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Lack of both androgen receptor and forkhead box A1 (FOXA1) expression is a poor prognostic factor in estrogen receptor-positive breast cancers

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Lack of both androgen receptor and forkhead box A1 (FOXA1) expression is a poor prognostic factor in estrogen receptor-positive breast cancers

Directed by Professor Seung Il Kim

The Doctoral 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

Seho Park

December 2016

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And last but not least, I would like to thank my parents and family for their tremendous encouragement as well as endless devotion until this day. I dedicate this doctoral dissertation to my better half, Yoon-Young Kim and my little princess, Seohyun Park with the deepest love in my heart.

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ABSTRACT

Lack of both androgen receptor and forkhead box A1 (FOXA1) expression is a poor prognostic factor in estrogen receptor-positive breast cancers

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(Directed by Professor Seung Il Kim)

Androgen receptor (AR) and forkhead box A1 (FOXA1) have been suggested to play an important role in breast cancer patients. However, the clinical significance of both biomarkers has not been established. The present study aimed to examine the associations between AR and FOXA1 and to investigate clinicopathological features and survival outcomes according to combined AR and FOXA1 status in estrogen receptor (ER)-positive breast cancers by web-based and clinical datasets analysis with an in vitro study. Using the cBioPortal for Cancer Genomics and Kaplan-Meier Plotter websites and tissue microarray (TMA) blocks of breast cancer patients treated at Severance Hospital between 1999 and 2005, genetic associations, clinicopathological characteristics and survival outcomes were evaluated according to mRNA and protein expressions of combined biomarkers by univariate and multivatiate analysis. T47D and ZR75-1 cells were used to explore the molecular connection between AR and FOXA1. Approximately 10% of samples in the cBioPortal website showed genetic alterations in ESR1, AR, and FOXA1 which generally co-occurred. The positive associations

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of mRNA expression were shown among 3 biomarkers. An in vitro study demonstrated that AR-overexpressing ER-positive cell lines decreased in cell proliferation through downregulation of ER mRNA and protein expression, but FOXA1 levels did not change. Overexpression of FOXA1 had no effect on ER activity and knockdown of FOXA1 resulted in a significant reduction of cell viability. In the immunohistochemical TMA study, AR positivity was significantly associated with FOXA1 positivity. The AR(-)/FOXA1(-) group frequently showed aggressive histopathological features and significantly poor survival outcomes in ER-positive patients. AR and FOXA1 mRNA levels were significantly higher in ER-positive than in ER-negative tumors by analysis of public datasets. Although the TCGA Provisional dataset analysis based on AR and FOXA1 mRNA status presented no statistical significance in survival outcomes of ER-positive breast cancer patients, AR-low/FOXA1-low tumors showed aggressive clinicopathological characteristics and poor disease-free survival in ER-positive cancers of the METABRIC dataset. The Kaplan-Meier Plotter analysis independently validated that patients with low AR/FOXA1 levels were significantly associated with lower relapse-free survival in ER-positive or luminal A subtype cancers. The present findings suggest that AR and FOXA1 are closely associated in breast cancers, and distinctive clinicopathological features according to combined biomarkers status are exhibited in ER-positive tumors. Importantly, lack of both AR and FOXA1 expression is an independently significant poor prognostic factor in ER-positive tumors. Clinical applications of AR and FOXA1 should be further studied to improve the survival of breast cancer patients.

Key words : androgen receptor, breast neoplasms, estrogen receptor, forkhead box A1, prognosis

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I. INTRODUCTION

Androgen receptor (AR) is a mainly ligand-dependent transcription factor that regulates target gene expression. The AR gene is located on the X chromosome. The 110-kDa AR phosphoprotein mediates diverse biological actions in the development and maintenance of the reproductive, musculoskeletal, cardiovascular, immune, neural, and hematopoietic systems and is involved in the development of malignancies in the prostate, bladder, liver, kidney, and lung.[1,2]

Recent attention has focused on the emerging roles of AR not only as a prognostic and predictive factor, but also as a therapeutic target in breast cancer patients.[3-5] A systematic review and meta-analysis showed that positive AR expression was significantly associated with better survival of patients with early breast cancer irrespective of estrogen receptor (ER) status.[6] However, in

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vitro evidence partly supported clinical studies and AR showed antiproliferative activity in only ER-positive breast cancers but rather AR signaling promoted tumor growth in ER-negative and human epidermal growth factor receptor 2 (HER2)-positive breast tumors.[3,7] Furthermore, Lehmann et al.[8] identified six subtypes of triple-negative breast cancer (TNBC), one of them being a luminal androgen receptor (LAR) subtype with distinct features among diversely heterogeneous TNBCs.[9] Many studies consistently suggest that AR is a favorable biomarker in hormone receptor-positive tumors, but the clinical or biological impact of AR has not been clearly defined. Therefore, additional approaches are necessary to clarify the various roles of AR and its control mechanisms according to ER status.

Forkhead box A1 (FOXA1), initially discovered as hepatocyte nuclear factor 3α (HNF3α), is a member of the FOX family transcription factors.[10] Because of a lack of the basic amino acids in FOXAs for chromatin compaction, binding of FOXAs to nucleosomes creates an open chromatin configuration that can recruit other transcriptional regulators.[11,12] Thus, FOXA1 belongs to a 'pioneering factor'.[13]

Recent meta-analyses of breast cancers demonstrated that high FOXA1 levels were positively correlated with ER-positive and progesterone receptor (PR)-positive tumors.[14] Patients with high FOXA1 expression showed better disease-free survival (DFS) and overall survival (OS).[15] A study by Hurtado et al.[16] supported that FOXA1 played a key role in differentially influencing interactions between ER and chromatin. It was required for almost binding events and transcriptional activities of ER in breast cancer cells. Genetic analysis of invasive lobular carcinomas, which were predominantly categorized as the luminal A subtype, exhibited recurrent FOXA1 mutations and correlation with high FOXA1 activity.[17] The data confirmed that FOXA1 was closely associated with the ER signaling pathway and suggested that FOXA1 may explain heterogeneous features of hormone receptor-positive tumors.

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In contrast, molecular apocrine breast tumors are characterized by apocrine features upon histopathological examination, ER-negativity, AR-positivity, and a high association with HER2 amplification.[18,19] Even though most molecular apocrine breast cancers are ER-negative, they have AR-driven, hormonally regulated transcriptional activities mediated by FOXA1, similar to ER-mediated transcription in luminal subtype breast cancers.[20] Immunohistochemically, AR and FOXA1 were expressed in 100% (54/54) and 93% (50/54) of histologically diagnosed, almost ER-negative apocrine breast cancers, respectively.[21] In hormone-dependent prostate cancers, FOXA1 is a global mediator of AR action and facilitates prostate cancer growth.[22] A recent study suggested that FOXA1 promotes cell proliferation through AR by activation of the Notch pathway in endometrial cancers.[23] An ancillary immunohistochemistry (IHC) study of AR and FOXA1 in 592 TNBCs from the UNICANCER PACS08 adjuvant multicenter trial suggested that co-expression of both markers seems to be associated with distinct clinicopathological features of luminal tumors compared to other TNBCs.[24] These findings implied a close molecular connection between AR and FOXA1 and the important clinical roles of both biomarkers in various cancer types, including breast malignancy. However, the clear genetic or clinical implications of these biomarkers on tumor biology and patient prognosis have not been fully explained according to ER status of breast cancer, especially in ER-positive tumors.

The purpose of the present study was to explore the genetic expression patterns and associations between AR and FOXA1 by ER status determined from web-based breast cancer genetic datasets. Next, it was to examine the influence among biomarkers through an in vitro ER-positive cell lines study. Finally, the present study aimed to investigate and validate clinicopathological characteristics and survival outcomes according to combined AR and FOXA1 protein and mRNA status in mainly ER-positive patients using clinical data of a single institution and public datasets.

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II. MATERIALS AND METHODS

1. Web-based bioinformatics analysis

A. The cBioPortal for Cancer Genomics

Genomic analysis was performed for investigating the associations between ESR1, AR, and FOXA1 through the cBioPortal for Cancer Genomics (http://www.cbioportal.org), which provides web-based visualization and access to large-scale cancer genomic datasets including The Cancer Genome Atlas (TCGA) data.[25,26] The Breast Invasive Carcinoma (TCGA, Provisional) and the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) datasets were selected for analysis.

Each dataset included RNA sequencing data and clinicopathological information of 1,105 samples obtained from 1,098 patients in the TCGA Provisional and of 1,980 samples obtained from 1,980 patients in the METABRIC datasets (June, 2016).[27] Expression by RNA Seq Version 2 [RNA-Seq by Expectation Maximization (RSEM)] and U133 microarray in each dataset, respectively, was selected to generate an OncoPrint in the cBioPortal website for visualizing the genetic alteration and to investigate the mutual exclusivity or co-occurrence of alterations among biomarkers in all analyzed samples. The network view was extracted using the TCGA Provisional dataset to show ESR1, AR, and FOXA1 in the context of biological interactions derived from public pathway databases. The interaction types were derived from the BioPAX to binary interaction mapping rules defined within Pathway Commons (http://www.pathwaycommons.org) and were shown closely connected between ESR1, AR, and FOXA1 on the website.

Raw data of AR and FOXA1 mRNA expression and clinical information in each dataset were downloaded from the cBioPortal website to explore the association of AR and FOXA1 status with clinicopathological

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characteristics and survival, mainly in immunohistochemically determined ER-positive breast tumors. The lower quartile cutoff values were selected to determine high and low expression levels of AR and FOXA1. Of 1,105 cases in the TCGA Provisional dataset, samples coded as metastatic disease (n = 22) or missing AR or FOXA1 data (n = 5) were excluded in the present study. Of 1,980 samples within the METABRIC dataset, cases with stage 0 disease (n = 12) or stage IV disease (n = 10) were excluded from analysis. Upon exclusion, 1,078 nonmetastatic invasive carcinomas from the TCGA Provisional dataset and 1,958 samples with stage I–III disease from the METABRIC dataset were analyzed for survival in this study.

B. The Kaplan-Meier (KM) Plotter

The probability of relapse-free survival according to AR and FOXA1 status including subgroup analyses was calculated using the KM Plotter (http://kmplot.com/analysis).[28] It is an online tool that allows analysis of the effects of 54,675 genes on survival by using 10,188 cancer samples, which includes 4,142 breast cancer patients with a mean follow-up duration of 69 months (June, 2016). Survival and gene expression data were derived from the Gene Expression Omnibus (Affymetrix microarray only), European Genome-phenome Atlas, and TCGA. The Affymetrix probe set IDs selected were 226197_at for AR and 204667_at for FOXA1 in the present study. Multiple genes were entered through a multigene classifier using the mean expression of selected biomarkers. To analyze the prognostic value of combined AR and FOXA1, the patient samples were split into two groups using the lower quartile as a cutoff value. Hazard ratio (HR) with 95% confidence interval (CI) and log-rank *p*-value were calculated, and survival curves were displayed on the webpage.

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2. In vitro cell lines study

A. Cell culture

Human breast cancer cell lines (T47D and ZR75-1) were obtained from the American Type Culture Collection (Manassas, VA, USA). All reagents related to animal cell culture were purchased from Life Technologies (Big Cabin, OK, USA). Cells were cultured in Dulbecco's modified Eagle's medium. All media contained 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cells were cultured at 37°C in a 5%-CO_2 humidified environment.

B. Cell counting

Cells (1×10^4 cells/well) were plated on 12-well plates and counted every 24 hours for 5 days using the ADAM-MC automatic cell counter (NanoEnTek Inc., Seoul, South Korea).

C. Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cultured cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For quantitative real-time RT-PCR, cDNAs were synthesized from 4 μg of total RNA using random hexamer primers and SuperScript reverse transcriptase II (Invitrogen) following the manufacturer's instructions. Diluted cDNAs were analyzed for qPCR using the SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) and gene-specific primers, and then subjected to RT-PCR quantification using the ABI PRISM 7300 RT-PCR System (Applied Biosystems).

