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Isoform-specific expression of *T*-type voltage-gated calcium channels and estrogen receptors in breast cancer reveals specific isoforms that may be potential targets

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ABSTRACT

Purpose: *T*-type voltage-gated calcium channels (TTCCs) have been implicated as novel targets in breast cancer treatment; however, the knowledge of isoform-specific expression of TTCCs in the pathology of breast cancer remains limited. Although estrogen receptor blockers are clinically beneficial, patients often show resistance to these compounds. Interestingly, TTCCs are modulated by estrogen in the brain and heterologous expression systems; however, whether TTCCs and estrogen receptors (ERs) interact in breast tissue is unknown. In addition, the isoform specific expression of TTCCs and ERs in breast cancer subtypes has not been determined.
Methods: We investigated the alterations in TTCC and ER gene isoforms, their co-occurrence and the association of alterations with patient survival in breast cancer and its subtypes by using The Cancer Genome Atlas (TCGA) dataset. To understand the functional roles of TTCC and ESR genes in breast cancer, we studied cell proliferation in the presence of a TTCC inhibitor and ER activator.
Results: Our results from the TCGA dataset indicated differential alterations in TTCC isoform expression in patients with breast cancer, including downregulation of CACNA1G and upregulation of CACNA1H, and CACNA1I. Among ER isoforms, upregulation of ESR1 and downregulation of ESR2 were observed. Survival analysis indicated that ESR2 may be a potential target, because its downregulation was associated with shorter overall survival. Subtype specific survival analysis revealed CACNA1G, CACNA1H and ESR2 as potential novel targets in luminal type breast cancer. Alterations in ESR2 co-occurred with alterations in CACNA1H and CACNA1I, thus warranting investigation of potential crosstalk. Experimental data indicated that targeting both TTCCs and ESR2 may be beneficial for luminal type breast cancer.
Conclusion: Our data provide novel insights into the altered expression of TTCC and ESR gene isoforms in breast cancer subtypes and suggest novel therapeutic targets.

Introduction

Breast cancer is a global challenge and is among the most common cancers in women (Harbeck and Gnant, 2017). Four major intrinsic molecular subtypes of breast cancer have been identified: luminal A, luminal B, human epidermal growth factor receptor 2 positive ($HER2^+$) and basal-like/triple-negative breast cancer (TNBC) (Prat et al., 2015). The luminal (type A and B) subtype is characterized by the presence of hormone receptors for estrogen and progesterone. The luminal A subtype lacks HER2, whereas HER2 may or may not be present in luminal type B (Yersal and Barutca, 2014). In the $HER2^+$ subtype, hormone receptors are absent, and the HER2 receptor is present. The TNBC subtype of breast cancer does not express hormone receptors or the HER2 receptor. The inherent complexity of breast cancer underscores the need for novel treatments. Steroid hormones such as estrogen and progesterone increase the risk of breast cancer by regulating the breast cancer stem cell population (Finlay-Schultz and Sartorius, 2015; Simões et al., 2015). Breast cancer tumors also exhibit estrogen dependency, and thus estrogen receptors (ERs) play critical roles in promoting the proliferation of breast cancer.

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ERs are classified into ER-alpha (ER-α) and ER-beta (ER-β), encoded by the genes ESR1 and ESR2, respectively. In breast cancer, the expression and activation of ER-α indicates a pro-proliferative role; consequently, therapy with estrogen receptor blockers is the choice of treatment for breast cancers expressing ER-α (Sharma et al., 2018). ER-β is abundant in normal breast epithelial cells but is present only in 20–30 % of breast cancer cases (Zhou and Liu, 2020). Studies suggest an anti-proliferative role of ER-β, although its exact functional role remains controversial (Fox et al., 2008; Leygue and Murphy, 2013; Tonetti et al., 2003; Lazennec et al., 2001).

Given the role of calcium in cancer progression, major players involved in maintaining calcium homeostasis, notably calcium channels, have been investigated. Two major types of calcium channels exist: store-operated calcium channels and voltage-gated calcium channels (VGCCs). Most prior research has focused on store-operated calcium channels, because VGCCs were thought to play a role in only excitable cells. However, VGCC's role in non-excitable cells is now well established (Pervarskaya et al., 2010; Phan et al., 2017). In addition, in the past decade, VGCCs have been implicated as potential targets in cancer. However, information on the exact roles of VGCC isoforms in breast cancer is lacking. Among VGCCs, *T*-type calcium channels (TTCCs) are low-voltage activated, meaning that a small depolarization in the plasma membrane can trigger the opening of these channels, thereby allowing calcium entry into the cells (Triggle and D., 1998). Consequently, TTCCs are excellent candidates for maintaining calcium homeostasis. Three genes, CACNA1G, CACNA1H and CACNA1I, encode the three TTCC isoforms, $Ca_V3.1$, $Ca_V3.2$ and $Ca_V3.3$, respectively. In addition, recent data have hinted at isoform-specific differential roles of TTCCs in breast cancer subtypes (Pera et al., 2016; Ohkubo and Yamazaki, 2012). Along these lines, *in vitro* studies have reported the over-expression of TTCCs in various cancers, including prostate, breast and ovarian cancer, whereas blocking these receptors has therapeutic effects (Antal and Martin-Carabello, 2019; Taylor et al., 2008). However, because most prior studies have used TTCC blockers that do not differentiate between different isoforms, the isoform-specific roles of TTCC isoforms remain unclear. In addition, in neurons and heterologous expression systems (Qiu et al., 2006), VGCCs are modulated by estrogen, but such data are lacking for breast cancer cells.

