Biochemical and Biophysical Research Communications xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

CHFR functions as a ubiquitin ligase for HLTF to regulate its stability and functions

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ARTICLE INFO

Article history:
Received 2 April 2010
Available online xxxx

Keywords:
CHFR
HLTF
Ubiquitination
PAI-1
Cell migration
Tumorigenesis

ABSTRACT

CHFR functions as a mitotic checkpoint by delaying entry into metaphase in response to mitotic stress. *CHFR* is frequently silenced by hypermethylation in human cancers, indicating that CHFR is a tumor suppressor. To further elucidate the role of CHFR in tumorigenesis, we studied the relationship between CHFR and a novel CHFR-interacting protein, HLTF, helicase-like transcription factor. Here we show that CHFR binds to and ubiquitinates HLTF, leading to its degradation. HLTF modulates basal expression of *PAI-1* involved in regulation of cell migration. Consistently, overexpression of CHFR inhibits cell migration, resulting from reduced HLTF followed by decreased *PAI-1* expression. HLTF expression is also higher in human breast cancer cells where CHFR is not expressed. Taken together, this is the first report identifying the regulatory mechanism of HLTF by CHFR, suggesting that CHFR-mediated downregulation of HLTF may help protect against cancer.

1. Introduction

The ubiquitin-proteasome system plays important roles in modulating various protein levels during cell-cycle progression, cell proliferation and differentiation, immune response, apoptosis, etc. [1,2]. Ubiquitination is accomplished by a multiple enzyme cascade composed of ubiquitin-activating (E1), conjugating (E2), and ligating (E3) enzymes. E3 ubiquitin ligases determine the substrate specificity and are classified into two groups based on the presence of either a HECT or a RING domain [1].

CHFR (checkpoint protein with FHA and RING finger domains), a RING type E3 ubiquitin ligase, was described as a mitotic checkpoint that delays cell-cycle progression to metaphase in response to mitotic stress [3]. CHFR controls intracellular levels of crucial mitotic proteins such as Plk1 and Aurora A, resulting in the regulation of cell-cycle progression and chromosomal stability [4,5]. CHFR has an N-terminal forkhead-associated (FHA) domain required for the phosphoprotein interaction [6], a RING finger domain contributed to the ubiquitin ligase activity [7], and a C-terminal cysteine-rich (CR) domain that its function has not been fully elucidated. CHFR also contains a nuclear localization signal (NLS) sequence essential for its cellular function [8]. Ubiquitin-specific protease, USP7/HAUSP deubiquitinates CHFR and prevents its degradation, resulting in the increased stability [9].

Several lines of evidence indicate that CHFR is a tumor suppressor. CHFR is frequently silenced by promoter hypermethylation in various cancers, although CHFR is ubiquitously expressed in normal tissues [3,10,11]. Loss of CHFR expression leads to tumorigenic phenotypes, i.e. the increased invasion and motility, the faster tumor development, and the higher mitotic index [12]. *Chfr* knockout mice have increased tumor incidence, aneuploidy, and defective chromosomal segregation and cytokinesis [4,13]. It has recently been reported that CHFR is also involved in tumorigenesis through downregulation of HDAC1 and inhibition of NF-κB activity [14,15]. To further understand biological functions of CHFR in tumorigenesis, we sought to find novel CHFR-interacting partners by immunoaffinity purification in combination with mass spectrometry. The list of CHFR-interacting proteins by LC–MS/MS analysis was previously reported [14] and we further focused on HLTF (helicase-like transcription factor; also known as HIP116, Zbu1, RUSH1, and SMARCA3) [16–18]. HLTF belongs to the SWI/SNF family of chromatin remodeling complex and contains a DNA-binding domain, a RING finger domain and seven helicase domains [19]. HLTF regulates *PAI-1* (plasminogen activator inhibitor-1) gene expression [19], which is involved in embryonic development, tumor invasion, and metastasis by controlling cell migration [20]. Although there are several studies showing that HLTF is either inactivated by its promoter methylation in human cancers or upregulated in immortalized cell lines and an estrogen induced-kidney tumor model system [21,22], the underlying mechanism of these controversial observations has not been fully understood. In the present study, we show that CHFR plays an important role in regulation of HLTF stability and protects cells against HLTF-mediated cell migration.