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Table 1. Primers used for real-time RT-PCR

Gene symbol	GenBank Accession No.	Sequence
ESR1	NM_000125	FW: ATGACTATGCTTCAGGCTACCATT RV: GTGGCTGGACACATATAGTCGTTA
AR	NM_000044	FW: CGACCAGATGGCTGTCATTC RV: TGTGCATGCGGTACTCATTG
FOXA1	NM_004496	FW: ACTCCTTCAACCACCCGTTC RV: GCGAGTATTGCAGTGCCTGT

D. Overexpression and knockdown assay

For the stable overexpression of AR, the fragment encoding the full-length cDNA of AR was cloned into the pLL-CMV-puro lentiviral vector. Plasmid DNAs and a lentiviral packaging mix containing an envelope and packaging vector were transfected into human embryonic kidney (HEK293T) cells according to the manufacturer's instructions to produce lentiviruses packed with AR cDNA cassettes. Positive cells harboring AR cDNA cassette were selected by 1 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA) selection after infection. For the knockdown assay, targeting small interfering RNA (siRNA) and non-targeting control siRNA were transfected into cells utilizing Lipofectamine RNAiMax reagent (Invitrogen) following the manufacturer's protocols. The sequences of targeting oligo duplex against FOXA1 were as follows: 5`–GAGAGAAAAAATCAACAGCTT–3`(sense) and 5`–GCTGTTG ATTTTTTCTCTCTT–3`(antisense) (Integrated DNA Technologies Inc., Coralville, IA, USA).

E. Cell viability assay

Cell viability was determined by EZ-Cytox Cell Viability Assay Kit (Daeil Lab Service, Seoul, South Korea) based on the cleavage of the tetrazolium salt to water-soluble formazan by succinate-tetrazolium reductase. Cells in suspension with siRNA mixtures were transferred to 96-well plate ($5x10^3$ cells/well) followed by medium changed the next day. After 48 hours,

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Ez-Cytox reagent (10 ul/well) was added and absorbance (OD_{450}) was detected at 450 nm after 4 hours.

F. Western blot analysis

Cultured cells were washed twice with ice-cold phosphate-buffered saline and harvested in whole-cell lysis buffer (1% sodium dodecyl sulfate, 60 mM Tris-HCl, pH 6.8). Protein concentrations were measured by the bicinchoninic acid assay. Equal amounts of protein extracts were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose transfer membranes (Whatman GmbH, Dassel, Germany). The membranes were blocked in 5% (w/v) non-fat Difco™ skimmed milk (BD Biosciences, San Jose, CA, USA), followed by incubation with the primary antibodies in 1% bovine serum albumin. The following antibodies were used: anti-AR (custom-made), anti-ERα (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and α-tubulin (Calbiochem, Brookfield, WI, USA).

G. Luciferase assay

Luciferase activities in whole cell lysates were measured using the Dual-Luciferase Reporter Assay System® (Promega Corp., Madison, WI, USA). Using a pRL-SV40 construct (Promega Corp.), luciferase activity was normalized to each cell lysate's *Renilla* luciferase activity levels.

3. Tissue microarray (TMA) study

A. Study population

A previous study cohort was selected to investigate the clinical implications of immunohistochemically determined AR and FOXA1 expression levels on breast cancer patients' survival outcomes. Immunohistochemical AR expression was evaluated from TMA blocks of 931 patients treated between

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November 1999 and August 2005.[29] Using consecutive slides of prior TMA blocks, FOXA1 expression was evaluated by IHC and was determined to be uninterpretable in 65 cases. The remaining patients (n = 866) who had both readable AR and FOXA1 expression were analyzed in the present study.

B. Clinicopathological parameters

Patient demographics, histopathology of primary tumor, treatment patterns and survival rates were retrospectively obtained from medical records. Patients were treated with either total mastectomy or breast conservation surgery and either sentinel lymph node biopsy or axillary lymph node dissection. After surgery, local radiotherapy or adjuvant systemic treatments were administered if a patient was able to tolerate them. Clinical follow-up included history-taking, physical examinations, laboratory tests, and radiologic imaging every 6–12 months in order to detect any signs of relapse. Tumor-node-metastasis (TNM) stage was determined from the 6th American Joint Committee on Cancer criteria. Histological grade was assessed by the modified Bloom-Richardson classification.

Local recurrence was defined as the reappearance of carcinoma in the treated remnant breast, skin, or chest wall. Events determining regional relapse were defined as recurrences to the ipsilateral axillary, supraclavicular, or internal mammary lymph nodes. Any recurrence at a distant site including the contralateral axillary or supraclavicular lymph nodes was considered to be a distant metastasis. DFS time was measured from the date of the first curative surgery to the date of the first local, regional, or distant recurrence or death without any type of relapse. OS time was measured from the date of the first operation to the date of the last follow-up or death from any cause.

C. Immunohistochemical staining

Prior TMA tumor blocks were constructed using formalin-fixed,

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paraffin-embedded tumor samples as detailed in procedure descriptions from a previous study.[29] TMA sections were deparaffinized and rehydrated. After treatment with 3% hydrogen peroxide solution for 10 minutes to block endogenous peroxidase, sections were pretreated in 10 mM citrate buffer for antigen retrieval in a microwave oven for 20 minutes. After incubation with primary antibodies against AR (AR 441, 1:100; Thermo Scientific, Fremont, CA, USA), FOXA1 (2F83, 1:4,000; Abcam, Cambridge, United Kingdom), ER (SP1, 1:100; Thermo Scientific), PR (PgR 636, 1:50; DAKO, Glostrup, Denmark), HER2 (polyclonal, 1:1,500; DAKO), and Ki-67 (MIB-1, 1:100; DAKO), immunodetection was performed with biotinylated anti-mouse/rabbit immunoglobulin, followed by peroxidase-labeled streptavidin using a labeled streptavidin biotin kit with 3,3'-diaminobenzidine chromogen as the substrate. The slides were counterstained with Harris hematoxylin. IHC was interpreted in a blind fashion, without any information regarding clinical parameters or outcomes.

Tumors with ≥ 10% positively nuclear-stained cells were considered positive for AR expression. Considering the proportion and staining intensity, FOXA1 expression was categorized as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). An arbitrary cutoff point of ≥ 2 was applied to determine FOXA1-positivity. Tumors with ≥ 1% nuclear-stained cells were considered positive for ER and PR based on the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines.[30] HER2 status was evaluated using the HercepTest™ (DAKO) and was interpreted as 0, 1+, 2+, or 3+ according to the ASCO/CAP guidelines.[31] In cases with HER2 2+ results, fluorescence in situ hybridization (FISH) was performed using a PathVysion HER2 DNA Probe Kit (Vysis, Downers Grove, IL, USA). HER2 gene amplification was classified as a case with either HER2 gene/chromosome 17 copy number ratio ≥ 2.0 or < 2.0, along with an average HER2 copy number ≥ 6.0 signals/cell as determined by ASCO/CAP guidelines.[31] HER2 was

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considered positive in cases with a 3+ IHC score or gene amplification by FISH regardless of the HER2 IHC result. Ki-67 levels were scored by counting the number of positively stained nuclei and were expressed as a percentage of total tumor cells.

Based on the IHC scores or FISH findings of ER, PR, HER2, and Ki-67 expression, breast cancer subtypes were categorized as follows: luminal A (ER+ and/or PR+, HER2–, and Ki-67 < 15%); luminal B (ER+ and/or PR+, HER2–, and Ki-67 ≥ 15% or ER+ and/or PR+ and HER2+ irrespective of Ki-67 expression); HER2-positive (ER–, PR–, and HER2+); and TNBC (ER–, PR–, and HER2–).

4. Statistical analysis

Web-based bioinformatics statistics including mutual exclusivity, correlation coefficient (*r*), HR with 95% CI, and a log-rank *p*-value were automatically calculated in each website and the results were displayed. An independent, two-sample *t*-test was used to compare the means of continuous numerical datasets. Differences between the groups were evaluated by a chi-square test. In order to analyze the downloaded TCGA Provisional and METABRIC datasets and the TMA study, survival curves were plotted using the Kaplan-Meier method and group differences in survival time were investigated by a log-rank test. A Cox's proportional hazards model was used to identify the variables that were independently associated with survival. All statistical tests were two-tailed and a p-value < 0.05 was considered statistically significant. SPSS version 23.0 (IBM Inc., Armonk, NY, USA) was used for all statistical analyses.

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III. RESULTS

1. Web-based bioinformatics analysis

A. Genetic alteration in ESR1, AR, and FOXA1

First, the mRNA expression status of ESR1, AR, and FOXA1 was explored using the TCGA Provisional and METABRIC datasets. Biomarker information was obtained in 1,100 samples from 1,093 patients of the TCGA Provisional dataset and in 1,980 samples from 1,980 patients of the METABRIC dataset. The TCGA Provisional dataset showed that genetic alteration of ESR1, AR, and FOXA1 was in 4%, 5%, and 2.3% of all analyzed samples, respectively (Fig. 1A). One or more genes were altered in 112 (10.2%) queried samples. There was no mRNA downregulation of biomarkers. In the METABRIC dataset, genetic alteration of AR and FOXA1 was noted in 4% and 11% of total cases, respectively (Fig. 1B). Queried gene set was changed in 245 (12.4%) samples. There was no alteration in ESR1 mRNA and only mRNA downregulation was presented in FOXA1. The METABRIC dataset was able to add a clinical attribute track and when the PAM50 subtype was applied, AR and FOXA1 were mainly altered in the basal subtype.

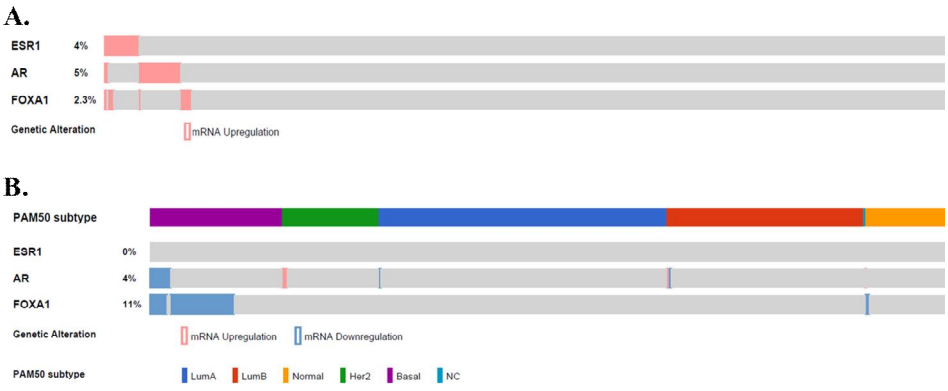

Figure 1. Genomic alteration by the OncoPrint. (A) The TCGA Provisional and (B) METABRIC datasets.

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When the mutual exclusivity or co-occurrence of alterations among these 3 biomarkers was investigated in the TCGA Provisional dataset, ESR1 showed a significant tendency towards co-occurrence with FOXA1 ($p < 0.001$; log odds ratio > 2.816). A tendency towards co-occurrence between AR and FOXA1 was also significant in all samples ($p = 0.038$; log odds ratio = 1.281). Alteration in ESR1 mRNA showed a weak trend of co-occurrence with AR, but there was no statistical significance ($p = 0.198$; log odds ratio = 0.618). In the METABRIC dataset, co-occurrence of alterations could be only calculated between AR and FOXA1 because there was no alteration in ESR1 mRNA. A significant tendency towards co-occurrence between AR and FOXA1 was determined in all queried samples ($p < 0.001$; log odds ratio = 2.474).

B. Associations of mRNA expression among 3 biomarkers

Figure 2 shows the positive associations of mRNA expression between ESR1, AR, and FOXA1. Pearson and Spearman correlation coefficient between biomarkers in each dataset is calculated in Figure 2. Among the 3 genes, the positive coefficient was the highest between ESR1 and FOXA1, subsequently between AR and FOXA1, and followed by ESR1 and AR.

