normal tissues, but it is broadly inactivated in 15-50% of the various human tumors through hypermethylation of promoters (Corn et al, 2003; Mizuno et al, 2002; Shibata et al, 2002). Despite its potential importance in human cancers, very few studies have addressed how CHFR is regulated.

Mitosis is a crucial cellular event which is a key process in the segregation of duplicated genetic material (Blow et al, 2005). Errors in mitosis could potentially lead to chromosomal instability or mutations. When genomic stability is threatened, the checkpoint delays cell cycle progression in eukaryotic cells (Meister et al, 2003). CHFR acts early in prophase before

chromosomes condensation (Matsusaka and Pines, 2004). It delays chromosome condensation during prophase into prometaphase, when centrosome movement is inhibited by microtubule-targeting stress (Scolnick and Halazonetics, 2000).

CHFR has an N-terminal fork head-associated (FHA) domain, a central RING finger (RF) domain and a C-terminal cysteine-rich (CR) region (Scolnick and Halazonetics, 2000). First, FHA domains bind phosphopeptides from other proteins, and appear to negatively influence ubiquitination activity (Durocher et al, 1999, 2000; Wang et al, 2000). From other studies that analysed CHFR and studies using methyl-ubiquitin to inhibit ubiquitination, it was known that the ubiquitination activity of CHFR is needed for delayed mitotic entry (Matsusaka et al, 2004; Bothos et al, 2003; Chaturvedi et al, 2002; Kang et al, 2002).

And the RING finger domain of CHFR confers ubiquitin E3 ligase activity, it is required for its auto-ubiquitination and acts as checkpoint (Joazeiro et al, 1999; Levkowitz et al, 1999). For example, Polo-like kinase 1 (Plk1) that is considered a proto-oncogene, can regulate mitotic entry through its function in the regulation of cyclin-dependent kinase activity (Van Vugt and Medema, 2005). In *Xenopus laevis* extracts, CHFR could

ubiquitinate Plk1, which is degraded by the proteasome. Also, this down-regulation of Plk1 causes a delay in the progression to mitosis (Kang et al, 2002). This result supports that CHFR could ubiquitinate a target protein for cell cycle delay through its RF domain. CHFR can also bind to Aurora A, a key kinase that is needed in cell division, through its CR domain. CHFR influences protein levels of Aurora A by ubiquitin-mediated destruction (Privette et al, 2008; Yu et al, 2005). Moreover, a recent study identified that CHFR was regulated by SUMOylation, which lead to proteasomal degradation by ubiquitination (Kwon et al, 2013).

Metabolic rewiring is an emerging hallmark of cancer. Cancer cells require increased glucose uptake and aerobic glycolysis to support rapid cell growth and proliferation (Hanahan et al, 2011). Recent studies revealed that this is largely regulated by reprogramming gene expressions in the metabolic network, and this is important for the roles of c-Myc, Sp1 and p53 (Yang et al, 2006; Chou and Hart, 2001; Han and Kudlow, 1997). All of these transcription factors have been found to be modified by *O*-GlcNAc, and this modification can regulate their functions either positively or negatively.

O-GlcNAc modification, or *O*-GlcNAcylation, is a dynamic

post-translational modification of many nuclear, cytoplasmic and other proteins by *O*-linked *N*-acetyl-β-D-glucosamine (*O*-GlcNAc) (Wang et al, 2010; Wells et al, 2002; Torres and Hart, 1984). In many eukaryote systems, the hexosamine biosynthesis pathway (HBP) converts imported glucose and glucosamine to UDP-GlcNAc (Kornfeld, 1967). These two enzymes modulate proteins by the addition of *O*-GlcNAc to the protein at the hydroxyl groups of serine and threonine residue. UDP-N-acetyl-β-D-glucosamine /peptide N-acetyl glucosamin yl transferase (OGT) catalyzes the addition of *O*-GlcNAc, and the removal of *O*-GlcNAc is catalyzed by a natural N-acetyl-β -glucosaminidase (*O*-GlcNAcase, OGA) (Boehmelt et al, 2000; Shafi et al, 2000; Mio et al, 1999).

Since its discovery, almost one thousand proteins have been identified that are modified with *O*-GlcNAc (Yang et al, 2006; Chou and Hart, 2001; Han and Kudlow, 1997). Indeed, *O*-GlcNAcylation plays a protective role against cellular stress and cell death. A large number of *O*-GlcNAc modified-proteins regulate intracellular signaling including transcription regulation, cytoskeletal networks, stress responses and the ubiquitin-proteasome system (Sayat et al. 2008; Hart et al. 2011; Ishihara et al, 2010). Also, *O*-GlcNAc modification

regulate cell cycle and cell growth, it raise the possibility of *O*-GlcNAcylation on CHFR.