Therefore, to identify the isoform-specific expression of TTCCs and their association with ERs in breast cancer, we used a publicly available patient dataset, The Cancer Genome Atlas (TCGA), which we analyzed with the cBioPortal and UALCAN online databases. This approach allowed us to study 1) isoform-specific expression and alterations in TTCC and ER isoforms in breast cancer subtypes; 2) whether alterations in TTCC isoforms tend to co-occur with alterations in ER isoforms, thus providing multiple targets in breast cancer that may interact; and 3) the effects of alterations in TTCC and ER isoforms on the survival of patients with breast cancer, to ascertain whether targeting them might be useful.

Methods

We used the cBioPortal and University of Alabama Cancer (UALCAN) databases for the analysis. cBioPortal is an open-access, open-source web resource hosting various projects and publication associated datasets for cancer genomics. The portal aids in the exploration of multidimensional cancer genomics data by enabling analysis of mutations and alterations in genes across the samples in a given study (Gao et al., 2013). TCGA PanCancer Atlas dataset, 2018, was chosen for this study, because it has recently been updated and normalized through a similar pipeline across different cancer types. We also compared the expression levels of the queried genes from TCGA dataset with the METABRIC dataset in the same portal. The TTCC isoforms CACNA1G ($Ca_V3.1$), CACNA1H ($Ca_V3.2$) and CACNA1I ($Ca_V3.3$), and the isoforms of estrogen receptors ESR1 and ESR2 were queried.

Major alterations in queried genes

The major genomic alterations observed across the queried genes were determined by choosing genomic level data including mutations, copy number alterations and mRNA expression relative to normal samples. Complete data for 994 samples were analyzed. Once queried, the alteration frequency of the respective genes can be found under the tab "cancer type summary".

Mutual exclusivity analysis between alterations in queried genes

To analyze whether the major alterations observed were mutually exclusive or co-occurring between each pair of the queried genes, we selected genomic profiles containing mutations, copy number alterations and mRNA expression relative to normal samples among patient samples containing complete sample data (994 samples). The statistical significance was denoted with p-values derived from one-sided Fisher exact test, and the q-values were derived with the Benjamini-Hochberg FDR correction procedure.

Expression analysis

Genomic profiles consisting of mRNA expression in normal samples under the mRNA sample data set (1082 samples) were probed. The expression levels of the genes were plotted with mRNA expression z-scores relative to normal samples (log RNA Seq V2RSEM) on the y-axis against the cancer study (TCGA-BRCA) on the x-axis. The upregulation or downregulation of the queried genes was determined on the basis of the median values. We also analyzed the genomic profiles consisting of mRNA expression relative to diploid samples in the "Breast cancer METABRIC" dataset. METABRIC is a well-annotated dataset consisting of 2509 samples, from which 1904 samples containing mRNA data across major breast cancer subtypes were considered for further analysis. The mRNA levels of the TTCC and ESR gene isoforms (in log intensity levels) were plotted on the y-axis, with breast cancer subtypes on the x-axis.

Survival curve analysis

The Kaplan-Meier estimators and plots for overall survival and disease-free survival were generated with cBioPortal by comparison of mRNA expression profiles from normal samples with the case set containing mRNA sample data (1082 samples). P-values were derived with the log-rank test, and q-values were derived with the Benjamini-Hochberg FDR correction procedure.

Subtype-specific expression analysis

UALCAN, an open-access web portal, was used for breast cancer subtype-specific analysis. UALCAN aids in gene expression, correlation and survival analysis of the TCGA dataset across various cancers and tumor subtypes (Chandrashekar et al., 2017). TCGA's breast invasive carcinoma dataset was chosen, and the expression of the TTCC and ESR gene isoforms was observed in major breast cancer subtypes, including luminal, $HER2^+$ and TNBC, and compared with that in normal tissue. The data were visualized in box-and-whiskers plots, and log-rank P values were calculated to determine statistical significance.

Subtype-specific survival curve analysis

The mRNA sample data (1082 samples) from the TCGA PanCancer Atlas dataset were downloaded and analyzed. The Kaplan-Meier survival plots for overall survival in patients with various breast cancer

subtypes associated with queried genes were obtained with the R packages "Survival" and "Survminer." $P \leq 0.05$ was considered significant.

Cell culture and quantitative PCR

T-47D and MDA-MB-231 cells were maintained in standard cell culture conditions. Total RNA was isolated from 40 to 50 % confluent cells with TRIzol reagent according to the manufacturer's instructions. cDNA synthesis and further steps are described in detail in the supplementary information.

Cell proliferation assays

T-47D cells were seeded in 96 well plates (40,000 cells/200 μl/well) and placed in a CO_2 incubator for 24 h. Blocker/agonist treatment was then administered. Cells were treated with the TTCC blocker NNC 55–0396 hydrate (NNC, 5.6 μM), ER β agonist diarylpropionitrile (DPN, 150 nM) or both. In combination treatments, 23 h after cell seeding, 1 h pre-incubation with NNC was performed before addition of fresh medium containing both NNC and DPN. At 24 h post treatment, the medium was replaced, MTT was added, and cells were incubated for 4 h. The medium was aspirated, and 200 μl DMSO was added to dissolve the formazan crystals formed by the cells. Absorbance was determined at 540 nm with a multimode plate reader.

Results and discussion

Estrogen receptor and T-type voltage-gated calcium channel alterations in breast cancer

To understand the expression and interaction of TTCCs and ESRs in breast cancer, we first determined the overall alteration frequency of TTCC isoforms and ESR gene isoforms in breast cancer. Alteration frequency is the number of cancer patients in which these genes are altered (amplification/deep deletion/mutation/fusion/multiple alterations) divided by the total number of cancer patients. TTCC has three isoforms: CACNA1G, CACNA1H and CACNA1I (Bidaud et al., 2006). ESR has two isoforms: ESR1 and ESR2 (Al-Bader et al., 2011). Fig. 1 shows the alteration frequency of all isoforms of TTCCs and ESR genes in breast cancer in TCGA PanCancer Atlas dataset, 2018. Table 1 shows the major alterations observed.