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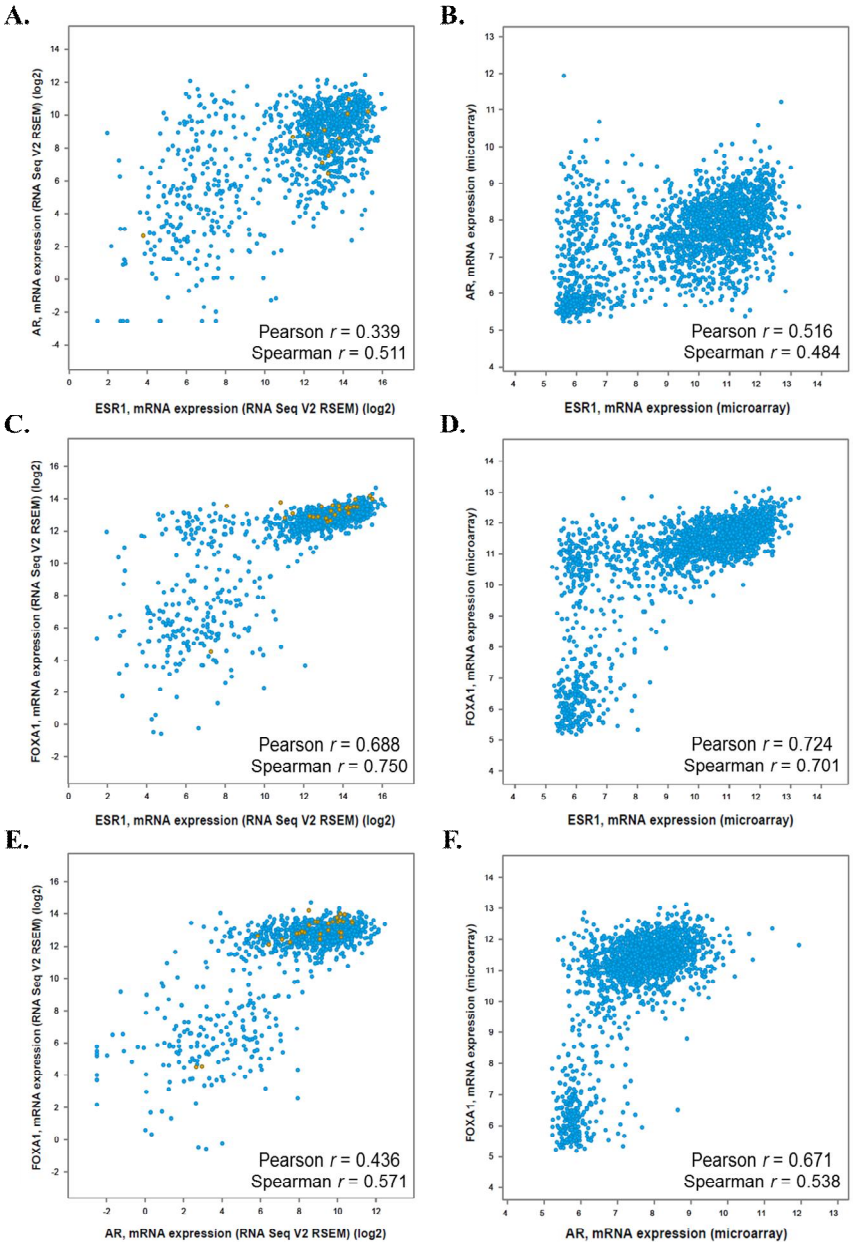

Figure 2. Associations of mRNA expression between ESR1, AR, and FOXA1. Plots shows the association (A, B) between ESR1 and AR, (C, D) between ESR1 and FOXA1, and (E, F) between AR and FOXA1. Blue dots indicate that neither gene is mutated and yellow dots express that one gene is mutated. (A, C, E) The TCGA Provisional and (B, D, F) METABRIC datasets.

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C. The network from public pathway databases

The cBioPortal website provides information of pathway and interaction data including drugs targeting genes in the network. Sources of the data are from the Human Protein Reference Database (HPRD), Reactome, National Cancer Institute (NCI)-Nature Pathway Interaction Database, and the Memorial Sloan Kettering Cancer Center (MSKCC) Cancer Cell Map, as all derived from Pathway Commons.[26] The network view, generated from the TCGA Provisional dataset, is presented in Figure 3. By setting the "Filter Neighbors by Alteration" to 10%, 3 query genes (ESR1, AR, and FOXA1) and 35 frequently altered neighbor genes (ABI1, APPBP2, AURKA, CARM1, CCND1, CREBBP, CSNK2A1, EBAG9, EFNA1, FOXM1, GNA13, HSF2, IKBKE, INPPL1, KAT5, KDM4C, MAPK1, MAPK3, MED1, NCOA2, NCOA3, NR1D1, PCNA, PHB2, PIP5K1A, PSEN2, RAC1, RORC, RPS6KB1, RXRB, SLC9A3R1, SLC9A3R2, SMAD4, SRC, and UBE2I) out of a total of 432 were displayed on the webpage. Interaction types were "controls state change of (23.7%)", "controls transport of (3.6%)", "controls phosphorylation of (9.6%)", "controls expression of (2.6%)", "catalysis precedes (1.7%)", "in complex with (10.3%)", "neighbor of (18.6%)", and "targeted by drug (29.9%)". Among them, Figure 3 shows 24 neighbor genes and 5 interaction types with known gene function in tumor cells or a close connection between ESR1, AR, and FOXA1.

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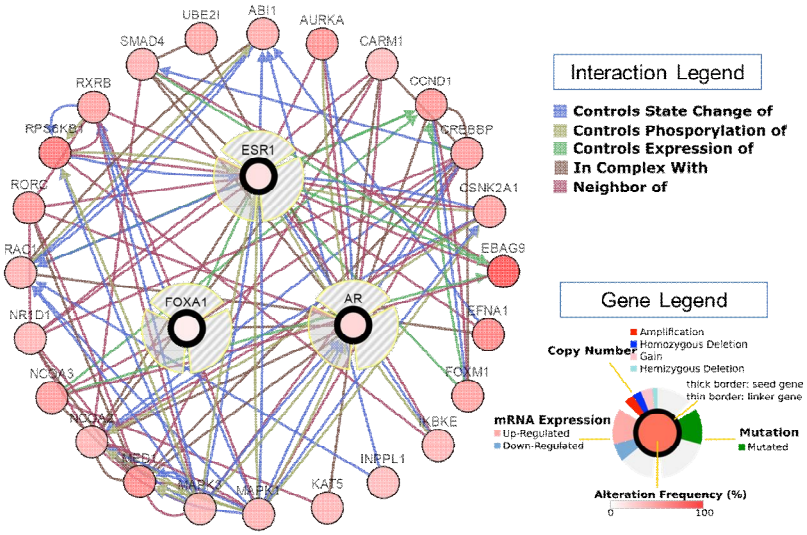

Figure 3. The network between the 3 query genes (ESR1, AR, and FOXA1) and frequently altered neighbor genes from the TCGA Provisional dataset. Copy number alteration and mutation of the query genes are not entered into the network analysis. Only interactions closely connected among the query genes are presented.

2. In vitro cell lines study

To investigate the association between AR and FOXA1 in ER-positive tumors, an in vitro study was performed using T47D and ZR75-1 breast cancer cell lines. As shown in Figures 4A and 4B, stable cell lines overexpressing AR in both T47D and ZR75-1 exhibited significant decrease in cell proliferation compared with negative control (mock) cells. Notably, Western blot and real-time RT-PCR analyses showed reduced expression levels of ER protein and mRNA in AR-overexpressing cancer cell lines, which suggested that downregulation of ER expression might affect cell proliferation (Figs. 4C and 5B). Next, in order to test the possibility that FOXA1 expression could be altered by AR overexpression, mRNA level of FOXA1 was checked. However, FOXA1 expression level was not changed by overexpression of AR (Fig. 5C).

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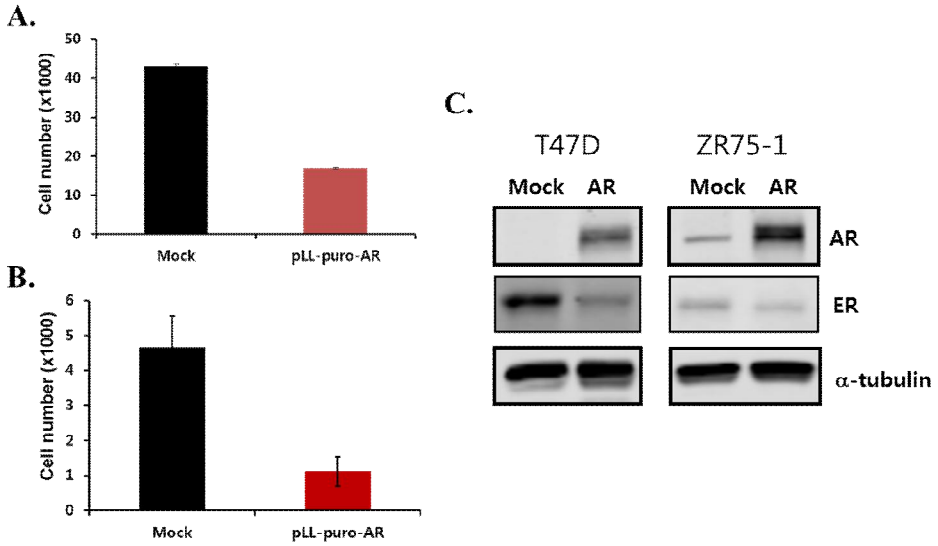

Figure 4. Decrease in cell proliferation of T47D and ZR75-1 cell lines by lentiviral overexpression of AR. Empty vector, pLL-CMV-puro was utilized for lentivirus production as a mock control. Stable AR overexpression of (A) T47D and (B) ZR75-1 cells decreased the number of cells at day 6. (C) Protein level of AR, ER, and α-tubulin by Western blot analysis. α-tubulin was detected as a loading control.

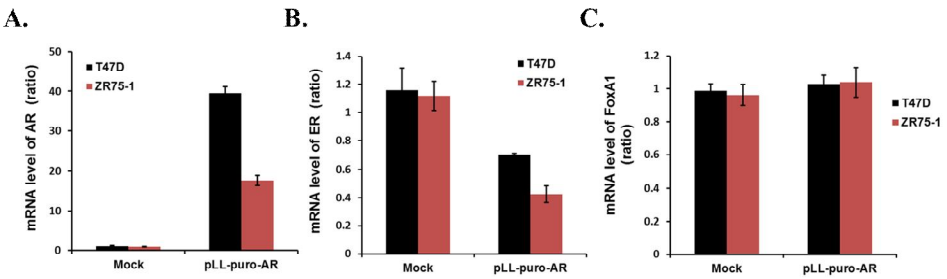

Figure 5. Levels of AR, ER, and FOXA1 mRNA in T47D and ZR75-1 cell lines. (A) mRNA levels of AR were measured by real-time RT-PCR analysis as described in Materials and Methods. (B) Overexpression of AR significantly decreased mRNA levels of ER. (C) No effects of AR overexpression on mRNA levels of FOXA1 were observed.

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Next, the effects of FOXA1 overexpression on ER activity were compared in mock- and AR-overexpressing T47D cell lines. As shown in Figure 6A, overexpression of FOXA1 in these cell lines had no effect on ER activity. However, knockdown of FOXA1 resulted in a significant reduction of cellular viability on day 5 (Fig. 6C), suggesting that FOXA1 has essential roles for viability of the ER-positive tumor cell lines, although there were no direct effects on ER and AR activities (Fig. 6).

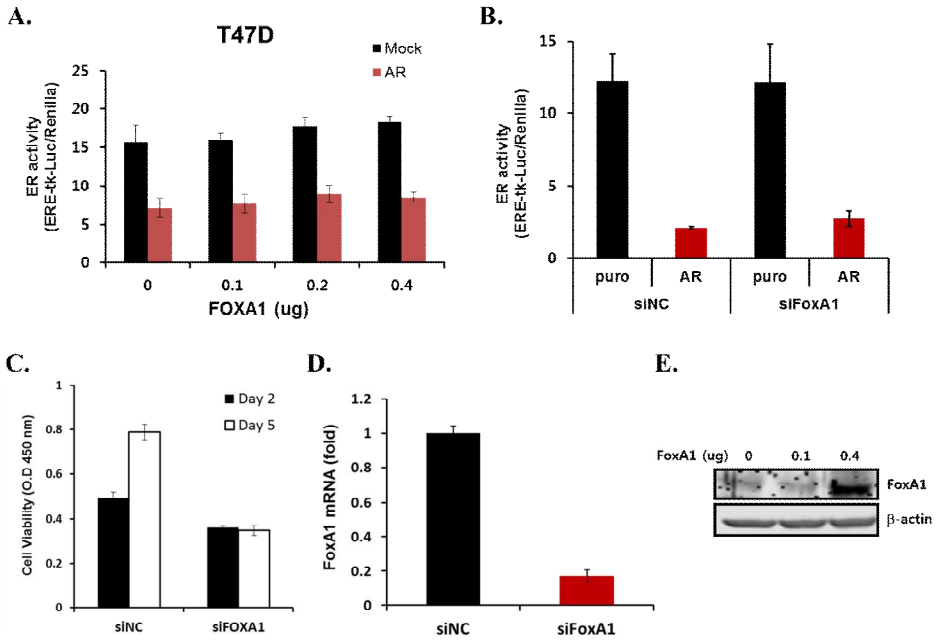

Figure 6. Effects of FOXA1 overexpression on ER activity. (A and B) ERE-tk-luciferase activity was normalized by the *Renilla* expression level. (C) Viability of T47D cells was measured at day 2 and day 5 after the treatments of siRNA against non-targeted sequence and FOXA1. (D) FOXA1 mRNA levels were evaluated after treatments of siRNA by quantitative real-time RT-PCR analysis. (E) Western blot analysis was performed to detect protein level of FOXA1. Expression of β-actin was analyzed as a loading control.