In this study, I demonstrated that CHFR modulated by *O*-GlcNAc through interaction with OGT. And, the level of O-GlcNAcylation on CHFR was increased by OGT and/or OGA inhibitor, Thiamet G (Scott et al, 2008). Using LC/LTQ-Orbitrap MS, i identified that Ser164 and Ser165 residues of CHFR are modified with O-GlcNAc. Moreover, the results of this study showed that O-GlcNAcylation on CHFR positively affects its stability.x

Materials and Methods

1. Cell culture, DNA transfection and plasmid.

MCF-7 (human breast carcinoma) and HEK 293 (human embryonic kidney) cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (Gibco), 100 units/ml penicillin and 100 μg/ml streptomycin at 37 ℃ in 5% CO_2. DNA transfections were performed by polyethylenimine (Sigma) according to the manufacturer's instructions. Wild type and mutant CHFR (human) inserts were cloned into the p3xFlag-CMV10 (Sigma) vector. CHFR 2SA mutant (Ser164, Ser165 mutated to Ala) was generated using the QuikChange site-directed mutagenesis Kit (Stratagene).

2. Antibodies and Reagents.

Antibodies against CHFR (rabbit polyclonal, Abcam), Xpress (mouse monoclonal, Invitrogen), FALG (DM-17, mouse monoclonal, Sigma), actin (AC-15, mouse monoclonal, Sigma), OGT (DM-17, rabbit polyclonal, Sigma) were used. The CTD110.6 (mouse monoclonal) antibody for *O*-GlcNAc detection was purchased from Covance. Thiamet G was kindly provided by Jin Won Cho (Yonsei University, seoul).

3. Immunoprecipitation and western blotting

Cells were lysed with buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, and 0.2% Triton X-100, and protease inhibitor cocktail). Lysate containing 1 mg protein was mixed with anti-CHFR antibody and rotate for 2 h at 4 ℃. Protein A sepharose beads (10 μl of 80% slurry, upstate) were added to the mixture before an additional 1 h rotation at 4 ℃. Immunoprecipitates were washed three times with lysis buffer, eluted with SDS sample buffer and the protein samples were subjected to 10% SDS-PAGE and transferred to a nitrocellulose (NC) membrane. The membrane was blocked with 3% bovine serum albumin (BSA). *O*-GlcNAcylation was detected with the CTD110.6 antibody.

4. Analysis of CHFR stability

FLAG-CHFR and HisMax-OGT was transfected into Hela cells, and treated with 10 μM Thiamet G. Cells were incubated for additional 12 h, and then treated with 200 μg/ml cycloheximide for indicated times. Cell lysates were analysed by immunoblot analysis with the anti-FLAG antibody. Relative band intensity was analysed by densitometry ImageJ software (ImageJ, US National Institutes of health).

5. Mapping of *O*-GlcNAc site using mass spectrometry

To map *O*-GlcNAcylation sites in CHFR, I used LC/LTQ-Orbitrap MS. Overexpressed FLAG-CHFR WT and HisMax-OGT proteins were purified using CHFR polyclonal antibody and subjected to SDS-PAGE. First, salt, SDS and coomassie brilliant blue were removed by repeated wash with 25 mM ammonium bicarbonate in 50% acetonitrile. Disulfide bridges were reduced with 10 mM dithiothreitol and alkylated with 55 mM iodoacetamide. In gel digestion with trypsin solution (10 ng/μL) was proceeded at 37 ℃ for 14-17 h. The resulting peptides were extracted with 1% formic acid in 50% acetonitrile. The peptide analysis was carried out using a Dual Cell Linear Ion Trap and Orbitrap Mass Spectrometer (Thermo Scientific).

RESULTS

1. CHFR is modified by *O*-linked *N*-acetylglucosamine

To determine whether CHFR is modified by *O*-linked *N*-acetylglucosamine, human breast carcinoma (MCF-7) cells were transfected to co-express FLAG-CHFR WT and OGT. Lysates from the MCF-7 cells were immunoprecipitated by polyclonal CHFR antibody, while the *O*-GlcNAc modification was detected by the *O*-GlcNAc modification specific antibody, CTD110.6 (Fig. 1a). I was showed that *O*-GlcNAcylation on CHFR was significantly detected by co-expressed OGT. Interestingly, when FLAG-CHFR WT, OGT and FLAG-OGA were co-expressed in MCF-7 cells, *O*-GlcNAcylation on CHFR was decreased by OGA (Fig. 1b). *O*-GlcNAcylation is reversibly regulated by the enzymatic activity of OGT and OGA. Therefore, FLAG-CHFR WT and OGT were co-transfected into MCF-7 cells, and then the cells were treated with 1, 10 μM Thiamet G (Fig. 1c). When the removal of *O*-GlcNAc was blocked by Thiamet G treatment (Scott et al, 2008), western blot analysis

with anti-*O*-GlcNAc antibody revealed that the levels of O-GlcNAc in total cell lysates was increased. In addition, immnunoprecipitats showed that *O*-GlcNAcylation on CHFR was markedly increased in does-dependent effect of Thiamet G. These results indicate that CHFR is modified by *O*-linked *N*-acetylglucosamine.