As shown in Table 1, the major alterations in patients with breast cancer were associated with altered expression of the genes, although other alterations were also present. In breast cancer, altered expression of TTCC and ESR genes has been reported in cell lines, but the data have varied (Ohkubo and Yamazaki, 2012; Taylor et al., 2008; Al-Bader et al., 2011; Bertolesi et al., 2002). Our data showing downregulation of CACNA1G in breast carcinoma are in agreement with those from Phan et al., who have observed similar findings in computational studies (Phan et al., 2017). High mRNA expression of CACNA1H, as seen in our analysis, has been reported in a drug-resistant ER^+ breast cancer cell line (Pera et al., 2016). Although several studies have reported overexpression of CACNA1H in breast cancer (Pera et al., 2016; Asaga et al., 2006), reports are conflicting regarding the expression of CACNA1G: Ohkubo et al. have reported overexpression of the CACNA1G gene (Ohkubo and Yamazaki, 2012), in contrast to our findings, whereas other studies have reported downregulation of CACNA1G. We speculate that this discrepancy might have been due to the heterogeneity of breast cancer cell lines and their subtypes. Nevertheless, a larger cohort of patient data may enable deeper understanding of the isoform-specific expression of TTCCs in breast cancer. Of note, many studies have not reported the expression of CACNA1I in their analyses, for unclear reasons. Similarly to CACNA1H, CACNA1I was upregulated in most cases (Table 1, Fig. 1).

ESR1 overexpression is observed in 20.6 % of breast cancer patient samples (Holst et al., 2007). Our data suggested a similar number of cases in which ESR1 was downregulated (Table 1, Fig. 1). ESR2 has been reported to be downregulated in experimental studies, whereas overexpression of ESR2 exhibits growth suppression in breast cancer cell lines (Fox et al., 2008; Chang et al., 2006). Our analysis also indicated downregulation of ESR2 in most breast cancer cases.

To determine the extent of gene expression changes, we compared the expression of TTCC or ESR gene isoforms with that in adjacent non-cancerous cells in patients with breast cancer. The expression levels in TCGA revealed that all queried genes had a wide range of expression across all patients; however, considering the median values of the data, we report the observed trends in Table 2 (Fig. 2, Table 2). Fig. 2 also shows the expression levels of genes in breast cancer subtypes. Notably, differential regulation of TTCC isoforms was observed, wherein CACNA1G was downregulated, and the other two isoforms, CACNA1H and CACNA1I, were upregulated. This finding is important because *T*-type inhibitor drugs do not differentiate between isoforms and perhaps might explain the varying results across several studies using either drugs or gene knockdown of pan TTCCs (Ohkubo and Yamazaki, 2012; Taylor et al., 2008). Therefore, understanding the isoform-specific roles of TTCCs in breast cancer will be both interesting and crucial.

Association between estrogen receptor and T-type voltage-gated calcium channel gene alterations in breast cancer (mutual exclusivity or co-occurrence)

Cancers have complex physiology, and deregulation of different genes or pathways is often observed in cancer. Because several of those genes or pathways are linked, investigation of the associations among relevant gene alterations is pertinent. Here, we observed whether the alterations in TTCC gene isoforms were concomitant or mutually exclusive with the alterations in ESR isoforms. Mutually exclusive genes are important targets that can be exploited to identify unknown mechanisms contributing to oncogenesis or cancer progression. In mutual exclusivity, events in genes associated with specific cancer tend to be mutually exclusive across a set of tumors. The opposite situation is co-occurrence, wherein genetic alterations occur in multiple genes in the same cancer sample. Co-occurring genes are important targets that can be jointly exploited to increase the effectiveness of therapies; these genes may interact with one another or may be involved in common pathways. Table 3 lists the tendency of mutual exclusivity or co-occurrence of alterations in TTCC and ESR isoforms in breast cancer tested in TCGA, PanCancer Atlas dataset, 2018, by using cBioPortal. First, we analyzed the association of mutations as well as copy number changes and mRNA expression together in both TTCC and ESR gene isoforms. A significant co-occurrence or mutual exclusivity is indicated by both p and q values ≤ 0.05.

We observed that in breast cancer, ESR1 gene alterations had a high likelihood of co-occurrence with alterations in CACNA1G. In the case of ESR2, the gene alterations had a high likelihood of co-occurrence with alterations in not only ESR1, but also CACNA1H and CACNA1I. Although not statistically significant, the co-occurrence of alterations in CACNA1H together with ESR1 indicated a tendency toward mutual exclusivity. All other combinations of alterations showed only a tendency toward co-occurrence. Overall, this novel observation indicates converging pathways in breast cancer that may involve ESR and TTCCs, given their association in other tissues. Experimentally determining the interaction between TTCC and ESR isoforms in breast cancer would be interesting and important.

Furthermore, because many experimental studies have reported changes only in the expression of these genes in breast cancer, we assessed associations between altered gene expression excluding mutations and copy number alterations in both TTCC and ESR isoforms (Table 4).