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3. Clinicopathological characteristics and patient survival in the TMA study

Using the breast cancer patient population treated at a single institute, the prognostic value of immunohistochemically determined AR and FOXA1 status was investigated. In all patients, AR and FOXA1 positivity was 55.8% and 72.1%, respectively. AR positivity was significantly associated with FOXA1 positivity ($p < 0.001$) and 384 (44.3%) patients had tumors that were AR and FOXA1 positive. AR and FOXA1 negativity was noted in 143 (16.5%) patients. Table 2 shows the clinicopathological characteristics according to AR and FOXA1 status. AR(+)/FOXA1(+) tumors were significantly associated with small tumor size, lower TNM stage, grade I/II, hormone receptors-positive expression, and luminal A subtype. Patients with AR(+)/ FOXA1(-) tumors showed the highest frequency of HER2-positive, low Ki-67 proliferative index tumors. Treatment patterns were not significantly different among groups except for endocrine therapy.

Table 2. Clinicopathological characteristics according to AR and FOXA1 expression in the whole population of the TMA study

Factor	AR(+)/ FOXA1(+) (n = 384, %)	AR(+)/ FOXA1(-) (n = 99, %)	AR(-)/ FOXA1(+) (n = 240, %)	AR(-)/ FOXA1(-) (n = 143, %)	*p*-value
Age (yrs)					
≤ 50	241 (62.8)	53 (53.5)	162 (67.5)	91 (63.6)	0.115
> 50	143 (37.2)	46 (46.5)	78 (32.5)	52 (36.4)	
Type					
Ductal	347 (90.4)	93 (93.9)	207 (86.2)	129 (90.2)	0.158
Lobular/special	37 (9.6)	6 (6.1)	33 (13.8)	14 (9.8)	
Tumor stage					
pT1	207 (53.9)	41 (41.4)	88 (36.7)	50 (35.0)	< 0.001
pT2-4	177 (46.1)	58 (58.6)	152 (63.3)	93 (65.0)	
Node stage					
pN0	204 (53.1)	46 (46.5)	120 (50.0)	66 (46.2)	0.424
pN1-3	180 (46.9)	53 (53.5)	120 (50.0)	77 (53.8)	

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Factor	AR(+)/ FOXA1(+) (n = 384, %)	AR(+)/ FOXA1(-) (n = 99, %)	AR(-)/ FOXA1(+) (n = 240, %)	AR(-)/ FOXA1(-) (n = 143, %)	*p*-value
Histologic grade					
I/II	325 (84.6)	77 (77.8)	145 (60.4)	82 (57.3)	< 0.001
III	59 (15.4)	22 (22.2)	95 (39.6)	61 (42.7)	
ER					
Positive	356 (92.7)	68 (68.7)	141 (58.8)	60 (42.0)	< 0.001
Negative	28 (7.3)	31 (31.3)	99 (41.2)	83 (58.0)	
PR					
Positive	307 (79.9)	61 (61.6)	122 (50.8)	49 (34.3)	< 0.001
Negative	77 (20.1)	38 (38.4)	118 (49.2)	94 (65.7)	
HER2					
Negative	293 (76.3)	64 (64.6)	180 (75.0)	117 (81.8)	0.023
Positive	91 (23.7)	35 (35.4)	60 (25.0)	26 (18.2)	
Ki-67 (n = 864)					
< 15%	345 (90.1)	89 (90.8)	156 (65.0)	73 (51.0)	< 0.001
≥ 15%	38 (9.9)	9 (9.2)	84 (35.0)	70 (49.0)	
Breast cancer subtypes (n = 864)					
Luminal A	267 (69.7)	52 (53.1)	98 (40.8)	42 (29.4)	< 0.001
Luminal B	89 (23.2)	17 (17.3)	53 (22.1)	26 (18.2)	
HER2-positive	18 (4.7)	20 (20.4)	21 (8.8)	16 (11.2)	
TNBC	9 (2.3)	9 (9.2)	68 (28.3)	59 (41.3)	
Type of surgery					
BCS	116 (30.2)	28 (28.3)	68 (28.3)	37 (25.9)	0.800
TM	268 (69.8)	71 (71.7)	172 (71.7)	106 (74.1)	
Radiation therapy					
Not done	201 (52.3)	49 (49.5)	125 (52.1)	75 (52.4)	0.964
Done	183 (47.7)	50 (50.5)	115 (47.9)	68 (47.6)	
Chemotherapy					
Not done	58 (15.1)	16 (16.2)	25 (10.4)	15 (10.5)	0.210
Done	326 (84.9)	83 (83.8)	215 (89.6)	128 (89.5)	
Endocrine therapy					
Not done	58 (15.1)	37 (37.4)	110 (45.8)	87 (60.8)	< 0.001
Done	326 (84.9)	62 (62.6)	130 (54.2)	56 (39.2)	

AR: androgen receptor, ER: estrogen receptor, PR: progesterone receptor, HER2: human epidermal growth factor receptor 2, TNBC: triple-negative breast cancer, BCS: breast conservation surgery, TM: total mastectomy.

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When examining the clinicopathological characteristics of ER-positive tumors based on AR and FOXA1 status, similar trends were observed between the AR(+)/ FOXA1(+) group and histopathological parameters, including small tumor size, node-negative disease, lower TNM stage, histologic grade I/II, and PR-positive expression (Table 3). The AR(-)/FOXA1(-) group frequently showed node metastasis, high grade, PR-negative expression, and high Ki-67 proliferation.

Table 3. Clinicopathological characteristics according to AR and FOXA1 expression in ER-positive breast cancer patients from the TMA study

Factor	AR(+)/ FOXA1(+) (n = 356, %)	AR(+)/ FOXA1(-) (n = 68, %)	AR(-)/ FOXA1(+) (n = 141, %)	AR(-)/ FOXA1(-) (n = 60, %)	*p*-value
Age (yrs)					
≤ 50	230 (64.6)	40 (58.8)	101 (71.6)	38 (63.3)	0.269
> 50	126 (35.4)	28 (41.2)	40 (28.4)	22 (36.7)	
Tumor stage					
pT1	199 (55.9)	32 (47.1)	51 (36.2)	26 (43.3)	< 0.001
pT2-4	157 (44.1)	36 (52.9)	90 (63.8)	34 (56.7)	
Node stage					
pN0	189 (53.1)	32 (47.1)	59 (41.8)	21 (35.0)	0.019
pN1-3	167 (46.9)	36 (52.9)	82 (58.2)	39 (65.0)	
Histologic grade					
I/II	309 (86.8)	58 (85.3)	109 (77.3)	46 (76.7)	0.030
III	47 (13.2)	10 (14.7)	32 (22.7)	14 (23.3)	
PR					
Positive	306 (86.0)	59 (86.8)	112 (79.4)	41 (68.3)	0.004
Negative	50 (14.0)	9 (13.2)	29 (20.6)	19 (31.7)	
HER2					
Negative	284 (79.8)	54 (79.4)	104 (73.8)	52 (86.7)	0.203
Positive	72 (20.2)	14 (20.6)	37 (26.2)	8 (13.3)	
Ki-67 (n = 623)					
< 15%	326 (91.8)	65 (97.0)	122 (86.5)	45 (75.0)	< 0.001
≥ 15%	29 (8.2)	2 (3.0)	19 (13.5)	15 (25.0)	
Type of surgery					
BCS	106 (29.8)	24 (35.3)	33 (23.4)	10 (16.7)	0.053
TM	250 (70.2)	44 (64.7)	108 (76.6)	50 (83.3)	

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Factor	AR(+)/ FOXA1(+) (n = 356, %)	AR(+)/ FOXA1(-) (n = 68, %)	AR(-)/ FOXA1(+) (n = 141, %)	AR(-)/ FOXA1(-) (n = 60, %)	*p*-value
Radiation therapy					
Not done	187 (52.5)	32 (47.1)	77 (54.6)	35 (58.3)	0.609
Done	169 (47.5)	36 (52.9)	64 (45.4)	25 (41.7)	
Chemotherapy					
Not done	55 (15.4)	11 (16.2)	12 (8.5)	10 (16.7)	0.191
Done	301 (84.6)	57 (83.8)	129 (91.5)	50 (83.3)	
Endocrine therapy					
Not done	30 (8.4)	9 (13.2)	20 (14.2)	12 (20.0)	0.031
Done	326 (91.6)	59 (86.8)	121 (85.8)	48 (80.0)	

During mean follow-up periods of 112.8 months [standard deviation (SD) = 39.9], 222 (25.6%) patients had pre-defined events and 183 (21.1%) patients died. DFS and OS curves according to AR and FOXA1 status showed no statistically significant prognostic value in all patients (Fig. 7).

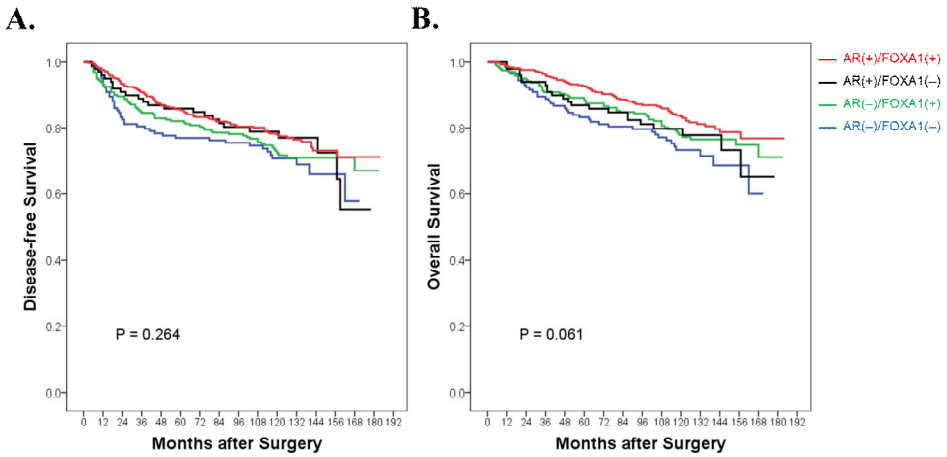

Figure 7. Survival curves according to AR and FOXA1 status in all patients of the TMA study. (A) Disease-free survival and (B) Overall survival curves.

However, when survival stratified by ER status was analyzed, AR(-)/FOXA1(-) tumors showed significantly worse DFS and OS than either AR(+) or FOXA1(+) tumors in ER-positive patients. Among AR(+) and/or FOXA1(+) tumors, there was no statistical difference in survival according to

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AR/FOXA1 status in patients with ER-positive tumors (Figs. 8A and 8B). No statistical difference in survival among groups was demonstrated in ER-negative tumors (Figs. 8C and 8D). Rather, AR(-)/FOXA1(-) tumors rather showed a trend toward better survival in the TMA study.