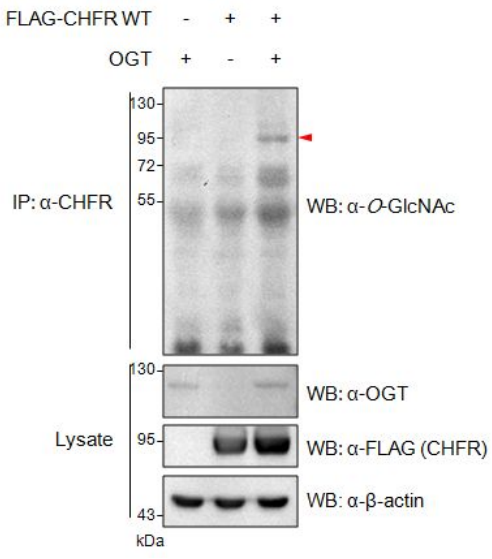
Figure 1. CHFR is modified with *O*-linked *N*-acetylglucosamine *in vivo*

(a) MCF-7 cells were co-transfected with FLAG-CHFR WT and OGT and harvested at 36 h after transfection. Cell lysates were immunoprecipitated by the anti-CHFR antibody and precipitates were analysed by immunoblotted with the anti-*O*-GlcNAc antibody (CTD 110.6) for *O*-GlcNAcylated CHFR.

(b) FLAG-CHFR WT, OGT and FLAG-OGA were co-transfecte d into MCF-7 cells.

(c) MCF-7 cells were co-transfected FLAG-CHFR WT and OGT for 24 h. And then cells were treated with Thiamet G (1, 10 μM) for 12 h. CHFR immunoprecipitates obtained from cellular extracts with the anti-CHFR antibody, and levels of *O*-GlcNAcylated CHFR were then analysed by western blot with anti-*O*-GlcNAc antibody (CTD 110.6).

a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OGT + - +
130
95
72
55
IP: α-CHFR
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130
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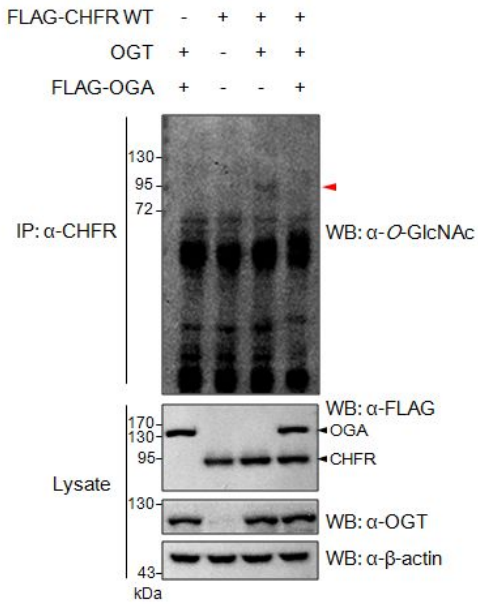
Lysate
95
WB: α-FLAG (CHFR)
WB: α-β-actin
43
kDa

b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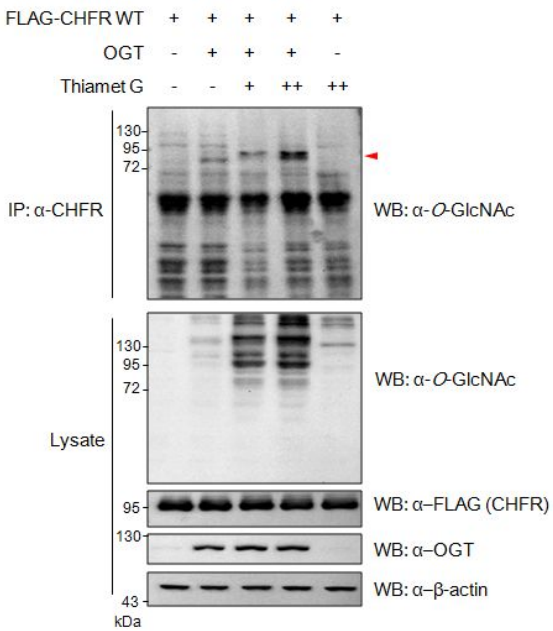
WB: α-β-actin
kDa

c

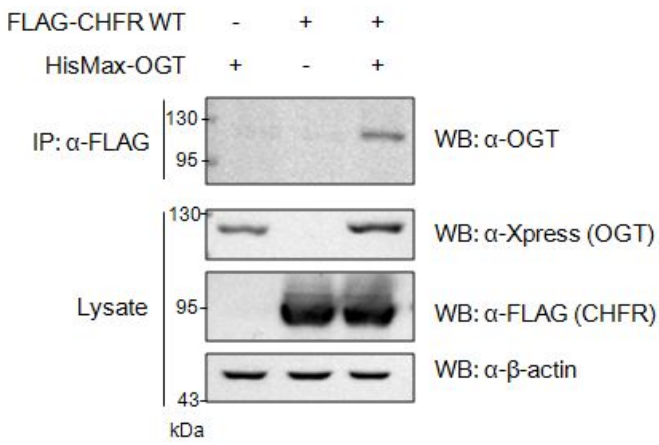

FLAG-CHFR WT + + + + +
OGT - + + + -
Thiamet G - - + ++ ++
IP: α-CHFR
130
95
72
WB: α-O-GlcNAc
Lysate
130
95
72
WB: α-O-GlcNAc
95
WB: α–FLAG (CHFR)
130
WB: α–OGT
43
WB: α–β-actin
kDa

2. CHFR interacted with OGT *in vivo*

To investigate whether CHFR could interact with OGT through co-immunoprecipitation, MCF-7 cells were transfected with FLAG-CHFR WT and HisMax-OGT. Cell lysates were incubated with anti-FLAG M2 resin that specifically recognized the Flag-tagged CHFR. And precipitates were immunoblotted with anti-OGT antibody. Interaction between CHFR and OGT was showed when the FLAG-CHFR WT and HisMax-OGT were co-expressed. (Fig. 2). Taken together, I showed that CHFR was modified with *O*-GlcNAc by interaction with OGT.