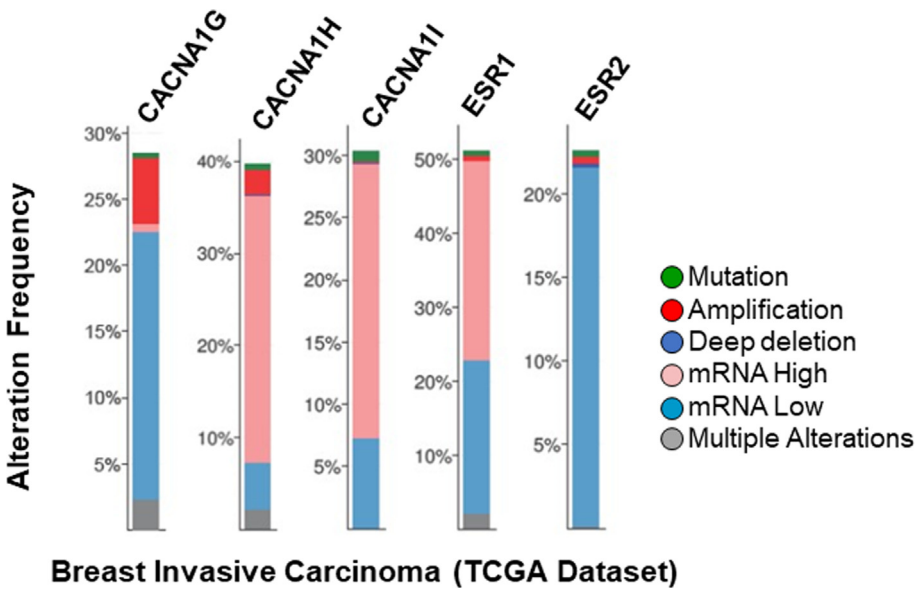

Fig. 1. Major alterations observed in the queried genes in breast cancer. Alteration frequencies of the queried genes are shown, including mutations, amplifications, deep deletions, low and high mRNA expression, and multiple alterations, as determined from the breast invasive carcinoma dataset in TCGA PanCancer Atlas.

Table 1
Major alterations observed in the queried genes in breast cancer.

Genes	Alteration type (% of cases)
CACNA1G	Low mRNA levels (20.22 %)
CACNA1H	High mRNA levels (29.07 %)
CACNA1I	High mRNA levels (22.03 %)
ESR1	High mRNA levels (27.06 %)Low mRNA levels (20.72 %)
ESR2	Low mRNA levels (21.53 %)

Table 2
Expression of TTCC and ESR gene isoforms in TCGA, PanCancer Atlas dataset, 2018.

Gene	Expression range (log scale)	Median expression (log scale)	Status based on median
CACNA1G	2.08 to (−3.37)	−1.26	Downregulation
CACNA1H	7.14 to (−4.18)	0.8	Upregulation
CACNA1I	5.53 to (−3.58)	0.35	Upregulation
ESR1	3.94 to (−5.28)	1.33	Upregulation
ESR2	0.48 to (−3.33)	−1.47	Downregulation

Similarly to the results in Table 3, when ESR1 gene expression was altered, a concomitant change was observed in the expression of CACNA1G. In addition, when ESR2 gene expression was altered, changes in the expression of ESR1 as well as CACNA1H and CACNA1I were observed. All other combinations of gene expression changes exhibited only a tendency of co-occurrence. Of note, the gene expression changes in CACNA1H showed a tendency to co-occur with ESR1 gene expression changes, in contrast to the mutual exclusivity tendency observed when all alterations were analyzed together (comparison of Table 3 and 4, 6th row). However, because the tendency was not statistically significant in both cases, the data might have varied; data from more patients might be required to draw a definitive conclusion. The similarity in the results reported in Tables 3 and 4 suggested that the major alterations reported in the queried genes were associated with gene expression changes; therefore, the tendency to co-occur or to be mutually exclusive may be governed by gene expression changes alone. The observations reported herein indicate pathways in breast cancer that may involve both TTCCs and ESRs and must be experimentally investigated.

Changes in the expression of estrogen receptor and T-type voltage-gated calcium channel genes in different subtypes of breast cancer

The observations described above included all subtypes of breast cancer grouped together. Because breast cancer subtypes are likely to differ in cell signaling, we investigated whether the altered expression of TTCC and ESR gene isoforms correlated with the pathology of a particular subtype of breast cancer. We plotted the expression levels of ESR and TTCC gene isoforms in different subtypes of breast cancer by using the UALCAN database. The expression levels of these genes were analyzed in luminal, $HER2^+$ and TNBC, compared with healthy breast tissue (Fig. 3, Table 5).

Interestingly, the CACNA1G gene was downregulated in all subtypes of breast cancer, and CACNA1H was upregulated only in the luminal type of breast cancer, whereas CACNA1I was upregulated in both luminal and TNBC subtypes of breast cancer. Unexpectedly, most studies reporting the overexpression of TTCCs did not mention CACNA1I; however, in our analysis, this was the only isoform of TTCCs upregulated in patients with TNBC. This finding again indicated isoform-specific regulation of TTCCs in different subtypes of breast cancer. Along with CACNA1H and CACNA1I, the ESR1 gene was also upregulated in the luminal subtype of breast cancer, thereby indicating that inhibition of these genes may be beneficial only in the luminal subtype of breast cancer but not all subtypes. Our results are in agreement with the findings of Pera et al., who have reported high expression of CACNA1H in luminal-like breast cancer cell lines such as MCF-7 and T-47D, thus indicating that CACNA1H may be a valid prognosis marker. We did not observe overexpression of TTCCs in the $HER2^+$ subtype, as reported in cell line SKBR3 (Pera et al., 2016). However, because the patient dataset for $HER2^+$ comprises data from only 37 patients, data from a larger $HER2^+$ patient cohort might address this discrepancy.

In addition, the ESR2 gene was consistently downregulated in all subtypes of breast cancer. Observing the subtype-specific role of