Abbreviations: CHFR, checkpoint protein with FHA and RING finger domains; HLTF, helicase-like transcription factor; PAI-1, plasminogen activator inhibitor-1; FHA, forkhead-associated; CR, cysteine-rich; GST, glutathione S-transferase.

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0006-291X/$ - see front matter © 2010 Published by Elsevier Inc.
doi:10.1016/j.bbrc.2010.04.052

2. Materials and methods

2.1. Cell culture and transfection

HeLa and MCF7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1 μg ml^{-1} streptomycin and 100 U ml^{-1} penicillin (Welgene) at 37 °C in a humidified 5% CO_2 atmosphere. All transient transfections were performed using polyethylenimine (Sigma) according to manufacturer's instructions.

2.2. Plasmids and antibodies

The p3xFLAG-CMV10-CHFR was described previously [14]. pcDNA4-HisMax-HLTF was cloned from pCMV-SPORT6-HLTF cDNA purchased from Benebiosis Co., Ltd. Antibodies used for experiments were as follows: anti-Xpress (Invitrogen); anti-FLAG and anti-β-actin (Sigma); anti-GAPDH (Cell Signaling Technology); anti-HLTF (Abcam); horseradish peroxidase-conjugated AffiniPure goat anti-rabbit and anti-mouse IgG (Bio-Rad Laboratory); rabbit polyclonal anti-CHFR antiserum (raised against a recombinant His-CHFR).

2.3. Assays for protein–protein interaction

Cells were lysed in buffer A (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 0.1 mM EDTA, and 0.2% Triton X-100) containing 1× complete protease inhibitor cocktail (Roche Applied Science). Cell lysates were incubated with anti-FLAG M2 resin (Sigma) or Ni–NTA resin (QIAGEN) for 2 h at 4 °C. The resins were collected by centrifugation and washed three times with buffer A and twice with buffer B consisting of 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.1 mM EDTA. Bound proteins were eluted by 0.2% SDS and subjected to SDS–PAGE followed by immunoblotting with appropriate antibodies. To analyze the interaction of CHFR and HLTF *in vitro*, GST-CHFR was purified from *Escherichia coli* and HisMax-HLTF was purified from HeLa cells. Purified GST-CHFR (1 μg) and HisMax-HLTF (1 μg) were incubated with Glutathione Sepharose 4 Fast Flow (GE Healthcare) for 1 h at 4 °C. The resins were washed three times with buffer A and twice with buffer B. Bound proteins were eluted and analyzed as described above.

2.4. Assays for ubiquitination and pulse-chase analysis

For the *in vitro* ubiquitination assay, purified HisMax-HLTF (1 μg) from HeLa cells was incubated at 37 °C for indicated times with E1 (0.5 μg), UbcH5 (0.5 μg), Ub (5 μg), 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, and 5 mM ATP in the presence or absence of GST-CHFR (3 μg) purified from *E. coli*. After incubation, samples were resolved by SDS–PAGE and immunoblotted with anti-HLTF antibody. For the pulse-chase analysis, FLAG-CHFR-transfected HeLa cells were treated with cycloheximide (200 μg ml^{-1}) for 0, 2, 4, or 6 h. Cell lysates were then analyzed by immunoblotting with indicated antibodies.

2.5. Reverse transcription-polymerase chain reaction

Total RNA was isolated from HeLa cells using the easy-BLUE™ total RNA extraction kit (iNtRON Biotechnology). Reverse transcription reactions were performed using RevertAid M-MuLV reverse transcriptase (Fermentas) and oligo (dT) primer, according to manufacturer's instructions. *PAI-1* and *GAPDH* mRNA were amplified by PCR using following oligos: for *PAI-1*, 5′-GTCTTTGGTGAAGGGTCTG-CTGTGCACCAT-3′ (forward) and 5′-TGAAAAGTCCACTTGCTTGACCG-TGCTCCG-3′ (reverse); for *GAPDH*, 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse).

