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Defective Signalling of the *BRCA1* Neighbouring gene, *NBR2*, leads to ER-α Negative tumours in breast cancer xenograft mouse models

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Abstract

Majority of breast and ovarian cancer treatment modalities are based on endocrine therapies like antiestrogens which are dependent on the hormone receptor status of tumours. Although BRCA1 is a major regulator of Estrogen Receptor (ER-α), BRCA1 mutations are largely limited to hereditary cases. Here, we show the role of NBR2 (neighbour of BRCA1) in regulating ER-α. We demonstrate a positive correlation between NBR2 & ESR1 in TCGA datasets. Further, the study revealed that shRNA-mediated knockdown of NBR2 led to the downregulation of ER-α in breast cancer cells, MCF7. Finally, the downregulation of ER-α in NBR2 knockdown xenograft tumours (in female NSG mice), which showed higher invasive properties than wild type tumours was demonstrated. Thus, we concluded that in ER-α negative tumours with NBR2 deficiency, biguanides such as metformin and phenformin, which are reported to have a better efficacy under NBR2 deficient conditions, could serve as more suitable alternatives to antiestrogens.

Keywords: *Breast cancer, ER-α, NBR2*

Therapeutic targeting of breast tumours and the effectiveness of existing androgen therapies is a major area of concern in the medical and veterinary world alike. Though endocrine therapies like antiestrogenic drugs have found massive success in the treatment of *ER-α* positive tumours (Pearson *et al.,* 1982), these tumours can become drug-resistant as cancer cells can undergo transformation to develop a Triple-Negative phenotype where the hormone receptors ER-α, PR and HER2 are absent (Bibu and Usha, 2016). These tumours also tend to be very aggressive and have a poor prognosis. Mutations in the Breast Cancer Susceptibility gene, *BRCA1* is known to be frequently associated with the Triple-Negative status in breast as well as ovarian

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cancers (Chen *et al*., 2018). However, *BRCA1* mutations are rare and hardly prevail in tumours without a family history (Blair *et al.,* 2018) and there are several other genes that can lead to mammary tumours (Christy *et al*., 2021). On the contrary, the frequency of *ER-α* negative breast tumours is around 25-35% and is not limited to hereditary cases (Putti *et al.,* 2005); Turner *et al*., 2004). In fact, several sporadic breast cancers manifest phenotypes similar to *BRCA1* mutated cancers, such as *ER-α* negative status which is termed as 'BRCAness' (Lord and Ashworth, 2016). This indicates the involvement of other factors that regulate the ER status in oncogenesis and study of these factors can help improve the prognosis of ER negative tumours.

Adjacent to *BRCA1*, there exists another gene, *NBR2* (Neighbour of BRCA1 gene 2), which utilises the *BRCA1* promoter for its expression (Xu *et al*., (1997). Encoding a long non-coding RNA and acting as a tumour suppressor (Liu *et al*., 2016), *NBR2* is often co-deleted with *BRCA1* in several breast and ovarian cancer cases (Garcia-Casado *et al*., 2011; van den Ouweland *et al*., 2009). Additionally, we have also observed loss of *BRCA1* expression when *NBR2* was downregulated (unpublished data). Based on this, we hypothesize that *NBR2* deficiency might be a factor that could cause the transformation of tumours to an ER negative phenotype. To that context, we developed *NBR2* knockdown cells and generated *NBR2* deficient tumours in mice and analysed their ER status.