Figure 2. CHFR interacts with OGT *in vivo*

MCF-7 cells were co-transfected with FLAG-CHFR WT and HisMax-OGT and harvested at 24 h after transfection. Cell lysates were immunoprecipitated by the anti-FLAG M2 resin and then immunoblotted with an anti-OGT antibody.


FLAG-CHFR WT - + +
HisMax-OGT + - +
IP: α-FLAG
130
95
WB: α-OGT
Lysate
130
95
43
kDa
WB: α-Xpress (OGT)
WB: α-FLAG (CHFR)
WB: α-β-actin

3. CHFR is modified by *O*-linked *N*-acetylglucosamine at Ser 164 and Ser165

To identified the *O*-GlcNAcylation site on CHFR, I first predicted the site of *O*-GlcNAcylation on CHFR using the YinOYang 2.1 program which is an *O*-GlcNAcylation site prediction program (Gupta and Brunak, 2002). From result of prediction program, I found that CHFR could be modified with *O*-GlcNAc at 8 serine and threonine residues, with the highest potential value for *O*-GlcNAc modification at Ser164 and Ser630 (Table 1, Fig. 3a). The higher the potential value is indicated the higher the tendency to be modified with *O*-GlcNAc. First, to determine the specific *O*-GlcNAc sites, I created deletion mutants of CHFR. When amino acids 1-520 (deletion of 521-664) were mutated, *O*-GlcNAc modified CHFR could be detected by immunoblot using the anti-*O*-GlcNAc antibody, whereas △260 (deleting amino acids 1-260) had no effect on *O*-GlcNAc detection (Fig. 3b). Taken together, I confirmed that CHFR is modified with *O*-GlcNAc at amino acids 1-260.

Based on the analysis by the YinOYang 2.1 program, CHFR may be modified with *O*-GlcNAc at Ser164, Ser165, Ser178, Thr179, Ser204, and Ser228 residues, with the highest potential

value of *O*-GlcNAc modification at Ser164. Thus, to map the *O*-GlcNAcylation site of CHFR, I performed LTQ-Orbitrap MS analysis with trypsin digested peptides of CHFR immunoprecipitates. MCF-7 cell extracts, which had overexpressed FLAG-CHFR and HisMax-OGT and been treated with Thiamet G, were immunoprecipitated with the anti-CHFR antibody. Immunoprecipitates were separated by SDS-PAGE (Fig. 4a). CHFR was excised from Coomassie brilliant blue-stained gels and in-gel digested with trypsin prior to LTQ-Orbitrap MS analysis. The *O*-GlcNAc-modified 161-200 peptide of CHFR was observed at $[M+2H]^{2+}$ m/z 4343.9 in the tryptic-digested peptides. This *O*-GlcNAc-modified 161-200 peptide of CHFR weighed approximately 304 Da more than the unmodified peptide. The mass spectrum and the peptide sequence of this *O*- GlcNAc-modified peptide are shown in Fig 4b. I identified two major and one minor possible *O*-GlcNAc sites from this peptide sequence: 161VPP(S*)(S*)PATQVCFEEPQ PSTSTSDLFPTASASSTEPSPAGR200 or 161VPPSSPA(T*)QVCFE EPQPSTSTSDLFPTASASSTEPSPAGR200. Moreover, Ser164 and Ser165 were predicted by the YinOYang 2.1 program. To determine the specific *O*-GlcNAc sites, I created site-specific point mutants of CHFR. When wild type and T168A (Threonine

converted to Alanine) were immunoprecipitated with anti-CHFR antibody, *O*-GlcNAc modification could be detected by immunoblot using the anti-*O*-GlcNAc antibody, whereas △260 mutant had no effect on *O*-GlcNAc detection (Fig. 4c). It suggests that the sites of *O*-GlcNAc modification on CHFR were Ser164 and Ser165. Indeed, I confirmed that CHFR 2SA (Ser164 and Ser165 substituted to Ala) was not *O*-GlcNAcylated compared to the CHFR wild type (Fig. 4d). Taken together, I identified that CHFR is modified with *O*-GlcNAc at Ser164 and Ser165 residues.

Table 1. Predicted *O*-GlcNAc modification sites on CHFR by YinOYang 2.1

'++' indicates a higher possibility of *O*-GlcNAcylation than '+'. The higher potential value has the higher tendency to be modified with *O*-GlcNAc.