2.6. In vitro wound-healing assay and statistical analysis

HeLa cells were transfected with appropriate expression vectors in 35-mm dishes. After cells became confluent, the cell monolayer was wounded using a sterile micropipette tip. Cells were placed back into the culture incubator for 20 h after removing floating cells. Then, wound regions were examined by visualization with a Zeiss microscope (Thornwood). The number of moved cells into the wounded area was counted excluding cells at time 0 h in four different fields per each group. Cell motility was expressed as mean ± SEM from four independent experiments and analyzed statistically by one-way analysis of variance (ANOVA) with Tukey's *post hoc* test for multiple comparisons using GraphPad Prism, version 5.0 (GraphPad Software, La Jolla). Statistical significance was defined as $P < 0.05$.

3. Results

3.1. CHFR interacts with HLTF in vivo and in vitro

Since HLTF was identified as a novel CHFR-interacting factor by immunoaffinity purification combined with mass spectrometry, co-immunoprecipitation assay of CHFR and HLTF was performed to examine their interaction *in vivo* in CHFR-transiently transfected HeLa cells, in which endogenous CHFR is not expressed. As shown in Fig. 1A, CHFR and HLTF interact each other. It was also confirmed in CHFR-stable HeLa cells (HeLa-CHFR) that CHFR binds to endogenous HLTF (Fig. 1B). To further validate whether this interaction between CHFR and HLTF is direct, GST pull-down assay was performed. HLTF was able to bind to CHFR directly (Fig. 1C). These results clearly show that CHFR binds to HLTF both *in vivo* and *in vitro*. Since CHFR interacts with its target proteins through different regions, i.e. FHA domain for Plk1 [5] and CR domain for Aurora A and HDAC1 [4,14], various CHFR-deletion mutants were generated to identify the interaction domain of CHFR with HLTF. When HeLa cells expressing FLAG-CHFR-deletion mutants that lack either FHA, CR, or N-terminus were assayed, CHFR-wild-type, -ΔFHA, and -ΔN mutants were able to interact with HLTF, but not CHFR-ΔCR mutant (Fig. 1D). Thus, the CR domain of CHFR is necessary for its interaction with HLTF.

3.2. CHFR negatively regulates HLTF by the ubiquitin-proteasome system

Given the fact that CHFR binds to HLTF directly and possesses an E3 ubiquitin ligase activity, the effect of CHFR on HLTF stability was analyzed. When HeLa cells were transiently transfected with wild-type, ubiquitination-defective mutant (which possesses point mutation in a RING finger domain, I306A hereafter), or HLTF binding-defective ΔCR mutant of CHFR, both CHFR-I306A and CHFR-ΔCR mutant did not affect the protein level of HLTF (Fig. 2A). Next, we investigated whether the reduction of HLTF was due to proteasomal degradation. As CHFR levels increased, HLTF levels decreased accordingly and proteasome inhibitor MG132 reverted the destabilizing effect of CHFR on HLTF stability, suggesting that this is mediated by ubiquitin-proteasome dependent proteolysis (Fig. 2B). This is also confirmed by the subsequent cycloheximide-chase assay that the half-life of HLTF was shortened in the presence of CHFR (Fig. 2C). CHFR-mediated degradation of HLTF is further delineated by the *in vitro* ubiquitination assay in the presence of CHFR. As shown in Fig. 2D, HLTF was directly ubiquitinated *in vitro* by CHFR. These results clearly demonstrate that CHFR binds to and ubiquitinates HLTF, resulting in its proteasomal degradation.

3.3. CHFR inhibits PAI-1 by destabilizing HLTF

Since HLTF is destabilized by CHFR, it is of interest to study the effect of CHFR on HLTF downstream targets. In order to investigate

