Estrogen-Related Receptors Gene Expression and Copy Number Alteration Association With the Clinicopathologic Characteristics of Breast Cancer

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ABSTRACT

PURPOSE: It has been suggested that dysregulation of transcription factors expression or activity plays significant roles in breast cancer (BC) severity and poor prognosis. Therefore, our study aims to thoroughly evaluate the estrogen-related receptor isoforms (ESRRs) expression and copy number alteration (CNA) status and their association with clinicopathologic characteristics in BC.

METHODS: A METABRIC dataset consist of 2509 BC patients' samples was obtained from the cBioPortal public domain. The gene expression, putative CNA, and relevant tumor information of ESRRs were retrieved. ESRRs messenger RNA (mRNA) expression in BC cell lines was obtained from the Cancer Cell Line Encyclopedia (CCLE). Association and correlation analysis of ESRRs expression with BC clinicopathologic characteristics and molecular subtype were performed. Kaplan–Meier survival analysis was conducted to evaluate the prognostic value of ESRRs expression on patient survival.

RESULTS: ESRRα expression correlated negatively with patients' age and overall survival, whereas positively correlated with tumor size, the number of positive lymph nodes, and Nottingham prognostic index (NPI). Conversely, ESRRγ expression was positively correlated with patients' age and negatively correlated with NPI. ESRRα and ESRRγ expression were significantly associated with tumor grade, expression of hormone receptors, human epidermal growth factor receptor 2 (HER2), and molecular subtype, whereas ESRRβ was only associated with tumor stage. A significant and distinct association of each of ESRRs CNA with various clinicopathologic and prognostic factors was also observed. Kaplan–Meier survival analysis demonstrated no significant difference for survival curves among BC patients with high or low expression of ESRRα, β, or γ. On stratification, high ESRRα expression significantly reduced survival among premenopausal patients, patients with grade I/II, and early-stage disease. In BC cell lines, only ESRRα expression was significantly higher in HER2-positive cells. No significant association was observed between ESRRβ expression and any of the clinicopathologic characteristics examined.

CONCLUSIONS: In this clinical dataset, ESRRα and ESRRγ mRNA expression and CNA show a significant correlation and association with distinct clinicopathologic and prognostic parameters known to influence treatment outcomes; however, ESRRβ failed to show a robust role in BC pathogenesis. ESRRα and ESRRγ can be employed as therapeutic targets in BC-targeted therapy. However, the role of ESRRβ in BC pathogenesis remains unclear.

KEYWORDS: Breast cancer, estrogen-related receptors, survival, gene expression, gene regulation, cBioPortal, CCLE

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Background

Breast cancer (BC) is one of the most commonly diagnosed tumors in the United States, affecting one in every eight women during their lifetime and is second to lung cancer as a cause of cancer death in women.[1] Although there was an advancement in disease management during the past years, the molecular mechanism behind BC development and progression is still not fully understood and consequently needs further investigations. Disease severity and prognosis are highly associated with factors such as the tumor stage, grade, biomarker expression, and dysregulations in various signaling pathways.[2] Six subtypes of BC have been identified based on gene expression clustering and histological stratification: Luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-positive, normal-like, basal-like, and claudin-low.[3,4] The molecular subtypes of BC provide information on prognosis and guide the treatment plan for better clinical outcomes.[4] For example, both luminal A and luminal B express estrogen receptor (ER), yet they react differently to hormone therapy and are associated with distinct clinical outcomes.[4] The presence of different molecular subtypes with diverse biological and pathological characteristics have added additional levels of complexity and challenges in BC management. Consequently, identifying novel biomarkers and key regulators in BC pathogenesis has become a critical component of disease characterization and treatment success in personalized medicine.

The orphan members of the nuclear receptor superfamily, estrogen-related receptors (ESRRs), act as transcription factors

and regulate a wide range of physiological and pathological processes.[5-11] The family includes ESRRα, ESRRβ, and ESRRγ. Unlike other classical nuclear receptors, ESRRs are not controlled by natural ligands.[12] Therefore, their expression and activity are regulated by other means such as co-regulators, post-translational modifications, and diverse cellular signaling pathways. ESRRs are ubiquitously expressed in various tissue types. Their gene expression is higher in metabolically active tissues such as the heart, brain, fat, skeletal muscles, and kidneys.[13-15] The genome of ESRRs shares a unique structural organization but has distinct roles. The ESRRs contain DNA-binding domain (DBD), an activation function (AF)-1 domain, a ligand-binding domain (LBD), and an AF-2 domain. The DBD is required for receptor binding to its estrogen-related response element (ESRRE) on target promoters. ESRRs share about 68% sequence homology in the DBD.[13] ESRRs bind to ESRRE as monomers, homodimers, or as heterodimers with co-activators.[16,17] In addition, ESRRs share significant sequence homology in the LBD with ER; therefore, cross-talk between ESRRs and ER has been established.[13] ESRRs can also bind to estrogen response element and, conversely, ERα, but not ERβ, can bind to ESRRE, implying shared transcriptional networks driven by both ESRRs and ERα.[18]

Transactivation of ESRRs is constitutive and the efficacy and potency of transactivation are cell and promoter-specific.[19] Similarly, co-regulators often play significant roles in ESRRs transcription by either altering their gene expression or activity. Co-regulators can physically interfere with ESRRs interaction with the transcriptional machinery and serve as bridging elements between ESRRs and DNA, regulate chromatin remodeling, and affect histone modifications. For example, cofactors such as SRC1, TIF-2/SRC2, PGC1 alpha and beta, TLE1, and PNRC2 interact with ESRRs and positively regulate their functions.[7,20-24] Conversely, RIP140/Nrip1, SHP, and NR0B2 are recruited to promoters by ESRRα and suppresses its transcriptional activity.[20,25,26]

