Differences in expression of metabolism-related markers between cancer cells and stromal cells according to the molecular subtypes of breast cancer

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Differences in expression of metabolism-related markers between cancer cells and stromal cells according to the molecular subtypes of breast cancer

Directed by Professor Ja Seung Koo

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This certifies that the Doctorial Dissertation of Min Ju Kim is approved.

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June, 2015

Min Ju Kim

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ABSTRACT

Differences in expression of metabolism-related markers between cancer cells and stromal cells according to the molecular subtypes of breast cancer

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Alteration of energy metabolism of cancer cells is described by Warburg effect, a phenomenon that tumor cells obtain energy by glycolysis rather than by oxidative phosphorylation. The 'reverse Warburg effect' that human breast cancer cells instruct the neighboring stromal fibroblasts to provide energy by aerobic glycolysis is suggested. Breast cancer shows great heterogeneity in tumor and stromal morphology, and it is categorized into molecular subtypes identified by analyzing the gene expresseion profile; luminal A, luminal B, HER-2 type, and basal-like type. The aim of this study is to evaluate the difference of metabolic interaction between cancer cells and stromal cells according to the molecular subtype of breast cancer by investigating the markers related to glycolysis, mitochondrial status, and autophagy status, and to analyze the relationship between the expression of these metabolic markers and clinic-pathological parameters.

Cell culture of six types of human breast cancer cell lines (MCF-7, MDA-MB-361, MDA-MB-453, MDA-MB-435S, MDA-MB-231 & MDA-MB-486), co-culture with fibroblasts with inhibition study by siRNA and Western blot analysis for metabolic markers (glycolysis; Glut-1, CAIX, mitochondrial dysfunction; GC1qR, BNIP3, and autophagy; beclin1, LC3A, LC3B) were performed. Tissue microarray from 740 cases of breast cancer samples which underwent mastectomy due to invasive breast cancer from 2002 to 2005 were constructed for immunohistochemical and FISH studies of markers related to molecular classification (ER, PR, HER2, Ki67) and metabolism-related markers (glycolysis; Glut-1, CAIX, MCT4, mitochondrial dysfunction; BNIP3, and autophagy; beclin1, LC3A, LC3B, p62), followed by statistical analysis. Breast cancer phenotypes were classified as *luminal A*

type (ER or/and PR positive and HER-2 negative and Ki-67 LI <14%), *Luminal B type [HER-2 negative]* (ER or/and PR positive and HER-2 negative and Ki-67 LI ≥14%), *Luminal B type [HER-2 positive]* (ER or/and PR positive and HER-2 overexpressed or/and amplified), *HER-2 type* (ER and PR negative and HER-2 overexpressed or/and amplified), *TNBC type* (ER, PR, and HER-2 negative). The metabolic subtypes were defined as *Warburg type* (tumor: glycolysis type, stroma: non-glycolysis type), *reverse Warburg type* (tumor: non-glycolysis type, stroma: glycolysis type), *mixed type* (tumor: glycolysis type, stroma: glycolysis type), *null type* (tumor: non-glycolysis type, stroma: non-glycolysis type).

In cell line study, the expression levels of metabolic markers [autophagy-related markers (beclin-1, LC3A, LC3B), mitophagy marker (BNIP3), and glycolysis-related markers (CAIX, GLUT-1)] were higher in stromal cells than in tumor cells in MCF-7, whereas tumor cells show higher expression levels of metabolic markers [autophagy-related markers (beclin-1, LC3B), mitophagy marker (GC1qR, BNIP3), and glycolysis-related markers (CAIX, GLUT-1)] than stromal cells in HER2 type and TNBC type. GLUT-1 and LC3B inhibition studies showed reduction in the cancer cell proliferation rate; in luminal type, the reduction rate of cancer cell proliferation was greater in stromal inhibition than in tumoral inhibition, in TNBC type, it was greater in tumoral inhibition than in stromal inhibition.

Human breast cancer tissues were classified into 298 (40.3%) cases of luminal A type, 166 (22.4%) cases of luminal B type, 69 (9.3%) cases of HER-2 type, and 207 (28.0%) cases of TNBC type. The clinicopathologic features and the expression levels of metabolism-related proteins are different according to these phenotypes. Tissues were composed of 298 Warburg type (40.3%), 54 reverse Warburg type (7.3%), 62 mixed type (8.4%), and 326 null type (44.0%). TNBC consisted dominant portion of Warburg and mixed types, and luminal A constituted mainly of reverse Warburg and null types ($P < 0.001$). The mixed type had a higher histologic grade, higher rate of ER negativity, higher rate of PR negativity, higher Ki-67 index, higher rate of activated tumor autophagy status, whereas the null type showed lower histologic grade, higher rate of ER positivity, higher rate of PR positivity, lower Ki-67 index and higher rate of non- activated tumor autophagy status ($P \leq 0.001$).

Breast cancer is heterogeneous in its metabolic status and the expression levels of metabolism-related markers are different according to molecular subtypes of breast cancer. The metabolic phenotypes of breast cancer have correlations with molecular subtypes along with biology of breast cancer.

Key word: breast cancer, molecular subtype, metabolism, stroma

Differences in expression of metabolism-related markers between cancer cells and stromal cells according to the molecular subtypes of breast cancer

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I. INTRODUCTION

Alteration of energy metabolism of cancer cells is described by Warburg effect, a phenomenon that tumor cells obtain energy by glycolysis rather than by mitochondrial oxidative phosphorylation (OXPHOS).[1] Breast cancer is a group of heterogeneous diseases displaying diverse tumor and stromal morphology and active tumor-stromal interactions may facilitate tumor growth and progression. Complex interaction in the metabolic processes may also exist, and a unique tumor-stromal metabolic interaction of breast cancer, known as reverse Warburg effect theory is proposed.[2-5] Shortly, human breast cancer cells instruct the neighboring stromal fibroblasts to provide energy by aerobic glycolysis. Cancer cells induce oxidative stress by generating reactive oxygen species such as nitric oxide, leading to activation of HIF-1a and NFkB in stromal fibroblasts, resulting in glycolysis, autophagy (mitophagy) and mitochondrial dysfunction. The glycolysis metabolites such as ketones and lactate are transferred to tumor cells and incorporated into OXPHOS to produce ATP, contributing to the tumor growth and progression.[2-5] Cancer associated fibroblasts (CAFs) that are tumor-supporting stromal fibroblasts showing a loss of caveolin-1 expression via increased autophagic degradation have been implicated in this interaction.[3-7] Proteins involved in glycolysis, mitochondrial function and autophagy status may be expressed differently in cancer cells and stromal cells according to the both

theories. Table 1 shows these differences in Warburg effect theory and reverse Warburg effect theory.

Table 1. Comparison of metabolism type, mitochondrial status, and autophagy status between Warburg effect theory and reverse Warburg effect theory

	Warburg effect theory	Reverse Warburg effect theory	
Cell compartment	Cancer cell	Cancer cell	Stromal cell
Metabolism	Glycolysis	OXPHOS	Glycolysis
Mitochondrial status	Dysfunctional	Functional	Dysfunctional
Autophagy status	Not included	Not activated	Activated

OXPHOS: oxidative phosphorylation.

Breast cancer is known to have significant clinical, histological and molecular genetic heterogeneity. Identification of breast cancer subtypes by analysing the gene expression profiles was performed and it categorized breast cancer into five molecular subtypes; luminal A, luminal B, HER-2, normal breast-like, basal-like type.[8,9] Aside from these five subtypes, triple-negative breast cancer (TNBC) refers to any breast cancer showing negativity for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2).[10] TNBC accounts for 10~15% of all breast cancers and tends to have a poor prognosis because of no known effective targeted therapies such as HER2-targeted therapies or hormonal therapies. Distinct histologic features, and differences in clinical parameters such as treatment response and survival were established according to these subtypes. Hence the metabolic interaction between cancer cells and stromal cells can be different according to these subtypes. The purpose of this study is to evaluate the difference of metabolic interaction between cancer cells and stromal cells according to the molecular subtype of breast cancer by investigating the markers on glycolysis [glucose transporter 1 (Glut-1),[11,12] carbonic anhydrase IX (CAIX),[12] monocarboxylate transporter 4 (MCT4)[13,14]], mitochondrial dysfunction [BCL2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3),[15,16] p32 (gC1q receptor)[17]], and autophagy [beclin1,[18] light chain 3α (LC3A),[19,20] light chain 3β (LC3B),[19,20] p62[21,22]] in cancer cells and stromal cells and to analyze the relationship between the expression of the metabolic markers and clinic-pathological parameters.

II. MATERIALS AND METHODS

1. In vitro cell line study

A. Cell culture

Five breast cancer cell lines, MCF-7, MDA-MB-453, MDA-MB-435S, MDA-MB-231 and MDA-MB-468 (all from the American Type Culture Collection) (ATCC, Manassas, VA, USA), were examined. For separating cancer cells and fibroblasts after co-culture, we established green fluorescent protein (GFP) stable cancer cell lines with them. GFP stable cancer cell lines were built by transducing pGIPZ non-silencing control lentiviral particles (GE healthcare life-Sciences, Buckinghamshire, UK) and were selected by puromycin. MCF7-GFP was maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM/ F12; Gibco, Rockville, MD, USA) without Phenol Red, but supplemented with 10μg/mL insulin (Sigma, St. Louis, MO, USA), 10% Fetal bovine serum (Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin (Gibco, Rockville, MD, USA). The other cells were maintained in DMEM/F12 (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum and 1% penicillin/streptomycin. Co-plated cells were fixed, stained anti-vimentin antibody for detecting fibroblasts, and analyzed by confocal microscopy (Figure 1).

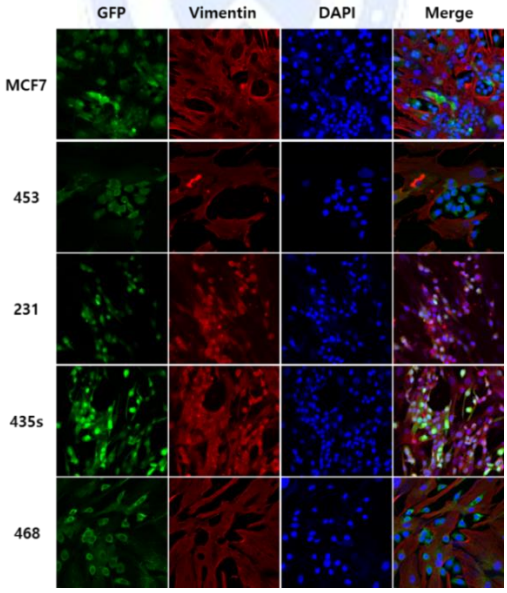


Figure 1. Confocal microscopy images of breast cancer cell lines. The first column shows green fluorescent protein (GFP) signal of cancer cells, the second column shows fibroblasts stained with vimentin (red), the third column shows cancer cell nuclei stained with 4',6-diamidino-2-phenylindole (DAPI) (blue), and the fourth colume the merged picture.

B. Isolation of primary fibroblasts

Human breast tumor specimens were obtained from patients through. Fresh tissues were cut or chopped into small pieces, placed in culture dish with digestion solution of enzyme cocktail (ISU ABXIS, Seoul, Korea) and incubated at 37℃ incubator overnight. Digested tissue was filtered through a 70 ㎛ cell strainer. The cells were suspended in medium: Ficoll (3:2) and separated by differential centrifugation at 90g for 2 minutes at room temperature. The supernatant containing fibroblasts was centrifuged at 485g for 8 minutes, resuspended in DMEM/F12 supplemented with 10% fetal bovine serum (Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin (Gibco, Rockville, MD, USA). They were cultured at 37℃ in a humidified atmosphere with 5% CO_2. The isolated fibroblasts were confirmed by immunofluorescence analysis of vimentin expression.

C. Co-cultures of breast cancer cells and fibroblasts

Fibroblasts and GFP (+) breast cancer cells were co-plated on 100cm culture dish. Briefly, GFP expressing cancer cells were seeded within 2 hours of fibroblast plating. The total seeding cell number was 2.2 x 10^5 per well. Experiments were performed at 5:1 fibroblast to cancer cell ratio. Mono-cultured fibroblasts and cancer cells were used as controls. The day after, media was changed to 1% DMEM/F12 with 1% FBS and cells were grown for additional days. All cells were cultured at 37℃ in a humidified atmosphere with 5% CO_2.

D. Inhibition study

(a) GLUT1 knockdown

Cells were seeded 24h prior transfection with siRNA to reach approximately 50% confluence. For GLUT1 knockdown, 20nM of IBONI siRNA and riboxx FECT (riboxx GmbH, Germany) were used as recommended by manufacturer instruction. GLUT1 knockdown was tested 48-72 h after transfection. To evaluate proliferation, GLUT1 siRNA transfected cells were mono- and co-cultured.

(b) LC3B knockdown

Cells were seeded 24h prior transfection with siRNA to reach approximately 50% confluence. For LC3B knockdown, 20nM of IBONI siRNA and riboxx FECT (riboxx GmbH, Germany) were used as

recommended by manufacturer instruction. LC3B knockdown was tested 48-72 h after transfection. To evaluate proliferation, LC3B siRNA transfected cells were mono- and co-cultured.

E. Immunocytochemistry

Cells grown on glass coverslips in 12-well plates were washed with PBS, fixed in 4% paraformaldehyde diluted in PBS for 10 minutes at room temperature, and permeabilized with 0.2% Triton X-100 in PBS for 10 minutes at room temperature. Then the cells were rinsed with PBS 3 times and blocked with 10% donkey serum (Jackson Immunoresearch Laboratories Inc., Baltimore, PA, USA) for 1 hour at room temperature. 1:100 diluted primary antibodies (vimentin) were incubated in PBS overnight at 4℃. After washing with PBS, cells were incubated for 1 hour with fluorochrome–conjugated secondary antibodies diluted in PBS. Finally, cells were washed with PBS, incubated with DAPI and mounted.