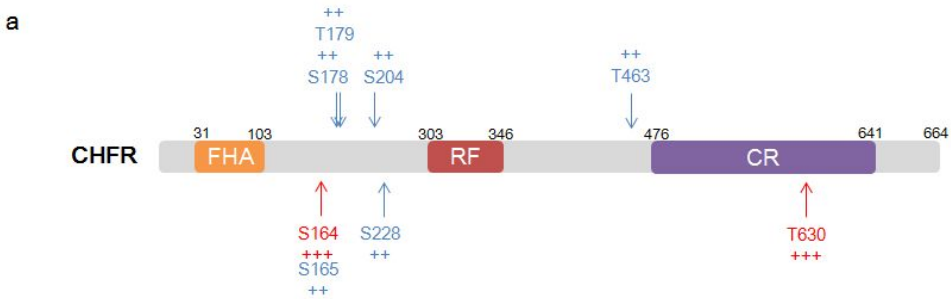
Table 1. Predicted O-GlcNAc sites of CHFR protein sequence by YinOYang 2.1

SeqName	Residue		O-GlcNAc result	Potential	Thresh.(1)	Thresh.(2)
CHFR	164	S	+++	0.69	0.4135	0.5078
CHFR	165	S	++	0.5654	0.4116	0.5052
CHFR	178	S	++	0.557	0.3956	4837
CHFR	179	T	++	0.592	0.4032	0.4939
CHFR	204	S	++	0.6008	0.4278	0.527
CHFR	228	S	++	0.685	0.4709	0.5852
CHFR	463	T	++	0.5792	0.4457	0.5512
CHFR	630	T	+++	0.6804	0.4636	0.5753

Figure 3. *O*-GlcNAcylation of CHFR is detected between residues 1-260

(a) Predicted *O*-GlcNAcylation sites of the CHFR by YinOYang 2.1

(b) MCF-7 cells were co-transfected with HisMax-OGT and FLAG-CHFR WT or 1-520, △260 mutants and treated with 10 μM Thiamet G for 12 hr. After incubation, cell lysates were immunoprecipitated with the anti-CHFR antibody and immunoblotted with the anti-*O*-GlcNAc antibody (CTD 110.6).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++
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S178
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S204
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T463
CHFR
31
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346
476
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FHA
RF
CR
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b
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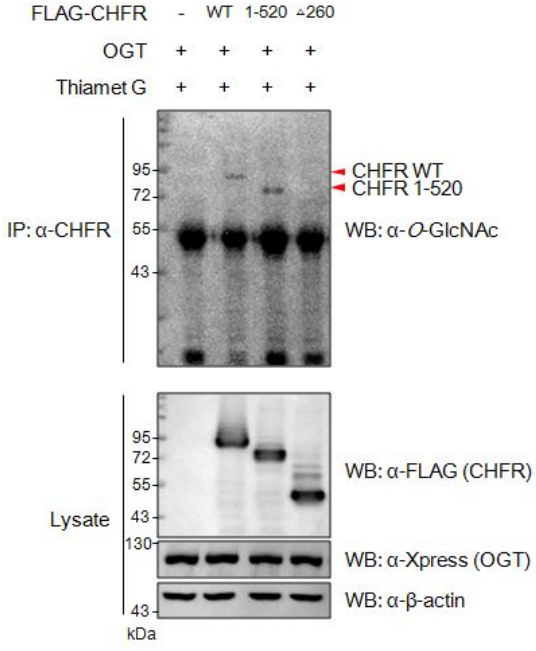
FLAG–CHFR △260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72
55
43
CHFR WT
CHFR 1-520
WB: α-O-GlcNAc
Lysate
95
72
55
43
130
43
kDa
WB: α-FLAG (CHFR)
WB: α-Xpress (OGT)
WB: α-β-actin

Figure 4. CHFR is modified by *O*-linked *N*-acetylglucosamine at Ser164 and Ser165

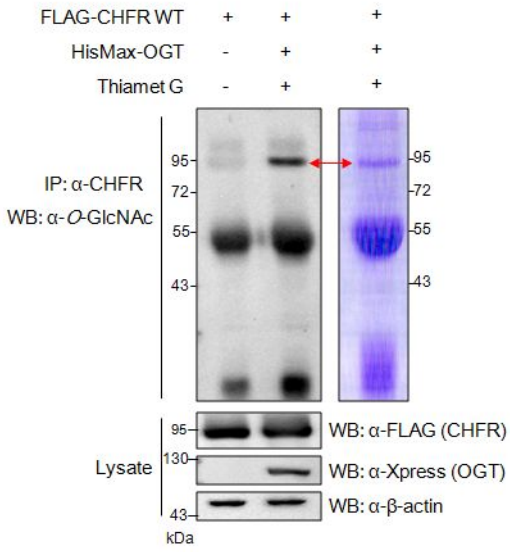
(a) *O*-GlcNAcylation on CHFR analysed by western blot with *O*-GlcNAc for immunoprecipitated CHFR from OGT-overexpressed MCF-7 cells in the presence of the Thiamet G, separated by SDS-PAGE, and visualized by coomassie brilliant blue (arrow). In-gel tryptic digestion was performed, and *O*-GlcNAc modification sites were mapped using LTQ-Orbitrap mass spectrometry, as described in the materials and methods.

