약학석사학위논문

유방암 진행과정의 에스트로겐 신호전달에서 Interferon, gamma-inducible protein 16의 역할 규명

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2014년 2월

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지도교수 이 미 옥

이 논문을 약학석사 학위논문으로 제출함

2014년 2월

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강 혜 림

강혜림의 석사 학위논문을 인준함

2014년 2월

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ABSTRACT

The Role of Interferon, gamma-inducible protein 16 in the Estrogen Signaling during Breast Cancer Progression

Hae-Lim Kang
College of Pharmacy
The Graduate School
Seoul National University

Interferon, gamma-inducible protein 16 (IFI16) is a member of the HIN-200 family that has been implicated in apoptosis and inflammation in breast cancer. Recently, it was reported that IFI16 forms complex with metastasis-associated protein 1 (MTA1) and class II histone deacetylases (HDACs) and the complex represses the transcription of estrogen receptor alpha (ERα). However, the role of IFI16 in breast cancer cell growth or tumor progression remains largely unknown. Therefore, we aimed to identify the effects of IFI16 on hormone-dependent proliferation and estrogen signaling in breast cancer cells. First, we established stable subline that expresses shRNA of IFI16 or MTA1 using ERα-negative breast cancer cell line,

MDA-MB-231, in order to investigate the cellular growth properties according to silencing of each gene. As reported, we confirmed the increase of mRNA and protein levels of ERα by knockdown of MTA1 or IFI16 in these stable sublines. Moreover, in cell proliferation assay, clonogenic survival assay and xenograft tumor growth experiment, we verified that treatment of tamoxifen, which is an anti-hormone drug targeting ERα, significantly reduced cell growth compared with shGFP-expressing control cell. Next, we profiled the estrogen receptor (ER) signaling-related gene expression pattern using the PCR array in MDA-MB-231 after knockdown or overexpression of IFI16. Nine genes were up- or down-regulated more than 1.5-fold. Among them, induction of Cytochrome P450, Family 19, Subfamily A, Polypeptide 1 (CYP19A1 or aromatase) by IFI16 was confirmed by qPCR in ERα -positive breast cancer cell line, MCF7. We also observed that concentration of estradiol in cell culture media was increased by 1.7-fold after transfection of IFI16 when measured by enzyme immunoassay (EIA). In addition, we demonstrated that IFI16 repressed the expression of AR, which is known to competitively suppress ERα-mediated transcription. When IFI16 was overexpressed, DNA binding of AR was reduced and that of ERα was induced at the promoter region of ERα-downstream gene, pS2 or progesterone receptor (PR), followed by increase of pS2 and PR mRNA levels. Furthermore, in MCF7 cell that stably overexpresses IFI16, cell growth was stimulated consistent with the activation of estrogen signaling. Taken together, our results indicate that IFI16 induces

tamoxifen resistance in ERα-negative breast cancer and activates estrogen signaling and cell proliferation via regulating expression of CYP19 and AR. This study extends understanding of the roles of IFI16 in breast cancer progression and suggests a new target for prevention and treatment of breast cancer.

keywords : IFI16, ERα, CYP19A1, AR, Breast cancer

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CONTENTS

LIST of FIGURES

LIST of TABLES

I . INTRODUCTION

Breast cancer is the most common cancer in women worldwide. While the mortality from breast cancer has fallen in western countries, the mortality in Asia has been still increasing annually (International Agency for Research on Cancer, Retrieved December 19, 2013 from http://www.iarc.fr). Many efforts have been contributed to identify biomarkers used to diagnose, choice therapeutic strategy and predict prognosis of breast cancer and as a result, breast cancer is classified into subtypes based on expression of estrogen receptor alpha (ERα), progesterone receptor (PR) and human epithermal growth factor receptor (Her2) (Onitilo et al., 2009).

Estrogen signaling is the most important regulatory pathway for hormone-dependent proliferation in ERα-positive breast cancer. Within the tumor tissue or surrounding daipose tissue, androgens are aromatized by Cytochrome P450, Family 19, Subfamily A, Polypeptide 1 (CYP19A1 or aromatase) and converted into estrogens such as estrone or estradiol (Bulun et al., 2005). Estradiol, which is biologically active estrogen, is secreted to adjacent epithelial cells and binds to ERα in the cytosol. Then Ligand bound-ERα translocalizes to nuclear and changes its conformation to make a transcriptional complex with coactivators. In consequence, hormone-mediated cell proliferation is activated (Johnston, 2010; Heldring et al., 2007). For

targeting the estrogen signaling, adjuvant drugs are used in combination with other anticancer drugs that kill cancer cells in breast cancer therapy (Figure 1; Bulun et al., 2005; Johnston, 2001). One of those is aromatase inhibitor reducing the activity of CYP19A1 that is a key enzyme of estrogen biosynthesis. The other one is selective estrogen receptor modulator (SERM), which binds to ERα competitively with estrogens but cannot activate ERα-induced transcription. Tamoxifen is a representative SERM. However, breast cancer cells that do not express ERα proliferate in hormone-independent manner and are resistant to tamoxifen. Since ERα is a critical determination factor for hormone therapy, there are numerous studies that investigate mechanisms of ERα repression and possibility of restoring susceptibility to anti-hormone drugs in ERα -negative breast cancer . It has been reported that expression of ERα is regulated mainly by epigenetics such as histone deacetylation and DNA methylation (Yang et al., 2001; Fan et al., 2008).

Our previous study showed that transcription of ERα is regulated by metastasis-associated protein 1 (MTA1) and class II histone deacetylases (HDACs) at the proximal region of ERα promoter named ERpro315 (Kang et al., to be published[1)]). Moreover, in order to figure out transcriptional factors that bind to ERpro315, DNA pull-down assay was carried out using ERpro315 DNA oligonucleotides and pull-downed proteins were analyzed by

1) Based in part on the thesis submitted by Kang HJ for the M.S. degree in College of Pharmacy, Seoul National University, Seoul, 2011.

LC/MS/MS. As a result, interferon gamma-inducible protein 16 (IFI16) was discovered with the highest match score. Further studies demonstrated that IFI16 binds with the complex composed of MTA1, class II HDACs and nuclear receptor corepressor (NCoR) to repress ERα transcription via histone deacetylation of ERpro315 region (Figure 2, 3).

IFI16 is a member of the HIN-200 family induced by interferon-gamma and well known for innate immune sensor of cytosolic and nuclear double-stranded DNA (Figure 4; Veeranki et al., 2012). Recent studies reported IFI16 as transcriptional repressor in cancer cells (Egistelli et al., 2009; Alimirah et al., 2007). In breast cancer, it is implicated in apoptosis and inflammation (Ouchi et al., 2008; Fujiuchi et al., 2004), but the role of IFI16 in breast cancer cell growth or tumor progression remains largely unknown. We therefore aimed to identify the effects of IFI16 on hormone-dependent proliferation and estrogen signaling in breast cancer cells.