F. Cell proliferation assay

Mono-cultured cells and co-cultured GFP (+) breast cancer cells were incubated with bromodeoxyuridine (BrdU) for 1 hour and then cells were sorted by FACs with a 488nm laser. Cells were washed in PBS, fixed in cold 70% ethanol and flow cytometry was used for analysis of DNA synthesis (BrdU incorporation).

G. Flow cytometry

After co-culture of GFP (+) breast cancer cells and fibroblasts, to separate each cell lines, GFP expressing co-cultured cells were sorted by FACS Calibur Flow Cytometer (Becton Dickinson, San Jose, CA) using a 488nm laser. Mono cultured GFP (+) breast cancer cells were used as a control.

H. Western immunoblotting

Mono-cultured and sorted co-cultured cells were washed twice with PBS and lysed with lysis buffer (50 mM Tris-HCL (pH 7.9), 100 mM NaCl, 1mM EDTA, 2% SDS, 0.1mM EDTA, 0.1mM EGTA, 0.1M and protease and phosphatase inhibitor cocktail) (Thermo scientific Inc., Bremen, Germany). Total protein (20 μg) treated with Laemmli sample buffer, heated at 100 ℃ for five minutes. Then it was loaded into each well and was resolved by 8% - 12% SDS-polyacrylamide gel electrophoresis

(PAGE) and electroblotted onto nitrocellulose membranes (GE Healthcare life-Sciences, Buckinghamshire, UK). Membranes were blocked in 5% non-fat dry milk in TBS-T for 1 hour at RT, and incubated with antibodies as described in table 2 overnight at 4℃, and then probed with peroxidase-conjugated goat anti-mouse IgG (1:2000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 hour at room temperature. The washes were repeated and the membrane was developed with enhanced chemiluminescent agent (ECL) (Amersham Life Science, Inc., Amersham Pharmacia Biotech, Buckinghamshire, UK). Band densities were measured using Image J software (National Institutes of Health, Bethesda, MD, USA).

Table 2. The antibodies used for western blotting

	Antibody	dilution	company
Autophagy	BECN1	1:5000	Abcam, Cambridge, UK
	LC3A	1:2000	Abcam, Cambridge, UK
	LC3B	1:2000	Abcam, Cambridge, UK
Mitochondrial status	p32 (GC1qR)	1:2000	Abcam, Cambridge, UK
	BNIP3	1:1000	Abcam, Cambridge, UK
Glycolysis	CAIX	1:1000	Abcam, Cambridge, UK
	GLUT1	1:500	Abcam, Cambridge, UK
	B-actin	1:5000	Sigma, St. Louis, MO, USA

BECN1: Beclin-1, LC3A: light chain 3α, LC3B: light chain 3β, GC1qR: gC1q receptor (p32), BNIP3: BCL2/adenovirus E1B 19-kDa interacting protein 3, CAIX: carbonic anhydrase IX, GLUT1: glucose transporter 1.

I. Statistical analyses of data

Data are presented as mean ± standard deviation (S.D.) from at least three independent experiments. Significant differences between groups were determined by Student's t-tests. Values of $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ were considered statistically significant.

2. Human breast cancer tissue microarray study

A. Patient selection

Patients who were diagnosed with invasive breast cancer and underwent surgical excision at

Severance Hospital from January 2002 to December 2005 were selected in this study. Patients who received preoperative hormonal therapy or neoadjuvant chemotherapy were excluded. This study was approved by the Institutional Review Board of Yonsei University Severance Hospital. Formalin-fixed and paraffin-embedded tissue specimens from 740 cases of primary breast cancer were included. All archival hematoxylin and eosin (H&E) stained slides for each case were reviewed retrospectively by 2 pathologists (Kim MJ, and Koo JS). The histological grade was assessed using the Nottingham grading system.[23] Clinicopathologic parameters evaluated in each breast cancer included patient age at initial diagnosis, lymph node metastasis, tumor recurrence, distant metastasis, and patient survival.

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. Tissue of invasive tumor was extracted. More than 2 tissue cores were extracted to minimize extraction bias. Each tissue core was assigned with a unique tissue microarray (TMA) location number that was linked to a database containing other clinicopathologic data.

C. Immunohistochemistry

The antibodies used for immunohistochemistry (IHC) in this study are shown in table 3. Five micrometer thick sections were obtained with a microtome, transferred into adhesive slides, and dried at 62℃ for 30 minutes. 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. The primary antibody incubation step was omitted in the negative control. Slides were counterstained with Harris hematoxylin.

Table 3. Source, clone, and dilution of used antibodies

antibody	clone	Dilution	company
Molecular subtype related			
ER	SP1	1:100	Thermo Scientific, San Diego, CA, USA
PR	PgR	1:50	DAKO, Glostrup, Denmark
HER-2	Polyclonal	1:1,500	DAKO, Glostrup, Denmark
Ki-67	MIB-1	1:150	DAKO, Glostrup, Denmark
Glycolysis related			
Glut-1	SPM498	1:200	Abcam, Cambridge, UK
CAIX	Polyclonal	1:100	Abcam, Cambridge, UK
MCT4	Polyclonal	1:100	Santa Cruz Biotechnology, Santa cruz, CA, USA
Mitochondrial status related			
BNIP3	Ana40	1:100	Abcam, Cambridge, UK
Autophagy related			
Beclin-1	Polyclonal	1:100	Abcam, Cambridge, UK
LC3A	EP1528Y	1:100	Abcam, Cambridge, UK
LC3B	Polyclonal	1:100	Abcam, Cambridge, UK
p62	SQSTM1	1:100	Abcam, Cambridge, UK

ER: estrogen receptor, PR: progesterone receptor, HER2: human epidermal growth factor receptor 2, Glut-1: glucose transporter 1, CAIX: carbonic anhydrase IX, MCT4: monocarboxylate transporter 4, BNIP3: BCL2/adenovirus E1B 19-kDa interacting protein 3, LC3A: light chain 3α, LC3B: light chain 3β.

D. Interpretation of immunohistochemical staining

All immunohistochemical markers were accessed by light microscopy. Pathologic parameters such as ER, PR, and HER-2 status were obtained from patients' pathologic report. A cut-off value of 1% or more positively stained nuclei was used to define ER and PR positivity.[24] HER-2 staining was analyzed according to the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines 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.[25] 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).

Glut-1, CAIX, MCT4, BNIP3, Beclin-1, LC3A, LC3B, and p62 immunohistochemical staining results were evaluated based on the proportion of stained cells and the immunostaining intensity. The proportion of stained cells was graded from 0 through 2 (0, negative; 1, positive in less than 30 %; and 2, positive in more than 30 % of tumor cells). Immunostaining intensity was graded from 0 through 3 (0, negative; 1, weak; 2, moderate; and 3, strong). The scores for the proportion of stained cells and the staining intensity were multiplied to provide a total score: negative (0–1) or positive (2–6). The Ki-67 labeling index (LI) was defined as the percentage of tumor cells exhibiting nuclear staining versus the total number of tumor cells.

E. Fluorescence *in situ* hybridization analysis

Before FISH analysis, invasive tumors were examined on H&E-stained slides. FISH was subsequently performed on the tested tumor. FISH was performed using a PathVysion *HER-2* DNA Probe Kit (Vysis, Downers Grove, IL, USA) according to the manufacturer's instructions. *HER-2* gene copy number on the slides was evaluated using an epifluorescence microscope (Olympus, Tokyo, Japan). At least 60 tumor cell nuclei in three separate regions were investigated for *HER-2* and chromosome 17 signals. *HER-2* gene amplification was determined according to the ASCO/CAP guidelines.[25] An absolute *HER-2* gene copy number lower than 4 or a *HER-2* gene/chromosome 17 copy number ratio (HER-2/Chr17 ratio) less than 1.8 was considered *HER-2* negative. An absolute *HER-2* copy number between 4 and 6 or a HER-2/Chr17 ratio between 1.8 and 2.2 was considered *HER-2* equivocal. An absolute *HER-2* copy number greater than 6 or a HER-2/Chr17 ratio higher than 2.2 was considered *HER-2* positive.

F. Classification of tumor phenotypes

In this study, we classified breast cancer phenotypes according to the IHC results for ER, PR, HER-2 and Ki-67 and FISH results for HER-2 as follows;[26] *luminal A type*: ER or/and PR positive and HER-

2 negative and Ki-67 LI <14%, *Luminal B type*: (HER-2 negative) ER or/and PR positive and HER-2 negative and Ki-67 LI ≥14% , (HER-2 positive) ER or/and PR positive and HER-2 overexpressed or/and amplified, *HER-2 type*: ER and PR negative and HER-2 overexpressed or/and amplified, *TNBC type*: ER, PR, and HER-2 negative.

G. Classification of tumor metabolic subtypes

Breast cancers in this study were categorized into the following categories according to the immunohistochemical staining results of metabolism-related proteins: glycolysis type: positive for Glut1 and/or CAIX; non-glycolysis type: negative for Glut1 and CAIX; dysfunctional mitochondrial status: positive for BNIP; functional mitochondrial status: negative for BNIP3; activated autophagy status: positive for two or more markers among beclin-1, LC3A, LC3B, and p62; and non-activated autophagy status: positive for less than two among beclin-1, LC3A, LC3B, and p62.

Metabolic subtypes were defined as follows: Warburg type, when the tumor exhibited a glycolysis signature while the stroma did not; reverse Warburg type, when the tumor exhibited a non-glycolysis signature while the stroma exhibited a glycolysis signature; mixed type, when both the tumor and stroma exhibited a glycolysis signature; and null type, when neither the tumor nor stroma exhibited a glycolysis signature.

H. 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 metastasis and time to survival. Multivariate regression analysis was performed using Cox proportional hazards model.

III. RESULTS

1. In vitro cell line study

A. Comparison of expression of metabolism-related proteins in breast cancer cell lines and primary fibroblasts after co-culture

Western blotting of metabolism-related proteins in five of human breast cancer cells and primary

fibroblast is presented in figure 2. The density of each protein was calculated relative to β-actin and assessed in relation to the molecular subtypes of the tested cell lines: MCF7, luminal type; MDA-MB-453, HER-2 type; and MDA-MB-435S, MDA-MB-231, and MDA-MB-468, TNBC type. We confirmed that these proteins were expressed in cell lines of the luminal A and B, HER-2, and TNBC types.

As seen in figure 2, only p32 expression of MCF7 is higher than fibroblast and LC3A expression of MDA-MB-453 is less than fibroblasts. The expression levels of autophagy-related markers (beclin-1, LC3A, LC3B), mitophagy marker (BNIP3), and glycolysis-related markers (CAIX, GLUT-1) were higher in fibroblasts than tumor cells in co-cultured MCF-7 (luminal type), whereas the expression levels of autophagy-related markers (beclin-1, LC3B), mitophagy marker (GC1qR, BNIP3), and glycolysis-related markers (CAIX, GLUT-1) were higher in tumor cells than fibroblasts in co-cultured MDA-MB 453 (HER2 type), MDA-MB-435S (TNBC type) and MDA-MB-486 (TNBC type).

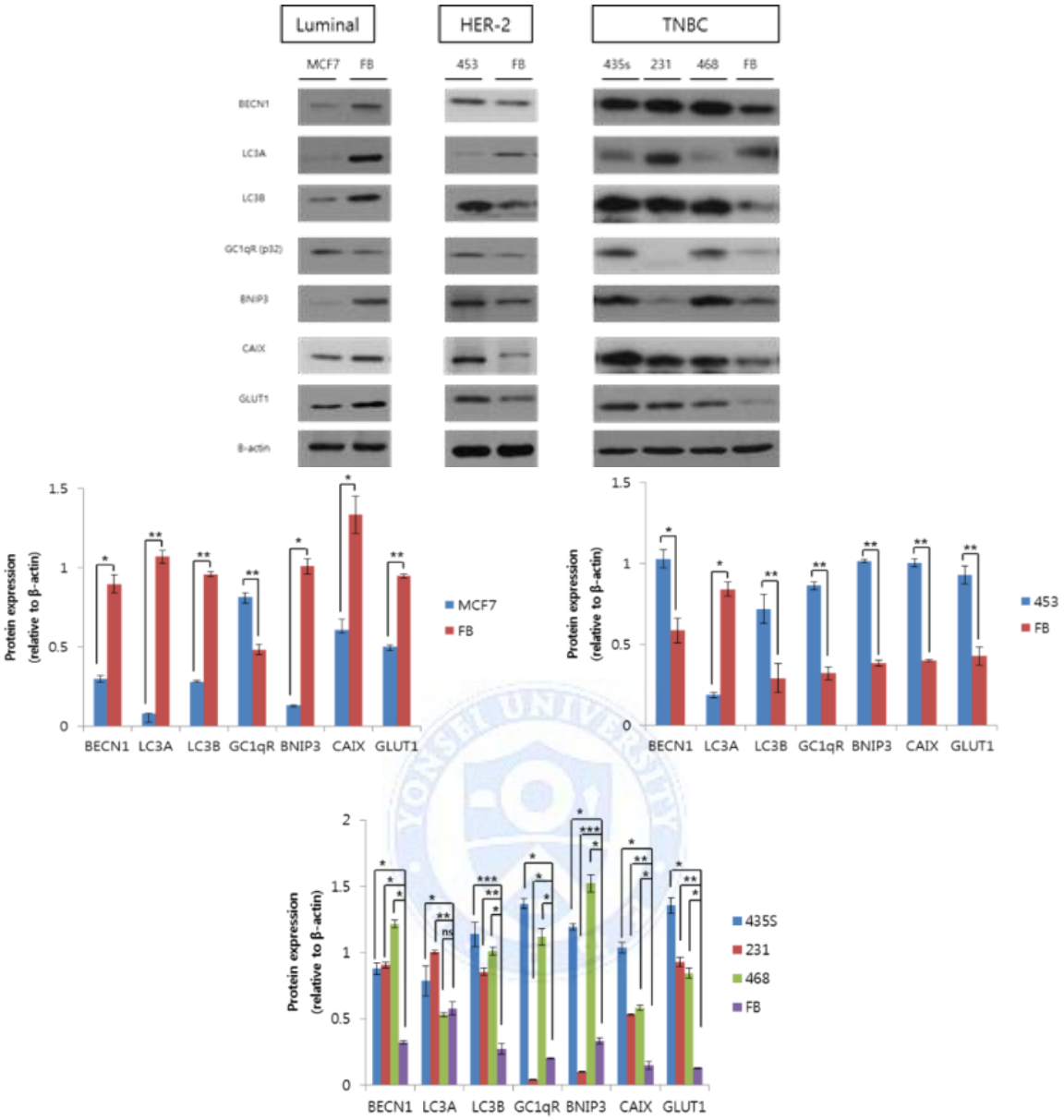


Figure 2. Expression of metabolism-related proteins in six human breast cancer cell lines and primary fibroblast. Only GC1qR (p32) expression of MCF7 is higher than fibroblast and LC3A expression of MDA-MB-453 is less than fibroblasts. BECN1, LC3B, CAIX and GLUT1 were expressed to greater extent in TNBC type.