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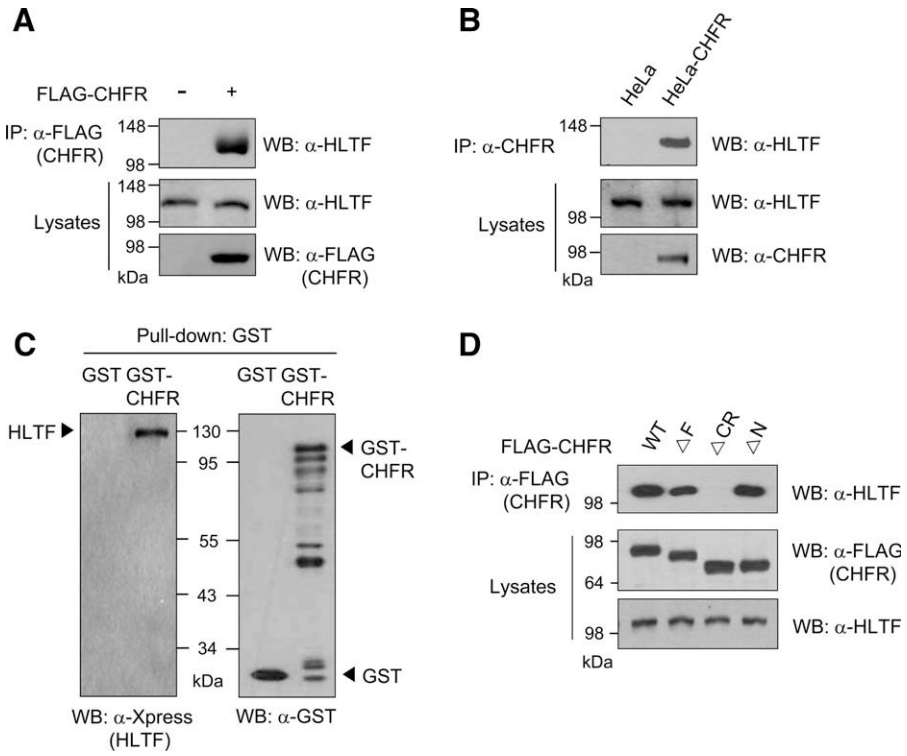

Fig. 1. CHFR interacts with HLTF *in vivo* and *in vitro*. (A) HeLa cells were transiently transfected with p3xFLAG-CHFR. Twenty-four hours after transfection, cells were treated with MG132 (2 μM) for 12 h. Cell lysates (1 mg) were immunoprecipitated with anti-FLAG M2 resin and immunoblotted with indicated antibodies. (B) Lysates from HeLa-mock or HeLa-CHFR cells stably expressing p3xFLAG-CMV10 or p3xFLAG-CHFR were immunoprecipitated with anti-CHFR antibody and immunoblotted with indicated antibodies. MG132 (2 μM) was treated for 12 h before harvest. (C) Both GST-CHFR and HisMax-HLTF were incubated with Glutathione Sepharose 4 Fast Flow. Precipitates were analyzed by SDS–PAGE followed by immunoblotting with anti-Xpress or anti-GST antibodies. (D) HeLa cells were transfected with various deletion mutants of FLAG-CHFR. Twenty-four hours after transfection, cells were treated with MG132 (2 μM) for 12 h. Cell lysates were immunoprecipitated and immunoblotted with indicated antibodies. WT, wild-type; ΔF, FHA domain deletion; ΔCR, CR domain deletion; ΔN, N-terminal deletion.