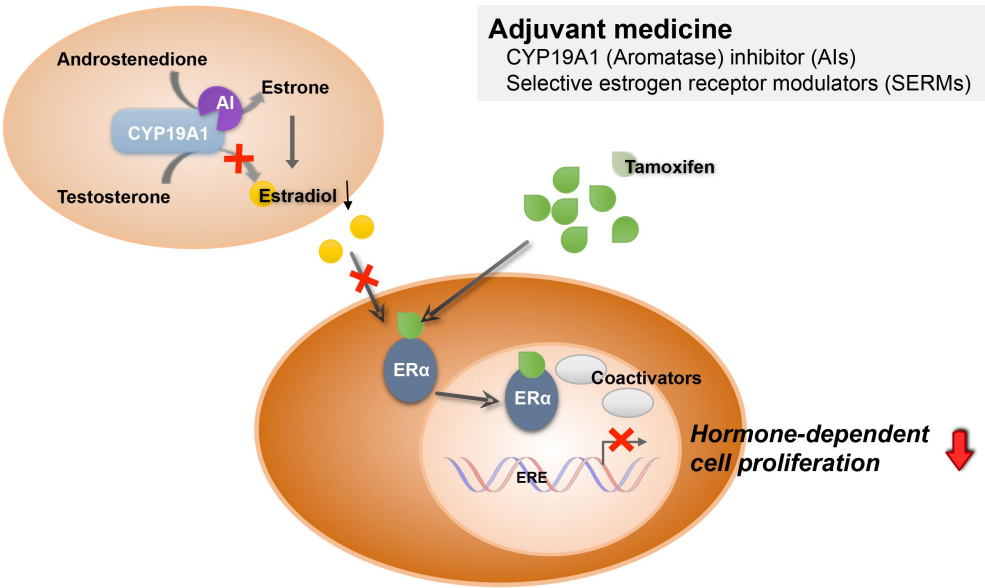


Figure 1. Estrogen signaling and breast cancer adjuvant chemotherapy

For ERα-positive breast cancer therapy, adjuvant drugs that target the estrogen signaling are used in combination with other anticancer drugs. Aromatase inhibitors reduce the activity of CYP19A1 and decrease the production of ligands of ERα. Meanwhile, SERMs compete with estrogens for binding to ERα and block the transcriptional activity of ERα. However, the proliferation of ERα -negative breast cancer cell don't be controlled by estrogens as well as not inhibited by tamoxifen.

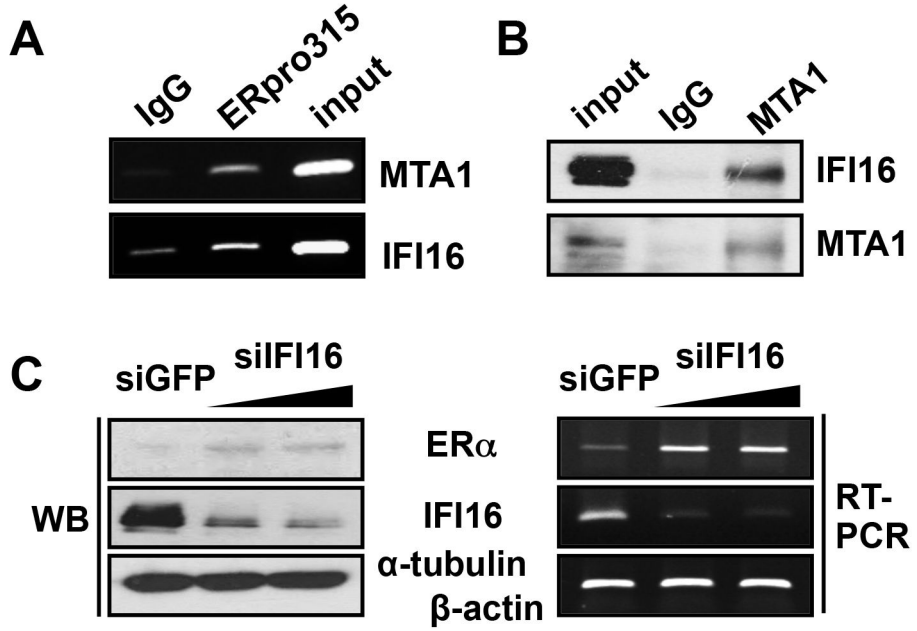


Figure 2. IFI16 interacts with MTA1 at the proximal region of ERα promoter and represses the transcription of ERα.

(A) DNA binding of MTA1 or IFI16 on the ERpor315 region was analyzed by chromatin immunoprecipitation (ChIP) assay. DNA fragments that immunuprecipitated by anti-MTA1 or anti-IFI16 antibody were amplified by PCR using primers for ERpro315. (B) Whole cell lysates were immunoprecipitated (IP) with normal IgG or anti-MTA1 antibody, and immunoprecipitates were fractionated and probed by western blotting (WB) using anti-MTA1 or anti-IFI16 antibody. (C) MDA-MB-231 cells were transfected with 50 or 100 pmoles of siIFI16 for 48 h. Expression levels of protein or mRNA of ERα and IFI16 were analyzed by western blotting (left) or RT-PCR (right), respectively.

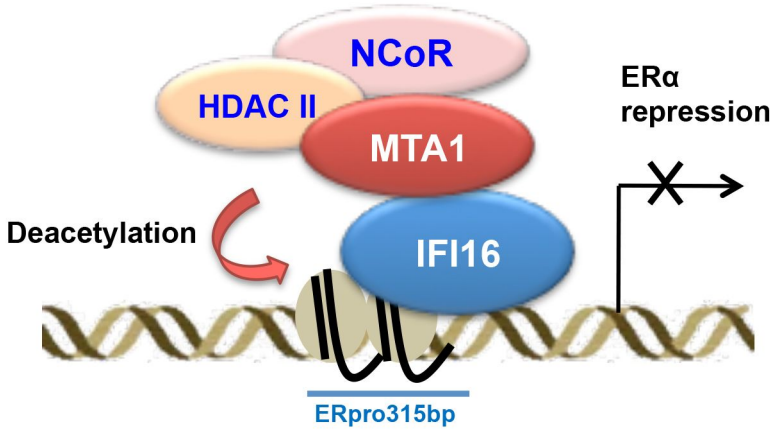


Figure 3. Schematic illustration of molecular mechanism of ERα regulation in ERα-negative breast cancer cell

In ERα-negative breast cancer cell, IFI16 binds with the complex composed of MTA1, class II HDAC and NCoR to repress transcription of ERα via histone deacetylation on ERpro315 region.

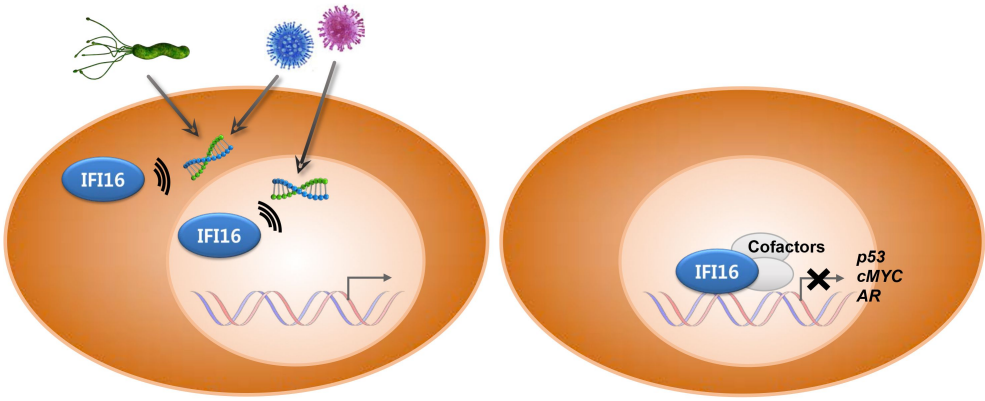


Figure 4. Physiological functions of IFI16

IFI16 is a member of the HIN-200 family induced by interferon-gamma. It is an innate immune sensor for cytosolic and nuclear double strand DNA that is inserted externally by bacteria or virus and induce inflammation response. IFI16 is also reported as transcriptional repressor in cancer cells.

Ⅱ. PURPOSE of the STUDY

In breast cancer, the expression of ERα is a criterion for hormone therapy. We previously figured out that IFI16/MTA1 complex represses transcription of ERα through histone deacetylation on the proximal region of promoter (Figure 3). In accordance with our previous studies, we aimed to demonstrate whether silencing of IFI16 or MTA1 would restore ERα expression and sensitize ERα-negative cells to tamoxifen that targets ERα. Thus in this study, we tried to establish MDA-MB-231 sublines that stably express shIFI16 or shMTA1 and examine susceptibility to tamoxifen. Moreover, we found that IFI16 is overexpressed in breast cancer tissue than non-cancerous breast tissue. Therefore, we aimed to figure out the role of IFI16 in ERα-positive breast cancer cell. For the purpose, we carried out PCR array that evaluate the expression of ERα signaling-related gene and investigated the regulation of estrogen signaling by IFI16.

