The expression of REDD-1 mediated mTOR pathway proteins and hypoxia related proteins in the human breast cancer

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Directed by Professor WooHee Jung

The Doctorial Dissertation submitted to the Department of Medicine, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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This certifies that the Doctorial Dissertation of Ja Seung Koo is approved.

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Ja Seung Koo

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

Abstract

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and hypoxia related proteins in the human breast cancer

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Breast cancer was mainly classified into 5 molecular subtypes, namely normal breast-like, luminal A, luminal B, HER2 overexpression, and basal-like phenotypes [triple negative breast cancer (TNBC)] according to gene profiling studies. Among these, HER-2 overexpression and TNBC show high tumor grade, increased proliferation activity and increase in the area of geographic tumor necrosis, representing hypoxic tumor microenvironment. In hypoxic condition, REDD1 (regulated in development and DNA damage response 1)-mediated regulation of the mammalian target of rapamycin (mTOR) pathway is involved in cell proliferation and survival. The purpose of present study was intended to investigate the expression of REDD1-mediated mTOR pathway proteins and hypoxia related proteins such as AMPK and HIF-1α in breast cancer through *in vitro* cell line and tissue microarray studies. For *in vitro* cell line studies, 4 types cell lines were selected showing breast cancer phenotype; MDA-MB-231(TNBC), SKBR3 (HER-2), MCF-7 (luminal A) and BT474 (luminal B). Through the western blot analysis and siRNA treatment for REDD1 and AMPK, the expressions of S6K1, phospho-S6KI, AMPK, REDD 1, and HIF-1α were evaluated in which hypoxic condition was made by cobalt chloride ($CoCl_2$) treatment

of 100, 200, 300, and 400 μM. Tissue microarray was done from the samples collected from 224 patients with breast cancer, and 30 cases of papilloma as the control group. The immunohistochemistry (IHC) for estrogen receptor (ER), progesterone receptor (PR), HER-2, epithelial growth factor receptor (EGFR), cytokeratin (CK) 5/6, Glut-1, HIF-1α, REDD 1, AMPK α 1, 14-3-3 σ, PTEN, phospho-Akt, phospho-mTOR, phospho-S6, and Ki-67 were performed. Phenotype classification of breast cancer was performed based on the results of IHC for ER, PR, and HER-2: luminal A (ER or/and PR +, HER-2 -), luminal B (ER or/and PR +, HER-2 +), HER-2 overexpression (ER or/and PR -, HER-2 +), and TNBC (ER or/and PR -, HER-2 -). In cell line study, the expression of HIF-1α and REDD 1were induced in all 4 cell lines under hypoxic condition. However, the expression of REDD1 was decreased or lost under severe hypoxic condition. The expression of HIF-1α was increased as hypoxic status was severe in MDA-MB-231. Under hypoxic condition the expression of phospho-S6K1 was higher with inhibition of REDD1 or AMPK by siRNA than without inhibition in MDA-MB-231 and MCF-7. The expression of HIF-1α was induced under hypoxic condition, irrespective of the inhibition of REDD1 or AMPK through siRNA. The human tissue microarray study showed that Glut-1 and HIF-1α were highly expressed in TNBC, HER-2 overexpression type breast cancer, and papilloma than luminal A and B type ($p < 0.000$). REDD1 expression was higher in papilloma than breast cancer ($p < 0.000$), but no difference was found among the 4 breast cancer phenotypes ($p = 0.307$). HIF-1α was significantly associated with poor disease free survival (DFS) and overall survival (OS) in multivariate Cox regression analysis (DFS; $p = 0.034$, odd ratio: 6.3), OS; $p=0.033$, odd ratio=5.2).
In conclusion, loss of REDD1 expression in TNBC and HER-2 overexpression type play important role to maintain tumor cell proliferation and survival under severe hypoxic condition. HIF-1α would be an important hypoxia related protein especially in high grade breast cancer

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Keywords: hypoxia, REDD1, mTOR, HIF-1α, breast neoplasm

The expression of REDD-1 mediated mTOR pathway proteins and hypoxia related proteins in the human breast cancer

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I. INTRODUCTION

Breast cancer is a heterogeneous disease which is diverse in behavior, outcome, and response to treatment. Recent studies by gene expression profiling to classify heterogeneous breast cancer enabled the identification of 5 different subtypes of breast cancer with distinct molecular signature and clinical implications: normal breast-like, luminal A, luminal B, HER2 overexpression, and basal-like phenotypes.[1-4] Immunohistochemical studies support this breast cancer classification with molecular signature to a certain extent.[5-7] Triple negative breast cancer (TNBC) has been used as a surrogate for basal-like phenotype because most basal-like phenotypes did not express ER, PR, and HER-2, and it has the advantage that those three stains are already used routinely in clinical work-up of breast cancer.[5, 8] Among these phenotypes, histological features of HER2 overexpression and TNBC type are reported to be associated with high tumor grade, increased proliferation activity and the area of geographic tumor necrosis.[5, 6, 8-12] According to histological features of geographic tumor necrosis with high mitotic index in HER2 overexpression and TNBC type, it could be assumed that tumor environment of HER2 overexpression and

TNBC type frequently was exposed to hypoxic condition. When normal cells were exposed to hypoxic condition, REDD1 (regulated in development and DNA damage response 1) was induced,[13] and induced REDD1 shuts 14-3-3 σ which was combined to TSC (tuberous sclerosis complex) 1-TSC2 complex. REDD1 mediated 14-3-3 σ shutting helped a TSC1-TSC2 complex recover its function.[14-17] A TSC1-TSC2 complex inhibits the mTOR pathway through inhibition of Rheb, and eventually gives rise to inhibition of cell growth and survival (Figure 1). However, the REDD1-mediated regulation of the mTOR pathway in human breast cancer has not been well studied. A previous *in vitro* cell culture study showed that cells under hypoxic condition demonstrated anchorage-independent growth when REDD1 was down-regulated, and study of human breast cancer tissue showed that 30% of breast cancers revealed significant down regulation of REDD1.[15] In addition, other hypoxia related proteins such as HIF-1α and AMPK are reported to be related with REDD1.[13, 18]

The purpose of this study was intended to investigate the expression of REDD1 mediated mTOR pathway related molecules and the hypoxia related proteins such as HIF-1α and AMPK in the breast cancer through the *in vitro* cell line study and human breast cancer tissue microarray study.

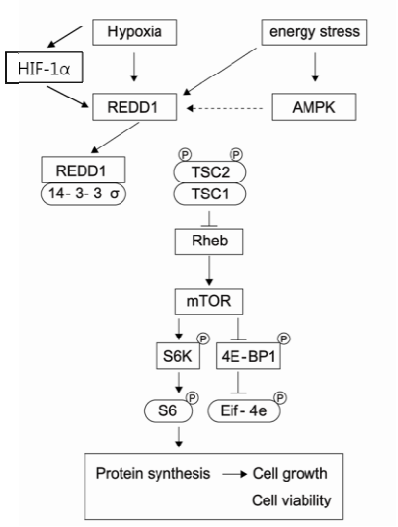

Figure 1. Regulation of hypoxia-mediated mTOR pathway.

II. MATERIALS AND METHODS

1. *In vitro* cell line study

A. Antibodies and chemicals

The rabbit polyclonal antibodies to S6K1, p-S6K1, and AMPK were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). REDD1 antibody was obtained from ProteinTech Group (Chicago, IL, USA), HIF-1α antibody was obtained from Novus Biologicals, and tubulin antibody was obtained from Sigma (St. Louis, MO, USA). Cobalt chloride ($CoCl_2$) was purchased from Sigma. siRNA targeting REDD1, AMPK and negative control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

B. Cells and cell culture

Human mammary carcinoma cell lines MDA-MB-231(TNBC; ER-, HER-2-), SKBR3 (HER-2 overexpression; ER-, HER-2+), MCF-7 (luminal A; ER+, HER-2-) and BT474 (luminal B; ER+, HER-2+) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (MDA-MB-231, MCF-7, and BT474) or McCoy's 5A medium (SKBR3) with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). All cells were maintained at 37°C in 5 % CO_2. In this study cobalt chloride ($CoCl_2$) was used to simulate hypoxic condition. $CoCl_2$ is commonly used hypoxic-mimetic agent, because like hypoxia, it can block the degradation of HIF-1α protein and thus induce the accumulation of HIF-1α protein, simulating hypoxic condition.[19] Cell counts were evaluated in second, fourth, and sixth day after treatment of 0, 200, 400, 600, 800, and 1000 μM to determine the optimum concentration of $CoCl_2$. For hypoxic experiments to evaluate the expression of S6K1, phospho-S6K1, REDD1, HIF-1α and AMPK, cells were treated with 100, 200, 300, and 400 μM $CoCl_2$ for 24 hours.

