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Regulation of *p53* Genes by Ferritin Light Chain: Preliminary Study

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= 국문초록 =

Ferritin에 의한 *p53* 유전자 조절 효과

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목 적 : TP53 단백질은 가장 흔한 종양억제 단백질이다. 그렇다면 암 세포에서 TP53을 억제하는 물질을 무엇인가? 대다수의 암들은 철분의 상승이 있고 또한 ferritin의 상승이 동반되어있다. 저장 철 형태인 ferritin의 구조는 heavy 및 light chain으로 구성되어 있고 이 중 light chain은 세포 내의 철분 저장의 주된 장소이다. 따라서 ferritin의 light chain은 세포 내에서 TP53 단백질의 활동을 조절하는 여러 단백질 중의 하나일 가능성이 있다. 본 연구는 ferritin의 light chain 단백질과 TP53 단백질의 상호 작용이 있고 *p53* 유전자의 기능을 억제한다는 가정을 세워 이의 관계를 증명하고자 하였다.

방 법 : FLAG-ferritin light chain plasmid을 만든 후, 몇 개의 *p53*유전자를 이용하여 *p53*-null H1299 세포에서 coimmunopreciptitation을 시행하였다. 전사 분석을 하기 위해 5 *p21*을 이용한 luciferase reporter gene assay를 이용하였다.

결 과 : *p53*-null H1299 세포에서 ferritin light chain과 wild-type 및 변종 *p53* 유전자들이 Coimmunoprecipitation을 이용하여 서로 반응하였다. 또한, ferritin light chain에 의해 *p53* 유전자의 transcriptional activity는 약 50% 정도 감소하였다.

결 론 : *p53* 유전자와 ferritin은 서로 반응하는 것을 확인하였으며 ferritin은 *p53* 유전자의 일부 활동을 억제하는 것으로 생각한다.

중심 단어 : Ferritin · Iron · *p53* genes · TP53 protein.

Introduction

TP53 protein is a tumor suppressor protein that plays a central role in the regulation of cellular responses to a variety of cellular stresses, including DNA damage, hypoxia and over-expression of oncogenes. *p53* genes exert its effects primarily by acting as a transcription factor. TP53 activates the expression of a large number of downstream genes, which can result in either cell cycle arrest or apoptosis, such as the cycline-dependent kinase inhibitor, $p21^{WAF1/CIP}$, *GADD45, Bax, Fas/Apo1, Killer/DR5, Noxa, Puma and p53R2.* TP53 is able to maintain cellular homeostasis by inducing cell cycle arrest, DNA repair, and apoptosis. Approximately 50% of all human cancers carry mutations in the *p53* gene that result in an

inactive TP53 protein. In the remaining cancer cases, the *p53* gene pathway is likely to be blunted or inactivated. Thus, it is important to identify and characterize additional proteins that inhibit TP53 protein function, which will broaden our knowledge of cancer biology and may lead to new targets for cancer therapy.

Ferritin is a protein essential for intracellular iron storage. Previous works have demonstrated that a large number of cancers are characterized by elevated levels of iron, a requirement for cellular proliferation[1-3]. Increased iron levels correlate with an increase in ferritin expression, which has been used as a prognostic marker. Reduction of ferritin levels by iron chelators results in growth arrest and apoptosis in cancer cells[4)5)]. However, the mechanism that is responsible for this and the precise contribution of TP53 protein to iron chelator-induced cell death have not been elucidated. It is hypothized, therefore, that TP53 protein is directly regulated by ferritin. Namely, ferritin as found in several types of cancers interacts with TP53 and inhibits function of *p53* gene.

Work presented here will for the first time describe a direct link between TP53 inactivation and its interaction with ferritin. The observation reported here that how cancer cell control of apoptotic pathway via interaction of TP53 protein and ferritin.

Materials and Methods

1. Cell culture and reagents

H1299 (*p53*-null cell) cells were routinely maintained in 37℃ incubator with 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen®, CA, US) supplemented with 0.1% penicillin/streptomycin (1ml), and 10% fetal bovine serum. Cells were maintained at 37℃ in a humidified atmosphere of 5% CO_2 in air, and media were replaced every 3-5 days.