The master regulators of energy metabolism, bone homeostasis, and their transcriptional pathways are known to be closely correlated with the cancer phenotype.[5,27] ESRRβ gene regulation and function in tissues are less studied compared with other isoforms. In a mouse model, it was reported that ESRRβ plays a critical role in the development and normal physiological function of several tissues and organ systems.[28] The expression of ESRRα and ESRRγ has also been explored as potential markers in various types of tumors such as endometrial, ovarian, colorectal, breast, and prostate cancers.[29-32] Although many studies have shown ESRRβ downregulation in BC and proposed its possible onco-suppressive action, others have reported mixed results on ESRRβ gene expression suggesting the predictive value of ESRRβ and its role in BC remain unclear.[32-34] ESRRα and γ appear to play opposite roles in cancer development and progression. Increased expression of ESRRγ correlates with better clinical outcomes and has been linked to progression-free survival.[34] Conversely, increased expression of ESRRα gene correlates with tumor aggressiveness, bad prognosis, and many other unfavorable clinical outcomes.[34]

Although ESRRs have been examined as prognostic markers for various tumors, their role and regulation in BC are far from being clearly understood. Therefore, the goals of this study are to comprehensively investigate ESRRs status in BC, evaluate their gene expression, examine their copy number alterations (CNAs), and assess potential correlations and associations of the expression of ESRRs with clinicopathologic characteristics of BC, survival, and clinical outcomes. Our findings will advance the current understanding of the functions of ESRRs in BC pathogenesis and help develop new pharmaceuticals and novel disease-treatment strategies.

Methods

Patients' data source and CCLE

A METABRIC dataset comprised of 2509 patients was obtained from the cBioPortal public domain (https://www.cbioportal.org/).[35-37] The demographic and clinicopathologic characteristics were previously described and summarized in Supplementary Table 1.[38] ESRRs messenger RNA (mRNA) and CNA were analyzed. Data regarding ESRRs mRNA gene expression were available for 1904 patients.

The gene expression values of ESRRs in 52 BC cell lines were obtained from the Cancer Cell Line Encyclopedia (CCLE; https://www.broadinstitute.org/cancer/cancer-program-scientific-tools-and-resources), developed by the Broad Institutes. The BC cell lines were stratified into four major molecular subtypes based on the classification by Jiang et al[39] and shown in Supplementary Table 2. The number of cell lines in each subtype is as follows: luminal A (n = 10), luminal B (n = 4), HER2-positive (n = 11), and basal-like (n = 27).

Statistical analysis

For data analysis, the IBM SPSS statistical package (IBM Corp. Version 23.0, Armonk, NY, USA) was used. Continuous variables are represented as mean ± standard deviation, or standard error of the mean, whereas categorical variables are represented as frequency and percentages (n, %). Pearson's χ^2-test of independence was used to compare categorical variables between groups. Assessment of correlations between continuous variables was applied using Pearson's correlation test. For association analysis of some categorical variables, dichotomization was considered and performed in advance of conducting statistical analysis. This approach was used to avoid small sample size on further stratification of data.[40] Consequently, the histologic grade was classified into grades (I/II) and grade III, whereas the TNM stage was categorized into early (I/II) and advanced (III/IV). Molecular subtype was grouped into luminal and non-luminal based on cut-points of previous reports.[38,41]

GraphPad Prism 8.0.1 software (GraphPad Software, San Diego, CA, USA) was used to generate Kaplan–Meier survival

Table 1. mRNA and copy number alteration of ESRR genes in breast cancer patients (N = 2509).

CHARACTERISTIC	ESRRα	ESRRβ	ESRRγ
mRNA log intensity, Mean ± SD	6.79 ± 0.39	5.47 ± 0.14	6.34 ± 0.83
mRNA expression[a]	n (%)		
Low	1084 (43.2)	953 (38)	1136 (45.3)
High	820 (32.7)	951 (37.9)	768 (30.6)
Missing	605 (24.1)	605 (24.1)	605 (24.1)
Copy number alterations (CNA)	n (%)		
Homozygous deletion	—	1 (0.00)	—
Hemizygous deletion	315 (12.6)	507 (20.2)	28 (1.1)
Neutral/no change	1705 (68.0)	1575 (62.8)	791 (31.5)
Gain	133 (5.3)	82 (3.3)	818 (32.6)
High-level amplification	20 (0.8)	8 (0.3)	536 (21.4)
Missing	336 (13.4)	336 (13.4)	336 (13.4)

ESRR, estrogen-related receptor; mRNA, messenger RNA.
[a]mRNA expression data are available for 1904 patients.

curves based on the expression status of ESRRs in BC patients. Cox proportional hazards models were fitted with overall survival (OS) as the outcome. All *P* values were two-sided, and values of $P < .05$ were considered statistically significant. The survival, correlation, and association analysis were conducted on patients with valid expression data for the three ESRR genes in the MATABRIC dataset.

The RNA gene expression log2 (TPM+1) of each BC cell line was examined, and statistical differences between molecular subtypes were assessed using the *t*-test. A *P*-value $< .05$ was regarded as statistically significant.