C. Small interfering RNA oligonucleotide duplexes (siRNA) and transient

transfections

The day before transfection, cells were seeded in six-well plates at 5 x 10^5 per well. Cells were transfected with siRNA at a final concentration of 50 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Briefly, the siRNAs were diluted in 100 µl of serum-free medium without antibiotic; after 5 min, Lipofectamine 2000 was added to the mixture and incubated at room temperature for 20 min. The mixture was added to cells (5 x 10^5 cells/well), and continued to incubate for 4 hours before switched to growing medium. After 48 hours of transfection process, $CoCl_2$ of 250 µM was added to the medium for 24 hours. After being cultured for another 24 hours, cells were collected for further experiments.

D. Cell extracts and western blot analysis of proteins

The cells were harvested in 2 days after transfection, washed twice in phosphate-buffered saline, and lysed in lysis buffer (1% SDS, 60 mM Tris-HCl, pH 6.8) containing, 1X Complete protease inhibitor mixture, 1X Phosstop phosphatase inhibitor (Roche Applied Science, Indianapolis, IN, USA). Protein concentration was determined by BCA assay (Thermo scientific Inc., Bremen, Germany). Equal amounts of proteins were separated by 8-10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a Hybond-ECL membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were then blocked in 5% non-fat dried milk in Tris-buffered saline containing 0.05 % tween 20 (TBST). Later, the membranes were incubated with primary antibodies, and the primary antibody was visualized using horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies and the SuperSignal West Pico chemiluminescence kit (Thermo scientific Inc. Bremen, Germany).

2. Human breast cancer tissue microarray study

A. Patient selection

From the Department of Pathology of Severance Hospital, we retrieved tissue samples of patients with breast invasive ductal carcinoma, which were filed between January 2000 and December 2001. This study was approved by the Institutional Review Board (IRB) of Severance Hospital. Formalin-fixed and paraffin-embedded tissue specimens from 224 cases of primary breast cancer were included. All archival hematoxylin and eosin (H&E) stained slides for each case were reviewed by 2 pathologists (Koo JS, and Jung W). The histological grade was assessed using the Nottingham grading system,[20] and nuclear grade was evaluated according to the modified Black′s nuclear grade (1 = low grade, 2 = intermediate grade, and 3 = high grade).[21] Histologic parameters such as histologic subtype, nuclear grade and histologic grade were evaluated from H&E stained slides. Clinical parameters which were evaluated for each tumor included patient age at initial diagnosis, tumor stage, nodal stage, cancer stage of American joint committee on cancer (AJCC) local recurrence, systemic recurrence, and patient′s survival. Thirty cases of intraductal papilloma with florid usual ductal hyperplasia were selected as a benign control group.

B. Tissue microarray

On H&E-stained slides of tumors, a representative area was selected and a corresponding spot was marked on the surface of the paraffin block. Using a biopsy needle, the selected area was punched out and a 3-mm tissue core was placed into a 6 x 5 recipient block. Tissues of invasive tumor were extracted. More than 2 tissue cores were extracted to minimize extraction bias. Each tissue core was assigned with a unique tissue microarray location number that was linked to a database containing other clinicopathologic data.

C. Immunohistochemistry (IHC)

The antibodies used for immunohistochemistry in this study are shown in Table 1. All immunostaining were performed using formalin-fixed, paraffin-embedded tissue sections. Five micro meter-thick sections were obtained with a microtome, transferred

to adhesive slides, and dried at 62°C for 30 min. The sections were then placed in a glass jar with 10 mM citrate buffer (pH 6.0), and they were irradiated in a microwave oven for 15 min. The sections were allowed to cool in the jar at room temperature for 20 min. The slides were then rinsed with Tris buffered saline (TBS). A blocking reagent was added for 10 min after quenching the endogenous peroxidase activity in 0.3% hydrogen peroxide for 10 min. After incubation with primary antibodies, immunodetection was performed with biotinylated antimouse immunoglobulin, followed by peroxidase-labeled streptavidin using a labeled streptavidin biotin kit with 3,3′-diaminobenzidine chromogen as substrate. Optimal primary antibody incubation time and concentration were determined via serial dilution for each immunohistochemical assay with an identically fixed and embedded tissue block. Slides were counterstained with Harris hematoxylin. The staining was interpreted by 2 pathologists (JS. Koo and W. Jung) under a multiview microscope. Different results were discussed and in case of persistent discordance, a third pathologist was consulted.

Table 1 Clone, dilution, and source of antibodies used

Antibody	*Clone*	*Dilution*	*Source*
Phenotype-related			
ER	SP1	1:100	Thermo Scientific, San Diego, CA, USA
PR	PgR	1:50	DAKO, Glostrup, Denmark
HER-2	Polyclonal	1:1500	DAKO, Glostrup, Denmark
Basal type-related			
EGFR	EGFR.25	1:50	Novocastra, Newcastle, UK
CK5/6	D5/16B4	1:100	DAKO, Glostrup, Denmark
Hypoxia-related			
Glut-1	SPM498	1:200	Abcam, Cambridge, UK
HIF-1 α	EP1215Y	1:100	Biocare, Yorba Linda , CA, USA
REDD 1	Polyclonal	1:50	Abcam, Cambridge, UK

mTOR pathway-related			
AMPK α1 (phospho T174)	Polyclonal	1:50	Abcam, Cambridge, UK
14-3-3 sigma	Polyclonal	1:1000	Abcam, Cambridge, UK
PTEN	Polyclonal	1:100	Invitrogen, Carlsbad, CA, USA
Phospho-Akt (Ser473)	736E11	1:50	Cell Signaling, Beverly, MA, USA
Phospho-mTOR (Ser2448)	49F9	1:50	Cell Signaling, Beverly, MA, USA
Phospho-S6 (Ser235/236)	D57.2.2F	1:50	Cell Signaling, Beverly, MA, USA
Proliferation-related			
Ki-67	MIB-1	1:150	DAKO, Glostrup, Denmark

D. Interpretation of IHC staining

All immunohistochemical markers were evaluated by light microscopy. Immunostained slides were scored according to the percentage of tumor cells exhibiting nuclear (ER, PR, HIF-1 α, and Ki-67), cytoplasmic (CK5/6, 14-3-3σ, PTEN, HIF-1 alpha, AMPK α1, phospho-Akt, phospho-mTOR, phospho-S6, and REDD1), and membrane (HER-2, EGFR, and Glut-1) staining. A cut-off value of 1% or more positively stained nuclei was used to define ER and PR positivity. HER-2 staining was analyzed according to the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines[22] using the following categories: 0 = no immunostaining; 1+ = weak incomplete membranous staining, less than 10% of tumor cells; 2+ = complete membranous staining, either uniform or weak in at least 10% of tumor cells; and 3+ = uniform intense membranous staining in at least 30% of tumor cells. HER-2 immunostaining was considered positive when strong (3^+) membranous staining was observed whereas cases with 0 to 1^+ were

regarded as negative. The cases showing 2+ HER-2 expression were evaluated for HER-2 amplification by fluorescent *in situ* hybridization (FISH). Immunohistochemical stain results of CK5/6 and EGFR were considered positive when more than 10% of tumor cells were stained. Immunohistochemical stain results of HIF-1α, Glut-1, and Ki-67 were scored as percentage of the antibody expressed tumor cells. Immunohistochemical stain results of 14-3-3 σ , PTEN, AMPK α1, phospho-Akt, phospho-mTOR, phospho-S6, and REDD1 were calculated by multiplying the percentage of tumor cells with antibody expression by the intensity of expression (1: weak, 2: moderate, and 3: strong).

E. Fluorescent *in situ* hybridization (FISH)

FISH analysis (Vysis pathvision c-erbB2 probe + DAKO FISH histology accessory kit) was manually performed. In brief, sections from formalin-fixed, paraffin-embedded tissue were mounted on Superfrost Plus slides, deparaffinized in xylene, and subsequently rehydrated in ethanol. Then, they were boiled for 10 min in pre-treatment solution, incubated with pepsin solution for 10 min, dehydrated in ethanol for 6 min, and finally air-dried. For hybridization, the buffered probe (Her-2/neu and centromere 17) was brought onto the slide and protected by a coverslip that was then sealed with rubber cement. For denaturation, slides were heated to 82°C and incubated overnight at 45°C in a dark humidified chamber. The rubber cement and coverslip were then removed, and the slides were transferred to stringent wash buffer for 10 min at 65°C. Afterward, they were dehydrated in ethanol for 6 min and air-dried. Finally they were counterstained with 4',6-diamidino-2-phenylindole (DAPI). The signals were evaluated using an epifluorescence microscope (Olympus, Japan) equipped with a fluorescein, Cy3, and DAPI filter set and 100 W mercury lamp. Counting was carried out according to the Vysis manual (the Her-2/neu gene appears as orange and centromere 17 as green). We counted signals in at least 20 tumor nuclei

in 2 separate regions of the tissue section according to the ASCO/CAP guideline. As proposed by the ASCO/CAP guideline,[22] an absolute HER-2 gene copy number lower than 4 or HER-2 gene/chromosome 17 copy number ratio (HER-2/Chr17 ratio) of less than 1.8 was considered HER-2 negative; an absolute HER-2 copy number between 4 and 6 or HER-2/Chr17 ratio between 1.8 and 2.2 was considered HER-2 equivocal; and an absolute HER2 copy number greater than 6 or HER-2/Chr17 ratio higher than 2.2 was considered HER-2 positive. Lymphocytes, fibroblasts, and normal ductal epithelial cells were used as internal controls.