B. GLUT1-knockdowned cells regulate proliferation of direct co-cultured cells

Directly co-cultured cells with GLUT1-knockdowned cells were affected on proliferation levels. In co-cultured cells with GLUT1 siRNA transfected fibroblasts, the extent of reduction of cancer cell proliferation was highest in MCF-7 (luminal type) than other types. In co-cultured GLUT1 siRNA

transfected cancer cells with fibroblasts, the extent of reduction of cancer cell proliferation was higher in MDA-MB 453 (HER2 type), MDA-MB 435S (TNBC type), MDA-MB-231 (TNBC type), and MDA-MB-468 (TNBC type) than in MCF-7 (luminal type) (Figure 3).

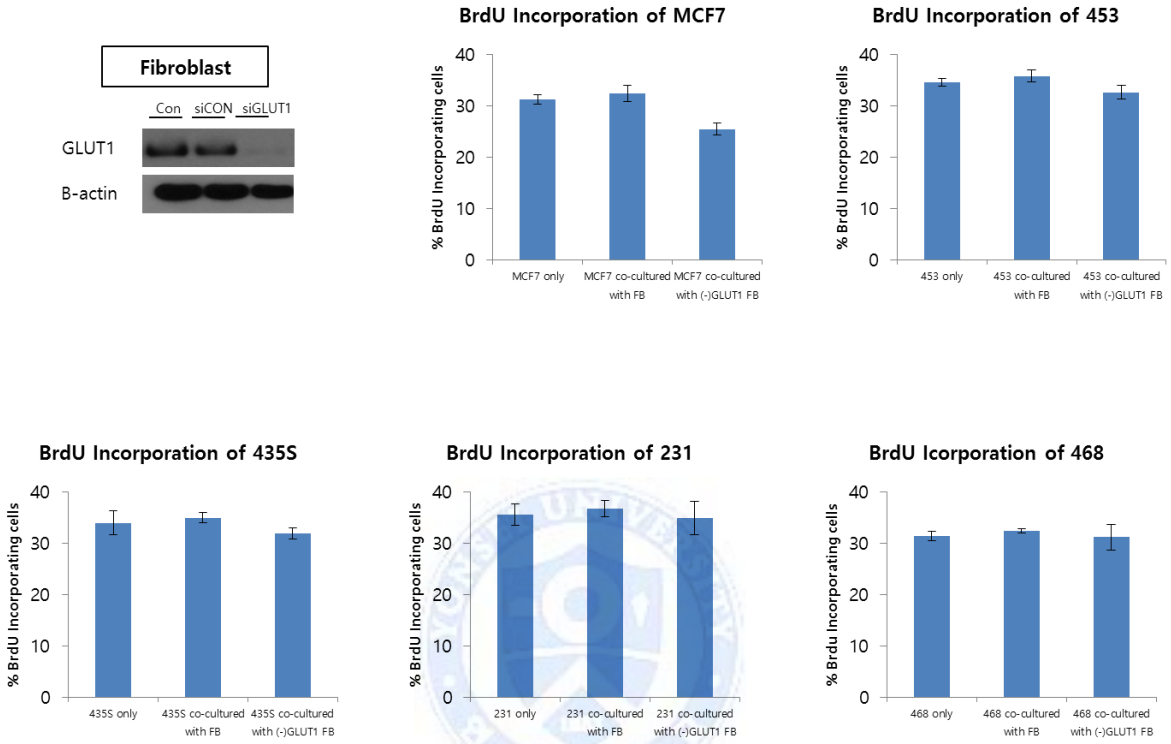


Figure 3 (a). Comparison of cancer cell proliferation assay (BrdU incorporation) among cancer cell only, cancer cell co-cultured with fibroblast (FB), cancer cell co-cultured with GLUT-1 knockdowned-fibroblast ((-) GLUT-1 FB). The MCF-7 (luminal type) showed significant reduction in the cancer cell proliferation rate whereas other types showed little differences in the rate of cancer cell proliferation.

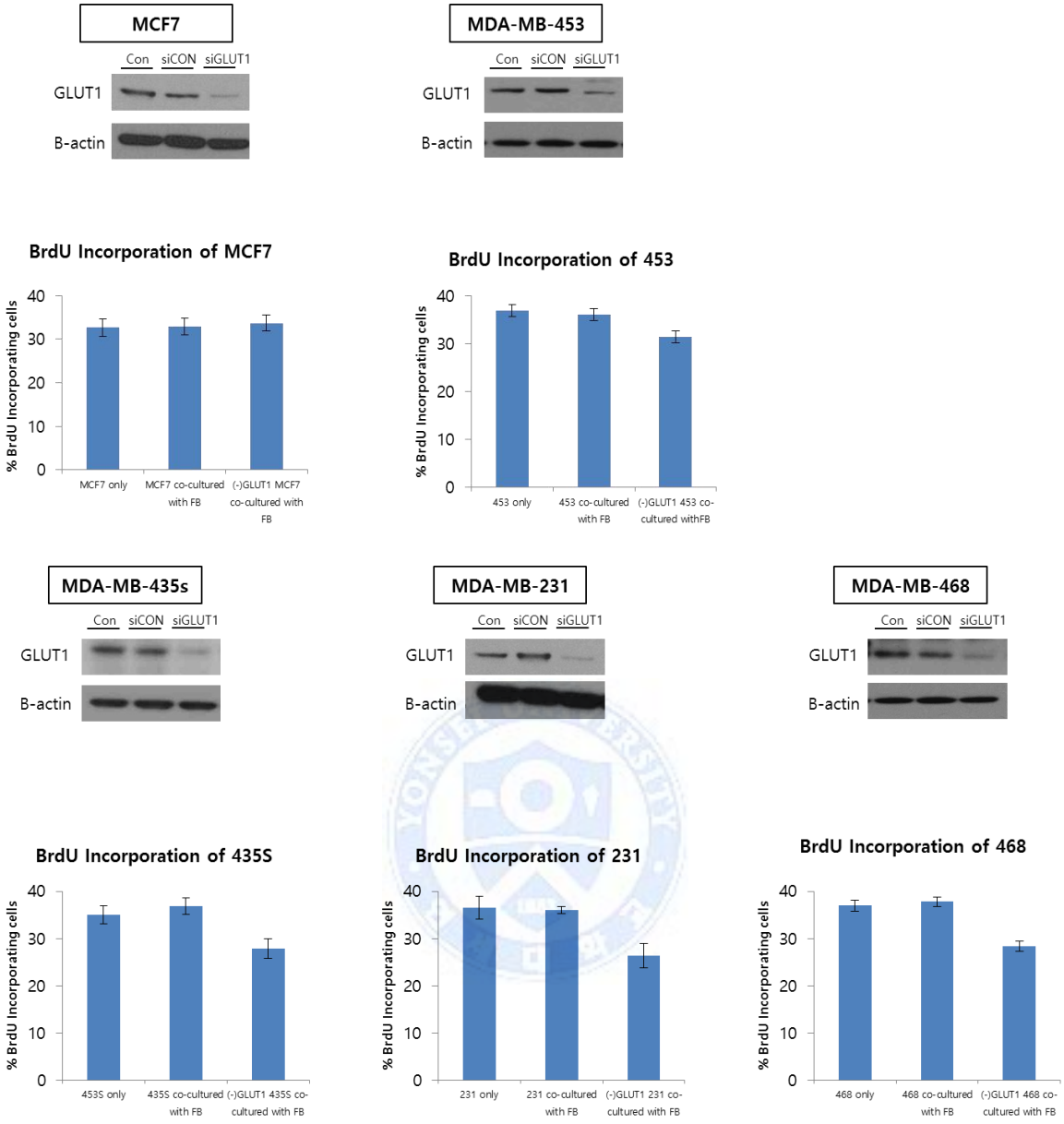


Figure 3 (b). Comparison of cancer cell proliferation assay (BrdU incorporation) among cancer cell only, cancer cell co-cultured with fibroblast (FB), GLUT-1 knockdowned ((-) GLUT-1)-cancer cell co-cultured with fibroblast (FB). The MCF-7 (luminal type) showed no difference in the cancer cell proliferation rate whereas all other types showed significant reduction in the rate of cancer cell proliferation.

C. LC3B-knockdowned cells regulate proliferation of direct co-cultured cells

Directly co-cultured cells with LC3B-knockdowned cells were also affected on proliferation levels. In co-cultured cells with LC3B siRNA transfected fibroblasts, the extent of reduction of cancer cell

proliferation was highest in MCF-7 (luminal type) than other types. In co-cultured LC3B siRNA transfected cancer cells with fibroblasts, the extent of reduction of cancer cell proliferation was higher in MDA-MB 435S (TNBC type), and MDA-MB-468 (TNBC type) (Figure 4).

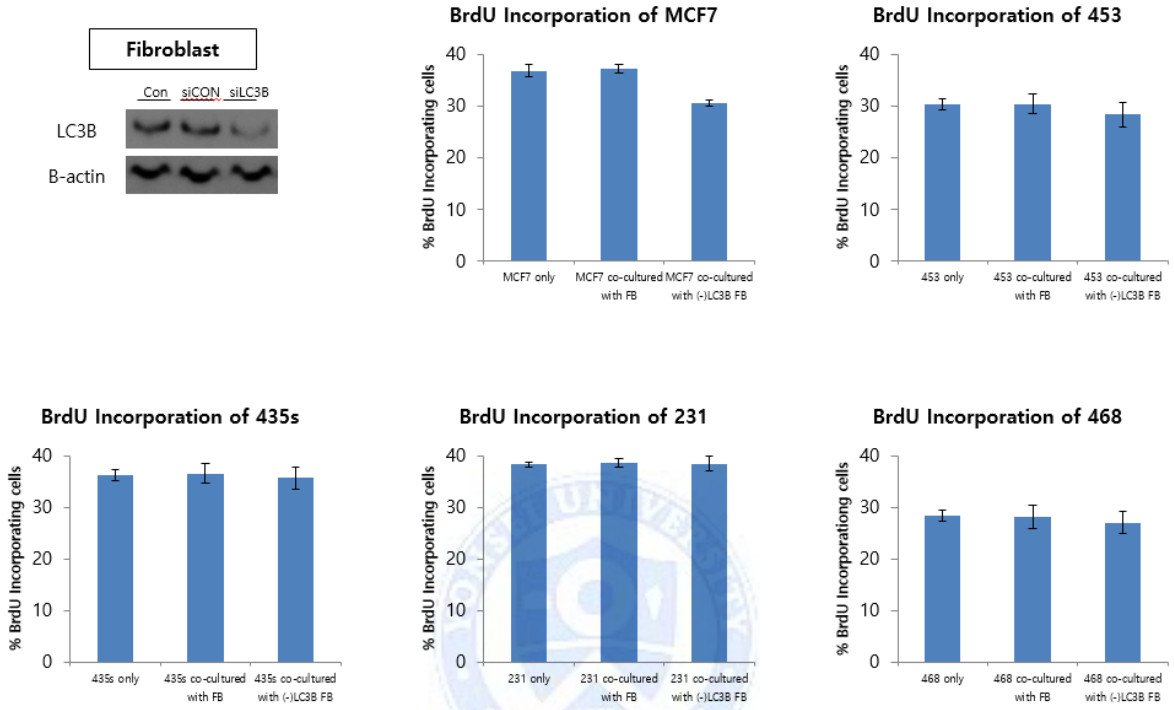


Figure 4 (a). Comparison of cancer cell proliferation assay (BrdU incorporation) among cancer cell only, cancer cell co-cultured with fibroblast (FB), cancer cell co-cultured with LC3B knockdowned-fibroblast ((-) LC3B FB). The MCF-7 (luminal type) showed significant reduction in the cancer cell proliferation rate whereas other types showed little differences in the rate of cancer cell proliferation.