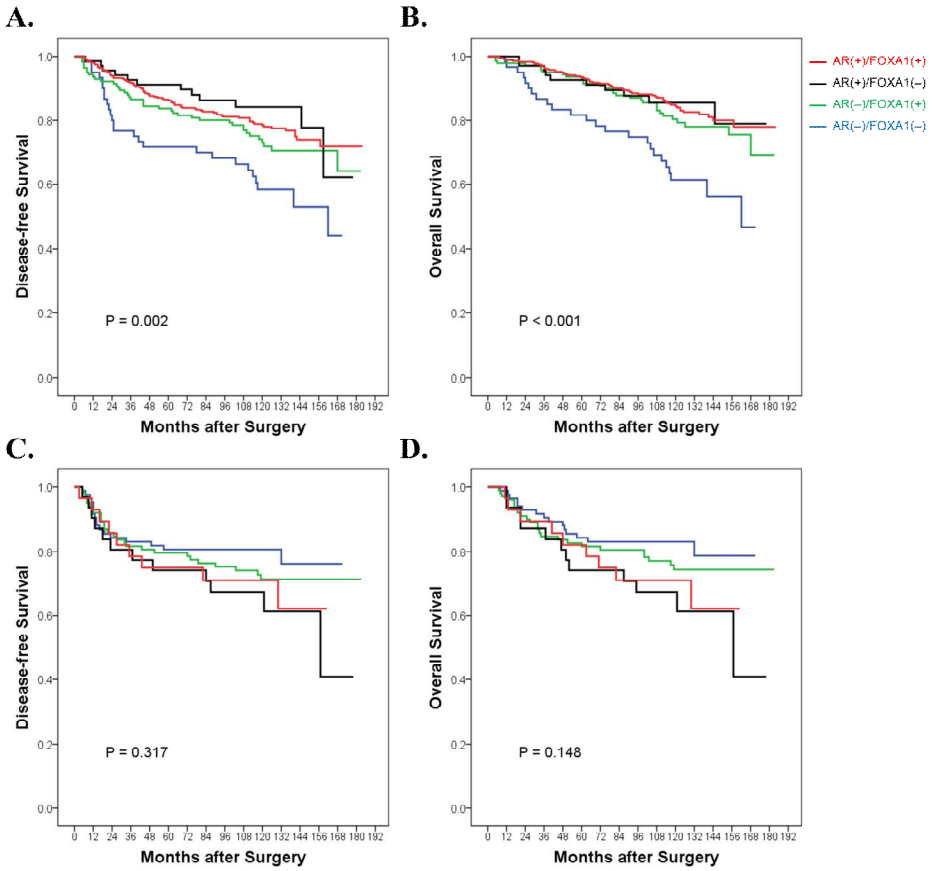

Figure 8. Survival curves according to AR and FOXA1 status stratified by ER expression in the TMA study. (A, B) ER-positive cancers and (C, D) ER-negative tumors.

In patients with ER-positive tumors, multivariate analysis revealed that AR(-)/FOXA1(-) tumors were independently poor prognostic factors for DFS and OS when age at diagnosis, tumor and node stage, histologic grade, HER2,

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Ki-67, and use of chemotherapy and endocrine therapy were adjusted (Table 4.) Node metastasis, HER2-positivity, and absence of chemotherapy were also significantly associated with increased risk of poor DFS and OS in the TMA study.

Table 4. Multivariate analysis for survival of ER-positive breast cancer patients in the TMA study

Factors	Disease-free survival			Overall survival		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
AR/FOXA1						
AR(-)/FOXA1(-)	Ref			Ref		
AR(-)/FOXA1(+)	0.579	0.348 - 0.964	0.036	0.451	0.261 - 0.780	0.004
AR(+)/FOXA1(-)	0.392	0.195 - 0.790	0.009	0.352	0.166 - 0.749	0.007
AR(+)/FOXA1(+)	0.552	0.349 - 0.875	0.011	0.417	0.255 - 0.682	< 0.001
Age (≤ 50 yrs)	0.921	0.651 - 1.303	0.641	0.754	0.510 - 1.114	0.157
Tumor stage (pT2-4)	1.307	0.922 - 1.852	0.133	1.451	0.977 - 2.155	0.065
Node stage (pN1-3)	2.846	1.912 - 4.237	< 0.001	2.642	1.696 - 4.113	< 0.001
Histologic grade (III)	1.000	0.653 - 1.530	0.999	0.840	0.512 - 1.379	0.491
HER2 (positive)	1.609	1.111 - 2.331	0.012	1.655	1.101 - 2.490	0.016
Ki-67 (≥15%)	1.295	0.767 - 2.184	0.333	1.196	0.657 - 2.177	0.558
CTx (not done)	2.390	1.441 - 3.963	0.001	2.282	1.316 - 3.955	0.003
EndoTx (not done)	1.132	0.703 - 1.822	0.611	1.211	0.713 - 2.057	0.478

HR: hazard ratio, CI: confidence interval, Ref: reference, CTx: chemotherapy, EndoTx: endocrine therapy.

4. Clinicopathological characteristics using the TCGA Provisional and METABRIC datasets

A. AR and FOXA1 mRNA expression in both datasets

Using data of mRNA levels and clinical attributes from the TCGA Provisional and METABRIC datasets, AR and FOXA1 mRNA expression patterns and clinicopathological characteristics according to AR/FOXA1 status were investigated. The median values of AR and FOXA1 mRNA were 467.67 [interquartile range (IQR), 906.43] and 6,530.17 (IQR, 4,753.36), respectively,

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from the TCGA Provisional dataset. The median values of AR and FOXA1 mRNA were 7.57 (IQR, 1.39) and 11.37 (IQR, 0.96), respectively, from the METABRIC dataset. The frequencies of AR and FOXA1 mRNA levels in each dataset are shown in Figure 9. The distribution of mRNA expression was unimodal for AR, but bimodal for FOXA1 in both datasets.

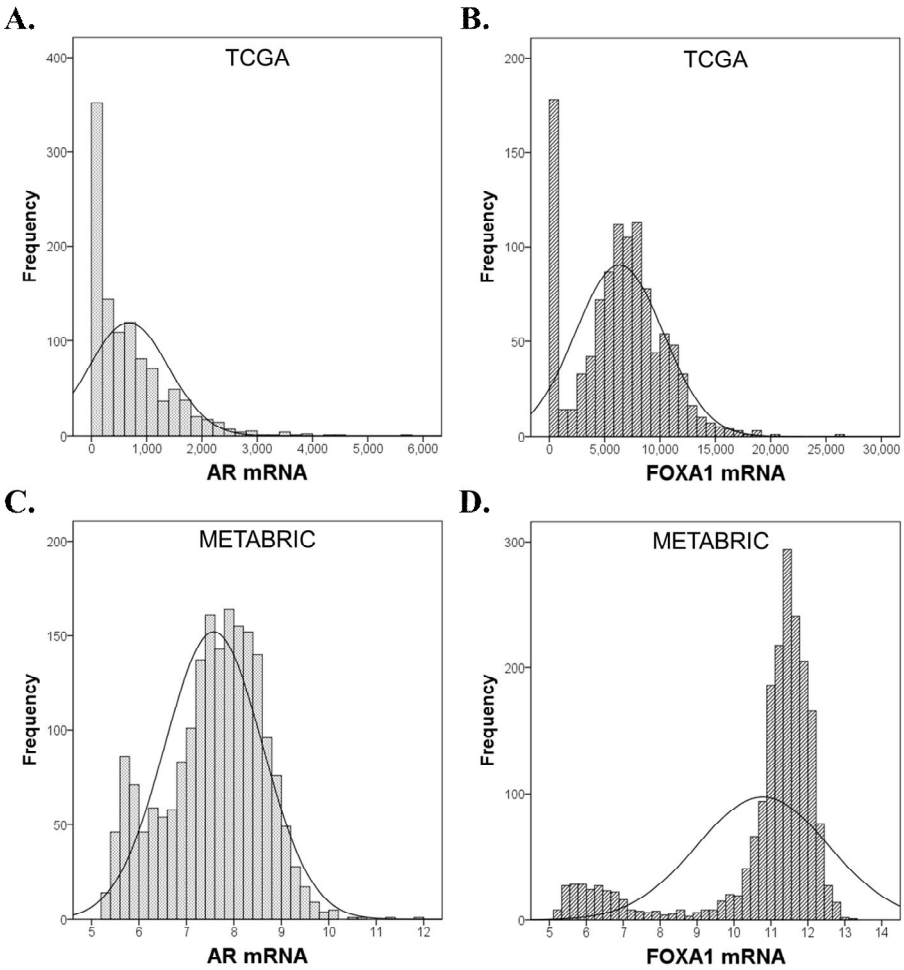

Figure 9. mRNA expression frequencies of AR and FOXA1. (A, B) The TCGA Provisional and (C, D) METABRIC datasets.

The clinical parameters of ER status by IHC were available in 1,030 cases of the TCGA Provisional and in 1,923 patients of the METABRIC

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datasets. The mean AR mRNA levels from the TCGA Provisional dataset were 788.96 (SD = 715.87) in ER-positive tumors and 296.35 (SD = 626.96) in ER-negative tumors ($p < 0.001$). The mean FOXA1 mRNA values from the TCGA Provisional dataset were 7,584.29 (SD = 3,217.99) in ER-positive cases and 1,950.41 (SD = 2,893.59) in ER-negative cases ($p < 0.001$). The mean AR mRNA levels from the METABRIC dataset were 7.80 (SD = 0.84) in ER-positive samples and 6.82 (SD = 1.22) in ER-negative samples ($p < 0.001$). The mean FOXA1 mRNA values from the METABRIC dataset were 11.40 (SD = 0.92) in ER-positive cancers and 8.72 (SD = 2.32) in ER-negative cancers ($p < 0.001$). In both datasets, AR and FOXA1 mRNA levels were significantly higher in ER-positive tumors than in ER-negative tumors (Fig. 10).

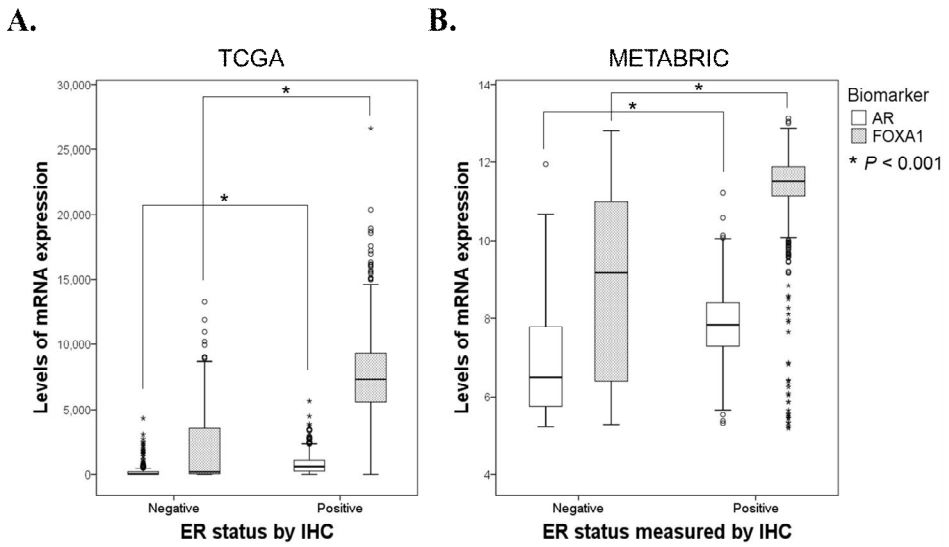

Figure 10. Levels of AR and FOXA1 mRNA expression according to ER status. (A) The TCGA Provisional and (B) METABRIC datasets.

The correlation between AR and FOXA1 mRNA levels in the TCGA Provisional dataset was weakly positive (Pearson $r = 0.297$; $p < 0.001$) in ER-positive tumors and moderately positive (Pearson $r = 0.594$; $p < 0.001$) in

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ER-negative tumors. Similarly, the correlation between AR and FOXA1 mRNA expression in the METABRIC dataset was moderately positive (Pearson r = 0.424; $p < 0.001$) in ER-positive cancers and strongly positive (Pearson r = 0.777; $p < 0.001$) in ER-negative cancers. Consistently in both datasets, the SD range in consideration with mean value was relatively wider in ER-negative than in ER-positive tumors, and most samples with high AR mRNA levels exhibited high FOXA1 expression in ER-positive cancers; therefore, the correlation coefficient was higher in ER-negative tumors.

B. Clinicopathological characteristics according to AR/FOXA1 mRNA status

The lower quartile cutoff values for defining high versus low mRNA expression of biomarkers were determined 99.47 for AR and 3,971.38 for FOXA1 from the TCGA Provisional dataset and 6.93 for AR and 10.81 for FOXA1 from the METABRIC dataset. The number of AR-high/FOXA1-high, AR-high/FOXA1-low, AR-low/FOXA1-high, and AR-low/FOXA1-low cases was 736 (68.3%), 73 (6.8%), 73 (6.8%), and 196 (18.2%), respectively, from the TCGA Provisional and 1,303 (66.5%), 170 (8.7%), 168 (8.6%), and 317 (16.2%), respectively, from the METABRIC datasets.