F. Tumor phenotype classification

In this study, we classified breast cancer phenotypes according to the immunohistochemistry and FISH results for ER, PR, and HER-2 as follows; *luminal A type*: ER or/and PR positive and HER-2 negative, *Luminal B type*: ER or/and PR positive and HER-2 overexpressed or/and amplified, *HER-2 overexpression type*: ER and PR negative and HER-2 overexpressed or/and amplified, *TNBC type*: ER, PR, and HER-2 negative.

G. Statistical analysis

Data were processed using SPSS for Windows, version 12.0 (SPSS Inc., Chicago, IL, USA). Student's *t* and Fisher's exact tests were used to examine any difference in continuous and categorical variables, respectively. Significance was assumed when $p < 0.05$. Kaplan-Meier survival curves and log-rank statistics were employed to evaluate time to tumor recurrence and time to survival. Multivariate regression analysis was performed using Cox proportional hazards model.

III. RESULTS

1. *In vitro* cell line study

A. Cell growth pattern under hypoxic condition

Cell growth pattern was evaluated with different concentration of $CoCl_2$ (0, 200, 400, 600, 800, and 1000 μM) in 4 cell lines (Figure 2). Cell growth was higher in MCF7 and MDA-MB-231 under normoxic condition. Cell growth decreased as the concentration of $CoCl_2$ was high in all 4 cell lines, but MDA-MB-231 showed similar cell growth under 200 μM $CoCl_2$ to that under normoxic condition, representing resistance to hypoxia in MDA-MB-231.

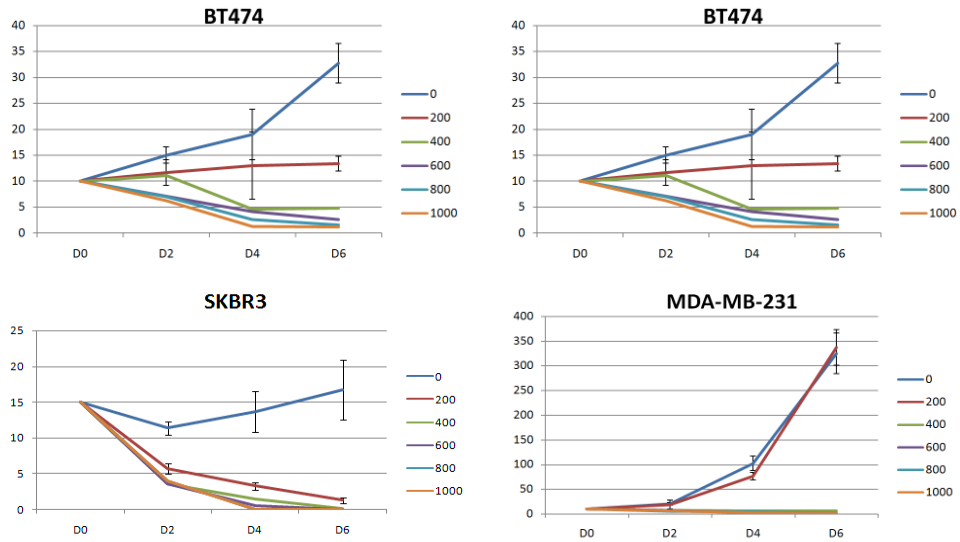

Figure 2. Cell growth pattern under hypoxic condition.

B. Hypoxic condition induces HIF-1α and REDD1 expression in breast cancer

To study the role of HIF-1α and REDD1 in mTOR pathway during hypoxic condition, breast cancer cells were exposed to $CoCl_2$ for 24 hours in various concentrations. As shown in figure 3, $CoCl_2$ induced the increased levels of HIF-1α and REDD1 protein and decreased expression level of phospho-S6K1, known as a downstream target of mTORC1in all breast cancer cell line. $CoCl_2$ induced the increased level of HIF-1α

especially in MDA-MB-231 cell line. In contrast, the expression level of REDD1 was increased in lower concentration of $CoCl_2$ and decreased in higher concentration of $CoCl_2$ in all of the four breast cancer cell lines.

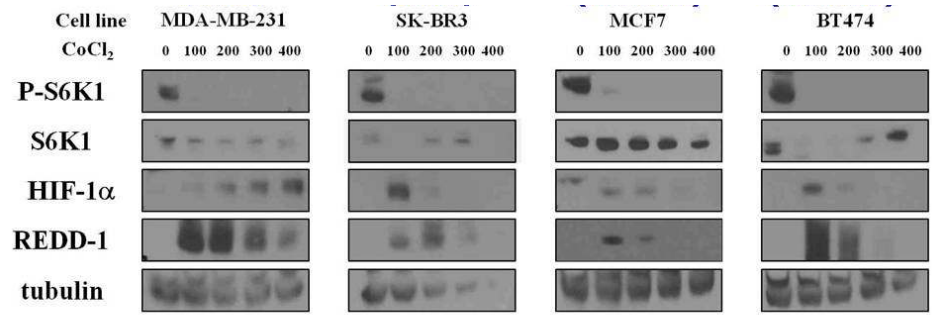

Figure 3. The impact of cobalt chloride on REDD1 mediated mTOR pathway proteins and hypoxia-related proteins in the breast cancer cell lines.

C. Expression of hypoxia related molecules after inhibition of REDD1 or AMPK

To examine whether reducing REDD1 or AMPK function alters hypoxia related molecules or mTOR pathway in breast cancer cells, we performed loss of function studies with siRNA knockdown targeting REDD1 or AMPK. Cell lysates were prepared 72 hours after transfection, and levels of mTOR pathway-related protein and hypoxia-related protein were determined by immunoblot analysis. As shown in figure 4, the siRNAs targeting REDD1 or AMPK efficiently blocked the $CoCl_2$-induced increase of REDD1 protein levels. Selective siRNA targeting of REDD1 or AMPK induced the expression of phospho-S6K1 under hypoxia in MDA-MB-231 and MCF7. HIF-1a was increased in hypoxic condition in all of the four breast cancer cell lines irrespective of REDD1 or AMPK inhibition. However, the expression level of HIF-1a was not increased in hypoxic condition after inhibition of AMPK in the MDA-MB-231 cell line.

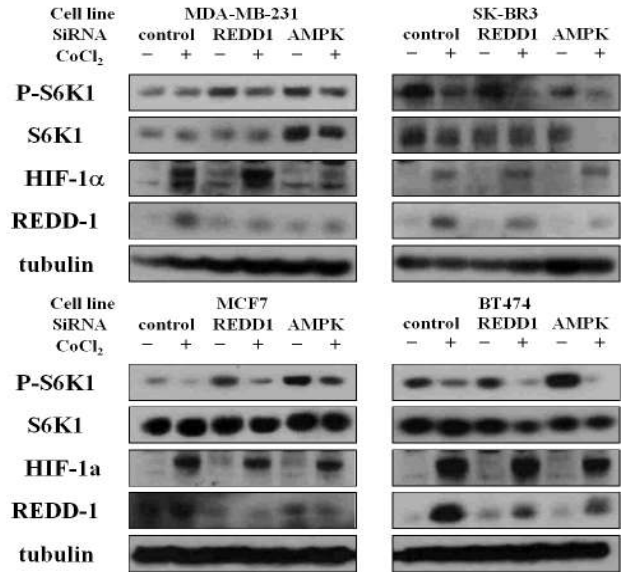

Figure 4. Effect of REDD1 and AMPK inhibition on REDD1 mediated mTOR pathway proteins and hypoxia-related proteins in breast cancer cell lines.