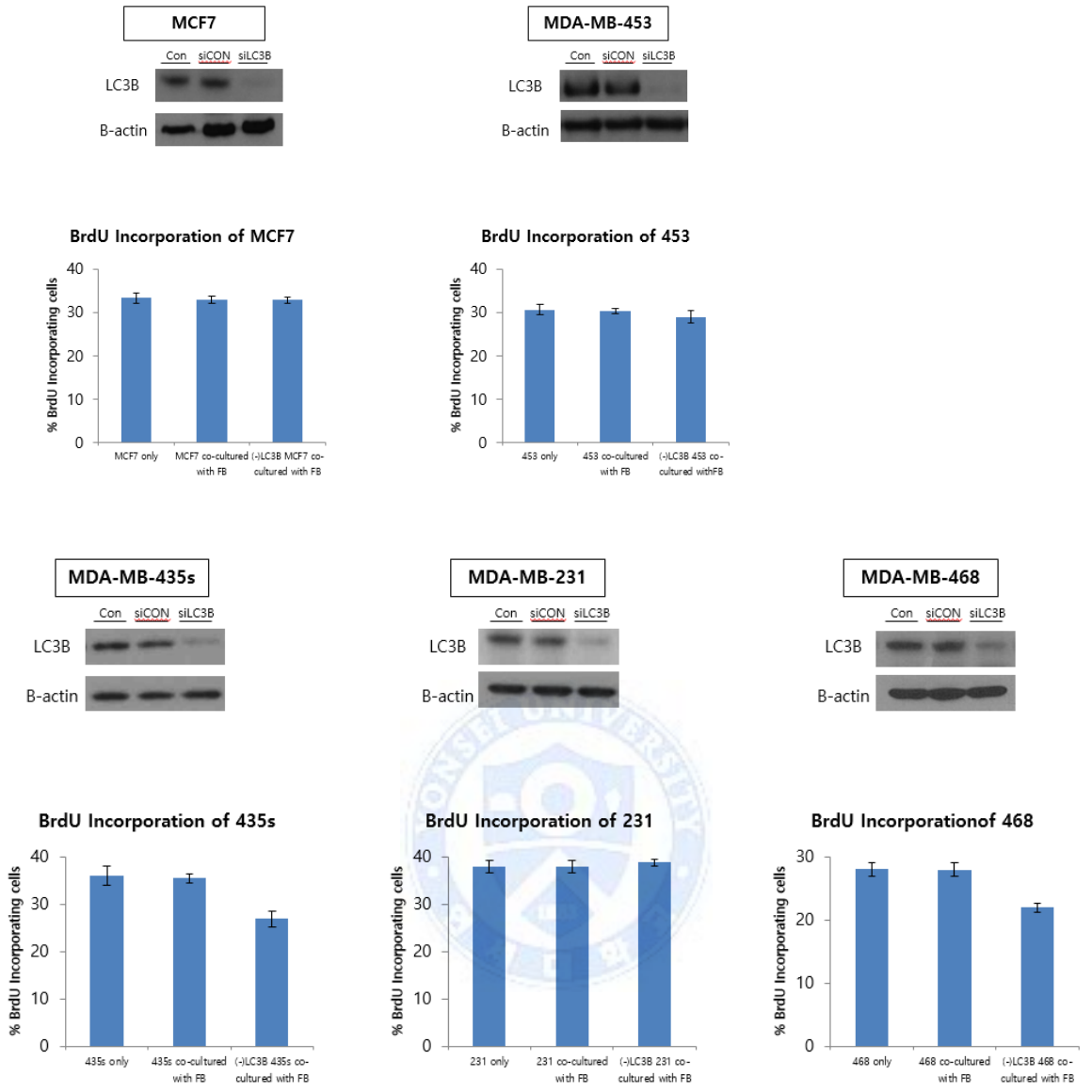


Figure 4 (b). Comparison of cancer cell proliferation assay (BrdU incorporation) among cancer cell only, cancer cell co-cultured with fibroblast (FB), LC3B knockdowned ((-) LC3B)-cancer cell co-cultured with fibroblast (FB). The MDA-MB 435S (TNBC type) and MDA-MB 468 (TNBC type) showed significant reduction in the cancer cell proliferation rate whereas other types showed little differences in the rate of cancer cell proliferation.

2. Human breast cancer tissue microarray study

A. Patients' characteristics according to the tumor phenotype

The clinicopathologic characteristics of the 740 patients, which comprised 298 (40.3%) cases of

luminal A type, 166 (22.4%) cases of luminal B type, 69 (9.3%) cases of HER-2 type, and 207 (28.0%) cases of TNBC, are shown in table 4. TNBC had the highest histologic grade, tumor stage, and Ki-67 LI ($p<0.001$, $p=0.002$, and $p<0.001$, respectively). HER-2 type and TNBC had higher tumor recurrence rates and numbers of patients' death ($p<0.001$ and $p<0.001$, respectively).

Table 4. Clinicopathologic characteristics of patients according to breast cancer phenotype

Parameters	*Total (n =740) (%)*	*Luminal A (n =298) (%)*	*Luminal B (n = 166) (%)*	*HER-2 (n =69) (%)*	*TNBC (n = 207) (%)*	*P-value*
Age (yr, mean ± SD)	49.7±11.0	50.6±10.5	48.5±10.1	52.8±9.8	48.4±12.4	0.007
Histologic grade						<0.001
I	118 (15.9)	90 (30.2)	18 (10.8)	1 (1.4)	9 (4.3)	
II	373 (50.4)	180 (60.4)	90 (54.2)	35 (50.7)	68 (32.9)	
III	249 (33.6)	28 (9.4)	58 (34.9)	33 (47.8)	130 (62.8)	
Tumor stage						0.002
T1	358 (48.4)	166 (55.7)	86 (51.8)	31 (44.9)	75 (36.2)	
T2	367 (49.6)	125 (41.9)	78 (47.0)	37 (53.6)	127 (61.4)	
T3	15 (2.0)	7 (2.3)	2 (1.2)	1 (1.4)	5 (2.4)	
Nodal stage						0.041
N0	436 (58.9)	168 (56.4)	91 (54.8)	42 (60.9)	135 (65.2)	
N1	200 (27.0)	90 (30.2)	43 (25.9)	13 (18.8)	54 (26.1)	
N2	66 (8.9)	27 (9.1)	17 (18.5)	10 (14.5)	12 (5.8)	
N3	38 (5.1)	13 (4.4)	15 (9.0)	4 (5.8)	6 (2.9)	
Estrogen receptor status						<0.001
Negative	286 (38.6)	5 (1.7)	5 (3.0)	69 (100.0)	207 (100.0)	
Positive	454 (61.4)	293 (98.3)	161 (97.0)	0 (0.0)	0 (0.0)	
Progesterone receptor status						<0.001
Negative	371 (50.1)	50 (16.8)	46 (27.7)	69 (100.0)	207 (100.0)	
Positive	369 (49.9)	248 (83.2)	120 (72.3)	0 (0.0)	0 (0.0)	
HER-2 status						<0.001
0	290 (39.2)	108 (36.2)	23 (13.9)	0 (0.0)	159 (76.8)	
1+	186 (25.1)	118 (39.6)	33 (20.0)	0 (0.0)	35 (16.9)	
2+	142 (19.2)	72 (24.2)	41 (24.7)	16 (23.2)	13 (6.3)	
3+	122 (16.5)	0 (0.0)	69 (41.6)	53 (76.8)	0 (0.0)	
Ki-67 LI (%, mean ± SD)	18.1±19.2	4.7±3.7	19.7±12.7	19.5±12.5	35.6±23.7	<0.001
Tumor recurrence	69 (9.3)	15 (5.0)	12 (7.2)	11 (15.9)	31 (15.0)	<0.001
Patients' death	67 (9.1)	14 (4.7)	11 (6.6)	12 (17.4)	30 (14.5)	<0.001
Duration of clinical follow-up (months, mean ± SD)	70.2±31.7	72.7±30.0	70.3±30.3	67.1±35.8	67.8±33.8	0.291

B. The expression of metabolism-related proteins according to the tumor phenotype

Metabolism-related protein expression according to the breast cancer phenotype is summarized in table 5. TNBC showed the highest expression rates of Glut-1, MCT4, and LC3A in tumor, whereas luminal A type showed the lowest rates of expression of these markers ($p<0.001$). The expression rates of CAIX, and MCT4 in stroma, and of cytoplasmic p62 in tumor were the highest in HER-2 type, and the lowest in luminal A type ($p=0.032$, $p<0.001$, and $p<0.001$, respectively). The expression rates of CAIX and LC3B in tumor were highest in TNBC, and lowest in luminal B type. ($P=0.008$, and $p=0.013$, respectively). Luminal A type had the highest expression rates of LC3A in stroma and nuclear p62 in tumor, whereas TNBC had the lowest rates of expression of these markers ($p<0.001$).

Table 5. The expression of metabolism related protein according to breast cancer phenotype

Parameters	*Total (n =740) (%)*	*Luminal A (n =298) (%)*	*Luminal B (n = 166) (%)*	*HER-2 (n =69) (%)*	*TNBC (n = 207) (%)*	*P-value*
Glut 1 in tumor						<0.001
Negative	504 (68.1)	260 (87.2)	124 (74.7)	47 (68.1)	73 (35.3)	
Positive	236 (31.9)	38 (12.8)	42 (25.3)	22 (31.9)	134 (64.7)	
Glut1 in stroma						0.103
Negative	724 (97.8)	296 (99.3)	162 (97.6)	66 (95.7)	200 (96.6)	
Positive	16 (2.2)	2 (0.7)	4 (2.4)	3 (4.3)	7 (3.4)	
CAIX in tumor						0.008
Negative	520 (70.3)	217 (72.8)	127 (76.5)	49 (71.0)	127 (61.3)	
Positive	220 (29.7)	81 (27.2)	39 (23.5)	20 (29.0)	80 (38.6)	
CAIX in stroma						0.032
Negative	627 (84.7)	264 (88.6)	137 (82.5)	52 (75.4)	174 (84.1)	
Positive	113 (15.3)	34 (11.4)	29 (17.5)	17 (24.6)	33 (15.9)	
BNIP3 in tumor						0.262
Negative	504 (68.1)	206 (69.1)	112 (67.5)	40 (58.0)	146 (70.5)	
Positive	236 (31.9)	92 (30.9)	54 (32.5)	29 (42.0)	61 (29.5)	
BNIP3 in stroma						0.262
Negative	700 (94.6)	281 (94.3)	159 (95.8)	62 (89.9)	198 (95.7)	
Positive	40 (5.4)	17 (5.7)	7 (4.2)	7 (10.1)	9 (4.3)	
MCT4 in tumor						<0.001
Negative	540 (73.0)	253 (84.9)	118 (71.1)	49 (71.0)	120 (58.0)	
Positive	200 (27.0)	45 (15.1)	48 (28.9)	20 (29.0)	87 (42.0)	

Parameters	*Total (n =740) (%)*	*Luminal A (n =298) (%)*	*Luminal B (n = 166) (%)*	*HER-2 (n =69) (%)*	*TNBC (n = 207) (%)*	*P-value*
MCT4 in stroma						<0.001
Negative	418 (56.5)	222 (74.5)	81 (48.8)	23 (33.3)	92 (44.4)	
Positive	322 (43.5)	76 (25.5)	85 (51.2)	46 (66.7)	115 (55.6)	
Cytoplasmic beclin-1						0.137
Negative	406 (54.9)	169 (56.7)	99 (59.6)	31 (44.9)	107 (51.7)	
Positive	334 (45.1)	129 (43.3)	67 (33.7)	38 (55.1)	100 (48.3)	
Nuclear beclin-1						<0.001
Negative	666 (90.0)	262 (87.9)	152 (91.6)	55 (79.7)	197 (95.2)	
Positive	74 (10.0)	36 (12.1)	14 (8.4)	14 (20.3)	10 (4.8)	
LC3A in tumor						<0.001
Negative	669 (90.4)	294 (98.7)	158 (95.2)	68 (98.6)	149 (72.0)	
Positive	71 (9.6)	4 (1.3)	8 (4.8)	1 (1.4)	58 (28.0)	
LC3A in stroma						<0.001
Negative	687 (92.8)	267 (89.6)	151 (91.0)	62 (89.9)	207 (100.0)	
Positive	53 (7.2)	31 (10.4)	15 (9.0)	7 (10.1)	0 (0.0)	
LC3B in tumor						0.013
Negative	475 (64.2)	186 (62.4)	124 (74.7)	42 (60.9)	123 (59.4)	
Positive	265 (35.8)	112 (37.6)	42 (25.3)	27 (39.1)	84 (40.6)	
LC3B in stroma						0.645
Negative	688 (93.0)	277 (93.0)	151 (91.0)	65 (94.2)	195 (94.2)	
Positive	52 (7.0)	21 (7.0)	15 (9.0)	4 (5.8)	12 (5.8)	
Cytoplasmic p62 in tumor						<0.001
Negative	274 (37.0)	131 (44.0)	51 (30.7)	15 (21.7)	77 (37.2)	
Positive	466 (63.0)	167 (56.0)	115 (69.3)	54 (78.3)	130 (62.8)	
Nuclear p62 in tumor						<0.001
Negative	532 (71.9)	180 (60.4)	131 (78.9)	44 (63.8)	177 (85.5)	
Positive	208 (28.1)	118 (39.6)	35 (21.1)	25 (36.2)	30 (14.5)	
Nuclear p62 in stroma						0.876
Negative	512 (69.2)	206 (69.1)	115 (69.3)	45 (65.2)	146 (70.5)	
Positive	228 (30.8)	92 (30.9)	51 (30.7)	24 (34.8)	61 (29.5)	

C. Correlation between metabolism-related proteins and clinicopathologic factors

Table 6 shows the correlation between the expression of metabolism-related proteins and clinicopathologic parameters. Tumoral Glut1 expression was correlated with higher histologic grade ($p<0.001$), ER negativity ($p<0.001$), PR negativity ($p<0.001$), higher T stage ($p<0.001$), higher Ki-67 LI ($p<0.001$), and tumor recurrence ($p=0.040$). Tumoral CAIX expression was correlated with higher Ki-67 LI ($p<0.001$). Tumoral MCT4 expression was correlated with higher histologic grade ($p<0.001$), ER negativity ($p<0.001$), PR negativity ($p<0.001$), higher T stage ($p<0.001$), and higher Ki-67 LI ($p<0.001$). Stromal MCT4 expression was correlated with higher histologic grade ($p<0.001$), ER negativity ($p<0.001$), PR negativity ($p<0.001$), HER-2 positivity ($p<0.001$), and higher Ki-67 LI ($p<0.001$). Tumoral LC3A expression was correlated with higher histologic grade ($p<0.001$), ER negativity ($p<0.001$), PR negativity ($p<0.001$), HER-2 negativity ($p<0.001$), and higher Ki-67 LI ($p<0.001$), and stromal LC3A expression was correlated with ER positivity ($p<0.001$), PR positivity ($p<0.001$), lower T stage ($p=0.040$), and lower Ki-67 LI ($p=0.008$). The expression of cytoplasmic p62 in tumor was correlated with HER-2 positivity ($p<0.001$), and the expression of nuclear p62 in tumor was correlated with lower histologic grade ($p<0.001$), ER positivity ($p<0.001$), PR positivity ($p<0.001$), and lower Ki-67 LI ($p<0.001$).