(b) MS/MS spectrum and sequencing result showed that an *O*-GlcNAc-bearing 161-200 peptide at $[M+2H]^{2+}$m/z 4343.9. The difference in molecular mass between the unmodified peptide and the *O*-GlcNAc-bearing peptide is 304 Da.

(c) FLAG-CHFR WT, T168A and △260 were co-expressed with HisMax-OGT in MCF-7 cells. Cell lysates were immunoprecipitated with the anti-CHFR antibody, and precipitates were immunoblotted by *O*-GlcNAc.

(d) FLAG-CHFR WT or FLAG-CHFR 2SA was co-expressed with HisMax-OGT in MCF-7 cells. Cell lysates were immunoprecipitated with the anti-CHFR antibody, and immunoprecipitates were detected with anti-*O*-GlcNAc antibodies.

a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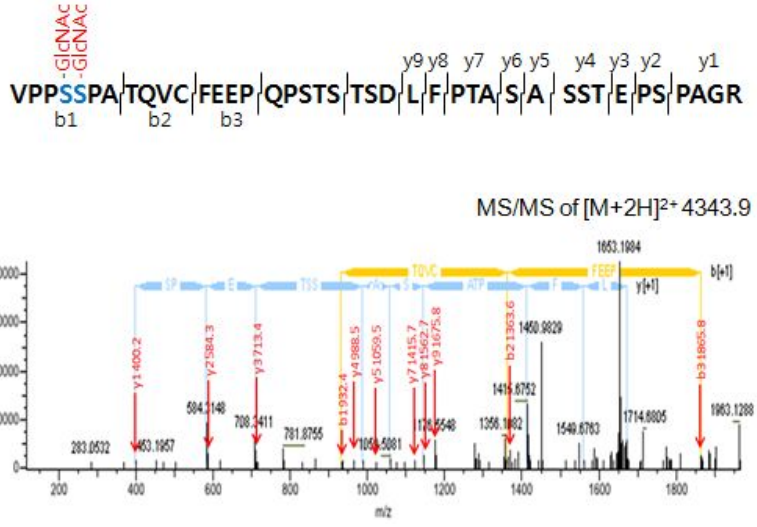
b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b1
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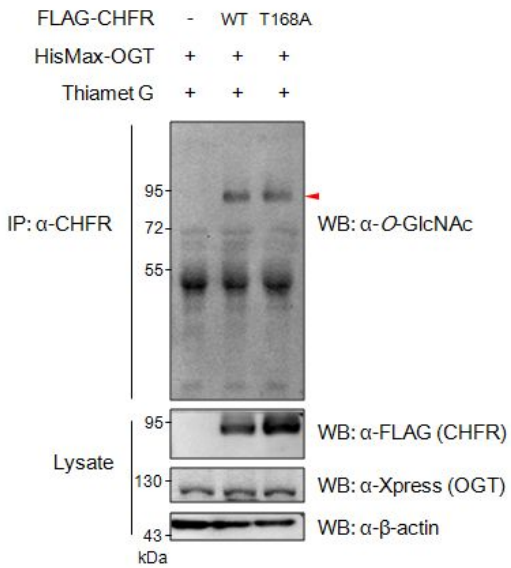
Intensity (Counts)
m/z

c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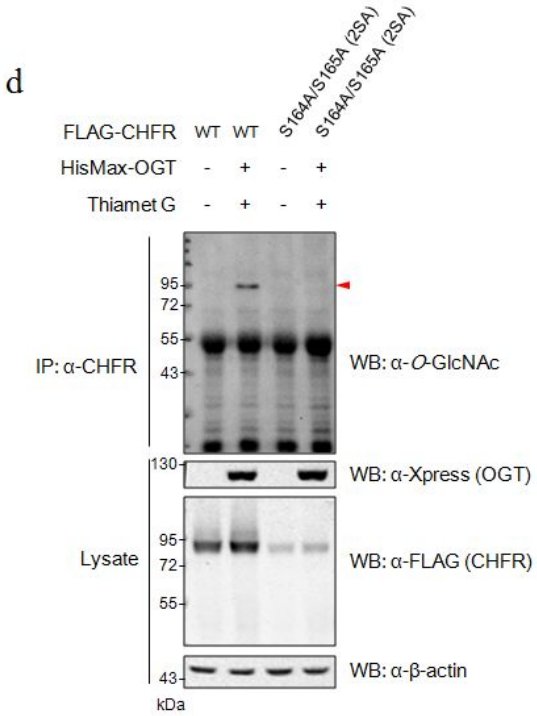
kDa

d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43
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130
WB: α-Xpress (OGT)
Lysate
95
72
55
WB: α-FLAG (CHFR)
43
WB: α-β-actin
kDa

4. Effect of *O*-GlcNAc modification on CHFR stability

O-GlcNAc modification regulates the activity and stability of target proteins (Yang et al, 2006; Chou and Hart, 2001; Han and Kudlow, 1997). To investigate the effect of *O*-GlcNAc modification on Ser164 and Ser165 residues of CHFR, I transfected FLAG-CHFR WT or 2SA with HisMax-OGT into MCF-7 cells. And at 24 h post-transfection, treated them with Thiamet G (Fig. 4d). After a 12 h incubation, cell extracts were immunoblotted with an anti-FLAG antibody. Wild type CHFR protein expression was little increased by *O*-GlcNAc modification.