2. Human breast cancer tissue microarray study

A. Clinicopathologic features of patients according to breast cancer phenotype

Table 2 shows clinicopathologic characteristics of patients. Forty three patients (19.2%) were not followed up after breast surgery. When breast cancer was classified according to ER, PR and HER-2 status, most medullary carcinomas (70%) and all metaplastic carcinomas (100%) were TNBC type ($p = 0.001$). The nuclear grade and histologic grade were higher in TNBC and HER-2 overexpression type than luminal A and B ($p < 0.000$). Most cytokeratin (CK) 5/6 (88.2%) or EGFR (62.5%) expressed breast cancer were TNBC ($p < 0.000$). Both CK5/6 and EGFR positivity were noted in 7 cases (5 cases: TNBC type, and 2 cases: HER-2 type). The numbers of metastatic lymph nodes were the highest in luminal B (5.5 ± 9.0, mean ± SD) and lowest in TNBC (1.0 ± 2.2, mean ± SD) ($p = 0.077$).

Table 2 Clinicopathologic characteristics of patients according to breast cancer phenotype

Parameters	*Total*	*TNBC*	*HER-2 type*	*Luminal A*	*Luminal B*	*P-value*
	(n = 224) (%)	*(n = 60) (%)*	*(n = 29) (%)*	*(n = 115) (%)*	*(n = 20) (%)*	
Age	48.7 ±	46.7 ±	48.2 ±	49.7 ±	49.6 ±	0.364
(yr, mean ± SD)	10.8	12.8	9.3	10.1	9.9	
Histologic subtype						0.001
IDC, NOS	212 (94.6)	51 (85.0)	26 (89.7)	115 (100.0)	20 (100.0)	
Medullary	10 (4.5)	7 (11.7)	3 (10.3)			
Metaplastic	2 (0.9)	2 (3.3)				
Tumor stage						0.558
T1	74 (33.0)	19 (31.7)	7 (24.1)	42 (36.5)	6 (30.0)	
T2	138 (61.6)	40 (66.7)	19 (65.5)	66 (57.4)	13 (65.0)	
T3-4	12 (5.4)	1 (1.7)	3 (10.3)	7 (6.1)	1 (5.0)	
Nodal stage						0.498
N0	119 (53.1)	36 (60.0)	15 (51.7)	59 (51.3)	9 (45.0)	
N1	66 (29.5)	17 (28.3)	8 (27.6)	37 (32.2)	4 (20.0)	
N2	22 (9.8)	5 (8.3)	3 (10.3)	11 (9.6)	3 (15.0)	
N3	17 (7.6)	2 (3.3)	3 (10.3)	8 (7.0)	4 (20.0)	
AJCC stage						0.219
Stage I	52 (23.2)	14 (23.3)	4 (13.8)	31 (27.0)	3 (15.0)	
Stage II	129 (57.6)	39 (65.0)	18 (62.1)	62 (53.9)	10 (50.0)	
Stage III	43 (19.2)	7 (11.7)	7 (24.1)	22 (19.1)	7 (35.0)	
Nuclear grade						0.000
1	14 (6.3)	1 (1.7)		12 (10.4)	1 (5.0)	
2	126 (56.3)	24 (40.0)	12 (41.4)	77 (67.0)	13 (65.0)	
3	84 (37.5)	35 (58.3)	17 (58.6)	26 (22.6)	6 (30.0)	
Histologic grade						0.000

I	32 (14.3)	4 (6.7)		25 (21.7)	3 (15.0)	
II	123 (54.9)	26 (43.3)	12 (41.4)	71 (61.7)	14 (70.0)	
III	69 (30.8)	30 (50.0)	17 (58.6)	19 (16.5)	3 (15.0)	
Estrogen receptor status						
Positive	131 (58.5)					
Negative	93 (41.5)					
Progesterone receptor status						
Positive	90 (40.2)					
Negative	134 (59.8)					
HER-2 status						
Positive	49 (21.8)					
Negative	175 (78.2)					
Cytokeratin 5/6						0.000
Positive	17 (7.6)	15 (25.0)	2 (6.9)			
Negative	207 (92.4)	45 (75.0)	27 (93.1)	115 (100.0)	20 (100.0)	
EGFR						0.000
Positive	24 (10.7)	15 (25.0)	6 (20.7)	3 (2.6)		
Negative	200 (89.3)	45 (75.0)	23 (79.3)	112 (97.4)	20 (100.0)	
The number of metastatic LN (mean ± SD)	2.4±7.0	1.0 ± 2.2	3.5 ± 7.1	2.4 ± 8.0	5.5 ± 9.0	0.077
Tumor recurrence	24 (10.7)	4 (6.7)	4 (13.8)	12 (10.4)	4 (20.0)	0.582
Distant metastasis	18 (8.0)	2 (3.3)	4 (13.8)	9 (7.8)	3 (15.0)	0.364
Patients' death	13 (5.8)	4 (6.7)	4 (13.8)	4 (3.5)	1 (5.0)	0.191
Duration of clinical follow-up (months,	89.6 ± 23.7	85.8 ± 24.1	81.3 ± 33.4	93.3 ± 19.9	89.9 ± 24.0	0.090

mean ± SD)

AJCC : American joint committee on cancer

B. Comparison of immunohistochemical features according to breast cancer phenotype

Table 3 and figure 5 demonstrate immunohistochemical results of hypoxia-mTOR pathway proteins and hypoxia related proteins according to breast cancer phenotype. Glut-1 and HIF-1 α were more highly expressed in TNBC, HER-2 overexpression type, and papilloma than luminal A and B type ($p < 0.000$, Figure 5 and figure 6). HIF-1α expression was noted in cytoplasm and nucleus in TNBC, and HER-2 overexpression type, however only in nuclei in papilloma (Figure 5). REDD1 and PTEN expression were higher in papilloma than any other breast cancer phenotypes ($p < 0.000$, Figure 5 and figure 6). The expression of Ki-67 was higher in TNBC and HER-2 overexpression type than other phenotypes and papilloma ($p < 0.000$). Phospho-mTOR and phospho-S6 were the most highly expressed in HER-2 overexpression type ($p = 0.005$, and < 0.000). In addition, the statistical analyses were performed for only 224 cases of breast cancer, not 30 cases of papilloma. Furthermore, the statistical analyses aimed at only TNBC, HER-2 overexpression type, and papillomas were performed because these 3 groups showed similar hypoxic tumor environment known from the histologic features and high Glut-1 expression. The corresponding statistical p-values were also presented in Table 3.

Table 3 Immunohistochemical characteristics of hypoxia-mTOR pathway-related protein according to breast cancer phenotype

Antibodies	*Total*	*Tumor phenotype (mean ± SD)*					*P-value*[1]	*P-value*[2]	*P-value*[3]
	(n = 224)	*TNBC (n = 60)*	*HER-2 (n = 29)*	*Luminal A (n = 115)*	*Luminal B (n = 20)*	*Papilloma (n = 30)*			
Glut-1	23.2 ± 28.7	39.5 ± 30.8	30.6 ± 30.8	14.3 ± 23.9	15.0 ± 20.9	41.6 ± 15.9	0.000	0.000	0.226
REDD 1	16.6 ± 28.0	12.5 ± 30.9	21.9 ± 25.7	18.4 ± 28.4	11.0 ± 17.4	51.0 ± 16.4	0.000	0.307	0.000
HIF-1 α	2.2 ± 7.4	3.1 ± 6.7	8.6 ± 16.7	0.3 ± 1.5	0.9 ± 1.8	3.5 ± 2.6	0.000	0.000	0.037
14-3-3σ	52.4 ± 33.7	43.8 ± 28.2	53.7 ± 34.1	55.8 ± 37.4	56.5 ± 20.0	48.5 ± 17.5	0.133	0.142	0.278
Ki-67	7.2 ± 10.4	13.2 ± 13.2	10.4 ± 13.0	3.9 ± 6.6	4.0 ± 3.8	4.2 ± 1.8	0.000	0.000	0.003
PTEN	13.2 ± 25.4	9.6 ± 22.0	17.2 ± 32.7	15.2 ± 26.2	6.5 ± 14.9	46.0 ± 20.9	0.000	0.262	0.000
Phospho-Akt (Ser473)	5.9 ± 19.0	8.0 ± 23.7	13.4 ± 32.6	3.4 ± 11.0	3.5 ± 8.7	7.2 ± 7.4	0.084	0.058	0.525

Phospho-mTOR (Ser2448)	87.7 ± 78.5	63.0 ± 61.6	109.3 ± 87.2	96.7 ± 82.0	79.2 ± 76.4	59.3 ± 31.1	0.005	0.019	0.003
Phospho-S6 (Ser235/236)	41.0 ± 58.2	51.5 ± 56.5	72.5 ± 79.9	25.6 ± 44.3	52.5 ± 72.2	35.6 ± 18.0	0.000	0.000	0.048
AMPK α1 (phospho T174)	45.0 ± 33.5	40.9 ± 31.5	52.0 ± 34.7	46.4 ± 35.2	39.5 ± 26.2	48.0 ± 20.4	0.571	0.405	0.228