In the whole population of the TCGA Provisional dataset, AR-high/FOXA1-high tumors were significantly associated with age at diagnosis > 50 years ($p = 0.022$), lower TNM stage ($p = 0.041$), ER-positivity ($p < 0.001$), and PR-positivity ($p < 0.001$). AR-low/FOXA1-low cases showed the highest frequency of ER-negative ($p < 0.001$), PR-negative ($p < 0.001$), and HER2-negative tumors ($p = 0.001$). The clinical attributes based on AR and FOXA1 status are presented in patients with ER-positive breast cancer from the TCGA Provisional dataset (Table 5). In ER-positive cases, AR-low/ FOXA1-low tumors were significantly associated with age > 50 years and PR-negativity. Although AR-high/FOXA-high cases demonstrated higher frequencies of lower TNM stage, there was no statistical difference.

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Table 5. Patient characteristics according to AR and FOXA1 mRNA status in patients with ER-positive breast cancer from the TCGA Provisional dataset

Factor	AR-high/ FOXA1-high (n, %)	AR-high/ FOXA1-low (n, %)	AR-low/ FOXA1-high (n, %)	AR-low/ FOXA1-low (n, %)	*p*-value
Age (yrs)					
≤ 50	175 (26.8)	17 (45.9)	25 (37.9)	9 (23.1)	0.019
> 50	479 (73.2)	20 (54.1)	41 (62.1)	30 (76.9)	
TNM stage					
Stage I	123 (19.2)	6 (16.2)	7 (10.8)	4 (10.3)	0.390
Stage II	363 (56.7)	19 (51.4)	39 (60.0)	26 (66.7)	
Stage III	154 (24.1)	12 (32.4)	19 (29.2)	9 (23.1)	
PR					
Positive	573 (87.9)	29 (78.4)	55 (83.3)	19 (48.7)	< 0.001
Negative	79 (12.1)	8 (21.6)	11 (16.7)	20 (51.3)	
HER2					
Negative	473 (78.7)	28 (80.0)	48 (82.8)	29 (82.9)	0.841
Positive	128 (21.3)	7 (20.0)	10 (17.2)	6 (17.1)	

In the whole population of the METABRIC dataset, which provides more information regarding histopathology and treatment patterns than the TCGA Provisional dataset, AR-low/FOXA1-low tumors were significantly associated with age at diagnosis ≤ 50 years ($p < 0.001$), high grade ($p < 0.001$), ER-negativity ($p < 0.001$), PR-negativity ($p < 0.001$), HER2-negativity ($p < 0.001$), and the basal-like subtype ($p < 0.001$). AR-high/FOXA1-low subgroup showed the highest frequency of stage III disease ($p = 0.004$), HER2-positive tumors ($p < 0.001$), and the HER2-enriched subtype ($p < 0.001$). The clinicopathological characteristics determined by to AR and FOXA1 status in ER-positive breast cancer patients of the METABRIC dataset are presented in Table 6. Similarly, in ER-positive tumors, AR-low/FOXA1-low cases were significantly associated with high grade, PR-negativity, the basal-like subtype, and chemotherapy administration. AR-low/FOXA1-low tumors also showed higher advanced stage and HER2-negativity, but without statistical significance.

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Table 6. Patient characteristics according to AR and FOXA1 mRNA status in patients with ER-positive breast cancer of the METABRIC dataset

Factor	AR-high/ FOXA1-high (n, %)	AR-high/ FOXA1-low (n, %)	AR-low/ FOXA1-high (n, %)	AR-low/ FOXA1-low (n, %)	*p*-value
Age (yrs)					
≤ 50	172 (14.7)	11 (11.3)	33 (22.3)	14 (19.4)	0.048
> 50	996 (85.3)	86 (88.7)	115 (77.7)	58 (80.6)	
TNM stage					
Stage I	336 (31.5)	28 (29.8)	60 (40.8)	26 (36.1)	0.085
Stage II	685 (59.0)	54 (57.4)	78 (53.1)	35 (48.6)	
Stage III	110 (9.5)	12 (12.8)	9 (6.1)	11 (15.3)	
Histologic grade					
I/II	701 (62.8)	44 (47.3)	79 (54.5)	34 (47.2)	0.001
III	415 (37.2)	49 (52.7)	66 (45.5)	38 (52.8)	
PR*					
Positive	823 (70.5)	48 (49.5)	81 (54.7)	15 (20.8)	< 0.001
Negative	345 (29.5)	49 (50.5)	67 (45.3)	57 (79.2)	
HER2*					
Negative	1,081 (92.6)	86 (88.7)	135 (91.2)	68 (94.4)	0.457
Positive	87 (7.4)	11 (11.3)	13 (8.8)	4 (5.6)	
PAM50 subtype					
Luminal A	613 (52.7)	19 (20.0)	56 (37.8)	4 (5.6)	< 0.001
Luminal B	373 (32.0)	20 (21.1)	62 (41.9)	6 (8.3)	
HER2	74 (6.4)	15 (15.8)	15 (10.1)	9 (12.5)	
Basal	9 (0.8)	14 (14.7)	5 (3.4)	30 (41.7)	
Normal	95 (8.2)	27 (28.4)	10 (6.8)	23 (31.9)	
Type of surgery					
BCS	459 (39.5)	38 (40.9)	70 (47.6)	30 (42.9)	0.296
TM	702 (60.5)	55 (59.1)	77 (52.4)	40 (57.1)	
Radiotherapy					
Not done	510 (43.7)	32 (33.0)	57 (38.5)	26 (36.1)	0.097
Done	658 (56.3)	65 (67.0)	91 (61.5)	46 (63.9)	
Chemotherapy					
Not done	1,073 (91.9)	92 (94.8)	128 (86.5)	55 (76.4)	< 0.001
Done	95 (8.1)	5 (5.2)	20 (13.5)	17 (23.6)	
Hormone therapy					
Not done	311 (26.6)	24 (24.7)	37 (25.0)	17 (23.6)	0.902
Done	857 (73.4)	73 (75.3)	111 (75.0)	55 (76.4)	

*Positive criteria of PR and HER2 are defined as expression status in the METABRIC dataset.

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5. Survival analysis using the TCGA Provisional and METABRIC datasets

A. The TCGA Provisional dataset

Mean DFS and OS follow-up periods were recorded as 37.6 months (n = 992; SD = 35.4) and 40.7 months (n = 1,076; SD = 38.6), respectively, of the TCGA Provisional dataset. Recurrent or progressive events and deaths occurred in 9.6% (104/992) and 12.7% (137/1076) patients, respectively. Survival curves according to AR and FOXA1 mRNA status showed no statistically significant prognostic value in the whole population, ER-positive, and ER-negative patients (Fig. 11).

B. The METABRIC dataset

The METABRIC dataset indicated the mean follow-up duration was 125.6 months (n = 1,958; SD = 76.1) and recurrent or progressive events and deaths were in 32.6% and 57.8% of patients, respectively. Figure 12 shows DFS and OS curves according to AR and FOXA1 mRNA status. Compared to other groups, the AR-low/FOXA1-low group showed the worst 5-year DFS with statistical significance in the whole population (p = 0.002). The OS curve demonstrated no statistical significance (p = 0.070). Similarly, in ER-positive patients, the AR-low/FOXA1-low group showed the worst 5-year DFS (p = 0.020) and the AR-low/FOXA1-high group exhibited the best statistically significant 5-year OS (p = 0.002). However, the AR-low/FOXA1-high group presented the worst 5-year DFS (p = 0.011) and 5-year OS (p = 0.002) in ER-negative tumors.

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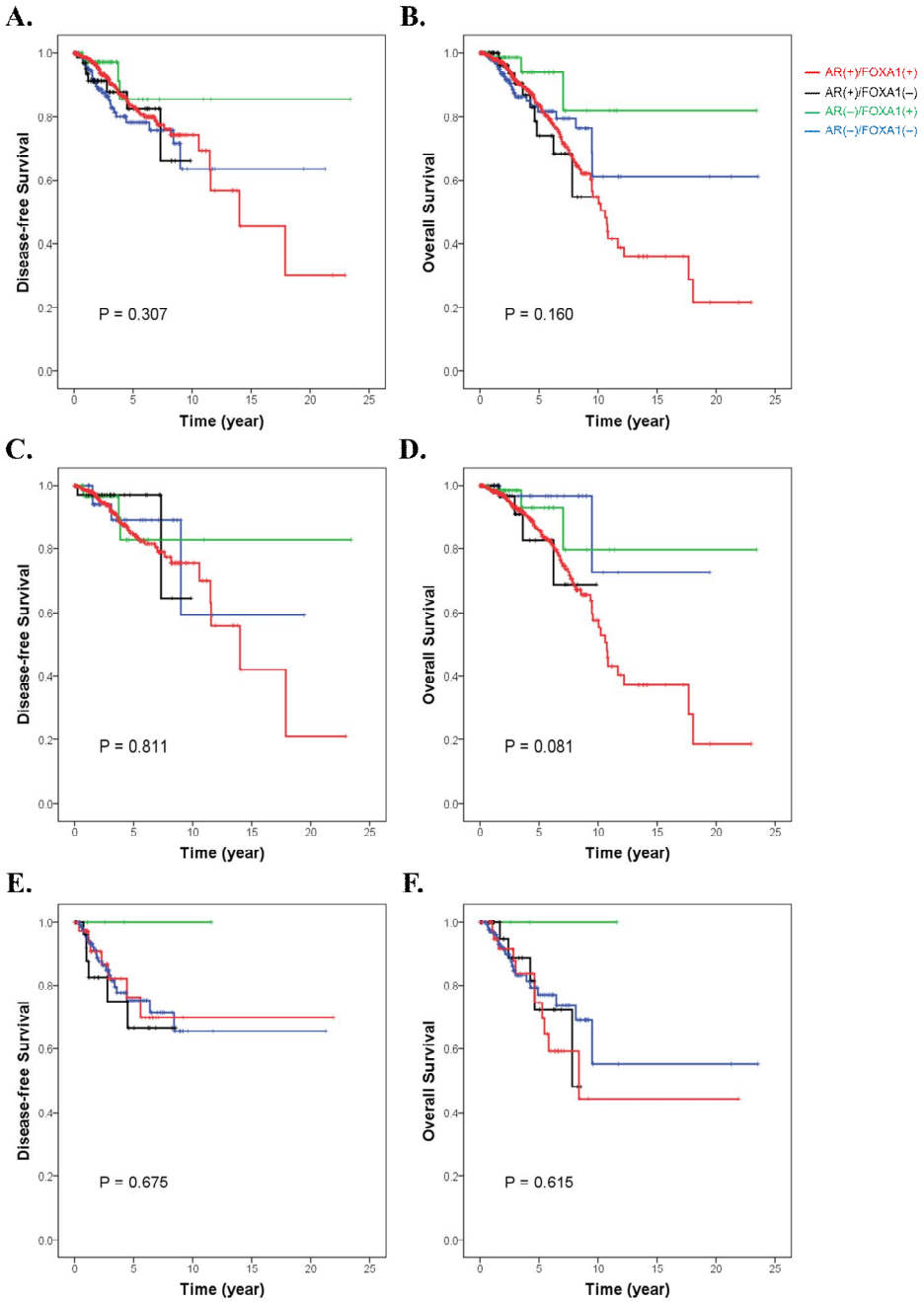

Figure 11. Survival curves according to AR and FOXA1 mRNA status of the TCGA Provisional dataset. (A, B) Whole study population, (C, D) ER-positive cancers, and (E, F) ER-negative tumors.