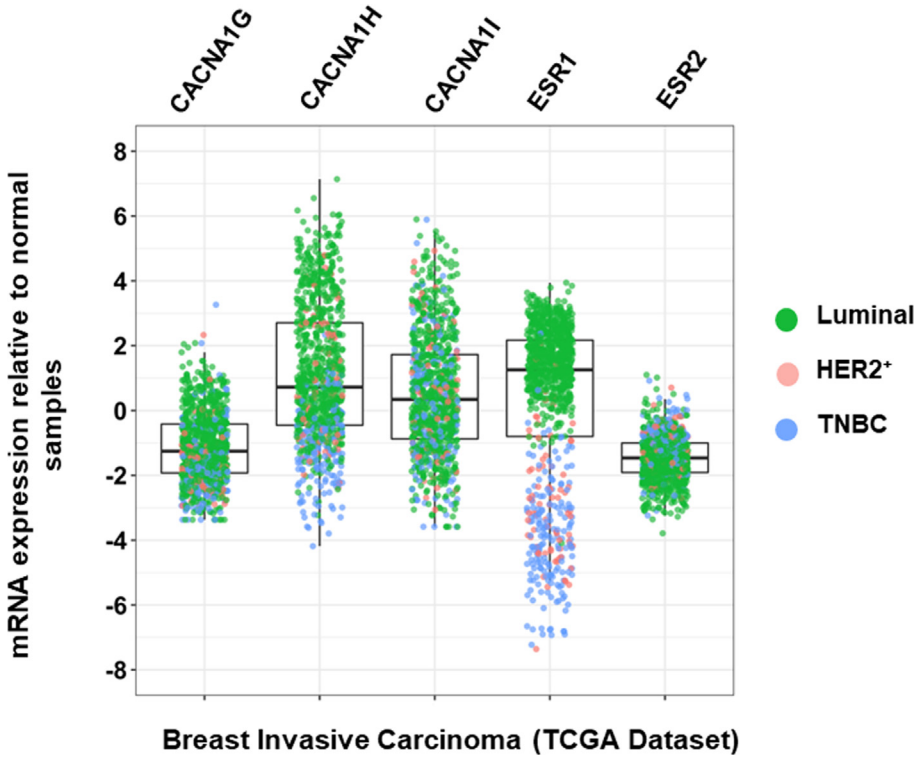

Fig. 2. Expression levels of TTCC and ESR genes in breast cancer cells. mRNA expression levels of the queried genes relative to normal samples, as determined from the breast invasive carcinoma dataset in TCGA Pan Cancer Atlas, are shown. Luminal, HER2$^+$ and TNBC subtypes of breast cancer are represented by green, pink and blue, respectively, for the queried genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Mutual exclusivity or co-occurrence of CACNA1G, CACNA1H, CACNA1I, ESR1 and ESR2 alterations (mutations/copy number changes/gene expression).

Test gene 1	Test gene 2	p value*	q value#	Tendency
CACNA1G	CACNA1H	0.09	0.122	Co-occurrence
CACNA1G	CACNA1I	0.068	0.117	Co-occurrence
CACNA1G	ESR1	< 0.001	< 0.001	**Co-occurrence**
CACNA1G	ESR2	0.168	0.186	Co-occurrence
CACNA1H	CACNA1I	0.098	0.122	Co-occurrence
CACNA1H	ESR1	0.456	0.456	Mutual exclusivity
CACNA1H	ESR2	0.002	0.01	**Co-occurrence**
CACNA1I	ESR1	0.07	0.117	Co-occurrence
CACNA1I	ESR2	0.019	0.047	**Co-occurrence**
ESR1	ESR2	0.011	0.037	**Co-occurrence**

* $p \leq 0.05$.
\# q represents false discovery rate.

Table 4
Mutual exclusivity or co-occurrence of CACNA1G, CACNA1H, CACNA1I, ESR1 and ESR2 gene expression changes.

Test gene 1	Test gene 2	p value*	q value#	Tendency
CACNA1G	CACNA1H	0.051	0.085	Co-occurrence
CACNA1G	CACNA1I	0.048	0.085	Co-occurrence
CACNA1G	ESR1	< 0.001	< 0.001	**Co-occurrence**
CACNA1G	ESR2	0.197	0.219	Co-occurrence
CACNA1H	CACNA1I	0.111	0.138	Co-occurrence
CACNA1H	ESR1	0.282	0.282	Co-occurrence
CACNA1H	ESR2	< 0.001	0.003	**Co-occurrence**
CACNA1I	ESR1	0.086	0.123	Co-occurrence
CACNA1I	ESR2	0.013	0.032	**Co-occurrence**
ESR1	ESR2	0.002	0.005	**Co-occurrence**

* $p \leq 0.05$.
\# q represents false discovery rate.