1 P-value was calculated in all 4 breast cancer phenotypes and papilloma.

2 P-value was calculated in all 4 tumor phenotypes.

3 P-value was calculated only in TNC, HER-2 types, and papilloma.

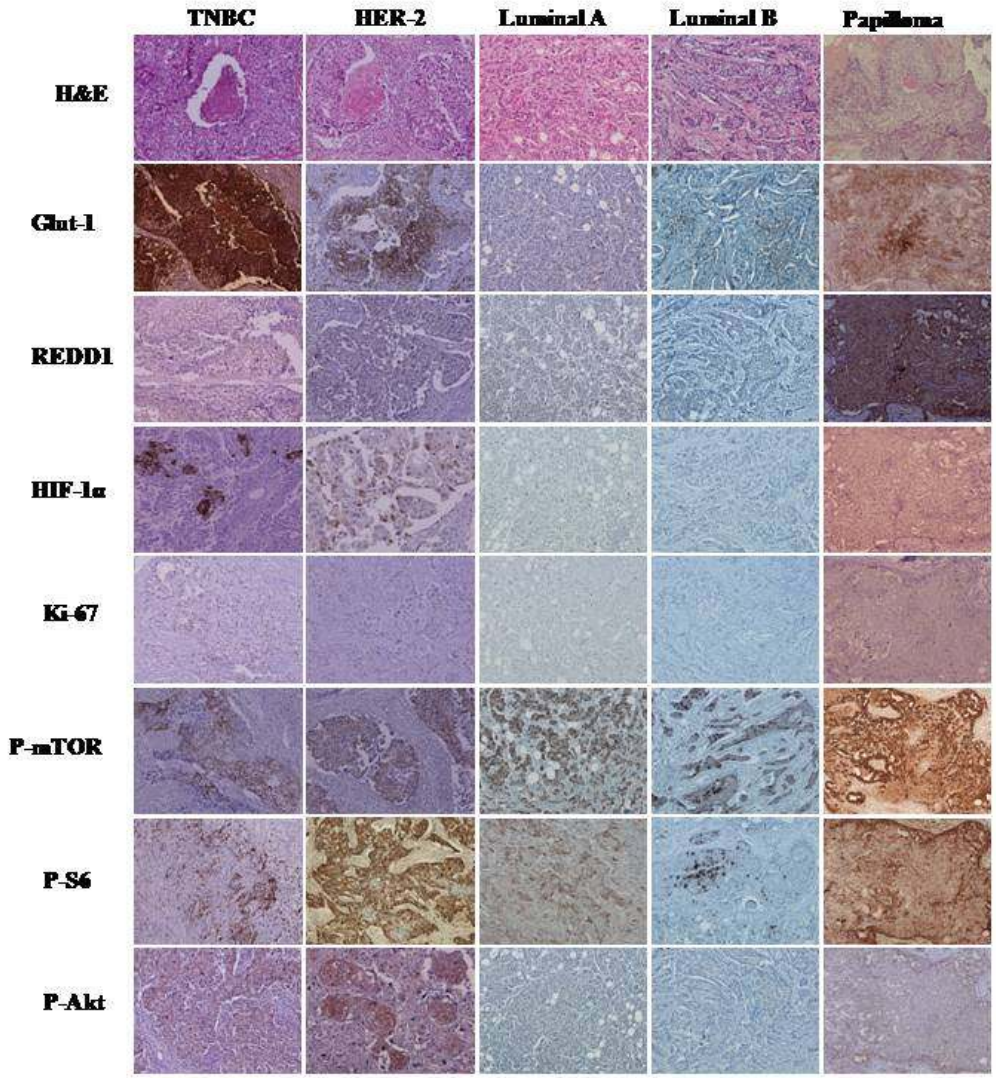

Figure 5. Histological and immunohistochemical features of breast cancer according to tumor phenotypes for ER, PR, and HER-2 status. TNBC, HER-2 overexpression type, and papilloma show the areas of geographic tumor cell necrosis, but luminal A and B type demonstrate no tumor cell necrosis. TNBC, HER-2 overexpression type and papilloma show more increased expression of Glut-1 than luminal A and B type, showing more hypoxic tumor environment. However, the expression of REDD1, a hypoxic-related protein, is not different among breast cancer phenotypes. In contrast,

papilloma shows diffuse expression of REDD1.

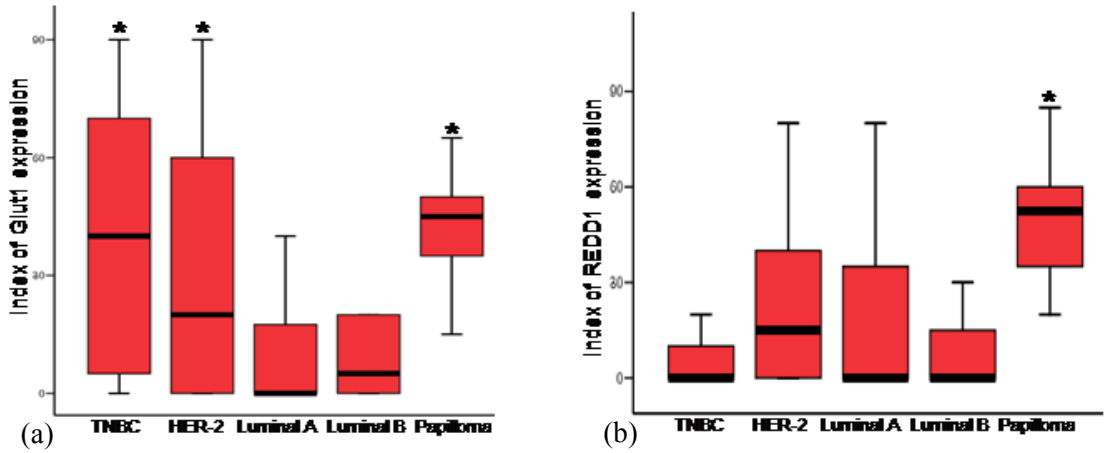

Figure 6. Comparison of expression index of Glut1 (a) and REDD1 (b) expression according to breast cancer phenotype. * $p < 0.05$

Table 4 shows the correlation of CK5/6 and EGFR expression with clinicopathologic and immunohistochemical results in TNBC type. CK5/6 positive TNBC showed higher Glut-1 expression ($p = 0.013$) and lower phospho-mTOR expression ($p = 0.049$) than CK 5/6 negative TNBC. CK5/6 positive TNBC showed lower REDD-1 expression than CK 5/6 negative TNBC without significance ($p = 0.188$). EGFR positive TNBC demonstrated higher expression of REDD-1 ($p = 0.024$) and AMPK α1 ($p = 0.021$) than EGFR negative TNBC. In addition, EGFR positive TNBC showed higher Glut-1 expression than EGFR negative TNBC without significance ($p = 0.063$).

Table 4 The correlation of CK5/6 and EGFR expression status with clinicopathologic and immunohistochemical results in triple negative breast cancer

Clinicopathologic parameters	*TNBC (n = 60) (%)*					
	CK5/6 positive (n = 15)	*CK5/6 negative (n = 45)*	*P-value*	*EGFR positive (n = 15)*	*EGFR negative (n = 45)*	*P-value*
Nuclear grade			0.369			0.672
1		1 (2.2)			1 (2.2)	
2	4 (26.7)	20 (44.4)		5 (33.3)	19 (42.2)	
3	11 (73.3)	24 (53.3)		10 (66.7)	25 (55.6)	
Histologic grade			0.489			0.953
I		4 (8.9)		1 (6.7)	3 (6.7)	
II	7 (46.7)	19 (42.2)		6 (40.0)	20 (44.4)	
III	8 (53.3)	22 (48.9)		8 (53.3)	22 (48.9)	
LN metastasis	2 (13.3)	17 (37.8)	0.078	6 (40.0)	13 (28.9)	0.423
Tumor recurrence	1 (6.7)	3 (6.7)	0.931	1 (6.7)	3 (6.7)	0.931
Distant metastasis	1 (6.7)	1 (2.2)	0.448	1 (6.7)	1 (2.2)	0.448
Patients' death	1 (6.7)	3 (6.7)	0.931	1 (6.7)	3 (6.7)	0.931
Immunohistochemical parameters	*TNBC (n = 60) (mean ± SD)*					
	CK5/6 positive (n = 15)	*CK5/6 negative (n = 45)*	*P-value*	*EGFR positive (n = 15)*	*EGFR negative (n = 45)*	*P-value*
Glut-1	56.3 ± 31.6	33.8 ± 28.8	0.013	52.3 ± 26.9	35.2 ± 31.2	0.063
REDD 1	3.3 ± 10.4	15.5 ± 34.8	0.188	28.0 ± 53.4	7.3 ± 16.2	0.024
HIF-1 α	2.0 ± 3.6	3.5 ± 7.4	0.441	5.6 ± 11.1	2.3 ± 4.2	0.095