Ⅲ. MATERIALS and METHODS

1. Cell culture and cell treatment

Human breast adenocarcinoma cell lines, MCF-7 and MDA-MB-231, were obtained from the American Type Culture Collection (ATCC). Cells were maintained in Dubelco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 / 95% air incubator. Tamoxifen citrate was purchased from Sigma-Aldrich (T9262). Puromycin was purchased from Santa Cruz Biotechnology (sc-205821).

2. Plasmids, siRNA duplexes, and transient transfection

FLAG-tagged IFI16 was constructed by inserting a PCR-amplified full-length human IFI16 into p3xFLAG-CMV10 (Sigma-Andrich). pLKO.1-TRC, pLKO.1-shGFP, psPAX2 and pMD2.G vectors were purchased from Addgene. The siRNA duplexes were synthesized and purified by ST PHARM. Sequences of siRNA duplexes used in this study are described in Table 1. Transient transfections of overexpression vector were performed using Polyfect® (Qiagen) for MCF7, and WelFect-EX™PLUS (WelGENE Inc.) for MDA-MB-231. The transfection of siRNA was performed with Lipofectamine™2000 reagent (Invitrogen) according to the manufacturer's manual.

3. Establishment of stable cell lines

To establish knockdown sublines, pLKO.1-shMTA1 and pLKO.1-shIFI16 were constructed by annealing oligomers described in Table 1 and inserting the annealed products into the AgeI-EcoRI site of the pLKO.1-TRC vector. The pLKO.1-shRNA vector, lentiviral packaging plasmids (psPAX2) and envelope plasmid (pMD2.G) were cotransfected into HEK293T cell using Lipofectamine^{TM}2000. After 60 h incubation, the lentivirus in the cell supernatant was collected and used to infect MDA-MB-231 cell with hexadimethrine bromide at a final concentration of 8 μg/mL. After puromycin selection at the concentration of 1.5 μg/mL for 4 weeks, stable clones were obtained and subsequently confirmed by western blotting as described below. To establish overexpression sublines, pLJM1-IFI16 was constructed by inserting a PCR-amplified full-length human IFI16 into the AgeI-EcoRI site of pLJM1-EGFP (Addgene). The production of lentivirus was performed as described above and transduction of virus was carried out to MCF7 cell. After puromycin selection at the concentration of 2 μg/mL for 4 weeks, stable clones were obtained and subsequently confirmed by western blotting as described below.

4. Western blotting

Cells were lysed in lysis buffer containing 150 mM NaCl, 50 mM Tris (pH 7.4), 5 mM EDTA, 1% NP-40, and protease inhibitors cocktail (Roche) for 30 min on ice, and whole-cell lysates were obtained by subsequent centrifugation. The protein concentration was

quantified by bicinchoninic acid assay (Pierce). In total, 20 μg of protein from whole-cell lysates were subjected to 8 % sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane. Blocking was performed in 5% (w/v) non-fat-dried milk in phosphate-buffered saline containing 0.1% Tween-20. The membrane was then incubated with specific antibodies against ERα (sc-543), MTA1 (sc-9445), IFI16 (sc-8023), CYP19 (sc-14245), AR (sc-816), actin (sc-1616) from Santa Cruz Biotechnology, or α-tubulin (calbiochem).

5. Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real time PCR

Total RNA was prepared using EASY-BLUE™ Total RNA Extraction Kit (iNtRON Biotechnology) according to the manufacturer's instructions. cDNA was synthesized from total RNA in a reaction mixture containing random hexamer (Invitrogen) and MMLV-Reverse transcriptase (Invitrogen). PCR reactions were performed with specific primers at the particular annealing temperature as described in Table 2. Quantitative real time PCR was performed using SYBR Green PCR mix (Applied Bioscience). The expression of β-actin was monitored as a control.

6. Cell proliferation assay

MDA-MB-231 shRNA stable sublines, shGFP, shMTA1, and shIFI16, were seeded at 2 X 10^5 cells/plate in triplicate into 60 mm plates.

After 24 h incubation, cells were treated with 10 μM tamoxifen or vehicle (DMSO and ethanol 1:1 mixture). Cell culture media containing drug were exchanged every 48 h. Cells were harvested with Trypsin-EDTA (Gibco®) at the described time after treatment and resuspended using culture media. Then we performed Trypan Blue staining (Gibco®) followed by cell counting using hematocytometer. The cell proliferation assay of MCF7 stable sublines was performed in the same way without drug treatment. Statistical significance was evaluated by two-way ANOVA followed by Bonferroni post-test.

7. Clonogenic survival assay

MDA-MB-231 shRNA stable cell lines, shGFP, shMTA1, and shIFI16, were seeded at 1000 cells/plate in triplicate into 35 mm plates. After 48 h incubation, cells were treated with 10 μM tamoxifen or vehicle for 12 days. At the end of treatment, colonies were fixed with methanol and stained with 0.5% crystal violet (Sigma-Aldrich). Colonies that composed of greater than 50 cells were counted. The clonogenic survival assay using MCF7 stable sublines was carried out in the same way without drug treatment. Statistical significance was evaluated by two-way ANOVA followed by Bonferroni post-test.

8. Xenograft experiment

Animal experiments were performed in accordance with guidelines of Seoul National University Animal Care and Use Committee. Female

five-week-old athymic (nu/nu) BALB/c mice were obtained from Orient Bio Inc. and housed in an air-conditioned room at a temperature of 22-24°C and a humidity of 37-64%, with a 12 h light/dark cycle. After one week of acclimatization, tumor inoculation was performed. Each 5 X 10^6 cells of MDA-MB-231 shGFP, shMTA1, or shIFI16 stable cell lines were mixed at a 1:1 ratio with Matrigel (BD Biosciences) and inoculated subcutaneously into the flanks of mice. When the tumor volume reached approximately 100 mm^3, mice were randomly divided into two groups. The experimental groups received a 21-day release tamoxifen pellet (25 mg/pellet) or a placebo pellet (Innovative Research of America) for two weeks. Tumor diameter was measured with caliper twice a week and tumor volumes were estimated using the following formula: tumor volume (cm^3) = (length X $width^2$) X 0.5. Statistical significance was evaluated by two-way ANOVA followed by Bonferroni post-test.

9. PCR array

A pathway focused Real-time PCR array comprised of probes for 84 estrogen receptor signaling related gene (PAHS-011, SABioscience) was used to investigate changes in gene expression when IFI16 was overexpressed or silenced. MDA-MB-231 (8 X 10^5 cells/plate) was seeded in 60 mm plate and incubated for 24 h. The cells were transfected with FLAG-IFI16 plasmid or siIFI16 and incubated for 24 h or 48 h. Total RNA was prepared using RNeasy® Mini Kit (Qiagen). cDNA was synthesized from the 1 μg of prepared RNA

using a RT^2 First Strand Kit (SA Bioscience). cDNAs were then mixed with the RT^2 Real-Time™ SYBR Green PCR master mix (SA Bioscience), and 45 polymerase chain reaction cycles were performed using a LightCycler® 480 System (Roche). Two array replicates were performed. Average value of five housekeeping genes (B2M, HPRT1, RPL13A, GAPDH and ACTB) was used for normalization, and the data was analyzed with the △△Ct method. The difference between the Ct values (△Ct) of each gene and the housekeeping gene was calculated for each sample. Then, the difference in the △Ct values between the experimental and control samples (△△Ct) was calculated. The fold-change in expression of the gene of interest between the two samples was equal to $2^{(-\triangle\triangle Ct)}$. Data from PCR array was represented as fold-regulation described as Table 3.

10. Estradiol ELISA assay

MCF7 (4 X 10^5 cells/well) were seeded in 6-well plate with phenol red-free DMEM containing 10% charcoal-stripped FBS. After 24 h incubation, the cells were transfected with FLAG-IFI16 plasmid and incubated more 12 h. Then we changed culture media with 1 mL of phenol red-free DMEM without FBS and after 36 h, the conditioned media were collected and briefly spun to sediment the dead cells. The concentration of estradiol in the cell supernatantwas assayed using an Estradiol Enzyme Immunoassay (EIA) kit (Cayman Chemical) according to the manufacturer's instructions. Experiment was performed in triplicate and the resulting supernatants were assayed in

duplicate.