Results

Study population description

The demographic and clinicopathologic characteristics of this dataset were previously described.[38] As shown in Supplementary Table 1, the mean age at the time of diagnosis was 60.42 ± 4.01 years. The average tumor size and the average number of positive lymph nodes were 26.22 ± 15.37 mm and 1.95 ± 4.02, respectively. Mean value of Nottingham Prognostic Index (NPI) was 4.03 ± 1.19, and the mean OS was 125.24 ± 76.11 months. One thousand five hundred and fifty-six patients (78.6%) were postmenopausal and 424 patients (21.4%) were premenopausal. ER expression was reported in 1817 (74.9%), and 1040 (52.5%) were identified as progesterone receptor (PR)-positive. Two hundred and forty-seven patients (12.5%) had HER2-positive status. Invasive ductal carcinoma (IDC) was the most common histology (76.2%). About 90% of patients had early-stage (I/II) disease, and almost half of them had high-grade tumor III (50.2%). Luminal A and luminal B were the most prevailing molecular subtypes representing 35.5% and 24.1%, respectively. Approximately two-thirds of BC patients had mastectomy and received hormonal and/or radiotherapy. Other clinicopathologic data for the study population are shown in Supplementary Table 1.[38]

Expression of ESRRs in BC patients

To examine ESRRα, β, and γ gene regulation, mRNA gene expression and CNAs for the three ESRRs were investigated in this cohort of BC patients. ESRRs mRNA expression data were available for 1904 patients. Average ESRRα, β, and γ mRNA expression log intensity were 6.79 ± 0.39, 5.47 ± 0.14, and 6.34 ± 0.83, respectively as shown in Table 1. To better understand the impact of ESRRα, β, and γ gene expression on BC pathogenesis, their mRNAs were stratified into low and high-expressing groups. The mean mRNA log intensity for each ESRR was set as a cutoff point of low (⩽ mean) or high (> mean) gene expression. Consequently, the total number of patients and the relevant percentages of high and low mRNA expression for each of the ESRRs are shown in Table 1. CNAs descriptive analysis in BC patients has also demonstrated that each of ESRRα, β, and γ genes has its distinct gene alteration, as presented in Table 1. Compared with other ESRR genes, ESRRγ CNAs were the most prevalent among patients, where 55.1% of patients had either hemizygous deletion, gain, or high amplification level. Although ESRRβ showed the highest proportion of patients with hemizygous deletion (20.2%), gene amplification was mostly seen in ESRRγ (21.4%).

Table 2. Correlation analysis of ESRR gene mRNA expression levels with clinicopathologic characteristics of breast cancer patients (N = 1904).

CHARACTERISTIC	ESRRα MRNA		ESRRβ MRNA		ESRRγ MRNA	
	R	*P*-VALUE	R	*P*-VALUE	R	*P*-VALUE
Age, years	−0.084	< .001*	−0.035	.130	0.084	< .001*
Tumor size, mm	0.059	.011*	0.025	.270	0.008	.741
Number of positive lymph nodes	0.061	.008*	−0.007	.759	−0.025	.273
Nottingham prognostic index (NPI)	0.166	< .001*	−0.017	.467	−0.119	< .001*
Overall survival (OS), months	−0.061	.008*	−0.006	.782	0.021	.353

ESRR, estrogen-related receptor; mRNA, messenger RNA; r, Pearson's correlation coefficient.
mRNA levels measured with log intensity.
*$P < .05$.

Association of the expression of ESRRs with clinicopathologic characteristics of BC patients

ESRRα mRNA expression was significantly and negatively correlated with the age of the patient at diagnosis ($P < .001$) and with OS ($P = .008$; Table 2). Alternatively, ESRRα expression was positively correlated with tumor size ($P = .011$), the number of positive lymph nodes ($P = .008$), and NPI ($P < .001$). The expression of ESRRγ was significantly and positively correlated with patients' age at the time of diagnosis ($P < .001$) and negatively correlated with NPI ($P < .001$) as shown in Table 2. Despite the significant findings, these correlations were considered weak in magnitude based on the accompanied correlation coefficient value for each of these bivariate analyses. No significant correlation was observed between ESRRβ mRNA expression and any of the criteria mentioned in Table 2.

As shown in Table 3, the expression of ESRRγ but not ESRRα or β showed a significant association with the patients' menopausal status ($P = .005$). A greater proportion of premenopausal patients had low ESRRγ expression, whereas a greater proportion of postmenopausal patients had a high expression status of the gene. With the exception of ESRRβ, neither ESRRα expression nor ESRRγ was significantly associated with tumor stage. The expression of both ESRRα and ESRRγ was significantly associated with tumor grade ($P < .001$). A greater proportion of BC patients who had high-grade (III) disease presented with a high expression status of ESRRα (53.1%), whereas most patients who have low and moderate grades had low expression of ESRRα (Table 3). Contrary to the findings with ESRRα, more than two-thirds of BC patients (66%) with high-grade (III) carcinoma had low gene expression of ESRRγ. Furthermore, the expression of ESRRα and ESRRγ was significantly associated with hormone receptor status. In this regard, more than two-thirds of patients who are ER-positive (65.4%) and PR-positive (68.1%) have low ESRRα gene expression. Interestingly, the ESRRγ low expression was associated with ER and PR regardless of their expression status compared with ESRRγ high expressing groups, as shown in Table 3. The association of ESRRα with HER2 status was also significant ($P < .001$). Approximately 66% of patients identified as HER2-positive, were also expressing a high level of ESRRα. The same pattern with ESRRα high expression and HER2 was also seen when patients were stratified based on their molecular subtypes (Table 3). On the contrary, the association between ESRRγ and HER2 was also significant ($P < .008$), but complex. Higher proportions of patients with low ESRRγ expression had HER2-negative disease (60.8%) compared with those with high expression (39.2%) of the gene. ESRRα and ESRRγ expressions were significantly associated with BC molecular subtypes ($P < .001$). Approximately two-thirds of normal-like (63.6%), luminal A (72.5%), and luminal B (60.3%) subtypes were identified as low expressing ESRRα. In comparison, approximately three-quarters of patients with HER2-positive (74.9%) and basal-like (72.4%) subtypes were identified as high ESRRα expressing patients. Unlike ESRRα, ESRRγ low expression was highly associated with luminal B (61.6%), basal-like (74.9%), and claudin-low (80.5%). No significant association was observed between ESRRβ and menopausal status, tumor grade, hormone receptors expression status, HER2 expression or BC molecular subtypes (Table 3).