14-3-3σ	52.6 ± 34.9	40.8 ± 25.4	0.164	52.0 ± 37.4	41.1 ± 24.4	0.199
Ki-67	11.8 ± 9.7	13.6 ± 14.2	0.640	14.7 ± 12.4	12.6 ± 13.6	0.609
PTEN	16.0 ± 30.6	7.5 ± 18.3	0.202	5.3 ± 12.4	11.1 ± 24.4	0.385
Phospho-Akt (Ser473)	6.6 ± 16.7	8.4 ± 25.7	0.804	3.3 ± 10.4	9.5 ± 26.6	0.383
Phospho-mTOR (Ser2448)	36.0 ± 46.6	72.0 ± 63.7	0.049	51.0 ± 61.5	67.0 ± 61.7	0.388
Phospho-S6 (Ser235/236)	44.3 ± 51.6	53.8 ± 58.3	0.575	69.3 ± 67.6	45.6 ± 51.7	0.160
AMPK α1 (phospho T174)	49.3 ± 24.9	38.1 ± 33.1	0.235	57.0 ± 36.7	35.5 ± 28.0	0.021

C. The impact of immunohistochemical results of hypoxia-mediated mTOR pathway proteins and hypoxia-related proteins on tumor recurrence, distant metastasis, and patient survival

Table 5 shows the influence of immunohistochemical stain results on tumor recurrence, distant metastasis, and patient survival. Breast cancers with tumor recurrence ($p = 0.033$), or distant metastasis ($p = 0.004$), or patient death ($p = 0.001$) showed higher incidence of HIF-1α expression than breast cancer without tumor recurrence or distant metastasis, or patient death. Breast cancers which resulted in tumor recurrence, distant metastasis, or patient death tended to show higher expression of phospho-mTOR and lower expression of phospho-Akt than breast cancer without tumor recurrence or distant metastasis, or patient death, but the results showed no statistical significance.

Table 5 The impact of immunohistochemical results in REDD1 mediated mTOR pathway proteins and hypoxia-related proteins on tumor recurrence, distant metastasis, and patient survival

Parameters	*Tumor recurrence (n = 182) (mean ± SD)*		*P-value*	*Distant metastasis (n = 182) (mean ± SD)*		*P-value*	*Patient survival (n = 182) (mean ± SD)*		*P-value*
	Present (n = 24)	*Absent (n = 158)*		*Present (n = 18)*	*Absent (n = 164)*		*Death (n = 13)*	*Survival (n = 169)*	
Glut-1	19.1± 23.1	23.0 ± 29.4	0.542	17.7 ± 21.5	23.0 ± 29.3	0.463	26.1 ± 26.3	22.2 ± 28.8	0.635
REDD 1	15.0 ± 23.5	17.6 ± 29.6	0.675	16.6 ± 23.7	17.3 ± 29.4	0.921	18.4 ± 26.0	17.2 ± 29.1	0.882
HIF-1 α	5.3 ± 16.0	1.7 ± 5.3	0.033	7.1 ± 18.3	1.6 ± 5.2	0.004	9.2 ± 21.3	1.6 ± 5.1	0.001
14-3-3σ	47.9 ± 27.6	54.3 ± 33.0	0.353	50.0 ± 29.5	54.0 ± 32.7	0.615	42.3 ± 30.3	54.5 ± 32.4	0.191
Ki-67	5.5 ± 9.0	6.8 ± 9.8	0.568	6.1 ± 10.3	6.7 ± 9.7	0.826	9.5 ± 12.6	6.4 ± 9.5	0.269
PTEN	8.7 ± 18.2	14.0 ± 27.1	0.356	10.0 ± 20.8	13.7 ± 26.7	0.568	8.4 ± 16.7	13.7 ± 26.7	0486
Phospho-Akt (Ser473)	1.2 ± 4.4	7.3 ± 21.9	0.179	1.6 ± 5.1	7.0 ± 21.6	0.293	1.5 ± 5.5	6.9 ± 21.3	0.366
Phospho-mTOR (Ser2448)	105.4 ± 87.9	89.3 ± 79.1	0.361	121.6 ± 86.1	88.1 ± 79.1	0.092	122.3 ± 79.9	89.0 ± 80.0	0.150

Phospho-S6 (Ser235/236)	36.4 ± 43.5	39.7 ± 60.3	0.796	41.6 ± 41.9	39.0 ± 59.9	0.859	40.3 ± 44.9	39.2 ± 59.3	0.947
AMPK α1 (phospho T174)	38.3 ± 30.5	46.4 ± 33.5	0.266	43.3 ± 32.1	45.6 ± 33.4	0.784	40.7 ± 29.5	45.7 ± 33.5	0.605

Table 6 demonstrates the univariate analyses of immunohistochemical results of REDD1 mediated mTOR pathway proteins and hypoxia related proteins on disease-free survival (DFS) and overall survival (OS). The immunohistochemical results were categorized according to the immunohistochemical scores as shown in Table 6. The results of univariate analyses of immunohistochemical markers on time to DFS revealed significance in HIF-1α ($p = 0.003$, Figure 7), and nodal stage ($p=0.015$). Multivariate Cox's proportional hazard model analysis also showed significance for HIF-1α ($p = 0.034$, odd ratio: 6.3). The results of univariate analyses of immunohistochemical markers on time to OS revealed significance of 3 parameters; ER ($p= 0.012$), Glut-1 ($p = 0.047$), and HIF-1α ($p = 0.000$, Figure 7). Multivariate Cox's proportional hazard model analysis showed significance for HIF-1α ($p = 0.033$, odd ratio: 5.2), and ER ($p = 0.034$, odd ratio: 3.8).

Table 6 – Univariate analysis of recurrence-free survival and overall survival by log-rank test according to clicopathologic and immunohistochemical factors in breast cancers

Parameters	*No. of patients (n = 182) (%)*			*Recurrence-free survival*		*Overall survival*	
	No. of cases	*Tumor recurrence*	*Patient death*	*Mean survival (95% CI) months*	*P - value*	*Mean survival (95% CI) months*	*P - value*
Tumor stage					0.976		0.674
T1	59	8	4	104 (99-110)		108 (104-112)	
T2	113	15	9	104 (99-109)		109 (105-113)	
T3-4	10	1	0	102 (84-120)		n/a	
Nodal stage					0.015		0.163
N0	92	6	3	111 (107-114)		113 (110-115)	
N1	57	11	6	95 (86-104)		104 (97-111)	
N2	20	3	3	101 (94-108)		103 (99-108)	
N3	13	4	1	88 (70-106)		106 (102-110)	
AJCC stage					0.319		0.308
Stage I	38	3	1	106 (101-111)		109 (106-111)	
Stage II	108	14	8	104 (98-109)		109 (105-113)	

Table 6 – Univariate analysis of recurrence-free survival and overall survival by log-rank test according to clicopathologic and immunohistochemical factors in breast cancers

Parameters	*No. of patients (n = 182) (%)*			*Recurrence-free survival*		*Overall survival*	
	No. of cases	*Tumor recurrence*	*Patient death*	*Mean survival (95% CI) months*	*P - value*	*Mean survival (95% CI) months*	*P - value*
Stage III	36	7	4	101 (92-109)		108 (105-112)	
Nuclear grade					0.855		0.200
1	12	2		94 (79-108)		n/a	
2	109	15	11	104 (99-109)		108 (104-112)	
3	61	7	2	105 (99-110)		111 (108-114)	
Histologic grade					0.541		0.535
I	29	2	1	104 (95-112)		107 (101-113)	
II	102	15	9	104 (99-109)		109 (105-113)	
III	51	7	3	103 (96-110)		109 (105-113)	
Estrogen receptor status					0.922		0.012
Positive	112	15	4	106 (101-111)		113 (111-115)	
Negative	70	9	9	102 (94-109)		104 (98-110)	
Progesterone receptor status					0.910		0.704

Table 6 – Univariate analysis of recurrence-free survival and overall survival by log-rank test according to clicopathologic and immunohistochemical factors in breast cancers