Table 6. Correlations between the expression of metabolism-related proteins and clinicopathologic parameters

Parameters	Glut1 in tumor			Glut1 in stroma			CAIX in tumor			CAIX in stroma		
	Negative n =504, (%)	Positive n = 236, (%)	p-value*	Negative n = 724, (%)	Positive n = 16, (%)	p-value*	Negative n = 520, (%)	Positive n = 220, (%)	p-value*	Negative n = 627, (%)	Positive n = 113, (%)	p-value*
Age (yr, mean ± SD)	50.5±10.7	48.1±11.4	0.024	49.7±110.	49.3±9.0	3.456	49.7±11.0	49.8±11.0	3.748	49.3±11.1	51.7±10.3	0.160
Histologic grade			<0.001			1.704			0.084			0.092
I/II	392 (71.8)	99 (41.9)		482 (67.3)	9 (56.3)		359 (69.0)	132 (60.0)		427 (68.1)	64 (56.6)	
III	112 (22.2)	137 (58.1)		242 (33.4)	7 (43.7)		161 (31.0)	88 (40.0)		200 (31.9)	49 (43.4)	
ER			<0.001			0.072			0.008			0.212
Negative	128 (25.4)	158 (66.9)		275 (38.0)	11 (68.8)		182 (35.0)	104 (47.3)		233 (37.2)	53 (46.9)	
Positive	376 (74.6)	78 (33.1)		449 (62.0)	5 (31.2)		338 (65.0)	116 (52.7)		394 (62.8)	60 (53.1)	
PR			<0.001			0.820			2.080			1.660
Negative	190 (37.7)	182 (77.1)		361 (49.9)	11 (68.8)		257 (49.4)	115 (52.3)		311 (49.6)	61 (54.0)	
Positive	314 (62.3)	54 (22.9)		363 (50.1)	5 (31.2)		263 (50.6)	105 (47.7)		316 (50.4)	52 (46.0)	
HER-2			0.136			1.284			0.056			0.240
Negative	386 (76.6)	197 (83.5)		572 (79.0)	11 (68.8)		397 (76.3)	186 (84.5)		502 (80.1)	81 (71.7)	
Positive	118 (23.4)	39 (16.5)		152 (21.0)	5 (31.2)		123 (23.7)	34 (15.5)		125 (19.9)	32 (28.3)	
Tumor stage			<0.001			3.584			3.744			0.608
T1	270 (53.6)	88 (37.3)		350 (48.3)	8 (50.0)		251 (48.3)	107 (48.6)		296 (47.2)	62 (54.9)	
T2/T3	234 (46.4)	148 (62.7)		374 (51.7)	8 (50.0)		269 (51.7)	113 (51.4)		331 (52.8)	51 (45.1)	
Nodal stage			0.368			3.072			2.976			3.024
N0	286 (56.7)	150 (63.6)		426 (58.8)	10 (62.5)		304 (58.5)	132 (60.0)		371 (59.2)	65 (57.5)	
N1/N2/N3	218 (43.3)	86 (36.4)		298 (41.2)	6 (37.5)		216 (41.5)	88 (40.0)		256 (40.8)	48 (42.5)	
Ki-67 LI (%, mean ± SD)	12.7±14.9	29.6±22.1	<0.001	18.0±19.3	22.5±14.7	1.428	16.1±17.5	22.7±22.1	<0.001	17.8±19.7	19.5±16.7	1.620

Tumor recurrence			0.040			1.544			3.548			3.400
Absent	467 (92.7)	204 (86.4)		655 (90.5)	16 (100.0)		471 (90.6)	200 (90.9)		568 (90.6)	103 (91.2)	
Present	37 (7.3)	32 (13.6)		69 (9.5)	0 (0.0)		49 (9.4)	20 (9.1)		59 (9.4)	10 (9.8)	
Death			0.080			1.540			2.240			2.900
Survival	467 (92.7)	206 (87.3)		657 (90.7)	16 (100.0)		475 (91.3)	198 (90.0)		571 (91.1)	102 (90.3)	
Death	37 (7.3)	30 (12.7)		67 (9.3)	0 (0.0)		45 (8.7)	22 (10.0)		56 (8.9)	11 (9.7)	

Parameters	BNIP3 in tumor			BNIP3 in stroma			MCT4 in tumor			MCT4 in stroma		
	Negative n =504, (%)	Positive n = 236, (%)	p-value*	Negative n = 700, (%)	Positive n = 40, (%)	p-value*	Negative n = 540, (%)	Positive n = 200, (%)	p-value*	Negative n = 418, (%)	Positive n = 322, (%)	p-value*
Age (yr, mean ± SD)	48.9±10.9	51.6±11.0	0.008	49.5±10.8	53.2±12.7	0.168	49.8±11.1	49.6±10.6	3.412	49.6±11.0	49.5±10.9	2.820
Histologic grade			1.116			2.928			<0.001			<0.001
I/II	341 (67.7)	150 (63.6)		463 (66.1)	28 (70.0)		386 (71.5)	105 (52.5)		314 (75.1)	177 (55.0)	
III	163 (32.3)	86 (36.4)		237 (33.9)	12 (30.0)		154 (28.5)	95 (47.5)		104 (24.9)	145 (45.0)	
ER			2.744			2.476			<0.001			<0.001
Negative	192 (38.1)	94 (39.8)		269 (38.4)	17 (42.5)		174 (32.2)	112 (56.0)		123 (29.4)	163 (50.6)	
Positive	312 (61.9)	142 (60.2)		431 (61.6)	23 (57.5)		366 (67.8)	88 (44.0)		295 (70.6)	159 (49.4)	
PR			3.252			1.668			<0.001			<0.001
Negative	255 (50.6)	117 (49.6)		349 (49.9)	23 (57.5)		232 (43.0)	140 (70.0)		179 (42.8)	193 (60.0)	
Positive	249 (49.4)	119 (50.4)		351 (50.1)	17 (42.5)		308 (57.0)	60 (30.0)		239 (57.2)	129 (40.0)	
HER-2			0.840			1.288			2.176			<0.001
Negative	404 (80.2)	179 (75.8)		554 (79.1)	29 (72.5)		422 (78.1)	161 (80.5)		356 (85.2)	227 (70.5)	
Positive	100 (19.8)	57 (24.2)		146 (20.9)	11 (27.5)		118 (21.9)	39 (19.5)		62 (14.8)	95 (29.5)	
Tumor stage			0.276			2.984			<0.001			1.496
T1	232 (46.0)	126 (53.4)		340 (48.6)	18 (45.0)		283 (52.4)	75 (37.5)		196 (46.9)	162 (50.3)	
T2/T3	272 (54.0)	110 (46.6)		360 (51.4)	22 (55.0)		257 (47.6)	125 (62.5)		222 (53.1)	160 (49.7)	

Nodal stage			1.188			0.120			3.468			1.464
N0	290 (57.5)	146 (61.9)		419 (59.9)	17 (42.5)		317 (58.7)	119 (59.5)		240 (57.4)	196 (60.9)	
N1/N2/N3	214 (42.5)	90 (38.1)		281 (40.1)	23 (57.5)		223 (41.3)	81 (40.5)		178 (42.6)	126 (39.1)	
Ki-67 LI (%, mean ± SD)	18.9±20.9	16.2±15.0	0.320	18.3±19.5	14.6±13.7	0.992	15.3±17.8	25.6±21.0	<0.001	13.2±16.5	24.3±20.7	<0.001
Tumor recurrence			0.004			0.656			2.688			2.096
Absent	445 (88.3)	226 (95.8)		632 (90.3)	39 (97.5)		491 (90.9)	180 (90.0)		376 (90.0)	295 (91.6)	
Present	59 (11.7)	10 (4.2)		68 (9.7)	1 (2.5)		49 (9.1)	20 (10.0)		42 (10.0)	27 (8.4)	
Death			1.088			2.900			2.268			0.488
Survival	454 (90.1)	219 (92.8)		636 (90.9)	37 (92.5)		493 (91.3)	180 (90.0)		374 (89.5)	299 (92.9)	
Death	50 (9.9)	17 (7.2)		64 (9.1)	3 (7.5)		47 (8.7)	20 (10.0)		44 (10.5)	23 (7.1)	

Parameters	Cytoplasmic beclin-1			Nuclear beclin-1			LC3A in tumor			LC3A in stroma		
	Negative n =406, (%)	Positive n = 334, (%)	p-value*	Negative n = 666, (%)	Positive n = 74, (%)	p-value*	Negative n = 669, (%)	Positive n = 71, (%)	p-value*	Negative n = 687, (%)	Positive n = 53, (%)	p-value*
Age (yr, mean ± SD)	48.6±10.5	51.1±11.4	0.008	49.6±11.1	50.8±9.9	1.584	50.2±11.0	45.6±9.8	0.004	49.7±11.0	49.6±9.2	3.744
Histologic grade			2.784			<0.001			<0.001			0.196
I/II	272 (67.0)	219 (65.6)		427 (64.1)	64 (86.5)		470 (70.3)	21 (29.6)		449 (65.4)	42 (79.2)	
III	134 (33.0)	115 (34.4)		239 (35.9)	10 (13.5)		199 (29.7)	50 (70.4)		238 (34.6)	11 (20.8)	
ER			0.160			1.528			<0.001			<0.001
Negative	143 (35.2)	143 (42.8)		261 (39.2)	25 (33.8)		226 (33.8)	60 (84.5)		278 (40.5)	8 (15.1)	
Positive	263 (64.8)	191 (57.2)		405 (60.8)	49 (66.2)		443 (66.2)	11 (15.5)		409 (59.5)	45 (84.9)	
PR			3.764			0.348			<0.001			<0.001
Negative	205 (50.5)	167 (50.0)		342 (51.4)	30 (40.5)		309 (46.2)	63 (88.7)		360 (52.4)	12 (22.6)	
Positive	201 (49.5)	167 (50.0)		324 (48.6)	44 (59.5)		360 (53.8)	8 (11.3)		327 (47.6)	41 (77.4)	
HER-2			2.356			0.064			<0.001			1.528
Negative	323 (79.6)	260 (77.8)		533 (80.0)	50 (67.6)		515 (76.9)	68 (95.8)		544 (79.2)	39 (73.6)	

Positive	83 (20.4)	74 (22.2)		133 (20.0)	24 (32.4)		154 (23.1)	3 (4.2)		143 (20.8)	14 (26.4)	
Tumor stage			0.008			1.312			3.212			0.040
T1	175 (43.1)	183 (54.8)		318 (47.7)	40 (54.1)		325 (48.6)	33 (46.5)		323 (47.0)	35 (66.0)	
T2/T3	231 (56.9)	151 (45.2)		348 (52.3)	34 (45.9)		344 (51.4)	38 (53.5)		364 (53.0)	18 (34.0)	
Nodal stage			2.612			0.688			1.248			3.092
N0	236 (58.1)	200 (59.9)		398 (59.8)	38 (51.4)		390 (58.3)	46 (64.8)		406 (59.1)	30 (56.6)	
N1/N2/N3	170 (41.9)	134 (40.1)		268 (40.2)	36 (48.6)		279 (41.7)	25 (35.2)		281 (40.9)	23 (43.4)	
Ki-67 LI (%, mean ± SD)	17.8±19.4	18.3±19.1	0.008	19.0±19.8	9.5±10.0	<0.001	15.7±17.2	39.6±23.1	<0.001	18.7±19.7	10.4±9.6	0.008
Tumor recurrence			0.168			0.548			0.772			3.236
Absent	360 (88.7)	311 (93.1)		600 (90.1)	71 (95.9)		610 (91.2)	61 (85.9)		622 (90.5)	49 (92.5)	
Present	46 (11.3)	23 (6.9)		66 (9.9)	3 (4.1)		59 (8.8)	10 (14.1)		65 (9.5)	4 (7.5)	
Death			3.192			0.036			2.048			1.860
Survival	368 (90.6)	305 (91.3)		600 (90.1)	73 (98.6)		610 (91.2)	63 (88.7)		623 (90.7)	50 (94.3)	
Death	38 (9.4)	29 (8.7)		66 (9.9)	1 (1.4)		59 (8.8)	8 (11.3)		64 (9.3)	3 (5.7)	