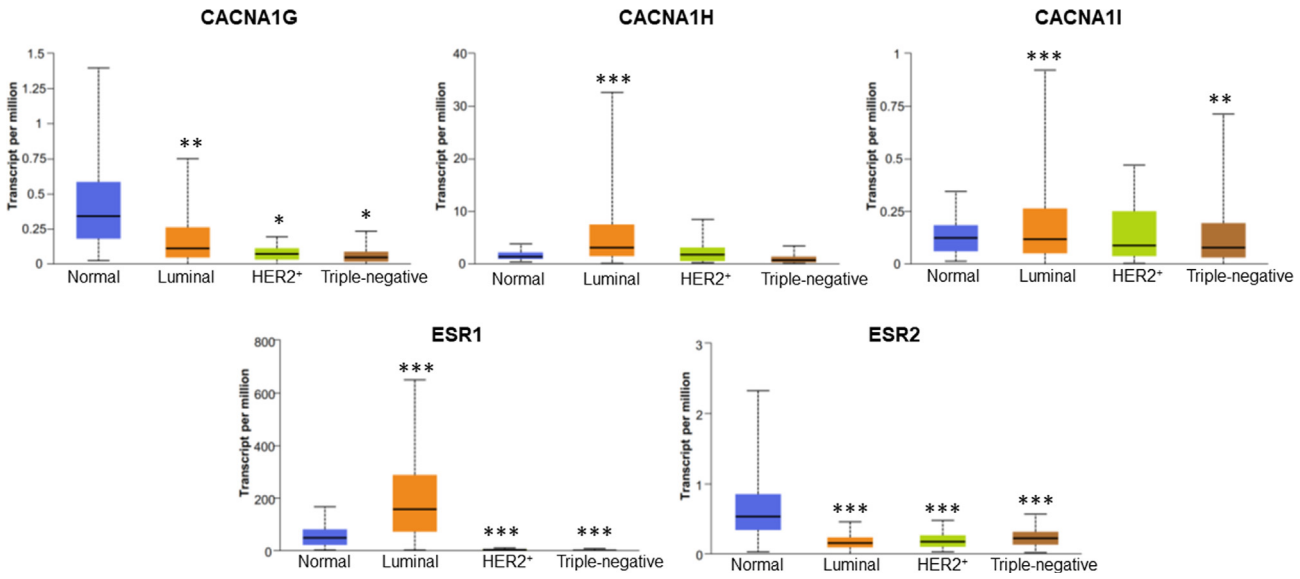

Fig. 3. Expression of TTCC and ESR gene isoforms in different subtypes of breast cancer, compared with healthy breast tissue. Expression levels of the queried genes are shown in different subtypes of breast cancer, as determined from the UALCAN database. Total number of samples for control = 114, luminal = 566, HER2$^+$ = 37 and triple-negative = 116. *p < 0.05, **p < 0.001 and ***p < 0.0001.

Table 5
Expression of TTCC and ESR gene isoforms in different subtypes of breast cancer compared with healthy breast tissue.

Breast cancer (subtype) (row) Gene (column)	Luminal (n = 566)	HER2$^+$ (n = 37)	Triple-negative (n = 116)
CACNA1G	Downregulated **	Downregulated*	Downregulated*
CACNA1H	Upregulated***	Similar	Similar
CACNA1I	Upregulated***	Similar	Upregulated**
ESR 1	Upregulated***	Downregulated***	Downregulated ***
ESR 2	Downregulated***	Downregulated***	Downregulated***

*p ≤ 0.05, **p ≤ 0.001, ***p ≤ 0.0001.

ESR2 in breast cancer pathology for which data are lacking should prove interesting.

The same trend of expression of TTCC and ESR gene isoforms was observed when subtype-specific expression analysis was conducted with the METABRIC dataset, thus again suggesting an isoform-specific alteration of TTCC and ESR genes in breast cancer subtypes (Supplementary Fig. S2). Significance in Supplementary Fig. S2 was determined by comparison of HER2$^+$ and triple-negative samples with the luminal type, because the METABRIC dataset does not have normal samples.

Effects of altered expression of estrogen receptor and T-type voltage-gated calcium channel gene isoforms on the survival of patients with breast cancer

In any disease, the therapeutic targets must crucially improve disease outcomes. One of the most important outcomes in cancer patients is survival time. Therefore, we analyzed whether the survival of patients with breast cancer correlated with altered expression of either ESR or TTCC gene isoforms. We first analyzed the overall survival, i.e., the number of months for which an individual survives after treatment (Fig. 4, Table 6). The data indicated that, among the five genes tested, changes in the expression (downregulation) of only ESR2 were associated with a significant decrease in the overall survival rate of patients with breast cancer (median survival altered ESR2 group = 122.83 m onths; median survival unaltered group = 130.16 months, Fig. 4E). Some trends were observed for CACNA1H and ESR1 genes, wherein upregulation of both genes were associated with a non-significant decrease in survival. The survival of patients with breast cancer was not affected by changes in the expression of CACAN1G and CACNA1I (Table 6, Fig. 4). The overall survival data indicated ESR2 as a promising target. We did not observe any changes in disease-free survival (Supplementary Fig. S1) and progression-free survival (data not shown) in patients with or without alteration of the ESR and TTCC genes.

Effects of altered expression of estrogen receptor and T-type voltage-gated calcium channel gene isoforms on the survival of patients according to breast cancer subtype

Because the expression of ESR and TTCC gene isoforms was breast cancer subtype-specific, we determined whether patient survival correlated with altered expression of either of the gene isoforms in a subtype-specific manner. The survival curves revealed four cases in which patient survival significantly correlated with the altered expression of the gene isoforms: 1) downregulation of CACNA1G in luminal type, 2) upregulation of CACNA1H in luminal type, 3) upregulation of ESR1 in luminal type and 4) downregulation of ESR2 in luminal type (Fig. 5). In all other subtypes and all other isoforms, survival did not appear to correlate with altered expression of gene isoforms (data not shown). ESR1 is already a target in luminal type breast cancer, thus validating our results. CACNA1G, CACNA1H and ESR2 emerged as potential new targets in luminal breast cancer. Thus, experimentally determining the contribution of each to the pathology of breast cancer should prove interesting.

Quantitative PCR of the estrogen receptor and T-type voltage-gated calcium channel gene isoforms in luminal type and triple-negative breast cancer cell lines

To determine whether the expression of TTCC and ESR gene isoforms, as indicated by the online database, might be reproducible in common cell lines used in scientific laboratories, we used the T-47D cell line as an example of luminal type breast cancer and the MDA-MB-231 cell line as an example of triple-negative breast cancer. The expression of gene isoforms in T-47D (Fig. 6A and 6B) and MDA-