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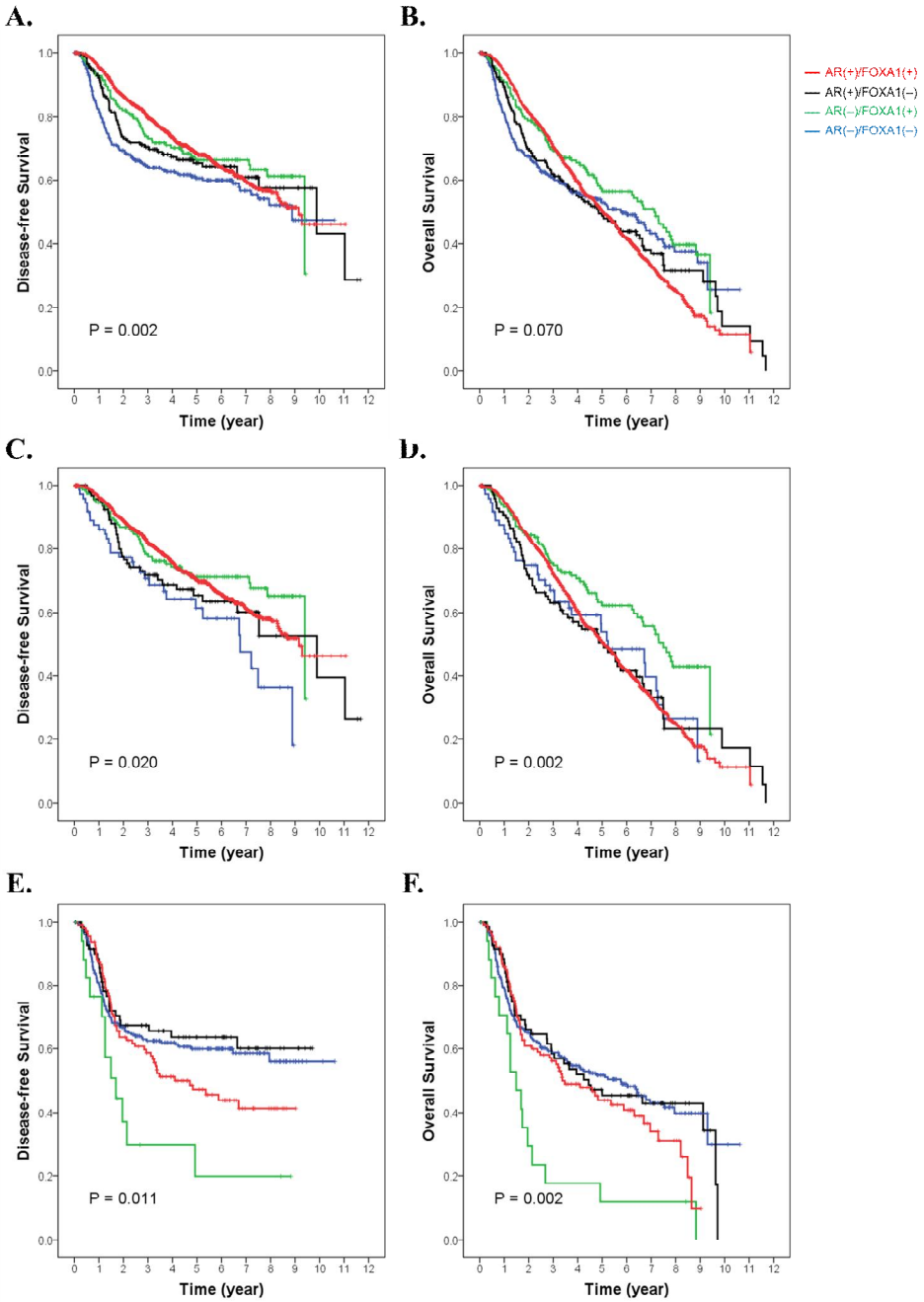

Figure 12. Survival curves derived from AR and FOXA1 mRNA status of patients in the METABRIC dataset. (A, B) Whole study population, (C, D) ER-positive cancers, and (E, F) ER-negative tumors.

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C. Multivariate analysis using the METABRIC dataset

To investigate the prognostic roles of AR and FOXA1 status in ER-positive breast cancers, multivariate analysis was performed using clinical variables of the METABRIC dataset (Table 7). The AR-low/FOXA1-low group was determined to be a significantly poor prognostic factor than the AR-low/FOXA1-high and AR-high/FOXA1-high groups for DFS and the AR-low/FOXA1-high group for OS when age, stage, grade, HER2, and use of chemotherapy and hormone therapy were adjusted.

Table 7. Multivariate analysis for survival rates of patients with ER-positive breast cancer from the METABRIC dataset

Factors	Disease-free survival			Overall survival		
	HR	95% CI	P-value	HR	95% CI	P-value
AR/FOXA1						
AR-low/FOXA1-low	Ref			Ref		
AR-low/FOXA1-high	0.566	0.351 - 0.912	0.019	0.661	0.440 - 0.995	0.047
AR-high/FOXA1-low	0.775	0.468 - 1.283	0.322	0.992	0.651 - 1.511	0.969
AR-high/FOXA1-high	0.648	0.443 - 0.950	0.026	0.996	0.713 - 1.392	0.982
Age (≤ 50 yrs)	0.664	0.491 - 0.896	0.008	0.407	0.314 - 0.527	< 0.001
TNM stage						
Stage I	Ref			Ref		
Stage II	1.654	1.287 - 2.126	< 0.001	1.604	1.348 - 1.909	< 0.001
Stage III	3.940	2.830 - 5.484	< 0.001	2.766	2.148 - 3.562	< 0.001
Histologic grade (III)	1.561	1.282 - 1.901	< 0.001	1.239	1.075 - 1.429	0.003
HER2 (positive)	1.871	1.395 - 2.510	< 0.001	1.474	1.156 - 1.880	0.002
CTx (not done)	0.672	0.489 - 0.924	0.015	0.840	0.636 - 1.110	0.220
EndoTx (not done)	1.067	0.826 - 1.379	0.619	0.960	0.802 - 1.148	0.651

6. Survival analysis using the KM Plotter

Finally, the KM Plotter analysis was performed to validate the prognostic value of combined AR and FOXA1 mRNA status. A multigene classifier uses the mean expression of the selected genes, and a new value [(gene X1 + gene X2 + ··· + gene Xn)/n] is computed for survival analysis of the KM Plotter. Figure 13

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shows relapse-free survival curves according to AR and FOXA1 levels in 1,660 patients with available data. Patients with low AR/FOXA1 expression levels demonstrated significantly lower survival in all patients (HR, 0.69; 95% CI, 0.58–0.83; log-rank p = 5.1e-0.5). This statistical significance was maintained in only 1,172 ER-positive tumors (HR, 0.72; 95% CI, 0.58–0.89; log-rank p = 0.003) but not in 488 ER-negative cancers (HR, 0.87; 95% CI, 0.64–1.19; log-rank p = 0.392).

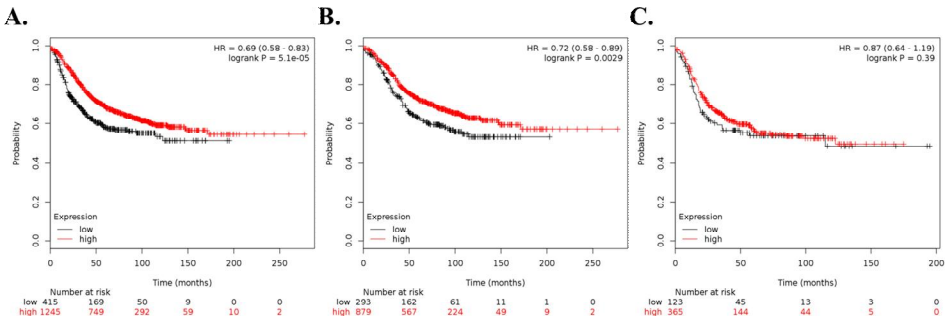

Figure 13. Relapse-free survival curves using a multigene classifier of the KM Plotter. (A) All patients, (B) ER-positive tumors, and (C) ER-negative cancers.

The KM Plotter provides subgroup analyses according to the intrinsic subtype based on the 2013 St. Gallen criteria using the expression of ESR1, HER2, and MKI67 as follows; luminal A (ESR1+/HER2–/MKI67 low), luminal B (ESR1+/HER2–/MKI67 high and ESR1+/HER2+), HER2-enriched (ESR1–/HER2+), and basal subtype (ESR1–/HER2–).[32] Stratification by the intrinsic subtypes is presented in Figure 14. Patients with low AR/FOXA1 expression exhibited poor relapse-free survival in 783 luminal A subtype tumors (HR, 0.65; 95% CI, 0.49–0.86; log-rank p = 0.002), but not in other subtypes (HR, 1.12; 95% CI, 0.77–1.63; log-rank p = 0.55 for 389 luminal B, HR, 1.3; 95% CI, 0.72–2.33; log-rank p = 0.39 for 149 HER2-enriched, and HR, 1.04; 95% CI, 0.71–1.53; log-rank p = 0.84 for 339 basal subtypes).

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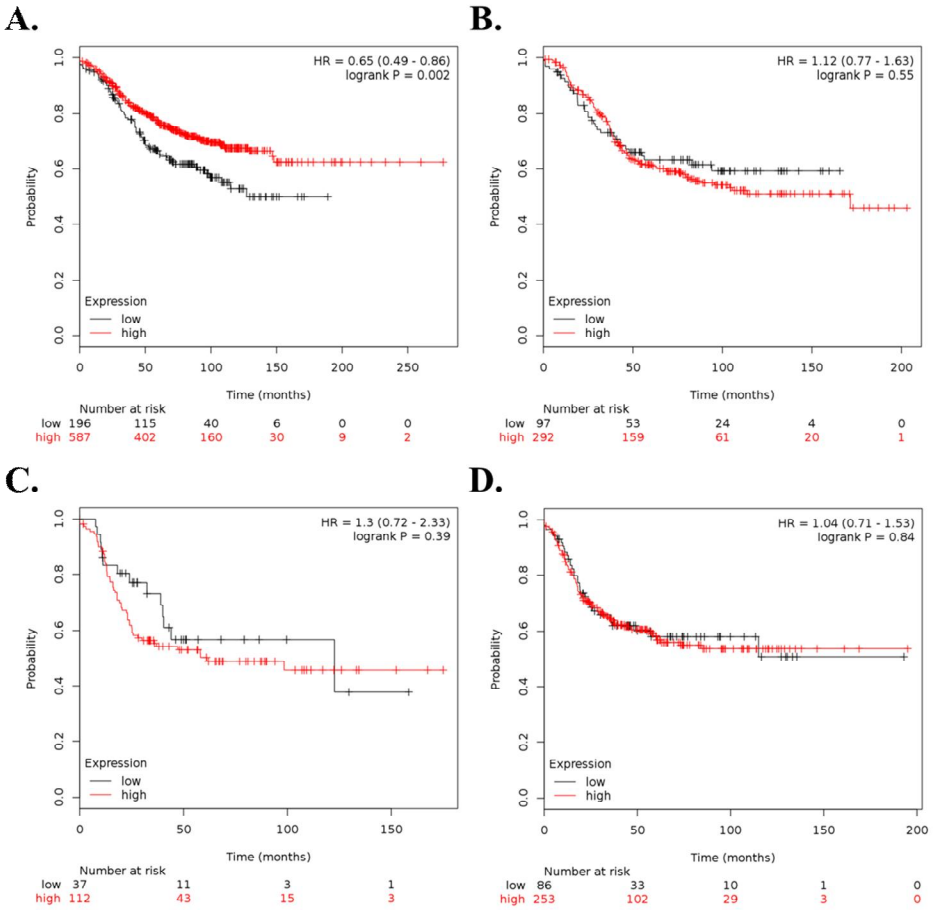

Figure 14. Relapse-free survival curves stratified by the intrinsic subtype in the KM Plotter. (A) Luminal A, (B) Luminal B, (C) HER2-enriched, and (D) Basal subtypes.

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IV. DISCUSSION

An exploration of the web-based genetic analysis in the present study showed that approximately 10% of breast cancers were altered in ESR1, AR, or FOXA1 genes and generally changes in genes concurrently occurred even though the frequency was low. It has been suggested that long length of CAG repeats in the exon 1 of the AR gene is associated with decreased efficacy of androgenic activity and the increased risk of breast cancer in women.[33] A recent meta-analysis partly supported this hypothesis and demonstrated that long CAG polymorphisms increased the risk of breast cancer only in Caucasian women.[34] Germline mutations in the AR gene have also been implicated in male breast cancer and long CAG repeats of the AR gene have been frequently found in male breast carcinomas.[35,36] Regarding the FOXA1 gene mutations, little clinical evidence has been reported even with recent advances in sequencing technologies.[37] The roles of changes in the AR or FOXA1 genes have not been much studied in female breast cancers. However, this study showed a close correlation between AR and FOXA1 expression levels. Clinically undetermined genetic networks between these markers have been proposed, suggesting combined biomarker studies may be critical. Additional basic and clinical researches are required to understand the genetic mechanisms and clinical behaviors related with AR and FOXA1, although the number of patients with alteration in AR and FOXA1 was very small.