11. Chromatin immunoprecipitation assay

ChIP assays were performed as described previously (Lee et al., 2012). Nuclear lysates were sonicated, and the lysates were immunoprecipitated using specific anti-ERα (sc-543x), anti-AR (sc-816x) and normal IgG antibodies for overnight at 4°C. DNA was extracted by phenol-chloroform extraction and amplified by PCR using specific primer for estrogen receptor element as described in Table 2 (Sabnis et al., 2009; Fritah et al., 2005). PCR products were resolved in 1.5% agarose gel.

12. Statistical analysis

Data are expressed as the mean ± SD or SEM. Statistical significance was determined by unpaired student's t-test or one-way ANOVA. Differences were considered statistically significant when p-value was < 0.05.

Table 1. Oligomer sequences of siRNA and shRNA

Gene		siRNA sequences
IFI16	Sense	5′-GCU GGU CCU AAC CAA ACG UTT-3′
	Antisense	5′-ACG UUU GGU UAG GAC CAG CTT-3′
GFP	Sense	5′-GUU CAG CGU GUC CGG CGA GTT-3′
	Antisense	5′-CUC GCC GGA CAC GCU GAA CTT-3′

Gene		shRNA sequences
IFI16	Sense	5′-CCG GAC GTT TGA GGT TCC AAA TAA ACT CGA GTT TAT TTG GAA CCT CAA ACG TTT TTT G-3′
	Antisense	5′-AAT TCA AAA AAC GTT TGA GGT TCC AAA TAA ACT CGA GTT TAT TTG GAA CCT CAA ACG T-3′
MTA1	Sense	5′-CCG GGC GCA TCT TGT TGG ACA TAT TCT CGA GAA TAT GTC CAA CAA GAT GCG CTT TTT G-3′
	Antisense	5′-AAT TCA AAA AGC GCA TCT TGT TGG ACA TAT TCT CGA GAA TAT GTC CAA CAA GAT GCG C-3′

Table 2. Primer sequences used for RT-PCR, qPCR and ChIP analysis

Gene	Primer sequences		Anneal Temp.
ERα	Forward	5′-ACT GTA GCA GAG TAT CTG GTG A-3′	55 ℃
	Reverse	5′-GGT CTG CAA GGA ATG TTC CTA-3′	
CYP19A1	Forward	5′-CAC ATC CTC AAT ACC AGG TCC-3′	60 ℃
	Reverse	5′-CAG AGA TCC AGA CTC GCA TG-3′	
AR	Forward	5′-CCT GGC TTC CGC AAC TTA CAC-3′	60 ℃
	Reverse	5′-GGA CTT GTG CAT GCG GTA CTC A-3′	
pS2	Forward	5′-ACC ATGGAG AAC AAG GTG AT-3′	60 ℃
	Reverse	5′-AAA TTC ACA CTC CTC TTC TG-3′	
PR	Forward	5′-ACA GGA CCC CTC CGA CGA AAA-3′	60 ℃
	Reverse	5′-AGC TGT CTC CAA CCT TGC ACC-3′	
β-actin	Forward	5′-CGT GGG CCG CCC TAG GCA CCA-3′	55 ℃
	Reverse	5′-TTG GCT TAG GGT TCA GGG GGG-3′	
pS2-ERE	Forward	5′-GGC CAT CTC TCA CTA TGA ATC-3′	55 ℃
	Reverse	5′-GGC AGG CTC TGT TTG CTT AAA-3′	
PR-ERE	Forward	5′-TAA CGG GTG GAA ATG CCA ACT-3′	55 ℃
	Reverse	5′-TCT GCT GGC TCC GTA CTG CGG-3′	

Ⅳ. RESULTS

1. IFI16 expression in breast cancer

In order to investigate the role of IFI16 in breast cancer, we first analyzed published microarray data sets that were provided publicly available on cancer gene expression. Since our previous study demonstrated that IFI16 represses the transcription of ERα (Figure 2C), we compared levels of IFI16 to those of ERα in the tissue of breast cancer patients. On six independent data sets that contain gene chip profiles obtained from Cancer Genomics Browser, UCSC web site, we found out that IFI16 mRNA levels are higher in ERα -negative breast carcinomas than ERα-positive breast carcinomas (Figure 5A; van de Vijver et al., 2002; van 't Veer et al., 2002; Neve et al., 2006; Chin et al., 2006; Desmedt et al., 2007; Yau et al., 2010). In addition, to gain a deeper insight about the role of IFI16 in breast cancer progression, we obtained another three data sets from ArrayExpress site, and examined the expression of IFI16 on several types of breast tissue. Figure 5B showed that IFI16 is more expressed in breast cancer tissue than non-cancerous tissue significantly (Poola et al., 2005; Liu et al., 2007; Chen et al., 2010). Through these results we had knowledge that IFI16 is induced in breast cancer and there is a negative correlation between IFI16 and ERα expression in cancer.

2. Loss of IFI16 or MTA1 provides susceptibility to tamoxifen-induced cell growth inhibition for ERα-negative breast cancer cells

To further validate knockdown effect of repressor proteins, IFI16 and MTA1, we established stable sublines derived from MDA-MB-231, ERα-negative breast cancer cell line, expressing shMTA1 or shIFI16 using the lentiviral delivery shRNA system and confirmed the silencing of these genes (Figure 6A). As shown in Figure 6B, the MDA-MB-231 stable lines lacking either MTA1 or IFI16 restored ER α expression like previous data of transient transfection. Tamoxifen treatment to these cells significantly reduced cell growth, while this hormone dependency was not seen in the shGFP control cells (Figure 7A). Clonogenic viabilities of both shIFI16 and shMTA1 MDA-MB-231 stable cells were significantly lower compared with shGFP control cells. Further survival fractions in response to tamoxifen treatment were dramatically reduced in shIFI16 and shMTA1 MDA-MB-231 stable cells (Figure 7B). To examine the in vivo susceptibility of the MDA-MB-231 stable cells to tamoxifen treatment, the shRNA MDA-MB-231 stable cells were inoculated to grow xenografts in athymic nuce mice. We found significant differences in tumor growth of shIFI16 and shMTA1 stable cells after tamoxifen treatment, whereas no difference in shGFP control cells (Figure 7C), strongly supporting the role of MTA1/IFI16 complex in the regulation of ERα expression. Taken together, our results raise the possibility that overexpression of IFI16 in ERα-negative breast

cancer mediates ERα negativity and hormone resistance during breast carcinogenesis (Figure 8).

3. IFI16 regulates expression of ERα signaling-related genes

Array data sets showing that IFI16 is induced in breast cancer tissue (Figure 6B) prompted us to clarify the point of action of IFI16 in breast cancer progression. For this purpose, a real-time PCR array, which includes 84 ERα signaling-related genes, was performed in MDA-MB-231 cell to study effects of induced IFI16 on gene expression. When IFI16 was overexpressed or silenced by transient transfection, expressions of nine genes were up- or down-regulated more than 1.5-fold in both samples (Table 3). Among them, CYP19A1 and androgen receptor (AR) showed the largest fold changes. To confirm the array data, mRNA and protein levels of each gene were examined after transient overexpression of IFI16 in ERα-positive breast cancer cell line, MCF7, because CYP19A1 and AR are known to participate in estrogen signaling of ERα-positive breast cancer (Bulun et al., 2005; Hickey et al., 2012). Consistently, expression of CYP19A1 was increased and that of AR was reduced by IFI16 (Figure 9A and B).

4. IFI16 increases estradiol level through induction of CYP19A1

Next, to verify the induction of CYP19A1 and its functionality,

concentration of estradiol in cell supernatant was measured by enzyme immunoassay. As shown in Figure 10, estradiol production was significantly increased in the present of IFI16. These data support that IFI16 induces the expression of functional CYP19A1 and it results in elevated realease of estradiol.