ESRRs expression in BC cell lines

The expression of ESRRs genes was further investigated in 52 BC cell lines. The cells were classified into four molecular subtypes as described in the Methods section and summarized in Supplementary Table 2. Among the three ESRRs, only ESRRα gene expression was significantly higher in HER2-positive cells compared with luminal A and basal-like subtypes ($P < .05$), as shown in Figure 1. No significant differences for the level of gene expression of each of ESRRβ and ESRR γ were observed across the molecular subtypes of BC cell lines (data not shown).

ESRRs CNAs in BC patients

Unlike mRNA gene expression analysis, the evaluation of the role of ESRRα, β, and γ CNAs alteration in BC pathogenesis

Table 3. Association between ESRR mRNA expression with clinicopathologic characteristics of breast cancer patients (N = 1904).

CHARACTERISTIC	ESRRα GENE EXPRESSION			ESRRβ GENE EXPRESSION			ESRRγ GENE EXPRESSION		
	LOW (N = 1084)	HIGH (N = 820)	*P*-VALUE	LOW (N = 953)	HIGH (N = 951)	*P*-VALUE	LOW (N = 1136)	HIGH (N = 768)	*P*-VALUE
Menopausal status			.144			.679			.005*
Premenopausal	221 (53.8)	190 (46.2)		202 (49.1)	209 (50.9)		270 (65.7)	141 (34.3)	
Postmenopausal	863 (57.8)	630 (42.2)		751 (50.3)	742 (49.7)		866 (58.0)	627 (42.0)	
TNM stage			.72			.026*			.527
In situ (stage 0)	1 (25.0)	3 (75.0)		1 (25.0)	3 (75.0)		1 (25.0)	3 (75.0)	
I	291 (61.3)	184 (38.7)		258 (54.3)	217 (45.7)		290 (61.1)	185 (38.9)	
II	459 (57.4)	341 (42.6)		373 (46.6)	427 (53.4)		473 (59.1)	327 (40.9)	
III	61 (53.0)	54 (47.0)		60 (52.2)	55 (47.8)		72 (62.6)	43 (37.4)	
IV	8 (88.9)	1 (11.1)		2 (22.2)	7 (77.8)		5 (55.6)	4 (44.4)	
Grade			< .001*			.354			< .001*
I	130 (78.8)	35 (21.2)		87 (52.7)	78 (47.3)		82 (49.7)	83 (50.3)	
II	476 (64.2)	265 (35.8)		357 (48.2)	384 (51.8)		399 (53.8)	342 (46.2)	
III	434 (46.9)	492 (53.1)		475 (51.3)	451 (48.7)		611 (66.0)	315 (34.0)	
ER			< .001*			.195			.014*
Positive	946 (65.4)	500 (34.6)		712 (49.2)	734 (50.8)		840 (58.1)	606 (41.9)	
Negative	121 (28.3)	307 (71.7)		226 (52.8)	202 (47.2)		277 (64.7)	151 (35.3)	
PR			< .001*			.311			< .001*
Positive	687 (68.1)	322 (31.9)		494 (49.0)	515 (51.0)		556 (55.1)	453 (44.9)	
Negative	397 (44.4)	498 (55.6)		459 (51.3)	436 (48.7)		580 (64.8)	315 (35.2)	
HER2			< .001*			.903			.008*
Positive	81 (34.3)	155 (65.7)		119 (50.4)	117 (49.6)		122 (51.7)	114 (48.3)	
Negative	1003 (60.1)	665 (39.9)		834 (50.0)	834 (50.0)		1014 (60.8)	654 (39.2)	
Molecular subtype			< .001*			.227			< .001*
Normal-like	89 (63.6)	51 (36.4)		59 (42.1)	81 (57.9)		69 (49.3)	71 (50.7)	
Luminal A	492 (72.5)	187 (27.5)		350 (51.5)	329 (48.5)		359 (52.9)	320 (47.1)	
Luminal B	278 (60.3)	183 (39.7)		232 (50.3)	229 (49.7)		284 (61.6)	177 (38.4)	
HER2-positive	55 (25.1)	164 (74.9)		108 (49.3)	111 (50.7)		109 (49.8)	110 (50.2)	
Basal-like	55 (27.6)	144 (72.4)		107 (53.8)	92 (46.2)		149 (74.9)	50 (25.1)	
Claudin-low	113 (56.5)	87 (43.5)		91 (45.5)	109 (54.5)		161 (80.5)	39 (19.5)	

ER, estrogen receptor; ESRR, estrogen-related receptor; HER2, human epidermal growth factor receptor 2; mRNA, messenger RNA; PR, progesterone receptor.
Data are presented as n (%).
Data represents frequency and valid percentage.
*$P < .05$.

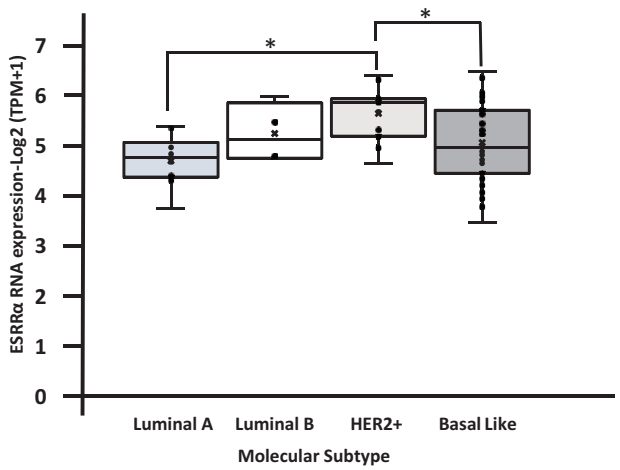

Figure 1. ESRRα gene expression status in BC cell lines: ESRRα RNA gene expression in BC cells from CCLE. Cell line classification and expression analysis are described in the Methods section. **P*-value < .05. BC, breast cancer; ESRR, estrogen-related receptor; CCLE, Cancer Cell Line Encyclopedia; HER2, human epidermal growth factor receptor 2; TPM, transcript per million.