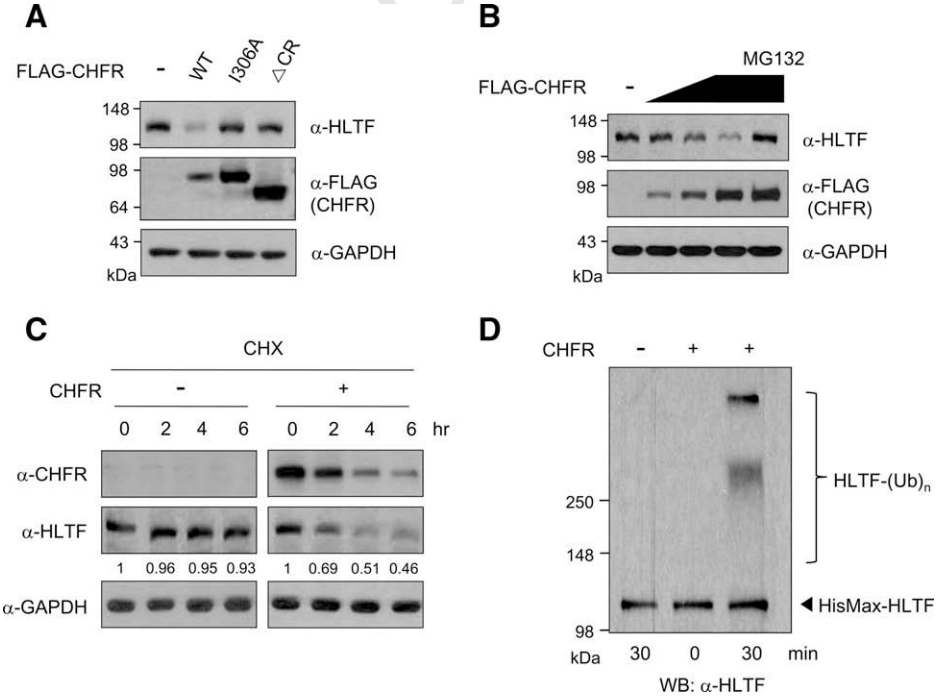

Fig. 2. CHFR negatively regulates and ubiquitinates HLTF. (A) HeLa cells were transfected with FLAG-CMV10 or FLAG-CHFR-deletion mutants. Cell lysates were subjected to SDS–PAGE followed by immunoblotting with indicated antibodies. (B) HeLa cells were transfected with FLAG-CHFR (0.5, 1, and 2 μg) plasmid. Cell lysates were subjected to SDS–PAGE followed by immunoblotting with anti-HLTF, anti-FLAG, or anti-GAPDH antibodies. Twenty-four hours after transfection, cells were treated with MG132 (20 μM) for 4 h. (C) HeLa cells were transfected with FLAG-CHFR. Twenty-four hours after transfection, cells were treated with cycloheximide for 0, 2, 4, or 6 h. Cell lysates were subjected to immunoblotting with indicated antibodies. The relative half-life of HLTF protein was quantified using ImageJ software (ImageJ, US National Institutes of Health). (D) Purified HisMax-HLTF was incubated for the indicated times with E1, E2 (UbcH5), and ubiquitin in the presence or absence of GST-CHFR. Each sample was then subjected to SDS–PAGE and immunoblotting with anti-HLTF antibody.

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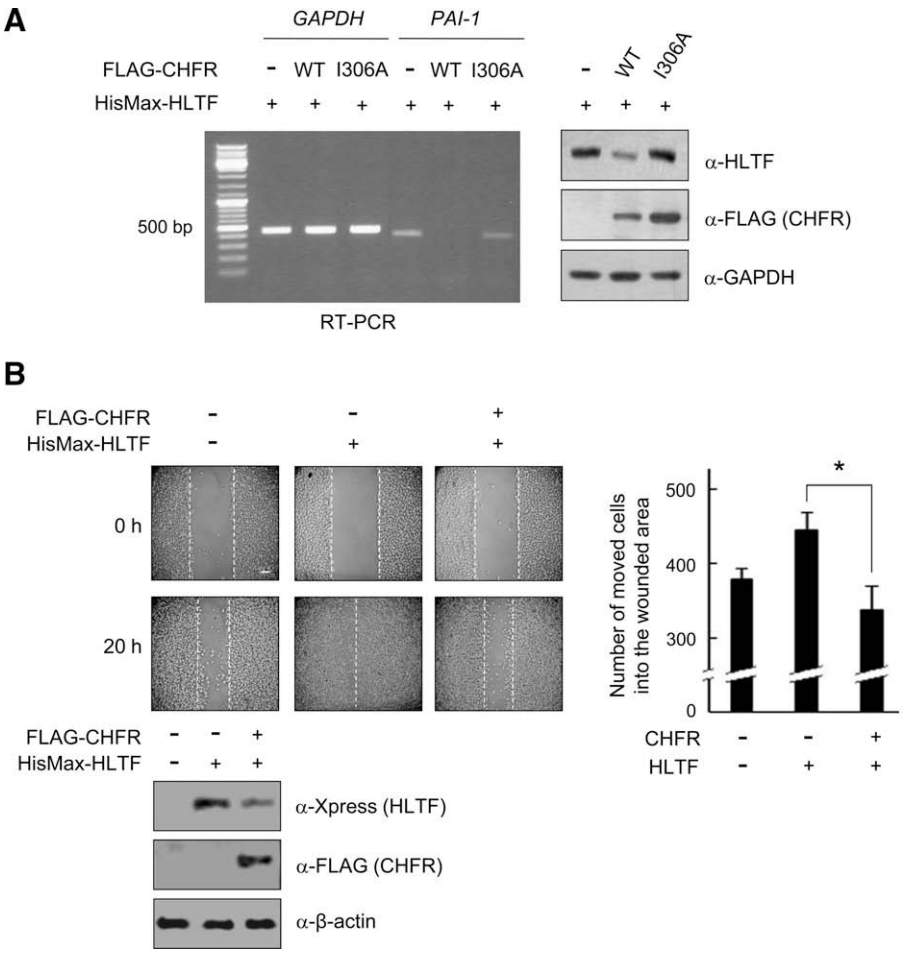