Parameters	LC3B in tumor			LC3B in stroma			Cytoplasmic p62 in tumor			Nuclear p62 in tumor			Nuclear p62 in stroma		
	Negative n =475, (%)	Positive n = 265, (%)	p-value*	Negative n = 688, (%)	Positive n = 52, (%)	p-value*	Negative n = 274, (%)	Positive n = 466, (%)	p-value*	Negative n = 532, (%)	Positive n = 208, (%)	p-value*	Negative n = 512, (%)	Positive n = 228, (%)	p-value*
Age (yr, mean ± SD)	49.4±10.4	50.4±12.0	1.095	49.6±11.0	51.1±10.6	3.755	49.4±10.3	49.9±11.4	2.465	49.4±10.9	50.6±11.2	0.885	49.5±11.2	50.3±10.4	1.605
Histologic grade			0.840			2.250			0.050			<0.001			4.330
I/II	324 (68.2)	167 (63.0)		459 (66.7)	32 (61.5)		198 (72.3)	293 (62.9)		322 (60.5)	169 (81.3)		341 (66.6)	150 (65.8)	
III	151 (31.8)	98 (37.0)		229 (33.3)	20 (38.5)		76 (27.7)	173 (37.1)		210 (39.5)	39 (18.8)		171 (33.4)	78 (34.2)	
ER			0.495			1.900			0.685			<0.001			3.415
Negative	173 (36.4)	113 (42.6)		269 (39.1)	17 (32.7)		96 (35.0)	190 (40.8)		227 (42.7)	59 (28.4)		195 (38.1)	91 (39.9)	
Positive	302 (63.6)	152 (57.4)		419 (60.9)	35 (67.3)		178 (65.0)	276 (59.2)		305 (57.3)	149 (71.6)		317 (61.9)	137 (60.1)	

PR			2.450			1.260			0.025			<0.001			4.060
Negative	234 (49.3)	138 (52.1)		350 (50.9)	22 (42.3)		119 (43.4)	253 (54.3)		293 (55.1)	79 (38.0)		259 (50.6)	113 (49.6)	
Positive	241 (50.7)	127 (47.9)		338 (49.1)	30 (57.7)		155 (56.6)	213 (45.7)		239 (44.9)	129 (62.0)		253 (49.4)	115 (50.4)	
HER-2			2.875			2.990			<0.001			3.820			3.135
Negative	371 (78.1)	212 (80.0)		540 (78.5)	43 (82.7)		238 (86.9)	345 (74.0)		421 (79.1)	162 (77.9)		406 (79.3)	177 (34.6)	
Positive	104 (21.9)	53 (20.0)		148 (21.5)	9 (17.3)		36 (13.1)	121 (26.0)		111 (20.9)	46 (22.1)		106 (20.7)	51 (22.4)	
Tumor stage			0.085			3.335			0.850			0.045			0.045
T1	214 (45.1)	144 (54.3)		331 (48.1)	27 (51.9)		142 (51.8)	216 (46.4)		241 (45.3)	117 (56.2)		231 (45.1)	127 (55.7)	
T2/T3	261 (54.9)	121 (45.7)		357 (51.9)	25 (48.1)		132 (48.2)	250 (53.6)		291 (54.7)	91 (43.8)		281 (54.9)	101 (44.3)	
Nodal stage			1.555			3.310			0.945			1.405			2.590
N0	273 (57.5)	163 (61.5)		407 (59.2)	29 (55.8)		170 (62.0)	266 (57.1)		320 (60.2)	116 (55.8)		306 (59.8)	130 (57.0)	
N1/N2/N3	202 (42.5)	102 (38.5)		281 (40.8)	23 (44.2)		104 (38.0)	200 (42.9)		212 (39.8)	92 (44.2)		206 (40.2)	98 (43.0)	
Ki-67 LI (%, mean ± SD)	18.2±19.8	17.8±18.2	3.835	18.4±19.4	18.9±17.6	3.755	16.0±19.2	19.3±19.2	0.135	21.4±20.8	9.5±10.6	<0.001	18.5±19.7	17.0±18.1	1.705
Tumor recurrence			4.480			2.325			4.485			0.795			4.715
Absent	430 (90.5)	241 (90.9)		622 (90.4)	49 (94.2)		248 (90.5)	423 (90.8)		477 (89.7)	194 (93.3)		464 (90.6)	207 (90.8)	
Present	45 (9.5)	24 (9.1)		66 (9.6)	3 (5.8)		26 (9.5)	43 (9.2)		55 (10.3)	14 (6.7)		48 (9.4)	21 (9.2)	
Death			2.125			3.070			3.460			2.390			3.400
Survival	435 (91.6)	238 (89.8)		624 (90.7)	49 (94.2)		251 (91.6)	422 (90.6)		481 (90.4)	192 (92.3)		467 (91.2)	206 (90.3)	
Death	40 (8.4)	27 (10.2)		64 (9.3)	3 (5.8)		23 (8.4)	44 (9.4)		51 (9.6)	16 (7.7)		45 (8.8)	22 (9.7)	

* p-values are corrected for multiple testing using the Bonferroni correction.

D. Correlation between tumor metabolic phenotype and clinicopathologic factors

The correlation between the breast cancer metabolic phenotype and clinicopathologic parameters is summarized in table 7 and figure 5. Tumors were classified into 298 Warburg type (40.3%), 54 reverse Warburg type (7.3%), 62 mixed type (8.4%), and 326 null type (44.0%). Histologic grade was highest in mixed type, and lowest in null type ($p<0.001$). Mixed type had the higher rates of ER negativity and PR negativity, whereas null type had the higher rates of ER positivity and PR positivity. ($p<0.001$). The rate of negative HER-2 status was highest in Warburg type ($p=0.006$). TNBC was the most common molecular subtype in Warburg type and mixed type, whereas luminal A was the most common molecular subtype in reverse Warburg type and null type ($p<0.001$). The rate of activated tumor autophagy status was highest in mixed type, whereas the rate of non-activated tumor autophagy status was highest in null type ($p=0.001$). Reverse Warburg type and mixed type had the higher rate of activated stromal autophagy status than in other types ($p<0.001$). The rate of positive expression of MCT4 in tumor was highest in Warburg type, and was lowest in null type ($p<0.001$). Stromal MCT4 expression rate was highest in mixed type and was lowest in null type ($P<0.001$). Ki-67 LI was highest in mixed type, and was lowest in null type ($p<0.001$).

Table 7. Clinicopathologic characteristics of patients according to metabolic phenotype

Parameters	Warburg type (n =298) (%)	Reverse Warburg type (n = 54) (%)	Mixed type (n =62) (%)	Null type (n = 326) (%)	P-value
Age (yr, mean ± SD)	48.5±11.7	52.0±10.2	51.3±10.2	50.1±10.5	0.052
Histologic grade					<0.001
I/II	169 (56.7)	41 (75.9)	23 (37.0)	258 (79.1)	
III	129 (43.3)	13 (24.1)	39 (72.2)	68 (20.9)	
Tumor stage					0.017
T1	123 (41.3)	29 (53.7)	34 (54.8)	172 (52.8)	
T2/T3	175 (58.7)	25 (46.3)	28 (45.2)	154 (47.2)	
Nodal stage					0.457
N0	177 (59.3)	27 (50.0)	40 (64.5)	192 (58.9)	
N1/N2/N3	121 (40.6)	27 (50.0)	22 (35.5)	134 (41.1)	
Estrogen receptor status					<0.001
Negative	152 (51.0)	15 (27.8)	39 (62.9)	80 (24.5)	
Positive	146 (49.0)	39 (72.2)	23 (37.1)	246 (75.5)	

Parameters	Warburg type (n =298) (%)	Reverse Warburg type (n = 54) (%)	Mixed type (n =62) (%)	Null type (n = 326) (%)	P-value
Progesterone receptor status					<0.001
Negative	181 (60.7)	22 (40.7)	40 (64.5)	129 (39.6)	
Positive	117 (39.3)	32 (59.3)	22 (35.5)	197 (60.4)	
HER-2 status					0.006
Negative	252 (84.6)	36 (66.7)	47 (75.8)	248 (76.1)	
Positive	46 (15.4)	18 (33.3)	15 (24.2)	78 (23.9)	
Molecular subtype					<0.001
Luminal A	91 (30.5)	22 (40.7)	12 (19.4)	173 (53.1)	
Luminal B	58 (19.5)	18 (33.3)	13 (21.0)	77 (23.6)	
HER-2	22 (7.4)	7 (13.0)	10 (16.1)	30 (9.2)	
Triple negative	127 (42.6)	7 (13.0)	27 (43.5)	46 (14.1)	
Tumor mitochondria status					0.217
Dysfunctional	94 (31.5)	20 (37.0)	26 (41.9)	96 (29.4)	
Functional	204 (68.5)	34 (63.0)	36 (58.1)	230 (70.6)	
Stroma mitochondria status					0.055
Dysfunctional	13 (4.4)	3 (5.6)	8 (12.9)	16 (4.9)	
Functional	285 (95.6)	51 (94.4)	54 (87.1)	310 (95.1)	
Tumor autophagy status					<0.001
Activated	168 (56.4)	28 (51.9)	45 (72.6)	117 (35.9)	
Non-activated	130 (43.6)	26 (48.1)	17 (27.4)	209 (64.1)	
Stroma autophagy status					<0.001
Activated	9 (3.0)	11 (20.4)	13 (21.0)	21 (6.4)	
Non-activated	289 (97.0)	43 (79.6)	49 (79.0)	305 (93.6)	
MCT4 in tumor					<0.001
Negative	180 (60.4)	38 (70.4)	40 (64.5)	282 (86.5)	
Positive	118 (39.6)	16 (29.6)	22 (35.5)	44 (13.5)	
MCT4 in stroma					<0.001
Negative	157 (52.7)	22 (40.7)	20 (32.3)	219 (67.2)	
Positive	141 (47.3)	32 (59.3)	42 (67.7)	107 (32.8)	
Ki-67 LI (%, mean ± SD)	24.6±22.5	13.2±11.1	25.2±18.6	11.5±14.1	<0.001
Tumor recurrence	38 (12.8)	6 (11.1)	4 (6.5)	21 (6.4)	0.043
Patients' death	36 (12.1)	5 (9.3)	6 (9.7)	20 (6.1)	0.081

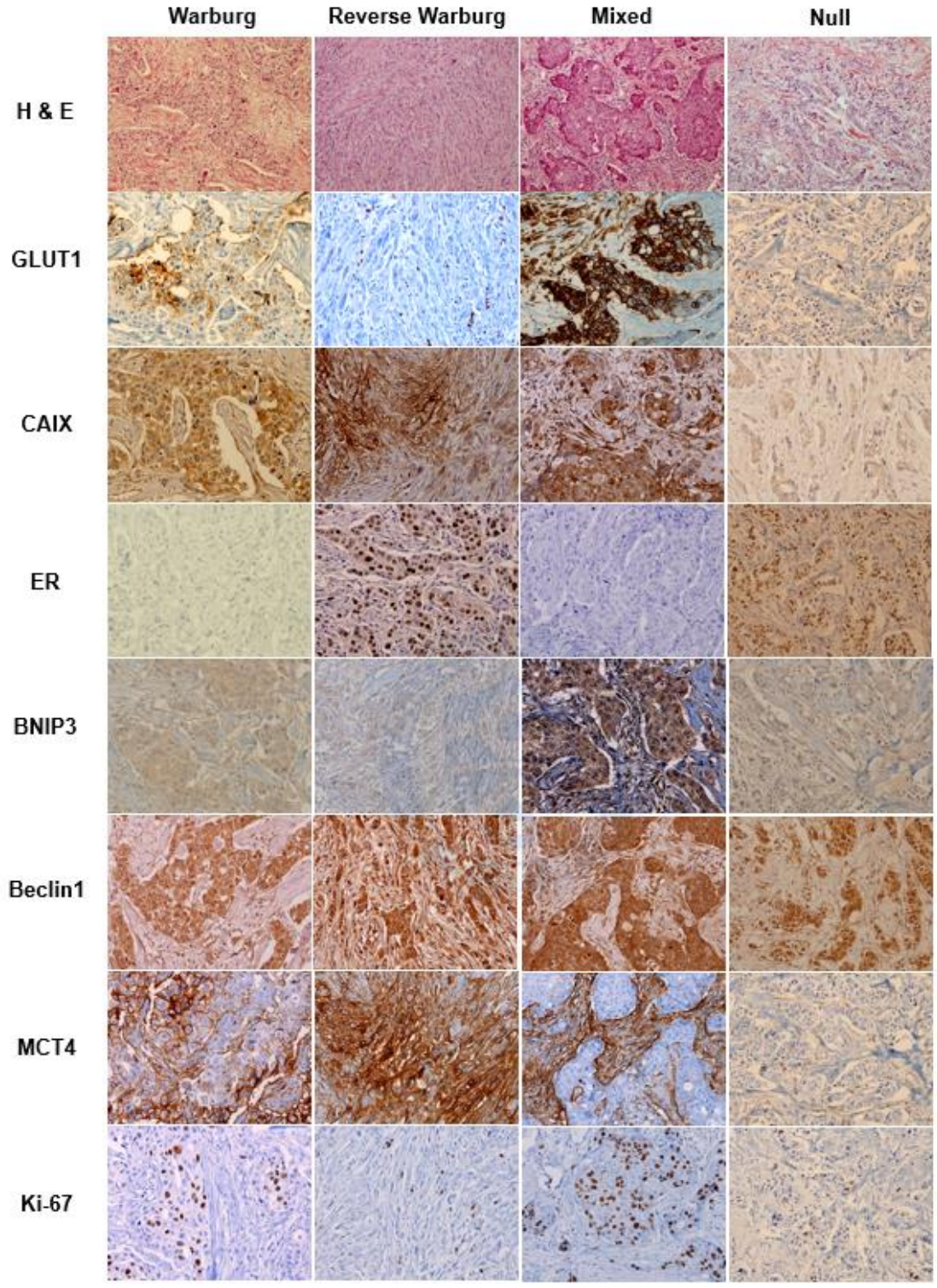


Figure 5. Histologic and immunohistochemical features according to metabolic phenotypes of breast cancer. The Warburg and mixed types show high histologic grade, estrogen receptor (ER) negativity and high Ki-67 labeling index (LI). In contrast, the reverse Warburg and null types show low histologic grade, ER positivity and low Ki67 LI.