2. Construction of ferritin light-chain with vector

Ferritin light-chain (FLC) of mouse (FLC, NM_010240 [gi:6753913])-expression plasmid in *E. coli* were get from company (NIH Mammalian Gene Collection, Invitrogen®, CA, US). They were grown in appropriate media (LB® media) with antibiotics for overnight. And then Miniprep® (QIAGEN® Inc. USA) was performed following the manufacture's protocol. After we get the DNA of FLC and polymerase chain reaction was performed by primers that have the restriction sites that digested by EcoR1 and Bgl II. The primers used are FLC S : 5'-GGT TCC GAA TTC AAG CTC CCA GAT TCG TCA GAA TTA TTC C-3' and AS : 5'-CAG GTC AGA TCT TTA GCT CTC ATC ACC GTG TCC AG-3'. The amplification reaction was performed in a 0.5 μL genomic DNA template, 1 μL (0.2mM/mL) of each primer, 1 μL (10mM/mL) of dNTP, 0.5 μL of *Taq* polymerase (0.5 μL), and 5 μL of 10X buffer, and 3 μL (25nmol/mL) of $MgCl_2$. The reaction conditions carried out by 50 cycles under the following condition : 30s at 95℃ for denaturation, 15s at 54℃ for primer annealing, and 4min at 60℃ for primer extension. All constructs were confirmed by DNA sequencing. And we cloned it into the FLAG-CMV vector. So we constructed full-length FLAG-tagged FLC expression plasmids

3. Cotransfection

H1299 cells transfected with the FLAG-FLC with *p53* or mutant *p53s*. Transfection procedures were performed using lipofectamine® following the manufacture's protocol. Briefly, cells were seeded in 3.5-mm wells at a concentration of cells/well. 4 μg of ferritin-FALG DNA and 1 μg of *p53* wt-type or *p53* mutant per tube was mixed with lopofectamine® Regent and Opti-MEM® Reduced Serum Medium and incubated for 30 minutes at room temperature. And that complex added to cells that were previously washed twice with phosphate buffered saline (PBS). After 5 hr, media (3.5- mm well) containing Dulbecco's modified Eagle's medium supplemented with 0.1% penicillin/streptomycin (1ml), and 10% fetal bovine serum were changed to each well.

4. Immunoprecipitation(IP) and western blot analyses

Cells were harvested overnight and lysed in 0.5% NP-40 lysis buffer [50mM HEPES (pH 7.2), 250mM NaCl, 5mM EDTA (pH 8.0), 0.5% NP-40, proteinase inhibitor, and kinase inhibitor]. For IP, lysates were treated by beads-FLAG antibody (purified using nickel-nitrotriacetic acid beads). Beads were washed with lysis buffer three times. Lysates and immune complexes were sepa-

rated by 10% SDS-polyacryamide gel electrophoresis (PAGE). After electro-transfer, polyvinylidene difluoride membranes were probed with primary antibody or/and secondary antibody conjugated to HRP. Antibodies used in these analyses were *α-p53* (FL-393 : sc-6243, Santa Cruz, CA, US) and *α*-FLAG (1° antibody-No. 200471 : Strategne,® US ; 2° antibody-No. NA931 : Amersham Life Science Inc.®, US).

5. Determine what protein region of FLC and *p53* are necessary for interaction

Identification of common protein domains and a structure-function analysis have not been performed to date for ferritin. Therefore, FLAG-tagged plasmids for the N- and C-terminal haves of FLC will be tested in co-IPs with full-length *p53*. The co-IP results will then dictate the design of additional mutants in either the transactivation, proline-rich, core DNA-binding, tetramerization or regulatory basic domains that are available in our laboratory. Also *p53* gene mutant plasmids is cotransfected with a full-length FLC plasmid into *p53* null cells and subjected to co-IP. It is expected that results identify specific region within the TP53 and FLC proteins that are necessary for interaction.

6. Reporter gene assay by luciferase

For identification of inhibition on transcriptional acitivity of *p53*, transient transfections were performed using Lipofectamine 2000 regent (Invitrogen®) following the protocol of the manufacturer. *H1299* cells were cotransfected in 6-well plates with FLAG-tagged *p53* wild type, FLC, luciferase reporter plasmid (5'p21 expression plasmid, 50ng/well) and renilla luciferase reporter (15ng/well). Twenty-four hours later, the luciferase activity was quantified by using a dual-luciferase reporter assay system (luminometer V3.1, SIRIUS®, USA).

Results

1. Immunoprecipitation(IP) between FLC and TP53 protein

This IP study showed that the FLC interacts with wild-type *p53* and several mutants in *p53*-null *H1299* cells (Fig. 1 and 2).

2. Reporter gene assay

5'p21 which is downstream gene of *p53* was cloned upstream of a luciferase reporter gene, and luciferase assays were performed in *H1299* cells by cotransfecting the reporter plasmid with plasmid expressing FLC and *p53* (Fig. 3). Luciferase activity of wild type *p53* decreased about 50% triggered by FLC. The FLC resulted in the loss of TP53 protein transciptional activity, suggesting that *p53*-dependent transactivation strictly depends on the FLC activity on *p53* gene.