Parameters	*No. of patients (n = 182) (%)*			*Recurrence-free survival*		*Overall survival*	
	No. of cases	*Tumor recurrence*	*Patient death*	*Mean survival (95% CI) months*	*P - value*	*Mean survival (95% CI) months*	*P - value*
Positive	79	11	5	106 (101-111)		111 (108-115)	
Negative	103	13	8	104 (98-110)		109 (105-113)	
HER-2 status					0.193		0.171
Positive	43	8	5	101 (92-110)		106 (99-114)	
Negative	139	16	8	106 (102-110)		111 (109-114)	
Glut-1					0.829		0.047
0	80	11	6	104 (98-110)		110 (106-114)	
1-30	46	5	0	108 (102-114)		n/a	
> 30	56	8	7	102 (94-109)		105 (99-111)	
REDD 1					0.696		0.968
0	110	16	8	102 (97-107)		108 (105-112)	
1-30	32	3	2	108 (101-116)		111 (106-117)	
> 30	40	5	3	105 (97-114)		109 (102-116)	

Table 6 – Univariate analysis of recurrence-free survival and overall survival by log-rank test according to clicopathologic and immunohistochemical factors in breast cancers

Parameters	*No. of patients (n = 182) (%)*			*Recurrence-free survival*		*Overall survival*	
	No. of cases	*Tumor recurrence*	*Patient death*	*Mean survival (95% CI) months*	*P - value*	*Mean survival (95% CI) months*	*P - value*
HIF-1 α					0.003		0.000
0	149	19	10	105 (101-110)		111 (108-113)	
1-30	30	3	1	106 (99-114)		110 (106-115)	
> 30	3	2	2	54 (12-97)		62 (25-98)	
14-3-3σ					0.210		0.343
< 100	172	24	13	104 (100-109)		110 (107-113)	
100 - 200	10	0	0	n/a		n/a	
> 200	0	0	0	n/a		n/a	
Ki-67					0.668		0.806
< 30	170	23	12	105 (101-109)		110 (108-113)	
≥ 30	12	1	1	96 (82-110)		96 (83-109)	
PTEN					0.705		0.170
0	123	15	8	106 (101-110)		111 (108-114)	

Table 6 – Univariate analysis of recurrence-free survival and overall survival by log-rank test according to clicopathologic and immunohistochemical factors in breast cancers

Parameters	*No. of patients (n = 182) (%)*			*Recurrence-free survival*		*Overall survival*	
	No. of cases	*Tumor recurrence*	*Patient death*	*Mean survival (95% CI) months*	*P - value*	*Mean survival (95% CI) months*	*P - value*
1-30	27	7	4	90 (77-103)		98 (88-108)	
>30	32	2	1	107 (102-112)		108 (103-112)	
Phospho-Akt (Ser473)					0.329		0.572
0	147	22	12	103 (98-108)		109 (106-113)	
1-30	24	2	1	109 (104-113)		111 (108-113)	
> 30	11	0	0	n/a		n/a	
Phospho-mTOR (Ser2448)					0.625		0.481
< 100	107	14	7	104 (99-110)		110 (107-114)	
100 - 200	41	4	2	106 (100-112)		109 (104-113)	
> 200	34	6	4	103 (94-112)		108 (100-115)	
Phospho-S6 (Ser235/236)					0.189		0.449

Table 6 – Univariate analysis of recurrence-free survival and overall survival by log-rank test according to clicopathologic and immunohistochemical factors in breast cancers

Parameters	*No. of patients (n = 182) (%)*			*Recurrence-free survival*		*Overall survival*	
	No. of cases	*Tumor recurrence*	*Patient death*	*Mean survival (95% CI) months*	*P - value*	*Mean survival (95% CI) months*	*P - value*
< 100	159	20	11	106 (101-110)		110 (108-113)	
100 - 200	16	4	2	90 (74-105)		98 (86-110)	
> 200	7	0	0	n/a		n/a	
AMPK α1					0.388		0.515
(phospho T174)							
< 100	177	24	13	105 (101-109)		110 (107-113)	
100 - 200	5	0	0	n/a		n/a	
> 200	0	0	0	n/a		n/a	

n/a, not available.

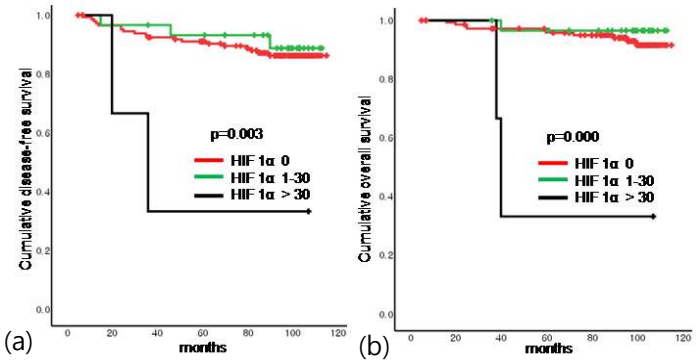

Figure 7. Disease-free survival (a) and overall survival curves (b) according to the extent of HIF-1α expression in breast cancer

IV. DISCUSSION

This study was designed to investigate the differences in the expression of REDD1-mediated mTOR pathway proteins and hypoxia-related proteins according to different breast cancer phenotypes through *in vitro* cell line and tissue microarray studies. TNBC, HER-2 type breast cancers, and papillomas showed higher expression of Glut-1, a hypoxia-regulated glucose transporter, compared to luminal A and B types ($p < 0.000$) in the tissue microarray study. This finding suggests that TNBC, HER-2 type, and papillomas were exposed to more severely hypoxic tumor environments, which was consistent with the histologic features (Figure 4). However, REDD1, which is known to be expressed in normal cells under hypoxia, was expressed less in TNBC and HER-2 types than papillomas ($p < 0.000$). REDD1 inhibits cell proliferation and survival by TSC1/TSC2 complex-mediated down regulation of the mTOR pathway through 14-3-3 σ shuttling.[15] Therefore, in spite of the severely hypoxic tumor environment, tumor cell proliferation and survival was not inhibited in TNBC and HER-2 type due to down regulation of REDD1. Moreover, the proliferation activity of TNBC and HER-2 type shown by Ki-67 immunohistochemistry was higher than that of other breast cancer phenotypes or papilloma ($p < 0.000$). A previous *in vitro*

cell line study demonstrated that loss of REDD1 signaling caused by REDD1 mutation promoted cell proliferation and anchorage-independent growth under hypoxic condition through mTOR dysregulation, and an *in vivo* mouse study showed that loss of REDD1 caused tumorigenesis.[15] In particular, 30% of human breast cancers were reported to show down regulation of REDD1, but the status of REDD1 expression has not been investigated according to breast cancer phenotypes.[15] In this study, TNBC and HER-2 type breast cancers with lower expression of REDD1 under hypoxia occupied 40% of breast cancers, which is a similar proportion to that of human breast cancer with REDD1 down-regulation in a previous study.[15] This study classified breast cancer phenotypes according to ER, PR and HER-2 status by using IHC and FISH methods. We believe that TNBC and HER-2 type in this study usually corresponded to basal-like type, HER-2 type, and normal breast-like type in gene profiling study. The incidence of basal-like type, HER-2 type, and normal breast-like type in gene profiling study was 18%, 14%, and 7.7%, respectively.[2] Therefore, the incidence of these three breast cancer phenotypes (39.7%) was similar to that of TNBC and HER-2 type in this study (40%). In our *in vitro* cell line study, REDD1 expression, which was absent under normoxic conditions regardless of the breast cancer phenotype, was increased in mild hypoxia along with a decrease in phospho-S6 expression and suppression of the mTOR pathway, and then decreased again with increasing levels of hypoxia. This implies that REDD1 expression is somehow induced under mildly hypoxic conditions in breast cancer with suppression of the mTOR pathway, but with severe hypoxia, REDD1 expression is lost and therefore the mTOR pathway is not suppressed. These findings were validated by immunohistochemical analysis of Glut-1 and Ki-67 expression in a tissue microarray study. The tissue microarray study showed in luminal A and luminal B breast cancers, the expression of REDD1 is low because the tumor microenvironment is not hypoxic, while in TNBC and HER-2 overexpression type breast cancers the loss of REDD1 expression helps the tumor cells to proliferate and survive despite the severely hypoxic tumor microenvironment.