was multifaceted. In this regard, and as shown in Tables 4 to 6, we performed the association analysis of ESRRα, β, and γ with BC clinicopathologic characteristics. ESRRα CNAs showed significant association with tumor stage, grade, HER2, and BC molecular subtypes. Hemizygous deletion of ESRRα gene was the most frequent CNA among BC patients in this cohort (Table 4). It was observed in 27% of patients with early-stage I/II compared with stage III (19.8%) as shown in Table 4. Tumor grades I/II also represent 22.2% of hemizygous deletion, whereas grade III represents 16.5%. The association between HER2 expression status and molecular subtypes with hemizygous deletion was more complex. Although both HER2-negative and positive have similar percentages of hemizygous deletions, the luminal B subtype represents 24% compared with gain and high-level amplification. Furthermore, no significant association was observed between ESRRα CNAs and hormone receptor status or menopausal status (Table 4).

The hemizygous deletion was also the most frequent ESRRβ CNA and represented approximately one-fifth of total patients (Table 1). Contrary to its mRNA gene expression, ESRRβ CNA was significantly associated with tumor grade, hormone receptor status, and molecular subtypes ($P < .001$), as shown in Table 5. A high proportion of patients with hemizygous deletion had high-grade III (33.9%), ER-negative (42.5%), and PR-negative (32.9%) disease. Strikingly, hemizygous deletion of ESRRβ was found in (59.5%) of patients having the basal-like subtype (Table 5). ESRRγ high-level amplification was the most prevalent CNA among all patients (n = 536, 21.4%), whereas hemizygous deletion was the least common one (n = 28, 1.1%), as shown in Table 1. The association of ESRRγ CNA with tumor stage, grade, hormone receptor status, and molecular subtypes was significant (Table 6). With exception to *in situ* (stage 0), the proportion of patients with gain and high-level amplification combined represented more than half of the total patients across the clinicopathologic characteristics presented in Table 6. However, no significant association between ESRRγ CNA was observed with menopausal status or HER2 expression.

ESRRα, β, and γ expression and OS of BC patients

The impact of ESRRα, β, and γ gene expression on OS was distinct as shown in Table 2. Only ESRRα mRNA gene expression showed a significant and negative correlation with OS (Table 2). Kaplan–Meier survival analyses revealed no significant differences in OS between low and high expressing groups of each of ESRRα, β, and γ genes among patients, as shown in Supplementary Figures S1 to S3. Notably, on data stratification based on prognostic factors and clinicopathologic characteristics, a higher survival rate was observed in ESRRα low expressing patients among premenopausal patients ($P = .0037$), tumor grade I/II ($P = .0454$), and early-stage ($P = .0445$) compared with ESRRα high expression patients as shown in Supplementary Figure S1B, F, and H. Alternatively, survival curves for patients with low or high expression of ESRRα were not significantly different among postmenopausal cases, molecular subtypes, grade III, and advanced stage as shown in Supplementary Figure S1C to E, G, and I. Survival curves were not significantly different for BC patients with high or low expression for each of ESRRβ and ESRRγ regardless of menopausal status, molecular subtypes, tumor grade, and stage as shown in Supplementary Figures S2 and S3. Hazard ratio (95% confidence interval (CI)) and *P*-values for the survival analysis of ESRRα, ESRRβ, and ESRRγ are summarized in Supplementary Table 3.

Discussion

BC development and progression are complex and still not fully understood. Efforts to unfold the mechanisms underlying the pathogenesis of BC will facilitate the development of precise cancer therapy, better disease management, and enhanced treatment outcomes. Here we have shown that ESRRα and ESRRγ gene expression and CNAs are significantly associated with BC clinicopathologic features. However, the role of ESRRβ is still unclear. This is in agreement with previous studies where ESRRα and ESRRγ have been proposed as potential markers in various types of tumors, play opposite roles in BC disease development and progression, and both are highly associated with the cancer phenotype.[5,27,29-31]

It has been suggested that age at diagnosis is one of the most important variables in determining BC outcomes.[42,43] BC of younger patients is characterized by a more aggressive tumor, less survival, and a higher incidence of negative clinicopathologic features.[44] Regarding this, we have shown that both ESRRα and γ, but not β, were significantly correlated with age

Table 4. Association analysis of ESRRα copy number alteration with clinicopathologic characteristics in breast cancer patients (N = 2173).

CHARACTERISTICS	ESRRα COPY NUMBER ALTERATION				
	HEMIZYGOUS DELETION (N = 315)	NEUTRAL/ NO CHANGE (N = 1705)	GAIN (N = 133)	HIGH LEVEL AMPLIFICATION (N = 20)	*P*-VALUE
Menopausal status					.159
Premenopausal	48 (11.3)	347 (81.8)	26 (6.1)	3 (0.7)	
Postmenopausal	245 (15.7)	1210 (77.8)	90 (5.8)	11 (0.7)	
TNM stage					.021*
In situ (stage 0)	0 (0.0)	14 (87.5)	1 (6.3)	1 (6.3)	
I	62 (11.7)	430 (81.4)	31 (5.9)	5 (0.9)	
II	133 (15.3)	670 (76.9)	62 (7.1)	6 (0.7)	
III	25 (19.8)	95 (75.4)	4 (3.2)	2 (1.6)	
IV	3 (30.0)	5 (50.0)	2 (20.0)	0 (0.0)	
Grade					< .001*
I	15 (8.6)	154 (88.5)	4 (2.3)	1 (0.6)	
II	116 (13.6)	693 (81.2)	37 (4.3)	7 (0.8)	
III	172 (16.5)	776 (74.3)	86 (8.2)	11 (1.1)	
ER					.306
Positive	250 (15.5)	1250 (77.6)	98 (6.1)	13 (0.8)	
Negative	59 (12.1)	393 (80.7)	30 (6.2)	5 (1.0)	
PR					.113
Positive	139 (13.4)	838 (80.6)	58 (5.6)	5 (0.5)	
Negative	154 (16.4)	719 (76.5)	58 (6.2)	9 (1.0)	
HER2					< .001*
Positive	34 (13.8)	188 (76.1)	18 (7.3)	7 (2.8)	
Negative	259 (14.9)	1369 (79.0)	98 (5.7)	7 (0.4)	
Molecular subtype					< .001*
Normal-like	21 (14.2)	121 (81.8)	4 (2.9)	2 (1.4)	
Luminal A	78 (11.1)	591 (84.4)	27 (3.9)	4 (0.6)	
Luminal B	114 (24.0)	310 (65.3)	47 (9.9)	4 (0.8)	
HER2-positive	31 (13.8)	179 (79.9)	11 (4.9)	3 (1.3)	
Basal-like	31 (14.8)	159 (76.1)	18 (8.6)	1 (0.5)	
Claudin-low	18 (8.3)	191 (87.6)	9 (4.1)	0 (0.0)	