Fig. 3. CHFR suppresses *PAI-1* gene expression by destabilizing HLTF. (A) HeLa cells were transfected with FLAG-CHFR (-wild-type or -I306A) and HisMax-HLTF. Cell lysates were subjected to immunoblotting with indicated antibodies. Total mRNA was isolated and subjected to RT-PCR for *PAI-1* and *GAPDH* mRNA. (B) HeLa cells were transiently transfected with HisMax-HLTF and/or FLAG-CHFR. The wound closure was examined using the light microscope (top left panel). A representative image is shown for each group. Scale bar, 100 μm. Cell lysates used in a wound-healing assay were subjected to SDS–PAGE followed by immunoblotting with indicated antibodies (bottom panel). $P = 0.03$ in overall ANOVA test and $^*P < 0.05$ [(−CHFR/+HLTF) vs. (+CHFR/+HLTF)] in *post hoc* Tukey's multiple comparison test.

the functional consequence of CHFR-dependent degradation of HLTF, we checked the expression of *PAI-1*, the best-characterized HLTF target gene [19]. *PAI-1* expression was not detected in the presence of CHFR-wild-type, while there is little effect of CHFR-I306A (Fig. 3A; left panel). This is mainly due to the CHFR-mediated proteasomal degradation of HLTF (Fig. 3A; right panel).

Although the exact mechanism by which PAI-1 influences cell migration remains unclear, it is well known that PAI-1 plays an important role in a variety of normal and pathological processes during cell migration [23]. As CHFR suppresses HLTF followed by the inhibition of *PAI-1* expression, we examined the effect of CHFR on cell migration by the *in vitro* wound-healing assay. Cell motility was increased in HLTF-expressing HeLa cells, compared with control cells. However, this stimulatory effect of HLTF on cell migration was abolished in the presence of CHFR (Fig. 3B; top left panel). Statistical analysis clearly showed that migrated cell numbers were significantly different. CHFR-mediated HLTF degradation was also validated by immunoblotting with cell lysates used in a wound-healing assay (Fig. 3B; bottom panel). Thus, these findings suggest that CHFR controls the expression and the function of HLTF downstream targets by destabilizing HLTF.

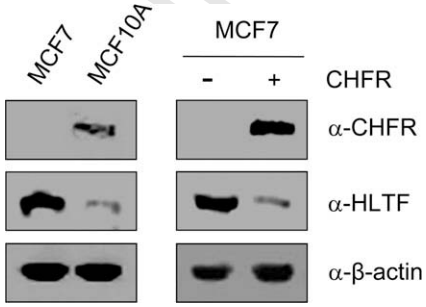

Fig. 4. CHFR and HLTF protein levels in breast cancer cells. Lysates of MCF7, MCF10A, and FLAG-CHFR transfected MCF7 cells were analyzed by immunoblotting with anti-CHFR, anti-HLTF, or anti-β-actin antibodies.

3.4. Loss of CHFR is correlated with stabilization of HLTF in human breast cancer cells

There is no or little expression of CHFR in MCF7 human breast cancer cells, while CHFR was detected in normal-like, non-tumorigenic breast epithelial cell line MCF10A [14]. To examine the biological consequences of CHFR deficiency in HLTF levels as well as in tumor progression, we examined the protein levels of HLTF (Fig. 4; left panel) in these cell lines. HLTF were upregulated in CHFR-deficient MCF7 cells compared with CHFR-proficient MCF10A cells. Furthermore, HLTF became unstable when CHFR was transfected to CHFR-deficient MCF7 cells, similar to MCF10A

cells (Fig. 4; right panel). These results clearly show that the expression of CHFR and HLTF is negatively associated.