Peters et al.[7] provided supporting data that growth inhibition of ER-positive breast cancer by androgens was directly mediated by AR and was derived from inhibition of the ER signaling pathway rather than via activation of AR-regulated target genes. Our in vitro study also confirmed the interplay between ER and AR. Overexpression of AR induced downregulation of ER expression and cellular proliferation. A subsequent cistrome study demonstrated that AR signaling was less likely than ER colocalization to rely on FOXA1 in

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ZR75-1 cells.[38] Approximately 20% of all peaks and > 60% of high-stringency sites showed a direct overlap between ER and FOXA1 binding sites. However, only 8% of all peaks and about 35% of high-stringency sites overlapped between AR and FOXA1 binding sites.[16,38] These findings suggest that AR along with FOXA1 exerts its function in specific cellular situations. Unfortunately, the present study exhibited no changes in FOXA1 mRNA levels by AR overexpression. In addition, FOXA1 overexpression did not affect ER activity in mock- and AR-overexpression cell lines but knockdown of FOXA1 induced marked loss of ER-positive cell viability as shown in Figure 6. These findings suggested that FOXA1, as a pioneering factor in downstream of the ER signaling pathway, played an important, complex role in tumor survival, as shown as a lineage-specific oncogene in luminal cancer cell lines.[16,39] Recently, the dual roles of FOXA1 in breast cancer as a growth stimulator and inhibitor have been considered controversial.[12,15,40] A comprehensive analysis and an individualized interpretation may be required for understanding the role of FOXA1.

Approximately 10–20% of all cases showed negative protein expression or low mRNA levels of both AR and FOXA1 in our study. These cases were significantly associated with aggressive tumor features such as ER-negative, PR-negative, TNBC, basal subtype, high grade, and high Ki-67 labelling index. These features were maintained in patients with ER-positive cancer. Habashy et al.[41] demonstrated that while a combined analysis was not performed, negative FOXA1 was significantly associated with negative ER, AR, and PR expression in both whole series and ER-positive cohorts. On the contrary, 15.2% of 460 patients with TNBC showed AR(+)/FOXA1(+) tumors, which were associated with frequent lobular histology, older age at diagnosis, lower nuclear grade, and less presentation of lymphocytic infiltration, pushing margin, syncytial architecture, and central fibrosis or necrosis from the UNICANCER PACS08 trial.[24] Of 54 apocrine carcinomas, 100% and 92.6%

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expressed AR and FOXA1, respectively.[21] Consistently, breast cancer cell lines with molecular apocrine features showed a significant functional cross-talk between AR and HER2 that involved FOXA1 activity.[42] These suggested that loss of AR and FOXA1 in ER-positive breast cancers and gain of AR and FOXA1 in ER-negative tumors were possible markers of distinct biological phenotypes.

Interestingly, a half of the apocrine carcinomas overexpressed the HER2 protein.[43] Regarding the association of AR/FOXA1 status with HER2 in the present study, TMA analysis and public datasets showed no statistical significance in ER-positive cancers. In ER-negative patients, however, positive HER2 was 67.9% of AR(+)/FOXA1(+), 67.7% of AR(+)/FOXA1(-), 23.2% of AR(-)/FOXA1(+), and 21.7% of AR(-)/FOXA1(-) tumors in the TMA study ($p < 0.001$). ER-negative cases of the TCGA Provisional dataset showed that HER2-positive tumors were 55.0% of AR-high/FOXA1-high, 25.8% of AR-high/FOXA1-low, 75.0% of AR-low/FOXA1-high, and 8.0% of AR-low/FOXA1-low breast cancers ($p < 0.001$). In ER-negative samples of the METABRIC dataset, positive HER2 was 57.9% of AR-high/FOXA1-high, 47.1% of AR-high/FOXA1-low, 44.4% of AR-low/FOXA1-high, and 6.8% of AR-low/FOXA1-low tumors ($p < 0.001$). Although the proportion of HER2-positive cases in ER-negative tumors was different among datasets, AR- or FOXA1-positive patients showed higher HER2-positive tumors. Therefore, further studies are necessary to understand the clinical implications of these networks in ER-negative breast cancers.

As a prognostic marker, AR is consistently reported to be associated with better survival outcomes.[6,29,44] Although somewhat conflicting results have been suggested, many studies demonstrate FOXA1 expression as a good prognostic factor.[14,15,45,46] However, since AR and FOXA1 could be closely connected as shown in protein and mRNA expression status analyses of the present study, the clinical impact of AR and FOXA1 on survival should be

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analyzed considering ER status. However, only a few studies have been conducted. This study demonstrated that patients with negative protein or low mRNA expression of both AR and FOXA1 showed independently poor survival outcomes in ER-positive cancers from the TMA study and the METABRIC dataset. This association was also validated in the analyses of the KM Plotter. The TCGA Provisional dataset did not present statistically significant associations in the present analysis, but a mean follow-up duration of the TCGA Provisional dataset was shorter than that of other datasets. Therefore, future analysis with longer follow-up periods might show different findings and should be conducted to confirm the hypothesis.

Interestingly, according to analyses of ER-negative tumors using the METABRIC dataset, 18 (4.1%) cases with AR-low/FOXA1-high tumor showed the worst survival and 114 (26.0%) with AR-high/FOXA1-high presented worse DFS with statistical significance. However, in ER-negative tumors from the TCGA Provisional dataset, 5 (2.1%) cases with AR-low/FOXA1-high tumor showed the best survival, though without statistical significance. In the TMA study, AR(-)/FOXA1(-) or AR(-)/FOXA1(+) subgroups demonstrated a trend of better survival in ER-negative patients, although no statistical significance was noted. Additional studies with larger sample sizes should be required to understand the different clinical impact of AR and FOXA1 status on survival in ER-negative breast cancers.

A potential limitation of the present study was inevitably the nonrandomized and retrospective nature of the clinical dataset. Difficulty in handling and manipulation of an in vitro study could not find out details of subcellular molecular mechanisms between AR and FOXA1 and many other ER-positive, luminal subtype breast cancer cell lines were not investigated. In addition, methodological problems were key issues. The evaluation and interpretation of immunohistochemical AR and FOXA1 expression were not standardized and the use of TMA tumor blocks with small sized cores may not

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have been able to represent the results of whole sections. Detection methods and cutoff values of public datasets were varied and arbitrary. Among the genomic profiles on the bioinformatics analysis website, the number of mutations, copy-number alterations, or methylations was not incorporated into the present study and only mRNA expression data were used. Results from the independent datasets could not be used to calculate the associations between protein and mRNA expression levels of biomarkers. Nevertheless, the present study had strengths to explore and validate the undisclosed role of combined AR and FOXA1 status in ER-positive breast cancers using the genetic and clinical datasets with in vitro cell lines study.

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V. CONCLUSION

The present results indicate that AR and FOXA1 are closely associated in breast cancers, and distinctive clinicopathological features are presented in ER-positive tumors according to AR and FOXA1 status. More importantly, loss of or decrease in both AR and FOXA1 expression is an independently significant poor prognostic factor in ER-positive tumors. Since different molecular mechanisms between AR and FOXA1 signaling pathways have been suggested in ER-negative breast cancers, the clinical implications of AR and FOXA1 status on patient prognosis should be further investigated to improve the survival of patients with heterogeneous breast cancers. Therefore, possible therapeutic strategies such as anti-androgens should be examined considering the AR, FOXA1, and ER status in breast cancer patients.

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ABSTRACT(IN KOREAN)

에스트로겐 수용체 양성 유방암에서 불량한 예후인자로서 안드로겐 수용체와 forkhead box A1 (FOXA1)의 발현 결핍

<지도교수 김 승 일>

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안드로겐 수용체와 forkhead box A1(FOXA1)은 유방암 환자에서 중요한 역할을 담당한다고 알려져 있으나, 두 생물표지자의 임상적 중요성은 아직 확립되어 있지 못하다. 본 연구는 웹 기반 데이터와 임상 자료 분석 및 체외 실험 연구를 통해 안드로겐 수용체와 FOXA1 사이의 연관성을 조사하고, 에스트로겐 수용체 양성 유방암에서 안드로겐 수용체와 FOXA1을 함께 고려했을 때 임상병리학적 특성과 생존 결과를 분석하고자 하였다. cBioPortal for Cancer Genomics와 Kaplan-Meier Plotter 웹사이트 분석 및 1999년에서 2005년 사이에 세브란스 병원에서 치료받은 유방암 환자의 조직 마이크로어레이 블록을 이용하여 두 생물학적표지자의 mRNA와 단백질 발현 상태에 따른 유전적 상관성, 임상병리학적 특성과 생존 결과를 단변량 및 다변량 분석을 통해 평가하였다. 또한 T47D와 ZR75-1 유방암 세포주를 이용하여 안드로겐 수용체와 FOXA1 사이의 분자생물학적 관련성을 탐색하였다. cBioPortal 웹 사이트에서 전체 샘플의 약 10% 정도에서 ESR1, AR, FOXA1 유전자의 변화가 관찰되었으며, 대개 함께 변하였다. 세 생물표지자의 mRNA 발현 정도는 서로 양의 상관성을 보였다. 체외 실험 결과 안드로겐 수용체를 과발현 시킨 에스트로겐 수용체 양성 유방암 세포주에서 에스트로겐 수용체 mRNA와 단백질 발현의 하향조절을 통해 세포 증식력이 감소되었다. 하지만 FOXA1은 안드로겐 수용체의 과발현에 상관없이 변화되지 않았다. FOXA1을

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과발현 시켰을 때 에스트로겐 수용체의 활성도는 변화 없었나, FOXA1의 발현을 제거하였을 때는 유의하게 세포 생존능력이 감소되었다. 면역조직화학염색법을 이용한 환자 조직 마이크로어레이 연구에서 양성 안드로겐 수용체 발현은 통계적으로 유의하게 FOXA1 발현 양성과 연관되었다. 에스트로겐 수용체 양성 종양에서 안드로겐 수용체-음성/FOXA1-음성군은 공격적인 조직학적 특성과 불량한 생존 결과와 유의한 상관성이 있었다. 웹 기반 유전자 데이터베이스 분석에서는 안드로겐 수용체와 FOXA1의 mRNA 발현 정도가 에스트로겐 수용체 음성 유방암보다 에스트로겐 수용체 양성 종양에서 유의하게 높았다. TCGA Provisional 데이터의 에스트로겐 수용체 양성 유방암 환자 분석에서는 안드로겐 수용체와 FOXA1의 발현 상태에 따라 통계적으로 유의한 생존율의 차이가 없었으나, METABRIC 데이터 세트의 에스트로겐 수용체 양성 유방암 환자 분석에서는 안드로겐 수용체-낮음/FOXA1-낮음 종양군에서 공격적인 임상병리학적 특성과 불량한 무병 생존 결과를 나타내었다. 에스트로겐 수용체 양성 종양이나 관형 A 아형 유방암 환자의 Kaplan-Meier Plotter 분석에서도 일관되게 안드로겐 수용체와 FOXA1의 발현이 낮은 환자군이 통계적으로 유의하게 낮은 무재발 생존율을 보였다. 결론적으로 본 연구는 유방암에서 안드로겐 수용체와 FOXA1이 밀접하게 연관되어 있으며, 에스트로겐 수용체 양성 종양에서 두 생물표지자의 상태에 따라 독특한 임상병리학적 특성이 발현됨을 시사한다 하겠다. 또한 에스트로겐 수용체 양성 유방암에서 안드로겐 수용체와 FOXA1의 발현이 없거나 낮은 경우 불량한 예후인자임이 입증되었다. 향후 유방암 환자의 치료 및 생존율 향상을 위하여 안드로겐 수용체와 FOXA1의 임상적용을 위한 연구는 지속 되야 할 것이다.

핵심되는 말 : 안드로겐 수용체, 유방암, 에스트로겐 수용체, forkhead box A1, 예후