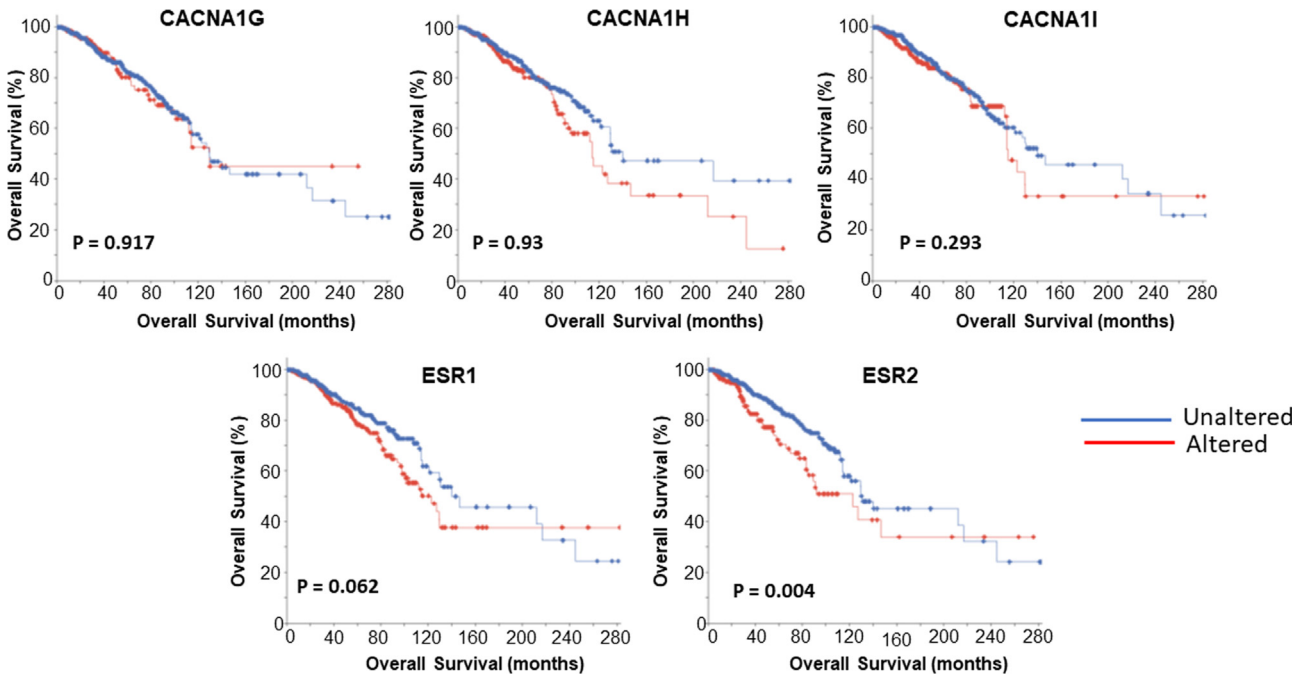

Fig. 4. Overall survival of patients with breast cancer with altered expression of the queried genes. Overall survival (%) is plotted for patients with breast cancer with alterations versus no alterations in the queried genes, as determined from the TCGA dataset. Total number of cases for CACNA1G: 244 altered, 838 unaltered; for CACNA1H: 389 altered, 693 unaltered; for CACNA1I: 323 altered, 759 unaltered; for ESR1: 541 altered, 541 unaltered; for ESR2: 232 altered, 850 unaltered. P values are given in the graphs.

Table 6
Median overall survival of patients with breast cancer with altered expression of the queried genes.

Gene	Median overall survival in months (unaltered)	Median overall survival in months (altered)	p value
CACNA1G	129.57	129.70	0.917
CACNA1H	140.28	114.15	0.093
CACNA1I	140.28	114.80	0.293
ESR1	140.28	122.83	0.062
ESR2	130.16	122.83	0.004*

* $p \leq 0.05$.

MB-231 (Fig. 6C and 6D), as detected by qPCR, was in accordance with the expression reported in Table 5 (comparison of Table 5 and Fig. 6), thus further validating our results.

Cell proliferation assays with T-type voltage-gated calcium channel blocker and estrogen receptor β activator in a luminal type breast cancer cell line

To determine the gene function of TTCC and ERβ, we performed proliferation experiments in luminal type cell line T-47D, wherein we inhibited TTCCs with NNC 55–0396 (unfortunately, a TTCC splice variant specific inhibitor does not exist). Because CACNA1G was downregulated in T-47D cells and was not detected (Fig. 6A), we speculated that NNC 55–0396 inhibited CACNA1H and CACNA1I, which are overexpressed in these cells. Inhibition of T-47D cell proliferation was observed in MTT assays. In addition, because ESR2 was downregulated in luminal type breast cancer, we activated ERβ (encoded by ESR2) with its specific activator DPN. As shown in Fig. 6E, we also observed a decrease in cell proliferation when we activated ERβ in

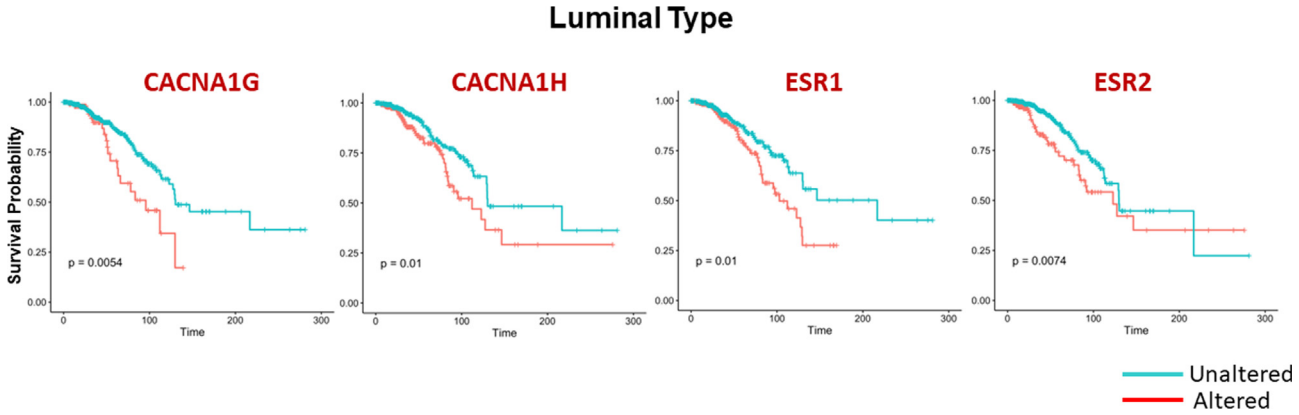

Fig. 5. Overall survival of patients with breast cancer with altered expression of the queried genes in luminal type breast cancer. Survival probability is plotted for patients with breast cancer with or without alterations in the indicated genes in luminal type breast cancer, determined from the TCGA dataset. Total number of cases for CACNA1G: 120 altered, 576 unaltered; for CACNA1H: 279 altered, 417 unaltered; for ESR1: 277 altered, 419 unaltered; for ESR2: 181 altered, 515 unaltered. P values are given in the graphs.