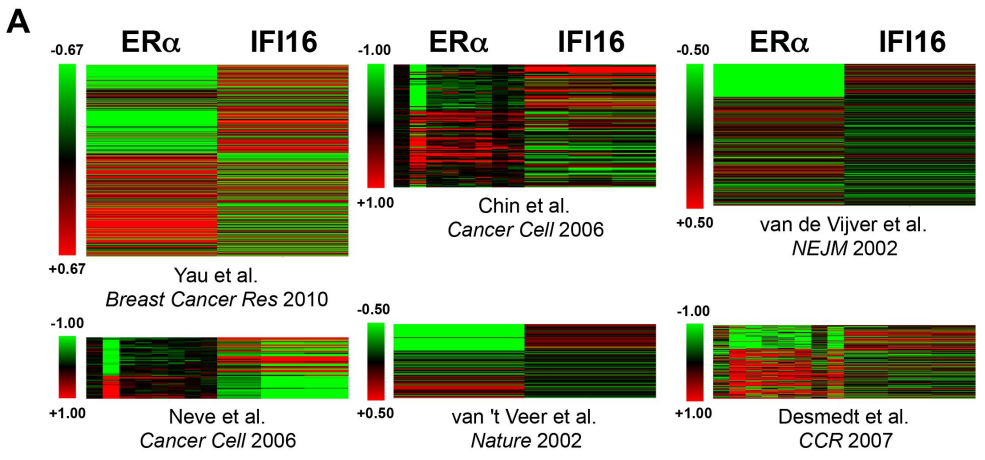
5. IFI16 affects recruitment of AR and ERα to ERα-response element

Earlier studies demonstrated that AR competes with ERα for binding to ERα-response element and represses ERα-dependent oncogenic activation in ERα-positive breast cancer (Hickey et al., 2012). Since IFI16 repressed AR expression (Figure 9), we tested whether IFI16 affect DNA binding of AR and ERα by ChIP assay. As expected, overexpression of IFI16 reduced AR recruitment to ERα-response element (ERE) site of ERα downstream, pS2 and PR, promoter (Figure 11A). By contrast, DNA binding of ERα was enhanced maybe due to disruption of competition with AR and increased level of ERα ligand (Figure 10). Consistent with this, we found that IFI16 induced the expression of pS2 and PR significantly (Figure 11B). These results indicate that IFI16 activates estrogen signaling by regulating CYP19A1 and AR levels.

6. Overexpression of IFI16 enhances proliferation of breast cancer cells

Finally, we established MCF7-derived stable subline that

overexpresses IFI16 to demonstrate the phenotype of IFI16 induction. CYP19A1 was more expressed and AR was repressed in IFI16-overexpressed subline as the results shown in transient transfection experiments (Figure 12A). Further we performed cell proliferation assay and clonogenic survival assay and it was found out that cell growth was significantly promoted in IFI16-overexpressed MCF7 subline comparing to control cell (Figure 12B and C). Taken together, our results indicate that the induction of IFI16 regulates expression of CYP19A1 and AR resulting in estrogen signaling activation and hormone-dependent proliferation during breast cancer progression (Figure 13).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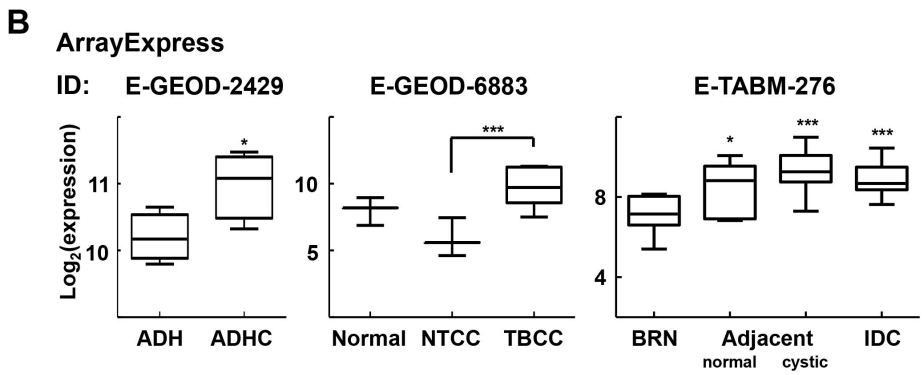
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ArrayExpress
ID:
E-GEOD-2429
E-GEOD-6883
E-TABM-276
Log2(expression)
11
10
ADH
ADHC
*
10
5
Normal
NTCC
TBCC
***
8
4
BRN
Adjacent
normal
cystic
IDC
*
***
***

Figure 5. Expression of IFI16 in human breast carcinoma

The public microarray data sets were analyzed for IFI16 expression in human breast tissue. (A) Heat maps present ERα and IFI16 expression level in the tissue of breast cancer patients. The microarray data sets were obtained and analyzed in Cancer Genomics Browser, UCSC (http://genome-cancer.ucsc.edu). (B) The published data sets were obtained from ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) with accession IDs: E-GEOD-2429, E-GEOD-6883 and E-TABM-276. The values of three probes for IFI16 from processed data were averaged and transformed as log2 expression that was presented in graph. Statistical significance was evaluated by one-way ANOVA followed by Tukey's post-test.* $P < 0.05$, ** < 0.01, and *** $P < 0.001$.

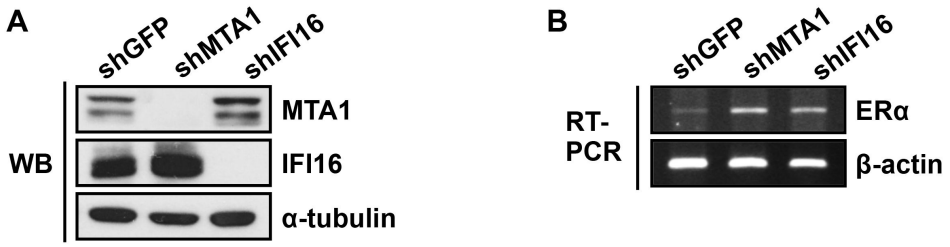

Figure 6. Knockdown of IFI16 or MTA1 restores ERα expression

(A) Establishment of the MDA-MB-231 stable sublines that expressing shGFP, shMTA1 or shIFI16 using lentiviral-delivered shRNA system. Knockdown of the corresponding proteins was confirmed by western blotting. (B) mRNA level of ERα in the MDA-MB-231 stable sublines was measured by RT-PCR.

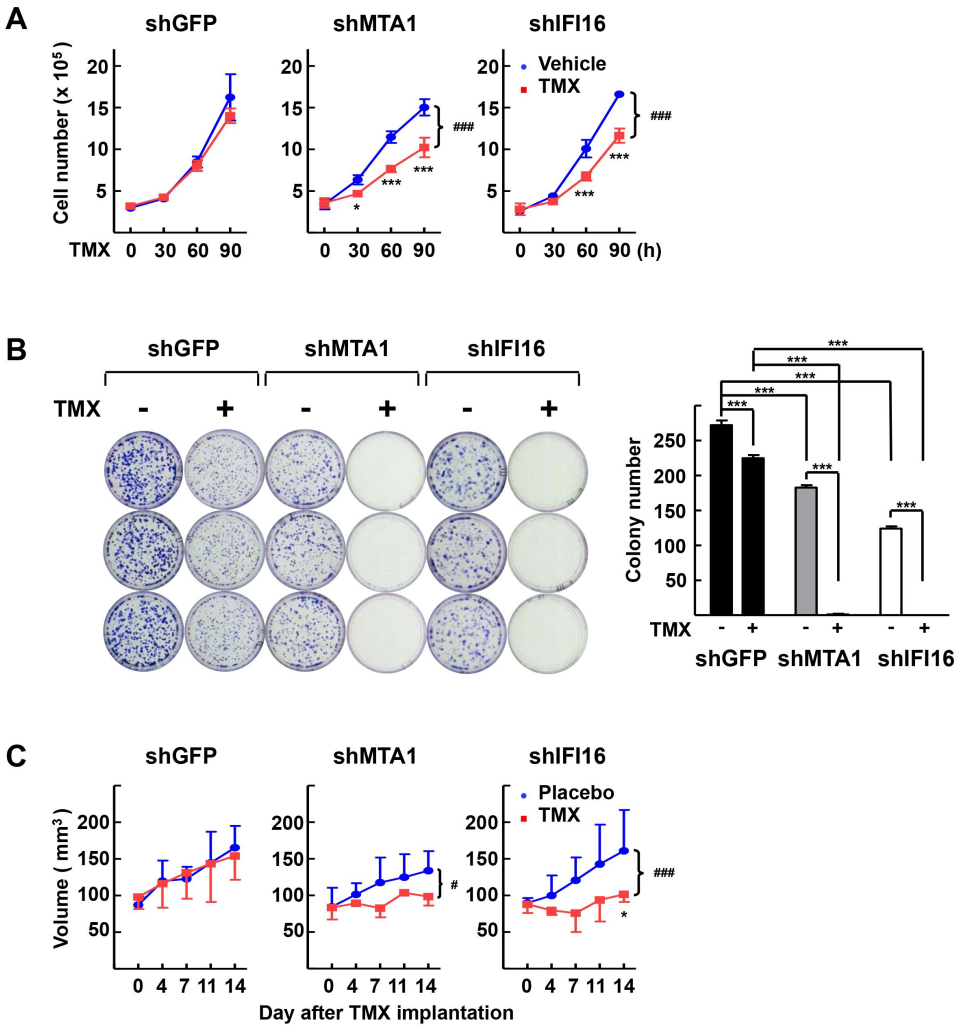
A
shGFP
shMTA1
shIFI16
Cell number (x 10^5)
Vehicle
TMX
TMX 0 30 60 90
0 30 60 90 (h)
###
B
shGFP
shMTA1
shIFI16
TMX
Colony number
C
Volume (mm^3)
Placebo
TMX
Day after TMX implantation