E. The impact of metabolism-related proteins on patient prognosis

Table 8 demonstrates the univariate analysis of the relationship between the expression of metabolism-related proteins and patient disease-free survival (DFS) and overall survival (OS). Parameters associated with a shorter DFS included tumoral Glut1 positivity (p=0.010), tumoral BNIP3 negativity (p=0.004), tumor phenotype (HER-2 and TNBC, p<0.001), and tumor metabolic type (reverse Warburg type, p=0.037, Figure 6 (a)). Parameters associated with shorter OS were tumoral Glut1 positivity (p=0.023), tumor phenotype (HER-2 and TNBC, p<0.001), and tumor metabolic type (mixed type, p=0.045, Figure 6 (b)). Multivariate Cox analysis (variables: histologic grade, T stage, N stage, ER status, PR status, HER-2 status, Tumor phenotype, Tumor metabolic phenotype, Glut1 in tumor) showed that ER negativity (OR: 2.7, 95% CI:1.7-4.5, p<0.001), N stage (N0 VS. N1/2/3, OR: 2.3, 95% CI: 1.4-3.8, p=0.001), and T stage (T1 VS. T2/3, OR: 2.4, 95% CI: 1.3-4.4, p=0.002) were significant independent factors for shorter DFS, and ER negativity (OR: 3.3, 95% CI: 2.0-5.5, p<0.001), and N stage (N0 VS. N1/2/3, OR: 2.3, 95% CI: 1.4-3.8, p=0.001) were significant independent factor associated with shorter OS. Further analyses of patient survival according to metabolic phenotypes in each molecular subtype are shown in figure 7. No significant differences in either disease-free survival or overall survival were observed.

Table 8. Univariate analysis of the expression of metabolism-related proteins in breast cancers and disease-free survival or overall survival by log-rank test

Parameters	Number of patients/ recurrence /death	Disease-free survival		Overall survival	
		Mean survival (95% CI) months	P - value	Mean survival (95% CI) months	P -value
Immunohistochemical factors					
Glut 1 in tumor			0.010		0.023
Negative	504/37/37	128 (125-131)		131 (128-134)	
Positive	236/32/30	119 (112-126)		123 (118-128)	
Glut1 in stroma			n/a		n/a
Negative	724/69/67	n/a		n/a	
Positive	16/0/0	n/a		n/a	
CAIX in tumor			0.740		0.222
Negative	520/49/45	126 (122-130)		130 (127-132)	
Positive	220/20/22	108 (102-113)		123 (117-130)	

Parameters	Number of patients/ recurrence /death	Disease-free survival		Overall survival	
		Mean survival (95% CI) months	P - value	Mean survival (95% CI) months	P -value
CAIX in stroma			0.927		0.496
Negative	627/59/56	125 (122-129)		129 (126-132)	
Positive	113/10/11	103 (98-108)		116 (109-123)	
BNIP3 in tumor			0.004		0.426
Negative	504/59/50	123 (119-127)		128 (124-131)	
Positive	236/10/17	123 (119-127)		131 (126-135)	
BNIP3 in stroma			0.191		0.973
Negative	700/68/64	125 (121-128)		128 (126-131)	
Positive	40/1/3	116 (111-121)		121 (112-129)	
MCT4 in tumor			0.550		0.451
Negative	540/49/47	125 (121-129)		129 (126-132)	
Positive	200/20/20	116 (111-121)		126 (120-131)	
MCT4 in stroma			0.673		0.262
Negative	418/42/44	123 (118-127)		127 (123-131)	
Positive	322/27/23	128 (124-132)		130 (126-133)	
Cytoplasmic beclin-1			0.169		0.566
Negative	406/46/38	124 (119-128)		129 (126-132)	
Positive	334/23/29	121 (118-124)		126 (123-130)	
Nuclear beclin-1			0.157		0.031
Negative	666/66/66	125 (121-128)		128 (125-131)	
Positive	74/3/1	111 (106-115)		136 (132-139)	
LC3A in tumor			0.085		0.299
Negative	669/59/59	126 (122-129)		129 (126-132)	
Positive	71/10/8	113 (103-122)		124 (115-133)	
LC3A in stroma			0.801		0.541
Negative	687/65/64	125 (122-129)		128 (126-131)	
Positive	53/4/3	65 (62-68)		66 (64-68)	
LC3B in tumor			0.990		0.271
Negative	475/45/40	125 (121-130)		130 (127-133)	
Positive	265/24/27	118 (113-123)		125 (120-130)	
LC3B in stroma			0.481		0.565
Negative	688/66/64	125 (122-129)		128 (126-131)	
Positive	52/3/3	63 (60-66)		64 (62-66)	

Parameters	Number of patients/ recurrence /death	Disease-free survival		Overall survival	
		Mean survival (95% CI) months	P - value	Mean survival (95% CI) months	P -value
Cytoplasmic p62 in tumor			0.958		0.528
Negative	274/26/23	121 (112-129)		129 (125-133)	
Positive	466/43/44	126 (122-130)		128 (125-131)	
Nuclear p62 in tumor			0.210		0.646
Negative	532/55/51	125 (122-129)		128 (125-131)	
Positive	208/14/16	117 (110-124)		128 (122-133)	
Nuclear p62 in stroma			0.720		0.387
Negative	512/48/45	126 (122-130)		129 (126-132)	
Positive	228/21/22	104 (99-109)		124 (118-130)	
Tumor phenotype			<0.001		<0.001
Luminal A	298/15/14	130 (126-133)		134 (131-137)	
Luminal B	166/12/11	129 (124-134)		130 (124-135)	
HER-2	69/11/12	111 (100-121)		119 (108-130)	
TNBC	207/31/30	116 (109-124)		120 (114-126)	
Metabolic status			0.037		0.045
Warburg type	298/38/36	119 (112-126)		124 (119-128)	
Reverse Warburg type	54/6/5	90 (83-96)		113 (106-121)	
Mixed type	62/4/6	105 (100-111)		112 (99-126)	
Null type	326/21/20	129 (126-133)		132 (129-136)	

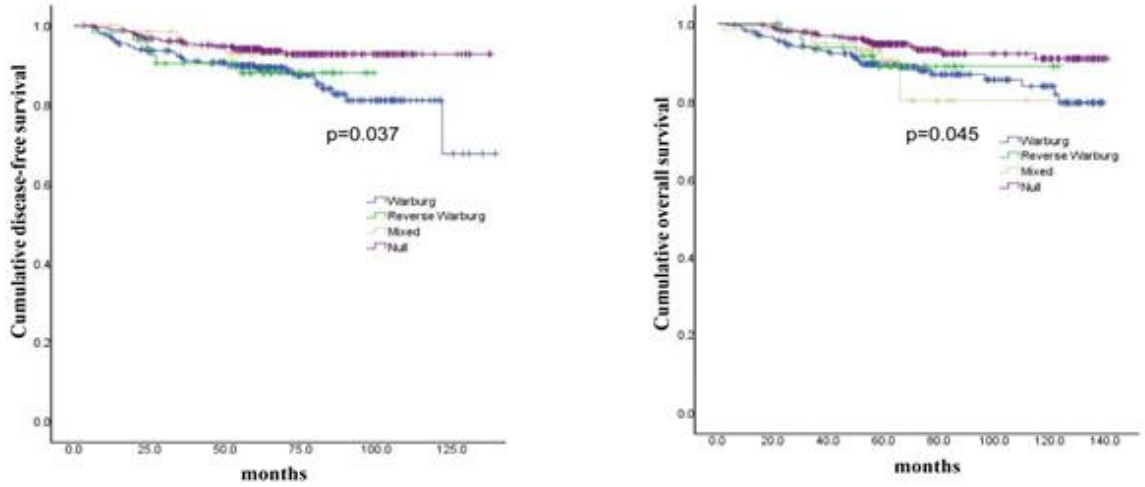
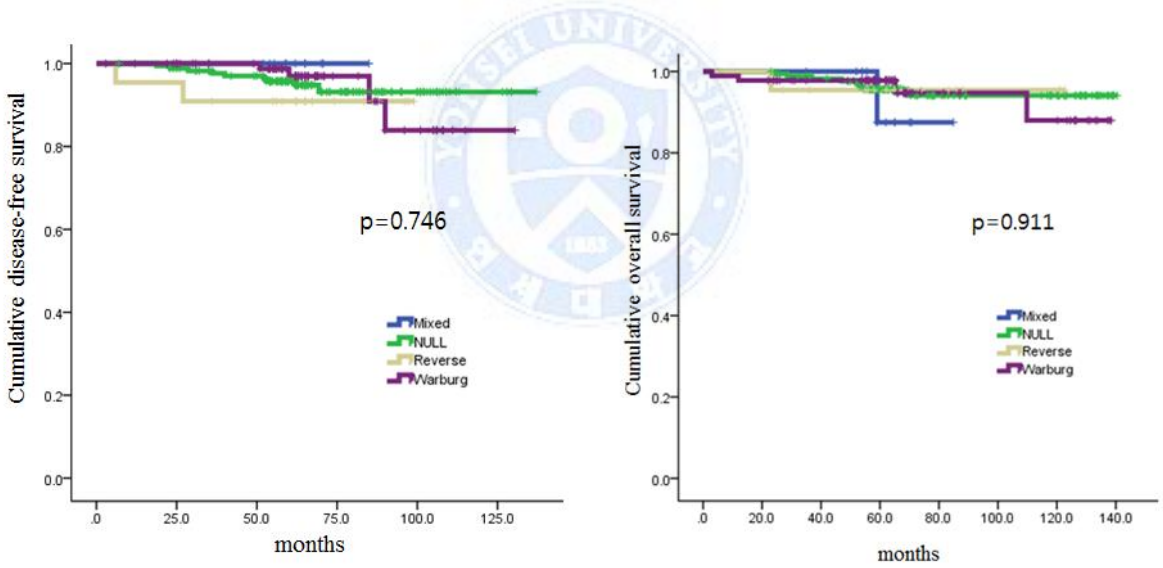
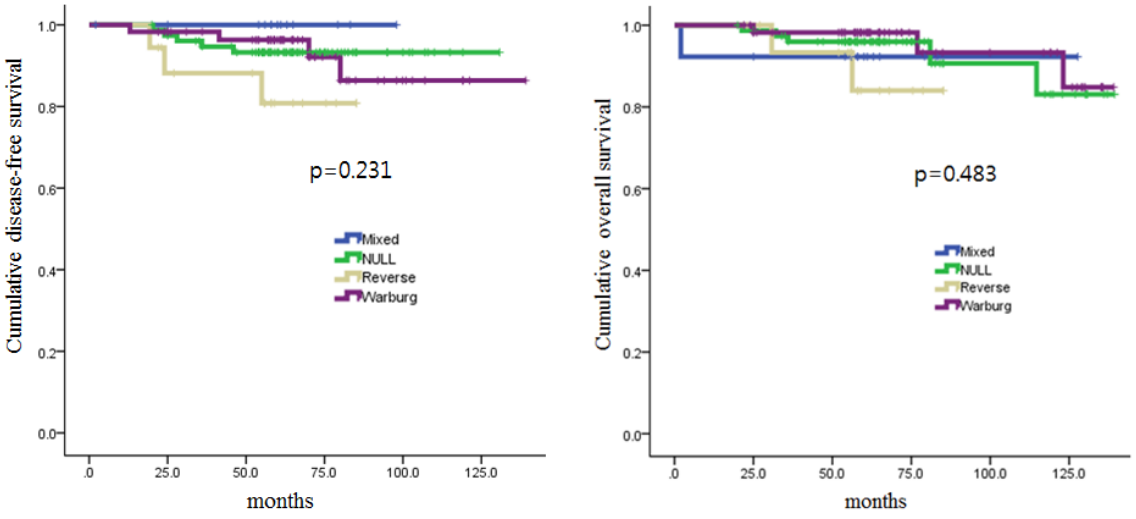


Figure 6. Disease-free survival and overall survival curves according to metabolic phenotypes of breast cancer.



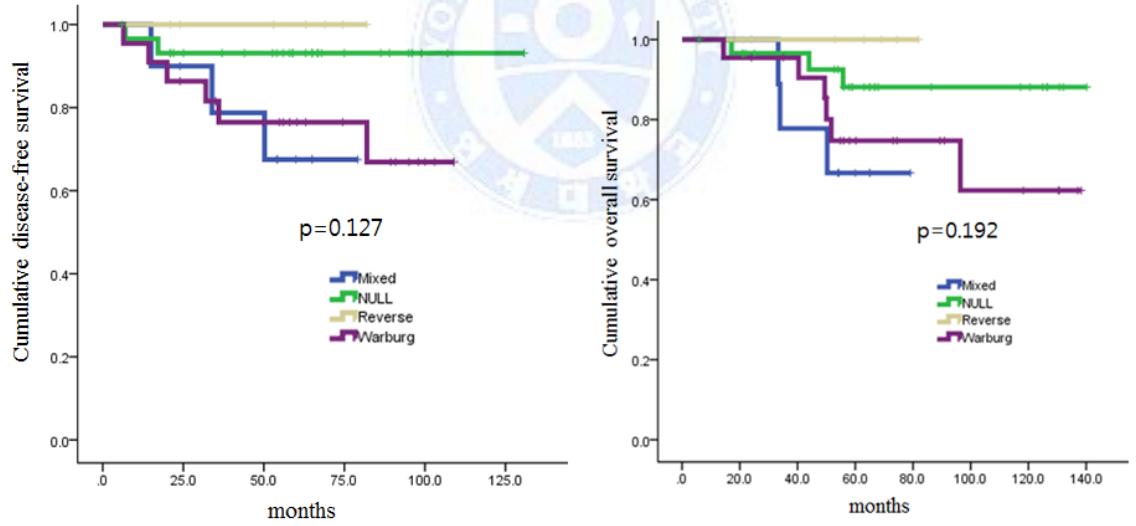
(a) Luminal A type

Figure 7 (a). Disease-free survival and overall survival curves according to metabolic phenotypes in luminal A type of breast cancer.