Discussion

TP53 protein is an important tumor suppressor protein

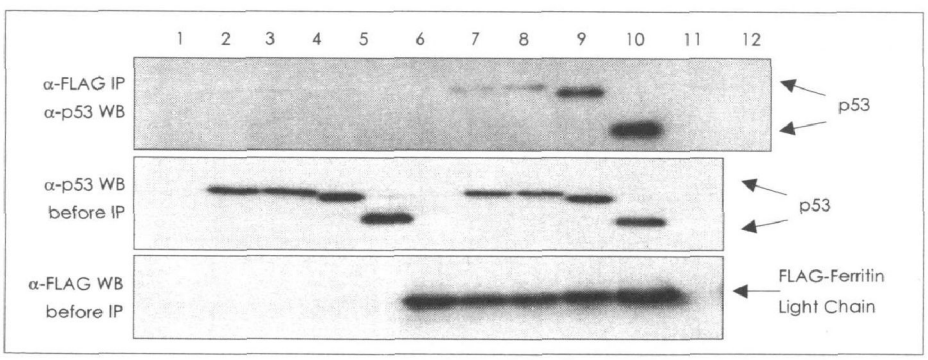

Fig. 1. *p53* coimmunoprecipitates with ferritin light chain. *p53*-negative *H1299* cells were cotransfected with FLAG-tagged ferritin and either wild-type or mutant *p53* plasmid. Lane 1. empty FLAG+parenteral vector, 2. empty FLAG +*p53*, 3. empty FLAG+*p53*-7 alanine mutant (lacking seven pholyphorylation sites in the *p53* transactivation domain), 4. empty FLAG+*p53* del(363−393), 5. empty FLAG+*p53* del(291−393), 6. FLAG-ferritin+parenteral vector, 7. FLAG-ferritin+*p53*, 8. FLAG-ferritin+*p53*-7 alanine mutant, 9. FLAG-ferritin+*p53* del (363−393), 10. FLAG-ferritin+*p53* del(291−393), 11. no DNA, 12. only beads+ELB. Results demonstrate a ferritin-p53 protein interaction in *H1299* cells.

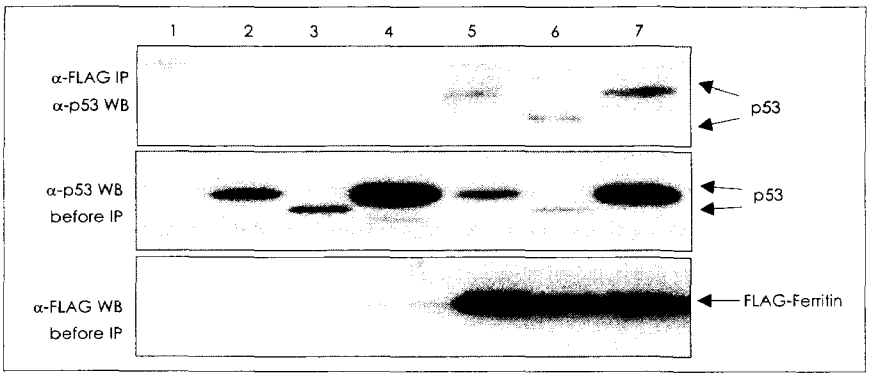

Fig. 2. *p53* coimmunoprecipitates with ferritin light chain. *p53*-negative *H1299* cells were cotransfected with FLAG-tagged ferritin and either wild-type or mutant *p53* plasmid. Lane 1. empty FLAG+parenteral vector, 2. empty FLAG+*p53*, 3. empty FLAG+*p53*-proline deletion, 4. empty FLAG+*p53 22Q/23S*, 5. FLAG-ferritin+*p53*, 6. FLAG-ferritin+*p53*-proline deletion, 7. FLAG-ferritin+*p53 22Q/23S*. Results demonstrate a ferritin-*p53* protein interaction in *H1299* cells.