Figure 7. Loss of IFI16 or MTA1 provides susceptibility to tamoxifen-induced cell growth inhibition for ERα-negative breast cancer.

(A) The MDA-MB-231 stable cells were treated with the 10 μM tamoxifen (TMX) and the number of cells was counted using hematocytometer. Experimental values are expressed as the mean ± SD of three independent experiments. Statistical significance was evaluated by two-way ANOVA followed by Bonferroni post-test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ *vs.* vehicle at each time; ### $P < 0.001$ *vs.* vehicle group. (B) The MDA-MB-231 stable cells were treated with 10 μM TMX for 12 days. At the end of treatment, colonies were fixed and stained with 0.5 % crystal violet (Left). Colonies that composed of more than 50 cells were counted (Right). Experimental values are expressed as the mean ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. (E) Female athymic nude mice were inoculated with the shRNA MDA-MB-231 stable cells. When tumor volume reached approximately 100 mm^3, placebo or TMX pellet (25 mg/pellet with 21 days release) was implanted subcutaneously. Tumor volume was measured during two weeks of treatment. Experimental values are expressed as the mean ± SD. The number of specimen of the experimental groups was as follow: shGFP-placebo (n = 4), shGFP-TMX (n = 5), shMTA1-placebo (n = 3), shMTA1-TMX (n = 3), shIFI16-placebo (n = 6), and shIFI16-TMX (n = 4). * $P < 0.05$ *vs.* placebo at that time; # $P < 0.05$ and ### $P < 0.001$ *vs.* placebo group.

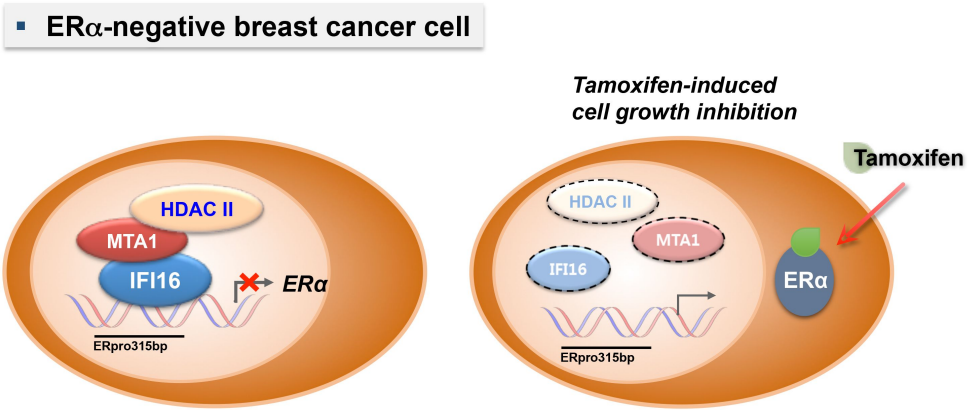

Figure 8. Schematic illustrations of function of IFI16 in the ERα-negative breast cancer

The IFI16/MTA1 complex represses ERα expression and loss of IFI16 or MTA1 sensitizes hormone-resistant cells to tamoxifen through inducing ERα re-expression.

Table 3. IFI16 regulates transcription of ERα signaling-related gene

Gene	Fold-regulation[2)]	
	Overexpression	Silencing
CYP19A1	1.97	-2.00
Complement component 3	-1.6	1.68
Interleukin 6 receptor	-1.74	3.73
GATA binding protein 3	-1.82	1.62
Estrogen receptor 1	-2.13	1.61
Serpin peptidase inhibitor, clade B (ovalbumin), member 5	-2.23	2.29
Nerve growth factor receptor	-2.27	1.66
Secretoglobin, family 2A, member 1	-2.39	1.58
Androgen receptor	-29.24	1.84

2) **Fold-regulation** represents fold-change results in a biologically meaningful way. Fold-change values greater than one indicate a positive- or an up-regulation, and the fold-regulation is equal to the fold-change. Fold-change values less than one indicate down-regulation, and the fold-regulation is the negative inverse of the fold-change.

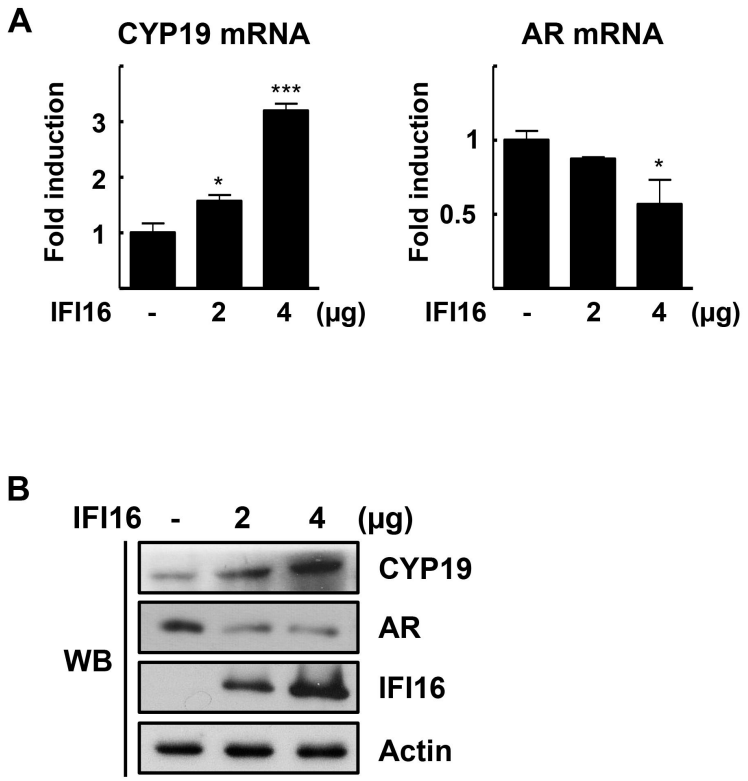

Figure 9. IFI16 overexpression alters expressional levels of CYP19A1 and AR

(A) MCF7 cells were transfected as described in panels. The mRNA levels of CYP19 and AR were analyzed by qRT-PCR and the values were normalized with β-actin expression. The values represent the means ± SEM of experiments in triplicate. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. (B) MCF7 cells were transfected as described in panels. The expression of CYP19, AR, IFI16 and a-tubulin were analyzed by western blot.