ER, estrogen receptor; ESRR, estrogen-related receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.
Data are presented as n (%).
Data represents frequency and valid percentage.
*$P < .05$.

at diagnosis. The negative correlation between ESRRα expression and age at diagnosis suggested a possible contribution of ESRRα to early development and severity of the disease. Conversely, the positive correlation between ESRRγ and age at diagnosis suggested that lower expression at a younger age might be associated with unfavorable outcomes in this group. This observation was further supported by OS analysis. Although the correlations were considered weak in magnitude

Table 5. Association analysis of ESRRβ copy number alteration with clinicopathologic characteristics in breast cancer patients (N = 2173).

CHARACTERISTICS	ESRRβ COPY NUMBER ALTERATION					
	HOMOZYGOUS DELETION (N = 1)	HEMIZYGOUS DELETION (N = 507)	NEUTRAL/ NO CHANGE (N = 1575)	GAIN (N = 82)	HIGH LEVEL AMPLIFICATION (N = 8)	*P*-VALUE
Menopausal status						.470
Premenopausal	0 (0.0)	100 (23.6)	306 (72.2)	15 (3.5)	3 (0.7)	
Postmenopausal	1 (0.1)	348 (22.4)	1145 (73.6)	59 (3.8)	3 (0.2)	
TNM stage						.356
In situ (stage 0)	—	2 (12.5)	14 (87.5)	0 (0.0)	0 (0.0)	
I	—	101 (19.1)	409 (77.5)	17 (3.2)	1 (0.2)	
II	—	204 (23.4)	623 (71.5)	38 (4.4)	6 (0.7)	
III	—	28 (22.2)	93 (73.8)	5 (4.0)	0 (0.0)	
IV	—	0 (0.0)	9 (90.0)	1 (10.0)	0 (0.0)	
Grade						< .001*
I	0 (0.0)	16 (9.2)	154 (88.5)	4 (2.3)	0 (0.0)	
II	0 (0.0)	123 (14.4)	692 (81.1)	36 (4.2)	2 (0.2)	
III	1 (0.1)	354 (33.9)	644 (61.6)	40 (3.8)	6 (0.6)	
ER						< .001*
Positive	1 (0.1)	286 (17.8)	1254 (77.8)	67 (4.2)	3 (0.2)	
Negative	0 (0.0)	207 (42.5)	263 (54.0)	14 (2.9)	3 (0.6)	
PR						< .001*
Positive	0 (0.0)	139 (13.4)	847 (81.4)	51 (4.9)	3 (0.3)	
Negative	1 (0.1)	309 (32.9)	604 (64.3)	23 (2.4)	3 (0.3)	
HER2						.283
Positive	0 (0.0)	68 (27.5)	172 (69.6)	6 (2.4)	1 (0.4)	
Negative	1 (0.1)	380 (21.9)	1279 (73.8)	68 (3.9)	5 (0.3)	
Molecular subtype						< .001*
Normal-like	0 (0.0)	22 (14.9)	121 (81.8)	4 (2.7)	1 (0.7)	
Luminal A	0 (0.0)	78 (11.1)	588 (84.0)	34 (4.9)	0 (0.0)	
Luminal B	1 (0.2)	112 (23.6)	340 (71.6)	20 (4.2)	2 (0.4)	
HER2-positive	0 (0.0)	61 (27.2)	154 (68.8)	7 (3.1)	2 (0.9)	
Basal-like	0 (0.0)	124 (59.3)	79 (37.8)	5 (2.4)	1 (0.5)	
Claudin-low	0 (0.0)	49 (22.5)	165 (75.7)	4 (1.8)	0 (0.0)	

ER, estrogen receptor; ESRR, estrogen-related receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.
Data are presented as n (%).
Data represents frequency and valid percentage.
*Statistical significance at $P < 0.05$.

based on the accompanied correlation coefficient value, the inverse correlation between ESRRα expression and OS was significant in our dataset and agreed with other studies where the expression of ESRRα was associated with poor prognosis in BC. Furthermore, in a small clinical study of 102 BC samples, Suzuki et al[45] have shown that a decrease in OS at 13 years was associated with an increase in ESRRα gene expression. This was also in agreement with another study conducted in

Table 6. Association analysis of ESRRγ copy number alteration with clinicopathologic characteristics in breast cancer patients (N = 2173).