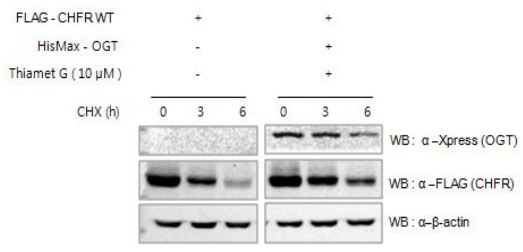
In a cycloheximide-chase assay, *O*-GlcNAc-modified CHFR was more stable than un-modified CHFR. CHFR WT protein levels were less decreased when co-expressed with HisMax-OGT (Fig. 5a). Consistent with this data, no effect was observed on the half-life and expression levels of the CHFR 2SA mutant (Fig. 5b). Thus, the results showed that *O*-GlcNAc-modified CHFR had increased stability by about 20% after treatment with the translational inhibitor (CHX). Our results showed that *O*-GlcNAcylation regulate CHFR stability.

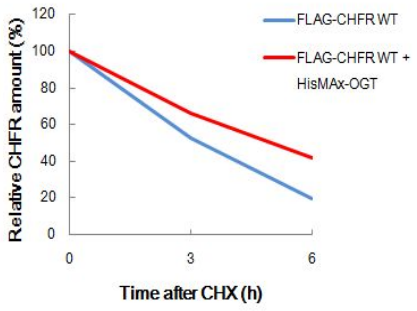
Figure 5. *O*-GlcNAc modification enhances the stability of CHFR

(a) Half-life of CHFR was increased by transfection of OGT in the presence of Thiamet G. Cells were treated with 200 μg/ml CHX for the indicated time and the CHFR levels were analysed by western blotting and densitometry.

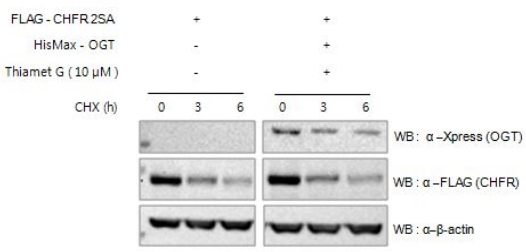
(b) Half-life of CHFR 2SA mutant was not changed by co-transfected with HisMax-OGT in the presence of Thiamet G. Cell lysates were subjected to immnoblotting with anti-Xpress, anti-FLAG and anti-β-actin antibodies.

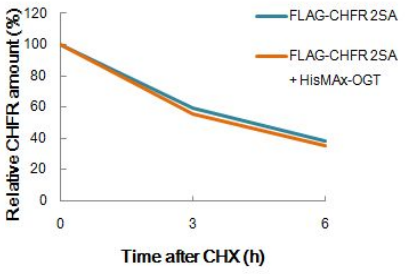
a


FLAG - CHFR WT
HisMax - OGT
Thiamet G (10 μM)
CHX (h)
0 3 6 0 3 6
WB : α –Xpress (OGT)
WB : α –FLAG (CHFR)
WB : α–β-actin


Relative CHFR amount (%)
120
100
80
60
40
20
0
0
3
6
Time after CHX (h)
FLAG-CHFR WT
FLAG-CHFR WT +
HisMAx-OGT

b


FLAG - CHFR 2SA
HisMax - OGT
Thiamet G (10 μM)
CHX (h)
0 3 6 0 3 6
WB : α –Xpress (OGT)
WB : α –FLAG (CHFR)
WB : α–β-actin


Relative CHFR amount (%)
120
100
80
60
40
20
0
0
3
6
Time after CHX (h)
FLAG-CHFR 2SA
FLAG-CHFR 2SA
+ HisMAx-OGT

DISCUSSION

CHFR was identified as a mitotic checkpoint protein with tumor-suppression function. And, several studies were characterized its expression patterns and regulation in normal and cancer cells and trying to elucidate its functional roles in cell biology (Corn et al, 2003; Mizuno et al, 2002; Shibata et al, 2002; Scolnick and Halazonetics, 2000). At present, It is just beginning to understand how CHFR stability is regulated. In a large number of studies, CHFR stability and activity are regulated by post-translational modifications such as ubiquitination or SUMOylation (Joazeiro et al, 1999; Levkowitz et al, 1999; Annemarthe et al, 2012; Kwon et al, 2013). For example, a recent study revealed that CHFR was modified by SUMOylation, leading to its proteasomal degradation by ubiquitination (Kwon et al, 2013).

O-GlcNAcylation is a ubiquitous post-translational modification, and its occurs at Serine and Threonine residues of nucleocytoplasmic protein (Ishihara et at, 2010). Many studies identified that a number of nutrient- and stress-responsive transcription factors, such as SP1, p53, NF-κB, CREB, CTRC2 and FOXO1 were regulated by *O*-GlcNAcylation (Yang et al,

2006; Chou and Hart, 2001; Han and Kudlow, 1997). And *O*-GlcNAcylation modulates functions of these transcription factors by many mechanisms influencing localization, stability, protein-protein interactions, phosphorylation, and transcriptional activity (Sayat et al. 2008; Hart et al. 2011; Ishihara et al, 2010).