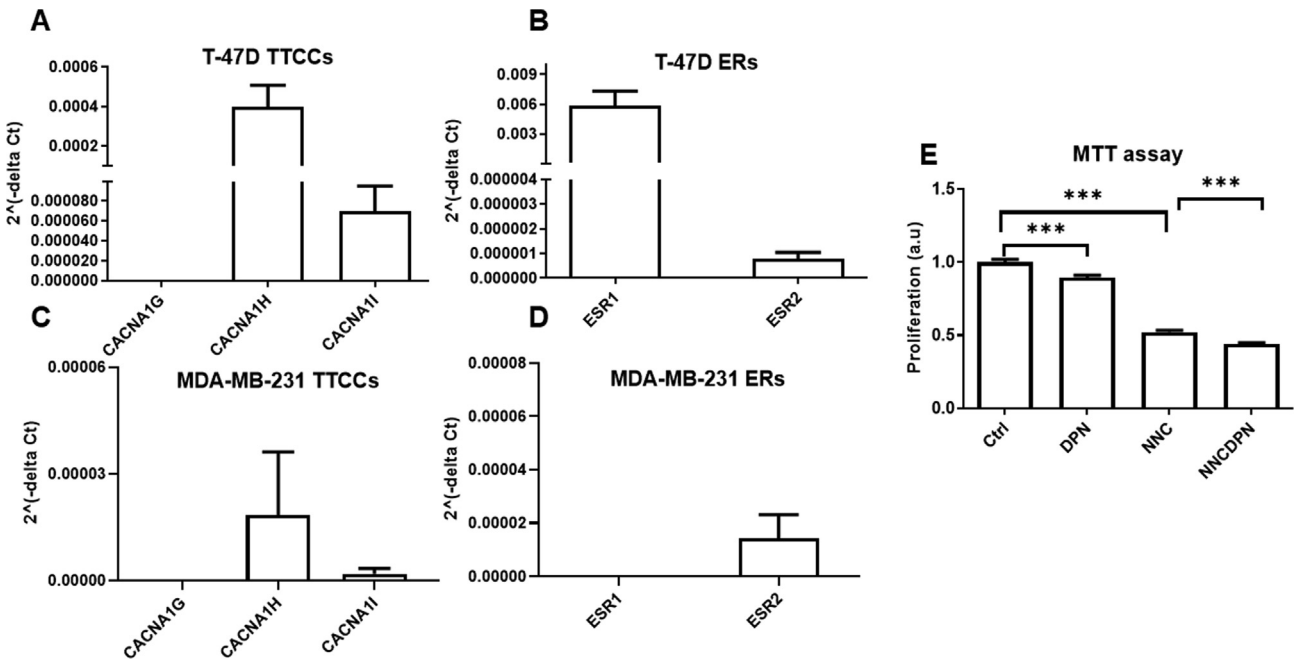

Fig. 6. Quantitative PCR experiments for TTCC and ESR gene isoform expression in T-47D and MDA-MB-231 cell lines and cell proliferation in T-47D. Gene expression of TTCC (A and C) and ESR (B and D) gene isoforms, as determined by qPCR, plotted relative to the actβ1 gene. CACNA1G was undetected in both cell lines. n = 3–15. (E) Cellular proliferation after treatment of T-47D cells with the TTCC blocker NNC 55–0396, ERβ activator DPN or both. *** $P < 0.0001$.

T-47D cells. When we inhibited TTCCs and activated ERβ simultaneously, we observed an additional effect of both drugs. These experiments indicated that inhibiting TTCCs (which are overexpressed) and activating ERβ (which is downregulated) in luminal type breast cancer may have therapeutic effects.

Conclusion

Our results revealed altered expression of TTCCs and ESRs in breast cancer, in a subtype-dependent manner. Expression and survival curve analysis suggested that CACNA1G, CACNA1H and ESR2 are likely potential targets in luminal type breast cancer. This conclusion was also supported by the finding that alterations in ESR2 co-occur with alterations in CACNA1H. ESR1, which is upregulated in only the luminal subtype, is already a target in breast cancer therapy. However, exploring the potential of ESR2 should be fruitful, given that ESR2 was the only isoform observed to be associated with the overall survival of patients, and activation of ERβ in a luminal type cell line decreased the proliferation of cancerous cells. Further experimental investigations of the breast cancer subtype-specific roles of TTCC and ESR isoforms, particularly the role of CACNA1I, will be essential. Determining whether activation of CACNA1G (which is downregulated in all subtypes of breast cancer) may be therapeutic should be particularly interesting. Overall, our data suggest that targeting calcium channels together with ESR2 may be beneficial; however, deeper knowledge regarding the signaling cascade and whether any interlinked pathways exist between these two sets of genes will be required for the development of better, more effective and specific breast cancer treatment options in the near future.

CRediT authorship contribution statement

Shwetha Sekar: Data curation, Formal analysis, Investigation, Methodology, Writing-first draft, Writing-reviewing and editing, Illustrations. **Yashashwini Subbamanda:** Data curation, Formal analysis, Investigation, Methodology, Writing - review & editing. **Narasimha Pullaguri:** Investigation. **Ankush Sharma:** Data curation, Writing - review & editing. **Chittaranjan Sahu:** Formal analysis, Investigation. **Rahul Kumar:** Investigation. **Anamika Bhargava:** Conceptualization, Funding acquisition, Formal analysis, Methodology, Supervision, Writing - original draft, Writing - review & editing.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics approval

This article does not contain any studies in human participants or animals performed by any of the authors.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and material

All data are available in cBioPortal and UALCAN, except qPCR and cell proliferation experiments.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crbiot.2022.09.009.

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