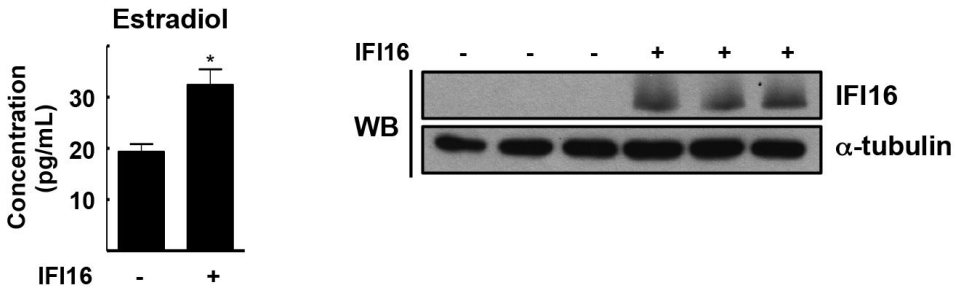

Figure 10. IFI16 increases estradiol level through induction of CYP19A1

MCF7 cells were transfected with 3 μg of FLAG-IFI16 or empty vector. The concentration of estradiol in cell supernatant was measured by enzyme immunoassay using standard curve. Experiment was performed in triplicate and the resulting supernatants were assayed in duplicate for absorbance reading. The values represent the means ± SD of experiments in triplicate. * P = 0.02 (Left). The overexpression of IFI16 was confirmed by western blotting (Right).

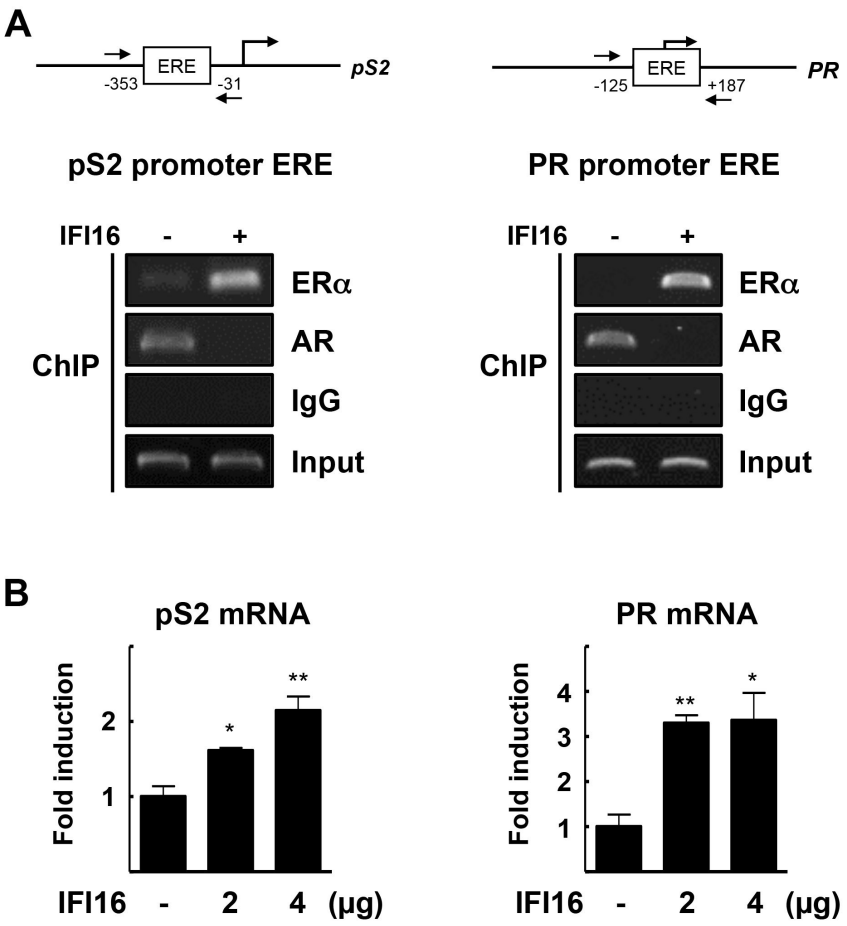
A
ERE
-353
-31
pS2
ERE
-125
+187
PR
pS2 promoter ERE
PR promoter ERE
IFI16 - +
ChIP
ERα
AR
IgG
Input
B
pS2 mRNA
PR mRNA
Fold induction
IFI16 - 2 4 (μg)

Figure 11. IFI16 affects recruitment of AR and ERα to ERα-response element

(A) Schematic representations of human pS2 and PR promoter with the known ERα-response element (Sabnis et al., 2009; Fritah et al., 2005) for ChIP assay (Top). MCF7 cells were transfected with FLAG-IFI16 or empty vector and DNA binding of ERα or AR on the ERE was analyzed by ChIP assay. DNA fragments that immunoprecipitated by anti-ERα or anti-AR antibody were amplified by PCR using primers for ERE site of each promoter (Bottom). (B) MCF7 cells were transfected as described in panels. The mRNA levels of pS2 and PR were analyzed by qRT-PCR and the values were normalized with β-actin expression. The values represent the means ± SEM of experiments in triplicate. * $P < 0.05$, and ** $P < 0.01$.

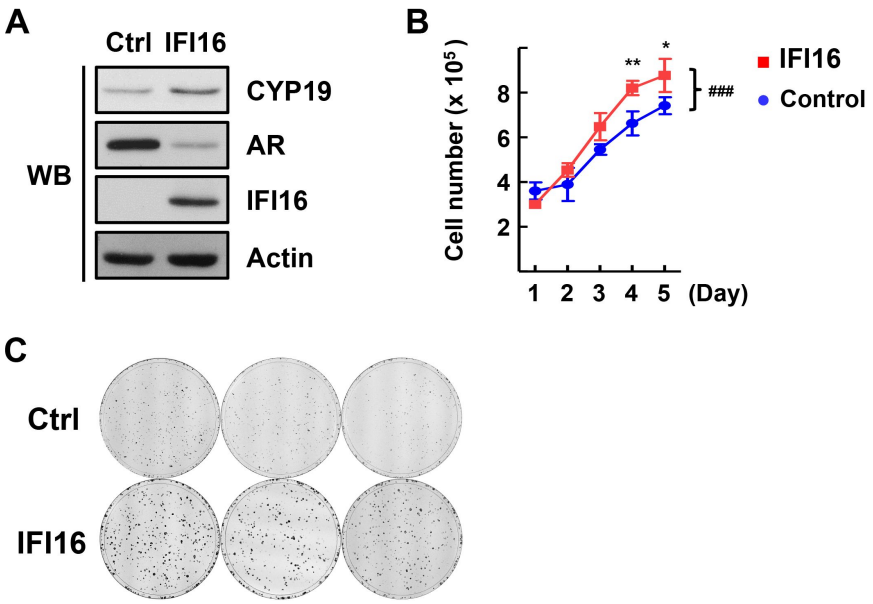

Figure 12. Overexpression of IFI16 enhances proliferation of breast cancer cell

(A) Establishment of the MCF7 stable subline that overexpressing IFI16 using lentiviral-delivering cDNA system. Expression levels of IFI16 and its putative targets, CYP19A1 and AR, were analyzed by western blotting. (B) The MCF7 stable cells (2 X 10^5 cells/plate) were seeded on 60 mm plate and incubated for 5 days. The number of cells was counted everyday using hematocytometer. Experimental values are expressed as the mean ± SD of three independent experiments. Statistical significance was evaluated by two-way ANOVA followed by Bonferroni post-test. * $P < 0.05$ and** $P < 0.01$ *vs.* vehicle at each day; ### $P < 0.001$ *vs.* control group. (C) The MCF7 stable cells were seeded on 35 mm plate and incubated for 12 days. Colonies were fixed and stained with 0.5 % crystal violet.