REDD1 which serves as a tumor suppressor is regulated by HIF-1α or AMPK on

transcription level, but, the functioning mechanism between HIF-1α and AMPK is different. HIF-1α causes REDD1 expression through activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway under acute hypoxia and high cell density.[23] However, AMPK is activated and causes REDD1 expression, independent of HIF-1α when ATP depletion occurs due to chronic hypoxia.[18] Post-translational regulation of REDD1 is manipulated by CUL4A (Cullin 4A)-DDB1 (DNA damage-binding protein 1)-ROC1 (regulator of cullins 1) ubiquitin ligase, which rapidly degrades REDD1 during the cellular recovery from hypoxic stress, resulting in restoration of the mTOR signaling pathway. [24] Because so many components are engaged in the regulation of REDD1, the exact mechanism of REDD1 down regulation in TNBC and HER-2 could not be determined in this study. However, in the *in vitro* cell line study, REDD1 expression was only seen when HIF-1α was expressed (Figure 2) and REDD1 expression was increased when HIF-1α expression was increased (Figure 3) in SK-BR3, MCF7 and BT474. Whereas in MDA-MB-231, which corresponds to TNBC, REDD1 expression was decreased and HIF-1α expression was increased under severely hypoxic conditions (Figure 2). In addition, when AMPK was inhibited by the siRNA method, HIF-1α expression was lost under hypoxic conditions in MDA-MB-231, suggesting that other regulatory mechanisms between REDD1, HIF-1α and AMPK may exist. Moreover, a tissue microarrary study showed that the expression rate of AMPK α1 was not different among different breast cancer phenotypes, but the expression of HIF-1α was higher in TNBC and HER-2 type ($p < 0.000$). REDD1 is induced by HIF-1α, and induced REDD1 regulates HIF-1α with negative feedback control through ubiquitin-proteasome-mediated degradation by reactive oxygen species (ROS)-dependent mechanism in mitochondria.[25] Therefore, when REDD1 is down-regulated, the expression of HIF-1α is enhanced as there is no negative feedback for HIF-1α, which in turn promotes tumorigenesis. This negative feedback mechanism can explain the phenomenon that the expression of HIF-1α increased but that of REDD1 decreased as the hypoxic condition is severe in MDA-MB-231 cell line corresponding to TNBC. In particular, our study demonstrated significant correlation between the expression of

HIF-1α and poor DFS (p = 0.003) and OS (p = 0.000). This correlation was concurrent with previous studies which reported that HIF-1α expression was associated with reduced DFS, reduced OS, high-grade tumor, ER loss, high Ki-67 expression, and HER-2 gene amplification.[26-28]

Interestingly, benign papilloma also demonstrated higher expression of HIF-1α than luminal A and B type (p < 0.000), but the HIF-1α expression pattern of benign papilloma was different from that of TNBC and HER-2 type. While papilloma mostly showed HIF-1α expression in nuclei, TNBC and HER-2 type demonstrated HIF-1α expression in nuclei and cytoplasm. In TNBC and HER-2 type, expressed HIF-1α protein by hypoxia was not degraded by ubiquitin-proteasome system due to REDD1 down regulation,[25] resulting in HIF-1α stabilization and expression in cytoplasm and nuclei. On the other hand, in papilloma, HIF-1α protein induced by acute hypoxia was degraded by ubiquitin-proteasome due to normally expressed REDD1, resulting in HIF-1α protein expression not in cytoplasm, but only in nuclei. The expression rate of HIF-1α was higher in HER-2 type than TNBC (p = 0.037). Because the expression of HIF-1α protein is enhanced by the HER-2 signaling pathway in human breast cancer,[29] eventually, in HER-2 type, the expression of HIF-1α protein is induced by not only enhanced intratumoral hypoxia, but also HER-2 signalling activation, which is also compatible with this study.

V. CONCLUSION

Through this *in vitro* human breast cancer cell line study, we could ascertain that REDD1 is not expressed in normoxic condition irrespective of cell line phenotype, that REDD1 starts to be expressed in mild hypoxic condition, and finally that the expression of REDD1 is lost in severe hypoxic condition. In the tissue microarray study, TNBC and HER-2 type were shown to be more severely hypoxic tumor microenvironment in comparison to luminal A and B types, and the expression of REDD1 was decreased in TNBC and HER-2 types in spite of the severely hypoxic tumor microenvironment. These results are in accord with the results from the *in vitro* human breast cancer cell line study, implicating an important role of REDD1

expression loss in the tumor cell proliferation and survival despite hypoxic condition. In addition, when the fact that the expression level of HIF-1α, one of the hypoxia-related molecules, is increased as the hypoxic status is severe in MDA-MB-231, which corresponds to TNBC in the *in vitro* breast cancer cell line study, the expression level of HIF-1α is higher in TNBC and HER-2 type in human tissue microarray study, and finally the fact that HIF-1α is an independent prognosis factor are altogether considered. From the results discussed so far it could be assumed that HIF-1α will function as an important hypoxia-related protein in high grade breast cancer but further research will be necessary.

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ABSTRACT(IN KOREAN)

인체 유방암에서 REDD1 매개 mTOR 경로 단백질과 저산소 관련 단백질의 발현

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유방암은 유전자 분석에 따라 normal breast-like, luminal A, luminal B, HER2 overexpression, 그리고 basal-like [triple negative breast cancer (TNBC)] 아형으로 분류할 수 있다. 이들 중에 HER2 overexpression 과 TNBC 는 조직학적으로 분화가 나빠 조직등급과 증식능이 높으며, 지역적인 종양세포의 괴사를 보여, 종양의 미세환경이 저산소증에 노출되어 있을 것으로 추측할 수 있다. 세포가 저산소 환경에 노출되면 REDD1 (regulated in development and DNA damage response 1)이 매개하는 mammalian target of rapamycin (mTOR) 경로가 세포의 성장과 생존에 관여한다. 이번 연구에서는 *in vitro* 유방암 세포주 연구와 인체 조직 microarrary 연구를 통해서 유방암에서 REDD1 매개 mTOR 경로 단백질과 저산소증과 연관된 단백질의 발현을 알아보고자 하였다. *In vitro* 유방암 세포주 연구에서는 유방암의 분자 아형에 따라 다음과 같이 4 가지 세포주를 선택하였다; MDA-MB-231(TNBC), SKBR3 (HER-2), MCF-7 (luminal A) and BT474 (luminal B). 저산소 환경은 100, 200, 300, 그리고 400 μM 농도의 $CoCl_2$ 를 이용해 만들었으며, western blot 분석과 siRNA 를 이용한 REDD1, AMPK 발현 억제를 통해서 S6K1, phospho-S6K1, AMPK, REDD 1, 그리고 HIF-1α 의 발현을 조사하였다.

인체 조직 microarrary 연구에서는 224 명의 침윤성 유방암과 대조군으로 30 예의 유두종을 대상으로 estrogen receptor (ER), progesterone receptor (PR), HER-2, epithelial growth factor receptor (EGFR), cytokeratin (CK) 5/6, Glut-1, HIF-1α, REDD 1, AMPK α 1, 14-3-3 σ, PTEN, phospho-Akt, phospho-mTOR, phospho-S6, 그리고 Ki-67 에 대한 면역조직화학염색을 시행하였다. ER, PR, 그리고 HER-2 에 대한 면역조직화학염색 결과에 따라 유방암을 luminal A, luminal B, HER-2 overexpression, 그리고 TNBC 로 분류하였다. *In vitro* 세포주 연구에서는 HIF-1α 과 REDD1 의 발현이 저산소 환경에서 모든 세포주에서 유도되었으나, 심한 저산소 환경에서는 4 개의 세포주 모두에서 REDD1 의 발현이 감소되거나 사라졌다. MDA-MB-231 세포주에서는 저산소 환경이 심할수록 HIF-1α 의 발현이 증가되었다. siRNA 를 이용한 REDD 1 과 AMPK 발현억제 연구에서는 MCF-7 과 MDA-MB-231 세포주에서 저산소 환경에서 REDD 1 과 AMPK 의 발현억제를 하지 않은 경우에 비하여 phospho-S6K1 의 발현이 증가하였다. 인체 조직 microarray 연구에서는 Glut-1 과 HIF-1α 의 발현이 luminal A 와 luminal B 에 비해 TNBC, HER-2 overexpression, 유두종에서 더 높았다 ($p<0.000$). 그러나 REDD 1 의 발현은 유두종에서 가장 높았으며 ($p<0.000$), 유방암 분자 아형 사이에서는 차이가 없었다 ($p=0.307$). HIF-1α 는 다변수 Cox 회귀분석에서 낮은 무병생존율과 전체 생존율과 연관이 있었다 (무병생존율; p=0.018, odd ratio=7.6, 전체생존율; p=0.002, odd ratio=13.7). 결론적으로, TNBC 와 HER-2 overexpression 아형에서 REDD1 발현의 상실은 심한 저산소 환경하에서 종양세포의 증식과 생존을 유지하는데 중요한 역할을 할 것으로 보이며, HIF-1α 는 유방암, 특히 고등급의 유방암에서 중요한 저산소 연관 단백질일 것으로 여겨진다.

핵심되는 말: 유방암, 저산소증, REDD1, HIF-1α, mTOR