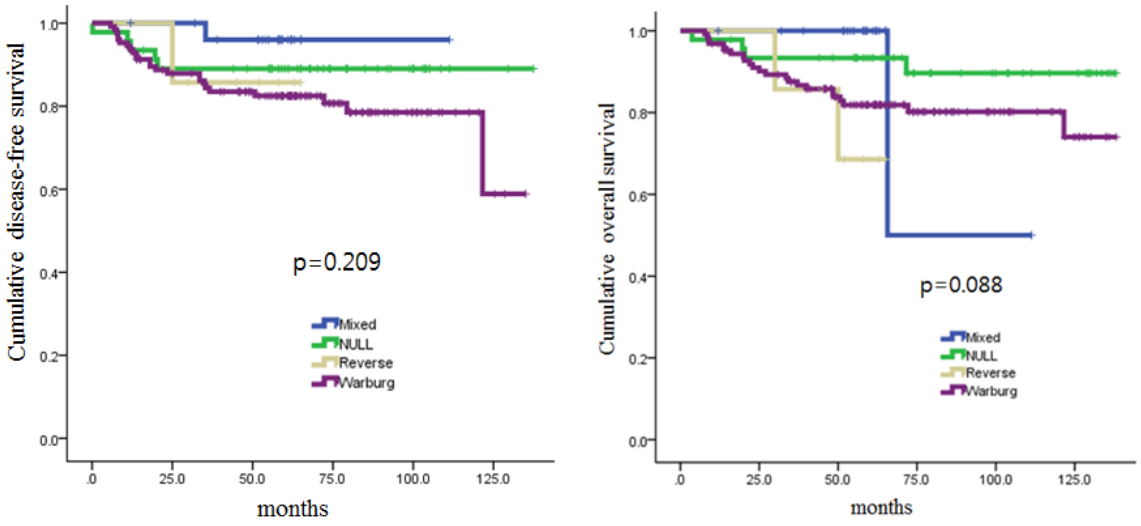
(b) Luminal B type

Figure 7 (b). Disease-free survival and overall survival curves according to metabolic phenotypes in luminal B type of breast cancer.



(c) HER2 type

Figure 7 (c). Disease-free survival and overall survival curves according to metabolic phenotypes in HER2 type of breast cancer.



(d) Triple-negative breast cancer type

Figure 7 (d). Disease-free survival and overall survival curves according to metabolic phenotypes in TNBC type of breast cancer.

IV. DISCUSSION

This study was designed to investigate the differences in the expression of metabolism-related markers between cancer cells and stromal cells according to the molecular subtypes of breast cancer through *in vitro* cell line and tissue microarray studies. In co-cultured MCF-7, which represents luminal type, the expression levels of most metabolic markers were higher in stromal cells than cancer cells, and siRNA inhibition for GLUT-1, and LC3B studies showed that the extent of reduction in the cancer cellu proliferation was greater when inhibition was performed in stromal cells than in cancer cells. Reversely, in co-cultured MDA-MB 453, representing HER2 type, and in MDA-MB-435S and MDA-MB-468, representing TNBC type, the expression levels of most metabolic markers were higher in cancer cells than stromal cells, and siRNA inhibition studies showed that the extent of reduction in the cancer cell proliferation were greater when inhibition was performed in cancer cells than in stromal cells. These results reveal that metabolic activities are different in tumor and stroma according to the molecular subtypes: in luminal type, the stromal cells have higher metabolic activity than the tumor cells, whereas in HER2 type and TNBC, the tumor cells have higher metabolic activity than the stromal cells. This result is consistent with former studies on reverse Warburg effect theory in

which a luminal type breast cancer cell line, MCF-7, was used *in vitro* cell line study, suggesting that in breast cancer, the stroma plays an energy supply role in cancer metabolism.[2-5] According to this theory, the metabolism of stromal cells is through glycolysis resulting from dysfunctional mitochondria caused by increased autophagy whereas that of tumor cells is through oxidative phosphorylation in functional mitochondria. This contrasts with the conventional Warburg effect theory which states that glycolysis is the major metabolic process in tumor cells. In the present study carried out with various cell lines shows that in HER2 type and TNBC, the tumor has more active metabolic status than the stroma, suggesting metabolic interaction between tumor cells and stromal cells differs according to the molecular subtypes. The common histologic features of TNBC such as high nuclear grade, high histologic grade, prominent necrosis, and increased mitotic activity corresponds the active metabolic status of this type of tumor,[10] and this was supported by the results of IHC in this study. Expression of glycolysis markers such as Glut-1, CAIX, and MCT-4 was highest in TNBC, and this is consistent with former studies revealing higher expression of Glut-1 and CAIX in basal-like breast cancer.[12] Tumoral expressions of Glut-1, CAIX, and MCT-4 were associated with factors reflecting higher metabolic activities. Tumoral expression of Glut-1 was associated with higher histologic grade ($P < 0.001$), ER negativity ($P < 0.001$), higher T stage ($P < 0.001$), higher Ki-67 LI ($P < 0.001$), and tumor recurrence ($P = 0.040$). Tumoral expression of CAIX was associated with higher Ki-67 LI ($P < 0.001$). Tumoral expression of MCT4 was associated with higher histologic grade ($P < 0.001$), ER negativity ($P < 0.001$), PR negativity ($P < 0.001$), higher T stage ($P < 0.001$), and higher Ki-67 LI ($P < 0.001$). In contrast, tumoral expressions of Glut-1, CAIX, and MCT-4 were lowest in luminal types. Luminal type tumors tend to show a lower histologic grade, less necrosis and lower mitotic count than HER-2 type or TNBC, thus showing non-active metabolic status of the tumor, and this was supported by the results of IHC.

The Warburg type (40.3%) and null type (44.0%) consisted major metabolic phenotypes in the present study. Each metabolic phenotype showed different characteristics. Mixed type had higher histologic grade, ER negativity, PR negativity, and higher Ki-67 index whereas null type had lower histologic grade, ER positivity, PR positivity, and lower Ki-67 index ($P < 0.001$). It could be suggested that mixed type that both tumor cells and stromal cells are glycolytic, consists of tumors showing high metabolic activity, and null type that both tumor cells and stromal cells are non-glycolytic, is a group

of tumors with lower metabolic activity. The results of this study reveal that mixed type had the highest percentage of activated autophagy whereas null type had the lowest percentage, thus supporting the hypothesis. In addition, different molecular phenotypes of breast cancer were classified into different metabolic subtypes. TNBC was the most common type in Warburg type and mixed type whereas luminal A was the most common type in reverse Warburg type and null type ($P < 0.001$). Warburg type and mixed type were classified into groups with a higher Ki-67 index, in contrast reverse Warburg type and null type were classified into groups with a lower Ki-67 index ($P < 0.001$). This study suggested that glycolysis of tumors significantly affects their metabolic and biological characteristics: Warburg type and mixed type were metabolically active and biologically aggressive, whereas reverse Warburg type and null type were metabolically inactive and biologically non-aggressive. The univariate analysis shows an association of Glut-1 with shorter DFS and OS, and this supports the hypothesis. One of the major limitations of this study is that the examination was quantitative analysis only and the effects of protein activities were not measured. Further research on the activity-based studies is required.

V. CONCLUSION

Breast cancer is heterogeneous in its metabolic status and metabolic interaction between tumor and stroma are different according to molecular subtypes of breast cancer. In luminal type, the expression levels of metabolism-related markers were higher in stroma than in tumor, and in HER2 type and TNBC, they were higher in tumor than in stroma. Luminal type was associated with reverse Warburg type and null type, whereas TNBC had strong associations with Warburg type and mixed type. This result suggests metabolic phenotypes of breast cancer have correlations with molecular subtypes along with biology of breast cancer.

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ABSTRACT(IN KOREAN)

유방암 분자아형에 따른 종양세포와 기질세포의 대사 관련 표지자 발현 차이

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암 세포에서 에너지 대사 작용에 대한 이론인 와버그 효과란, 암 세포에서는 일반적인 세포와 같이 산화적 인산화를 통해서가 아니라 호기성 해당작용을 통해 에너지를 얻는다는 것이다. 유방암의 경우 암 세포가 그 주변의 기질에 존재하는 섬유아세포에서 호기성 해당작용을 하도록 유도하여 에너지를 얻는다는 '역 와버그' 효과 이론이 제기되었다. 유방암은 형태학적으로 종양과 기질이 매우 다양한 모습으로 관찰되며, 유전자 발현에 대한 분석을 통해 luminal A, luminal B, HER-2 type, basal-like type 등의 분자아형으로 분류된다. 이 연구의 목적은 유방암의 분자아형에 따라 종양세포와 기질세포 사이의 대사작용에 어떠한 차이가 있는지 알아보고자 하는 것으로, 이를 위해 각 아형 별로 해당 작용, 미토콘드리아 상태, 자가 탐식 상태에 관련된 단백 표지자 발현의 차이를 분석하고 그 차이와 임상-병리학적 지표와의 관계를 살펴보았다. *in vitro* 유방암 세포주 연구를 위해 유방암 분자아형 별로 다섯 가지의 세포주(MCF-7, MDA-MB-453, MDA-MB-435S, MDA-MB-231, MDA-MB-486)를 선택하였고, 섬유아세포와 공동배양 후 Western blot 분석으로 대사 관련 표지자(해당작용; Glut-1, CAIX, 미토콘드리아 기능부전; GC1qR, BNIP3, 자가 탐식; beclin1, LC3A, LC3B)의 발현을 조사하였다. 각 세포주에서 종양세포와 기질세포에 각각 siRNA 를 이용한 Glut-1, LC3B 발현 억제 후 세포증식률을 측정하여 비교하였다. 인체 조직 microarray 연구는 2002 년부터 2005 년까지 침윤성 유방암으로 수술적 절제를 시행한 740 예를 대상으로 분자아형 분류에 관한 표지자(ER, PR, HER2, Ki67)에 대한 면역조직화학염색검사와 형광제자리부합검사, 대사 관련 표지자(해당작용; Glut-1, CAIX, MCT4, 미토콘드리아

기능부전; BNIP3, 자가 탐식; beclin1, LC3A, LC3B, p62)에 대한 면역조직화학염색검사를 시행하였다. 분자아형 분류에 관한 표지자(ER, PR, HER2, Ki67)의 결과에 따라 유방암을 luminal A, luminal B, HER-2 형, 삼중음성유방암으로 분류하였다. 유방암의 대사아형을 와버그형(종양: 해당 작용, 기질: 비-해당작용), 역 와버그형(종양: 비-해당작용, 기질: 해당작용), 혼합형(종양과 기질 모두 해당 작용), 비해당형(종양과 기질 모두 비-해당작용)과 같이 정의하고 이에 따라 분류하였다. 결과로 얻어진 자료와 환자의 임상-병리학적 지표를 통계학적으로 분석하였다. 세포배양 결과, 다섯 가지의 세포주중 MCF-7 에서는 종양세포보다 기질세포에서 대부분의 대사 관련 표지자가 더 높게 발현되었고, 나머지 HER2 형과 삼중음성유방암에 해당하는 세포주에서는 그와 반대로 기질세포보다 종양세포에서 더 높게 발현되었다. Glut-1, LC3B 발현을 억제한 후, 세포증식률을 측정한 결과, MCF-7 에서는 종양세포에 대한 억제를 시행한 경우보다 기질세포를 억제하였을 때 세포증식률이 더 많이 떨어졌고, 삼중음성유방암에서는 기질세포를 억제한 경우보다 종양세포를 억제하였을 때 세포증식률이 더 많이 떨어졌다. 인체 유방암 조직 microarray 검사 결과, luminal A 가 298 예(40.3%), luminal B 가 166 예(22.4%), HER2 형이 69 예(9.3%), 삼중음성유방암이 207 예(28%)였다. 분자아형 별로 임상병리학적 지표와 대사 관련 표지자 발현의 정도가 달랐다. 전체 조직 중 와버그형이 298 예(40.3%), 역 와버그형이 54 예(7.3%), 혼합형이 62 예(8.4%), 비해당형이 326 예(44%)였다. 와버그형과 혼합형에서는 삼중음성유방암이 가장 흔한 유형이었고, 역 와버그형과 비해당형에서는 luminal A 가 가장 흔한 유형이었다($P<0.001$). 혼합형은 높은 조직학적 등급, 높은 ER 음성률, 높은 PR 음성률, 높은 Ki67 지표를 보였고, 종양에서의 자가 탐식 상태의 활성화 비율이 높았다. 이에 반해 비해당형은 낮은 조직학적 등급, 높은 ER 양성률, 높은 PR 양성률, 낮은 Ki67 지표를 보였고, 종양에서의 자가 탐식 상태의 비활성화 비율이 높았다($P\leq0.001$). 유방암은 대사 작용에서도 다양성을 보이는 종양으로, 분자아형 별로 대사 관련 표지자의 발현 정도는 서로 달랐다. 유방암의 대사아형과 분자아형은 상관관계를 보였으며, 유방암의 임상양상과도 관련이 있었다.

핵심되는 말 : 유방암, 분자아형, 대사, 기질