4. Discussion

In the present study, we aimed to provide a possible mechanistic explanation for the tumor suppressive role of CHFR. CHFR not only functions as a mitotic checkpoint [3], but also plays an important role in tumorigenesis and tumor metastasis by downregulation of HDAC1 [14]. CHFR is often found to be silenced by CpG island hypermethylation, which is a key feature of tumorigenesis [3,10,11]. It has been reported that CHFR deficiency in mice and cell culture models promotes genomic instability and tumor growth [4,12,13]. A newly identified CHFR-interacting protein, HLTF is also silenced by promoter hypermethylation in colorectal, gastric, and cervical cancers [21]. Although HLTF is thought to be involved in tumorigenesis, it is still not clear whether HLTF inactivation is a cause or a consequence of tumor development. In addition, HLTF expression is upregulated in transformed cell lines and is detected at the initial step of an estrogen-induced renal carcinogenesis model system [22]. Here we showed that CHFR is essential for regulation of HLTF stability by the ubiquitin-proteasome system. Consistently, there is a reverse correlation between HLTF and CHFR. HLTF level is higher in MCF7 and HeLa cells where CHFR is not present. Also, there is no HLTF promoter methylation in CHFR-deficient cell lines including RKO and HT29 [11,22]. Therefore, this represents the first report of HLTF regulation that HLTF levels are largely dependent on the presence of CHFR. This suggests that CHFR expression gets lost during tumor progression and the subsequent stabilization of HLTF might contribute to tumorigenesis. Since both CHFR and HLTF get methylated and inactivated in human cancers, it would be of particular interest to investigate whether CHFR inactivation always comes first in the early stage of tumor development. If it turns out to be true, then the combinatorial methylation analysis of both CHFR and HLTF could serve as a potential tumor diagnostic marker.

PAI-1 can promote cancer progression and its increased level in tumor is an independent poor prognostic marker in many forms of cancer [24]. Since HLTF regulates PAI-1 expression [19], it is plausible that elevated HLTF could activate PAI-1 transcription, resulting in the initiation of tumor formation [25]. Thus, reduced HLTF could block PAI-1-mediated tumor progression. Consistent with our hypothesis, CHFR was able to inhibit cell migration by suppressing PAI-1. Although CHFR abolished HLTF-mediated cell migration, we cannot exclude the possibility that CHFR could block cell migration in a HLTF/PAI-1-independent manner. These findings are consistent with previous reports that CHFR negatively regulates cell proliferation, cell motility, and invasion in tissue culture models [12,14,15,26]. The anti-proliferative effect of CHFR is independent of the E3 ligase activity mediated by the RING finger domain [26]. However, CHFR-mediated HLTF destabilization and PAI-1 suppression were based on the ubiquitin ligase activity. CHFR-I306A abolishes the effect of CHFR on HLTF destabilization. This is also mediated by direct interaction between CHFR and HLTF, therefore, CHFR-ΔCR mutant also failed to destabilize HLTF since CR domain is necessary for their interaction.

CHFR downregulates HDAC1, leading to upregulation of its target genes such as *p21*, *KAI1*, and *E-cadherin* and a decrease of invasiveness [14]. These findings are the first illustration that CHFR plays a crucial role in regulation of gene expression via ubiquitin-dependent proteolysis. Here we added a new line of evidence that CHFR also inhibits *PAI-1* gene expression. Recently, it has been reported that CHFR suppressed *IL-8* transcription through inhibition of NF-κB regardless of the presence of a RING domain in human cancer cells [15].

Taken together, we showed here for the first time that CHFR functions as an E3 ubiquitin ligase responsible for the regulation of HLTF stability. CHFR-mediated destabilization of HLTF inhibits PAI-1 expression and cell migration. Thus, CHFR plays a crucial role in regulation of HLTF stability and blocks HLTF-mediated cell migration. Although our observations will require further confirmation in other model systems, this exciting initial finding may expand our understanding how CHFR protects cells against the development of human cancer.

Conflicts of interest

None declared.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST), Korea Science and Engineering Foundation Grant (M10533010001-05-N-3301-00100) and Research Center for Functional Cellulomics (2009-0063-019). J.M.K., E.N.C., Y.E.K., and S.J.B. are the recipients of Brain Korea 21 fellowships supported by the Korea Ministry of Education, Science, and Technology.

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