CHARACTERISTICS	ESRRγ COPY NUMBER ALTERATION				
	HEMIZYGOUS DELETION (N = 28)	NEUTRAL/NO CHANGE (N = 791)	GAIN (N = 818)	HIGH LEVEL AMPLIFICATION (N = 536)	*P*-VALUE
Menopausal status					.582
Premenopausal	6 (1.4)	155 (36.6)	168 (39.6)	95 (22.4)	
Postmenopausal	19 (1.2)	553 (35.5)	585 (37.6)	399 (25.6)	
TNM stage					.044*
In situ (stage 0)	0 (0.0)	10 (62.5)	3 (18.8)	3 (18.8)	
I	1 (0.2)	212 (40.2)	186 (35.2)	129 (24.4)	
II	17 (2.0)	298 (34.2)	333 (38.2)	223 (25.6)	
III	1 (0.8)	50 (39.7)	46 (36.5)	29 (23.0)	
IV	0 (0.0)	2 (20.0)	3 (30.0)	5 (50.0)	
Grade					.019*
I	1 (0.6)	60 (34.5)	59 (33.9)	54 (31.0)	
II	5 (0.6)	311 (36.5)	316 (37.0)	221 (25.9)	
III	22 (2.1)	373 (35.7)	411 (39.3)	239 (22.9)	
ER					< .001*
Positive	14 (0.9)	552 (34.3)	605 (37.6)	440 (27.3)	
Negative	14 (2.9)	199 (40.9)	194 (39.8)	80 (16.4)	
PR					< .001*
Positive	9 (0.9)	329 (31.6)	397 (38.2)	305 (29.3)	
Negative	16 (1.7)	379 (40.3)	356 (37.9)	189 (20.1)	
HER2					.363
Positive	2 (0.8)	81 (32.8)	92 (37.2)	72 (29.1)	
Negative	23 (1.3)	627 (36.2)	661 (38.1)	422 (24.4)	
Molecular subtype					< .001*
Normal-like	1 (0.7)	72 (48.6)	56 (37.8)	19 (12.8)	
Luminal A	4 (0.6)	201 (28.7)	265 (37.9)	230 (32.9)	
Luminal B	4 (0.8)	147 (30.9)	190 (40.0)	134 (28.2)	
HER2-positive	3 (1.3)	75 (33.5)	93 (41.5)	53 (23.7)	
Basal-like	8 (3.8)	71 (34.0)	94 (45.0)	36 (17.2)	
Claudin-low	5 (2.3)	141 (64.7)	51 (23.4)	21 (9.6)	

ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.
Data are presented as n (%).
Data represents frequency and valid percentage.
*$P < .05$.

ovarian cancer patients, where survival analysis showed that the ESRRα-positive group has a reduced OS.[46]

Stein et al[47] showed that although stable knockdown of ESRRα expression in MDA-MB-231 ER-negative BC cells had no impact on cell proliferation *in vitro*, a reduction of tumor growth rate was significant when these cells were implanted as xenograft tumors. Other studies showed that inverse agonists of ESRRα inhibit cell proliferation, induce cell

death, and reduce tumorigenicity.[48-50] These findings are consistent with our data, where the tumor size is positively correlated with ESRRα expression suggesting its role in tumor growth and proliferation. Our results also revealed a positive correlation between the number of positive lymph nodes and ESRRα gene expression. ESRRα has been shown to trigger the migration and invasion of cancer cells in various tumors such as endometrial cancer, colorectal cancer, oral squamous cell carcinoma, and BC.[48,51-54]

The NPI is a widely accepted scoring method for BC prognosis.[55] We have demonstrated the ESRRα gene expression to be positively correlated with NPI. Conversely, ESRRγ is inversely correlated with NPI. Although the correlations are considered weak, they are highly significant. These findings are in agreement with previous reports that linked ESRRα and ESRRγ to the pathology and prognosis of several solid tumors including BC.[45,47,56-59] Taken together, the above results clearly support the role of ESRRα and ESRRγ in BC pathogenesis. ESRRα expression is correlated with poor prognosis and unfavorable clinical outcomes, whereas ESRRγ is associated with a favorable prognosis and outcomes. Importantly, in our clinical dataset, we could not observe a significant correlation between ESRRβ gene expression and tumor size, the number of lymph nodes, NPI, and OS, which undermine its roles in BC pathogenesis but warrant further investigation.

The association analysis of ESRRα, β, and γ gene expression with BC clinicopathologic characteristics was also distinct among these isoforms. The significant association of ESRRα with tumor grade, hormone receptor status, HER2 expression, and molecular subtypes was generally in agreement with previous reports. In a small study conducted on 33 ovarian cancer samples and 12 samples from normal ovaries, Sun et al demonstrated that a great number of cancer samples had ESRRα gene expression. Furthermore, they demonstrated a positive correlation between ESRRα expression and advanced tumor stage and grade.[46] ERα and PR expression are considered good prognostic markers for patients with BC.[60] However, it has been shown that in patients with breast and ovarian cancers, the expression of ESRRα is inversely correlated with ER and PR gene expression and that high ESRRα is associated with an increased rate of recurrence and poor prognosis.[30,45,61] In another study using samples from various cohorts of patients with BC, Jarzabek et al have shown that ESRRα gene expression is positively correlated with HER2 oncogene expression and inversely correlated to ER and PR. These observations also supported the ESRRα expression in different BC cell lines and molecular subtypes. ESRRα high expression was associated with HER2-positive and basal-like molecular subtypes in patients. Similarly, ESRRα expression was significantly associated with HER2-expressing BC cell lines.