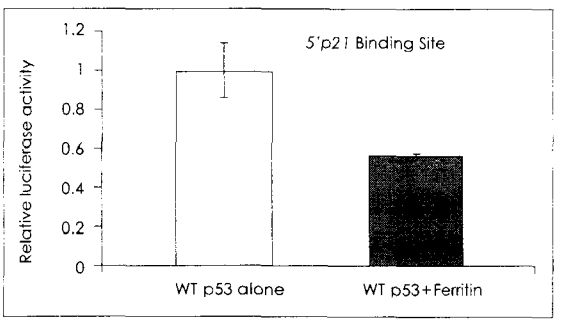

Fig. 3. Reporter gene assay of *p53*. *H1299* cells were transfected with constructs containing the promoter region of *5'p21*, ferritin and wild type *p53*. Ferritin decreased by about 50% the luciferase activity of *p53*. Each data set represents an average value from triplicate transfections.

and exerts its effects primarily as a transcription factor. In addition to its function as a transcription factor, TP53 protein regulates cellular processes by transcription-independent mechamisms[6-10]. TP53 protein is able to activate the intrinsic apoptotic pathway via localization to the mitochondrial membrane[7]. This is well known as transcription-independent *p53*-induced apoptosis. Once localized to the mitochondria, TP53 protein is able to interact with pro-apoptotic proteins, such as Bax, resulting in the release of cytochrome C[7][11]. The relative importance and timing of *p53*-mediated transcription-dependent and -independent apoptosis was recently addressed by showing that TP53 protein mediated apoptosis occurs in two phases[12]. A rapid first wave of apoptosis is caused by transcription-independent accumulation of TP53 protein at mitochondria, which is then followed by a second slower wave of apoptosis that is TP53 protein transcription-dependent.

Iron plays an important role in a variety of cellular functions including cell growth and respiration. Iron is transported in the serum bound to transferring[4]. This complex then bind to the transferrin receptor localized at the cell surface and becomes internalized by the process at receptor-mediated endocytosis. The elevated synthesis of transferrin receptor was seen in most carcinomas including pancreatic cancer, sarcoma, some lymphoma and leukemia[13]. Once iron is internalized, it is either incorporated into essential iron-containing proteins or is stored complex, ferritin. In eukaryotic cell, ferritin consists of 24 subunits of the heavy (H) chains (21kDa) and light (L) chains (19kDa) ferritin species and is able to concentrate as many as 4,000 iron atoms. The ratio of H and L subunits is various on the type of tissue and the physiologic state of cells. In the absence of iron, cells are not able to progress through the G1 to S restriction point of the cell cycle[4][14].

Several studies have suggested that a close relationship may exist between ferritin and cancer. Neoplastic cells, with their frequently more rapid growth rates, might be expected to show alteration in iron metabolism when compared to their normal cells. The serum ferritin level is frequently elevated in patients with cancer, and some neoplastic cell revealed a markedly altered ferritin component or content when compared to their normal cells[15][17].

For example, breast cancer cells can contain up to six-fold more ferritin than normal cells[2]. In addition, breast tumors with the highest levels of ferritin were found to be the most anaplastic. Thus ferritin levels have been used as a prognostic marker for a number of cancers includeing breast, prostate, renal, lung, and colon as well as myeloid leukemias[18].

So I presumably can suggest that ferritin interact with TP53 protein in some pathway. Recently, Greene and colleagues demonstrated that iron chelator-induced cellular apoptosis occurred via a mitochondrial-dependent apoptotic pathway[5]. In addition, studied by Sansome and colleagues demonstrated that treatment of myeloid leukemia cells with iron chelator deferoxamine resulted in the localization of wild-type endogenous *p53* to the mitochondria[19]. To date, the exact mechanism and outcome of *p53* localization to the mitochondria after iron chelator treatment remains unknown.

We made the FLC-FLAG expression plasmid was constructed and sequenced. To demonstrate the transcription-independent action of FLC on *p53* Co-IP was done. That experiment showed that the proteins of FLC interacts with wild-type *p53* and several *p53* gene mutants(deletion and point mutations) in *p53*-null *H1299* cells. These data suggested the possibility that the FLC contributes to TP53 protein inactivation through sequestration of *p53* gene. Also we investigated which domain of TP53 protein interacts with FLC. We used *p53* gene mutants including mutants with some deletions or switch one amino acid. So we identified specific regions within the *p53* gene and FLC protein that are necessary for interaction. And this demonstrated that the FLC effect on *p53* gene directly. It could be the intrinsic apoptotic pathway on mitochondrial membrane. Also, this study shows FLC interact with *p53*-transcritional activity via luciferase reporter gene assay. It revealed to be partly loss of transcriptional activity by FLC. This is the first study to demonstrate that the FLC correlate to the *p53* gene at both the transcription and protein level.

In conclusion, these results are consistent with the hypothesis that ferritin functionally inhibits *p53* genes, especially FLC. And this result suggested a new mechanism of TP53 protein inactivation by ferritin that affects transcriptional-independent apoptosis. This study has the potential to significantly advance our knowledge of how cancer cells neutralize this central apoptotic pathway and may result in new strategies that exploit the abnormal iron metabolism of cancer cells.

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