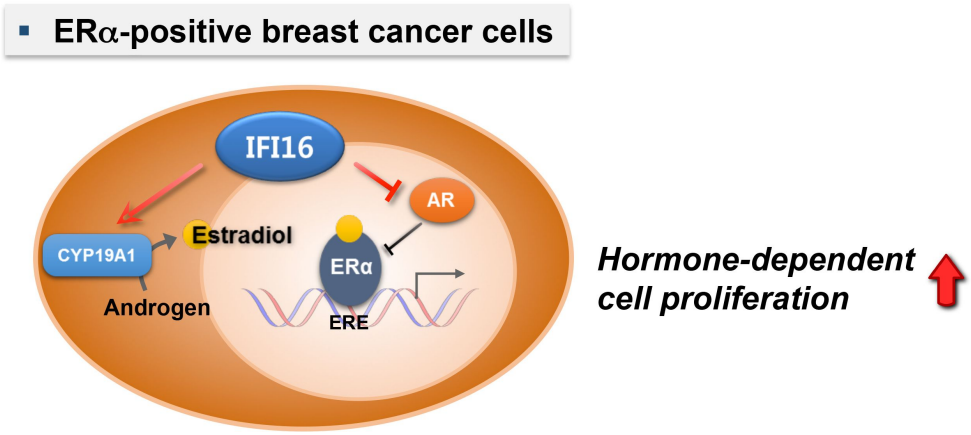

Figure 13. Schematic illustration of function of IFI16 in the ERα-positive breast cancer

IFI16 induces expressional level of CYP19A1 and reduces that of AR. Subsequently, the production of estradiol is increased and promoter binding of AR is decreased. As a result, ERα is activated and recuitment to downstream promoter is stimulated enhencing hormone-dependent cell proliferation.

V. DISCUSSION

Estrogen signaling plays a vital role in hormone-dependent proliferation of ERα-positive breast cancer and that is a reason for targeting estrogen signaling in breast cancer chemotherapy. The production of estradiol and the expression and activation of its receptor, ERα, make a main stream of estrogen signaling (Manavathi et al., 2012). However, there are no target-directed therapies for ERα -negative breast cancer although it is more clinically malignant and has poor prognosis. Thus, the strategy that restore ERα in expressional level has been suggested for hormone-independent breast cancer therapy and lots of researches studying ERα reactivation were performed (Cai et al., 2011; Linares et al., 2010; Li et al., 2013).

We hypothesized that silencing of IFI16 induces ERα re-expression on the basis of meta analysis of public expression data (Figure 5A) and our previous data describing that IFI16 is a member of repressive HDAC complex (Figure 2; Kang et al., to be published). We established shIFI16 or shMTA1-expressing MDA-MB-231 stable sublines and identified that ERα was up-regulated and the chemosensitivity to tamoxifen was enhanced in these cell line (Figure 6 and 7). Our results suggest that epigenetic regulation of ERα by IFI16/MTA1 complex is one of the molecular mechanisms that results

in the absence of ERα and hormone resistance in breast cancer cell.

In addition, we carried out PCR arrays to investigate another roles in breast cancer because IFI16 expression is higher in some breast carcinoma than normal tissue and inflammation, in which IFI16 is largely involved, is related to unfavorable prognosis in breast cancer (Ham et al., 2013). Here we demonstrated that IFI16 regulated expressions of CYP19A1 and AR and estrogen signaling was activated by IFI16 overexpression, leading to promote cancer cell proliferation (Figure 12). Interferon-gamma, which is well known to induce IFI16 (DeYoung et al., 1997), plays a critical role in immune response. Although many reports have shown its anti-tumor effect (Ikeda et al., 2002; Alshaker et al., 2011), several studies associated interferon-gamma with tumor development (Xiao et al., 2009; Hanada et al., 2006; Matsuda et al., 2005; Reiners et al., 1989). In the present study, we found that induction of IFI16 was oncogenic in ERα -positive breast cancer, suggesting that interferon- gamma could effect on activation of estrogen signaling and hormone dependency in inflammatory tumor microenvironment. For the follow research, we need to figure out the mechanism how IFI16 regulates expression of CYP19A1 and AR and whether treatment of interferon-gamma actually acts on IFI16-induced estrogen signaling and cell growth in breast cancer.

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국 문 초 록

Interferon gamma-inducible protein 16 (IFI16)은 인터페론으로 유도되는 핵단백질로서 HIN-200 family에 속하며, 유방암에서는 세포사멸과 염증반응에 관여하는 것이 알려져 있다. 최근 IFI16은 전이활성인자 (MTA1)와 함께 히스톤 탈아세틸화효소 (HDAC) 복합체를 이루어 에스트로겐 수용체 알파 (ERα)의 전사를 억제한다는 것이 보고되었다. 그러나 유방암 세포의 성장이나 암 진행과정에서 IFI16의 역할은 거의 연구된 바가 없다. 따라서 본 연구에서는 IFI16이 유방암 세포에서 호르몬-의존적 증식과 에스트로겐-신호전달계에 미치는 영향과 그 기전을 규명하고자 하였다. 먼저 IFI16 또는 MTA1의 shRNA를 발현하는 ERα-음성 유방암 세포주 (MDA-MB-231)를 구축하여 각 유전자 발현억제에 따른 세포증식 양상을 관찰하였다. 이 세포주들에서는 보고된 바와 같이 ERα의 mRNA 전사가 증가되어 있는 것을 확인하였다. 그리고 ERα를 타겟으로 하는 항호르몬제인 타목시펜을 처리하였을 때, 대조군에 비해 세포의 성장이 억제되는 것을 cell counting, clonogenic survival assay, 그리고 *in vivo* 이종이식(xenograft) 종양 성장 실험을 통해 확인하였다. 다음으로, MDA-MB-231 세포주에 IFI16을 과발현 시키거나 silencing한 후, PCR array를 통해 84 개의 ERα 시그널링 관련 유전자의 발현 변화를 분석한 결과, 9개의 유전자가 대조군에 비해 1.5배 이상 발현이 변화하였다. 그 중, IFI16에 의한 방향화효소 (CYP19A1 또는 aromatase) 발현의 증가를 qPCR을 통해 ERα-양성 유방암 세포주 (MCF7)에서도 확인하였다. ELISA를 이용하여 세포배양액의 estradiol 농도를 측정하였을 때,

IFI16을 과발현 시킨 경우 대조군에 비해 농도가 1.7 배 증가하여 방향화효소의 활성이 증가되었음을 알았다. 또한 IFI16이 ERα의 활성을 억제한다고 알려진 안드로겐 수용체 (AR)의 발현을 조절한다는 것을 MCF7에서 확인하였다. IFI16이 발현될 때, ERα의 타겟 유전자인 pS2와 프로게스테론 수용체의 프로모터에서 경쟁적으로 작용하는 AR의 결합은 감소하고, ERα의 DNA 결합이 증가되었음을 크로마틴 면역침강법으로 증명하였으며, 이들 타겟 유전자의 mRNA 발현이 증가함을 보였다. 마지막으로, IFI16을 과발현하는 MCF7 세포주를 구축하여 세포의 증식 양상을 확인한 결과, 대조군에 비해 증식 속도나 colony 형성이 유의적으로 증가한 것을 볼 수 있었다. 이상의 결과로 IFI16이 ERα 음성 유방암에서 항호르몬제에 대한 내성을 유도하고, ERα 양성 유방암에서는 방향화효소와 안드로겐 수용체의 발현을 조절하여 ERα 시그널링을 활성화시키며, 세포증식을 촉진시킨다는 사실을 알았다. 본 연구결과는 유방암에서 IFI16의 역할에 대한 이해를 넓히며, 유방암의 예방과 치료에 새로운 타겟을 제시할 수 있다.

주요어: IFI16, 에스트로겐 수용체 알파, 방향화효소, 안드로겐 수용체, 유방암

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아울러 2년 동안 함께 공부하며 저에게 힘이 되어주고 격려와 충고로 석사과정 잘 마칠 수 있도록 해준 좋아와 사랑하는 제 친구들 인화, 미경이, 나연이, 현경이, 세희에게도 고맙다는 인사와 응원을 보냅니다.

끝으로 항상 저를 믿고 전폭 지원해주시며 응원해 주시는 부모님과 동생 혜인이에게 진심으로 감사드리며 이 논문을 마칩니다.