The significant association of ESRRγ with tumor grade, hormone receptor status, HER2, and BC molecular subtypes was also in agreement with previous studies and further supported its role as a prognostic factor. In one study where the Oncomine cancer database was investigated, Tiraby et al[62] have shown that reduced ESRRγ expression is significantly correlated with higher BC grade, metastasis, recurrence, and unfavorable outcome. Using MDA-MB-231 BC cells expressing human ESRRγ, Tiraby et al[62] also showed that ESRRγ suppressed cell invasiveness *in vitro* and inhibited tumor growth *in vivo* using BC xenograft mouse model. Tumors overexpressing ESRRγ are frequently hormone receptor-positive.[34] Furthermore, in BC co-expressing ER and PR, ESRRγ induces E-cadherin expression and promotes the mesenchymal-to-epithelial transition, resulting in tumor growth inhibition.[34,62] As a result, the co-expression of hormone receptors and ESRRγ may reflect better hormonal sensitivity and a favorable clinical outcome.[34] However, with exception to tumor stage, we have shown that ESRRβ expression was not significantly associated with the clinicopathologic characteristics of BC. Contrary to our findings, a previous study has demonstrated that ESRRβ expression is associated with ERβ and that ESRRβ levels inversely correlate with the S-phase fraction. Consequently, the authors suggested that ESRRβ inhibits cellular proliferation, or possibly promotes cellular differentiation.[34] In another report, it was also found that ESRRβ can act as a proliferative gene.[62] Thus, the potential role of ESRRβ in BC remains unclear and needs further investigation.

Here we have demonstrated that only ESRRα gene expression showed a significant correlation with OS. This finding agrees with previous studies where it was revealed that increased expression of ESRRα was associated with risk of recurrence and poor prognosis in breast, colorectal, and ovarian cancer[30] patients.[45-47] Interestingly, the Kaplan–Meier analysis did not show a significant difference in survival in ESRRα low expressing patients compared with high expressing ones. However, using individual prognostic factors, the impact of ESRRα gene expression levels on survival was evident for many. The premenopausal status, tumor grade I/II, and early-stage have shown a negative correlation with survival in patients expressing high levels of ESRRα. The premenopausal status has often been used synonymously with age in evaluations of women with BC. This is also aligned with our finding that ESRRα expression is negatively correlated with the patients' age at diagnosis. Previous studies have shown that patients under 40 years of age are associated with a higher risk of relapse and death, even with the administration of more aggressive therapies.[63,64] It has also been shown that ESRRα can increase local estrogen production by induction of the aromatase gene expression, which may increase the risk of malignant transformation of breast epithelium.[65]

Using gene expression profiling, Anders et al[66] had shown that younger patients (45 years) had higher Myc and PI3 K pathway dysregulation than older patients (65 years). However, when the analysis was adjusted to BC molecular subtypes, no distinct molecular aberrations were found related to age.[44] The

significant correlation between ESRRα high expression and low survival in premenopausal status, tumor grade, and early disease stage can be explained, in part, by its ability to induce the expression of several oncogenes and/or by activation or dysregulation of pathways responsible for BC pathogeneses. Alternatively, additional factors involved in regulating ESRRα activity may be required for the pathogenesis of BC. Indeed, the activity of ESRRα is regulated by the expression of its attendant co-activator/co-repressor proteins. Using a gene signature derived from ESRRα-activated genes, Chang et al[67] reported a significant negative correlation of ESRRα activity and relapse-free survival in multiple BC datasets.

Associations of ESRRα, β, and γ CNAs with BC clinicopathologic characteristics were evident and distinct for each gene and somewhat different when compared with mRNA gene expression analysis. Although the association of ESRRs with cancer is apparent, few studies are out there to address their amplified or reduced expression in BC. Deblois et al[54] have shown that in a mouse model of ERBB2-induced mammary tumors, ESRRα homozygous deletion caused a significant delay in tumor development. Another study conducted in oral squamous cell carcinoma showed that genomic amplification upregulates ESRRα and its depletion inhibits oral squamous cell carcinoma *in vivo*.[52] These findings support the role of ESRRα in tumorigenesis. Here we have shown a significant association of ESRRα CNAs with tumor stage, grade, HER2 expression, and molecular subtypes. Interestingly, the proportion of hemizygous deletion was higher than gene amplification in this patient dataset. Contrary to gene expression association analysis, ESRRβ CNAs showed significant association with many clinicopathologic characteristics; however, the proportion of patients with deletions or amplification was low to draw a concrete conclusion. ESRRγ CNAs were significantly associated with many clinicopathologic characteristics; however, here we observed that approximately one-quarter of patients had shown gene amplification. A previous study demonstrated that both ESRRγ mRNA and protein expression are upregulated compared with normal samples in human BC specimens,[34] and exogenously transfected ESRRγ increased BC cell proliferation.[34] This suggested that gene amplification of ESRRγ alone is not enough to induce tumor suppression activity and needs further investigation.[62]

Conclusions

Here we have demonstrated that ESRRα, β, and γ gene expression and CNAs are modulated in BC but have distinct roles in its pathogenesis. ESRRα gene expression is an adverse prognostic factor and correlated with negative clinical outcomes. Conversely, ESRRγ gene expression is associated with positive outcomes and could serve as a good prognostic factor for BC patients. ESRRα and ESRRγ CNAs also showed a significant association with many clinicopathologic characteristics aligned with gene expression. Although ESRRβ gene expression has failed to show any association with BC clinicopathologic characteristics, the ESRRβ CNAs was significant but not conclusive due to the small number of patients who had either homozygous deletions or high-level amplification. Only ESRRα increased expression showed a shorter OS. Stratification of ESRRα gene expression into high and low groups showed no significant difference in patients' OS between the two groups. However, significant impact and shorter survival of high ESRRα expression among premenopausal patients, tumor grade I/II, and stage compared with low expressing patients. To conclude, ESRRα and ESRRγ could be utilized as therapeutic targets in BC therapy. Nevertheless, the potential of ESRRβ in cancer therapy needs further investigation.

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Author Contributions

AS contributed to the conception, design, and writing of the article. NMA contributed to the analysis, design, and reviewing of the article. AEA and DRI contributed to the coding and analysis of the article.

Availability of Data and Material

Clinical data are available on cBioPortal (https://www.cbioportal.org/). Cell line expression data are available on Cancer Cell Line Encyclopedia ((CCLE) https://www.broadinstitute.org/cancer/cancer-program-scientific-tools-and-resources) public domains.

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Supplemental Material

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