This study showed that CHFR could be modified with *O*-GlcNAc. The steady state level of the protein *O*-GlcNAcylation is dependent on the rate of addition, catalyzed by OGT, as well as its rate of removal, catalyzed by OGA (Dong and Hart, 1994). I observed that CHFR was modified with *O*-GlcNAc via overexpressed OGT and this modified-CHFR was decreased by OGA. Moreover, Thiamet G is known as a competitive inhibitor of OGA, and the removal of *O*-GlcNAc through Thiamet G leads to significant increases incellular *O*-GlcNAc levels in isolated cardiomyocytes (Scott et al, 2008). When MCF-7 cells were treated with Thiamet G, *O*-GlcNAc modified-CHFR was increased in a dose-dependent fashion. Futhermore, it was identified that Ser164 and Ser165 are critical sites of *O*-GlcNAcylation on CHFR using mass spectrometry analysis. Also, using site-directed mutagenesis, it was confirmed that Ser164 and Ser165 were modified with *O*-GlcNAc on

CHFR.

The present study showed that when CHFR was modified with *O*-GlcNAc, CHFR WT levels were increased, but CHFR 2SA mutants was not changed. Moreover, such a tendency was confirmed with the cycloheximide-chase assay (Fig 5). Although effects of *O*-GlcNAcylation on CHFR stability has not yet been investigated *in vivo,* it could provide novel insights into how CHFR is regulated.

In addition, As *O*-GlcNAcylation on CHFR stability, most E3 ubiquitin ligases are generally regulated at the protein level by auto-ubiquitination; thus, auto-ubiquitination of CHFR may be affected by *O*-GlcNAcylation. Another possible effect is the interplay between *O*-GlcNAcylation and phosphorylation, which may be important for CHFR activity. In most cases, *O*-GlcNAcylation has extensive cross-talk with phosphorylation, and the two modifications can compete for occupancy at the same site or at adjacent residues (Hart et al, 2007).

In conclusion, the present study shows that CHFR is modified with *O*-GlcNAc at Ser164 and Ser165 residues, and this modification contributes the stability of CHFR.

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국문 초록

O-linked β-N-acetylglucosamine (*O*-GlcNAc) 변형은 세포내 신호 변형, 전사, 번역, proteasome을 통한 분해 등과 같은 세포내 과정을 조절하는데 중요한 역할을 하고 있다. 이 과정은 OGT와 OGA 두 효소에 의해 조절이 된다.

종양 억제 단백질인 CHFR (checkpoint with FHA and RING domain)은 세포 주기 진행을 조절하는 검사 단백질로서 역할을 하는 것으로 알려져 있다. 지금까지 CHFR의 발현과 기능에 관한 많은 연구가 진행되어 왔으나, 이 CHFR을 조절하는 상위 기작에 관해서는 잘 알려지지 않았다.

본 연구에서는 *O*-GlcNAc에 의해 CHFR이 변형됨을 확인하였고, *O*-GlcNAc 변형된 CHFR이 OGT나 OGA를 막는 약물인 Thiamet G에 의해 증가하는 것을 확인할 수 있었다. 그리고 Mass spectrometry 분석을 통해 이 변형이 CHFR의 Ser164와 Ser165 잔기에서 일어나는 것을 확인하였다. 또한 세포내 단백질로의 번역을 저해하는 약물인 cycloheximide를 처리한 실험을 통하여, CHFR이 *O*-GlcNAc 변형에 의해 안정성이 증가되는 것을 확인할 수 있었다. 이러한 결과를 통해 CHFR의 *O*-GlcNAc에 의한 변형이 세포주기 조절과 암 억제에 중요한 역할을 하고 있는 CHFR의 조절작용으로서의 제시 될 수 있을 것이다.

주요어 : CHFR, Tumor suppressor, *O*-GlcNAcylation, OGT, OGA

학 번 : 2011-23247

감사문

가장 먼저, 저에게 항상 사랑을 주시는 부모님께 감사의 말씀 드립니다. 저를 낳아 주시고 대학원을 마치는 지금까지 항상 기도해 주시고 믿어주셔서 이렇게 대학원을 무사히 마칠 수 있었습니다. 아빠, 엄마 사랑합니다. 사랑 한다 동생 은혜야. 앞으로도 우리가족 모두 건강하고 행복하게 살았으면 좋겠습니다.

그리고 부족한 저를 가르쳐 주시고 끝까지 믿어주신 저희 설재홍 교수님 정말 감사드립니다. 앞으로 열심히 선생님의 자랑스러운 제자로서 살아가도록 하겠습니다. 건강하세요.

그리고 실험실에서 많이 가르쳐 주시고 도와주신 김명진 박사님 감사합니다. 또, 실험실에서 항상 많이 도와주신 성준오빠, 주미언니, 영은언니 선배님들 정말 감사했습니다. 선배님들이 계셔서 정말 든든했습니다. 그리고 후배님들도 감사합니다. 다들 좋은 결과 있기를 바라겠습니다.

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