For generating stable knockdown of *NBR2*, we cloned shRNA (5'-GCAGCGAAGG ATAAATTTATACCTGACCCATATAAATTTATCC TTCGCTGCTTTTT-3') targeting NBR2 into the shRNA vector pLVTHM {a kind gift from Didier Trono (Addgene plasmid #12247; http://n2t.net/addgene:12247; RRID: Addgene_12247)}, transfected the clones into the ductal breast carcinoma cell line, MCF7 using Lipofectamine 3000 (Invitrogen) and FACS sorted with FITC filter to establish the stable cell line, following which we performed qRT-PCR and immunoblots to check for NBR2 and *ER-α* expression. To generate xenograft tumours, $1x10^6$ tumour cells were orthotopically implanted into the fourth mammary fat-pad of 8-12 weeks aged female NOD SCID Gamma (NSG) mice as a 1:1 mixture of PBS and Matrigel. The implanted tumours were harvested 3 weeks later and analysed by Haematoxylin & Eosin Staining, and Western Blotting. All animal experiments were performed with the approval of Institutional Animal Ethics Committee (IAEC) (Protocol No. IAEC/807/PRY/2020). For TCGA (The Cancer Genome Atlas) analysis the cBioportal for cancer genomics was utilized to correlate the expression levels of NBR2, and ESR1 in

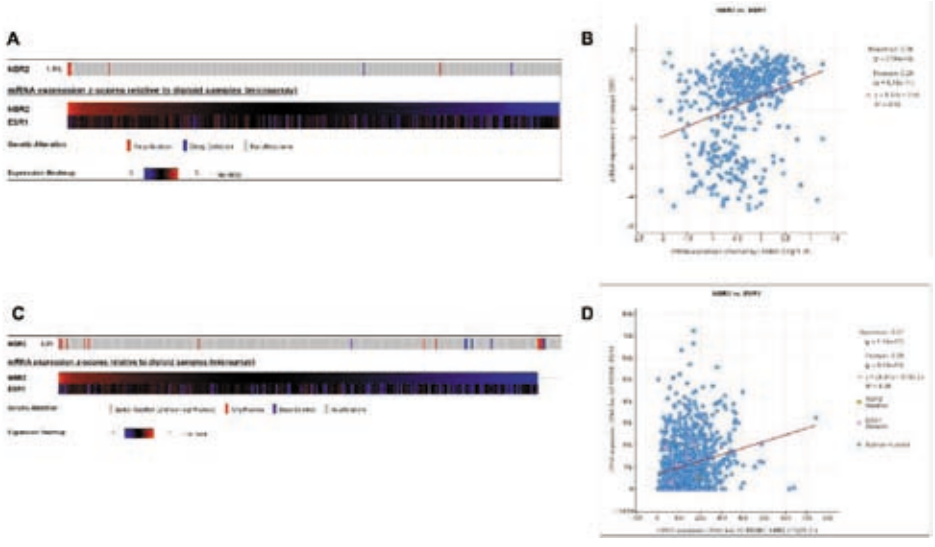


Figure. 1. **A** & **C** represent heatmaps of NBR2 and ESR1 from two human breast cancer patient datasets TCGA Nature 2012 (825 samples) and TCGA Firehouse Legacy (1108 samples) respectively. **B** & **D** depict co-expression analysis of *NBR2* and *ESR1* mRNA levels from the same datasets.

breast cancer tissue samples using Pearson's Correlation Analysis (http://www.cbioportal.org/). For all other statistical analyses involving quantification of western blots and qRT-PCR and measurement of explanted tumour volumes, 3 independent experiments were performed and mean±SD was taken. GraphPad Prism Software V8 was used to perform One-Way ANOVA with Bonferroni correction to calculate the level of significance.

In order to investigate whether *NBR2* has any correlation with *ER-α* status, we first checked the transcript data from two human breast cancer patient datasets in TCGA. Both *NBR2* and *ESR1* transcript heatmaps (Fig1 A & C) and co-expression analysis (Fig1 B & D) showed a positive correlation between *NBR2* and *ESR1*.

To confirm this result *in-vitro*, we generated stable *NBR2* knockdown in luminal breast cancer cell line, MCF7 via cloning of the shRNA targeting *NBR2* and its stable transfection into the target cells (Fig2 A-C). We confirmed the downregulation of *NBR2* upon knockdown (Fig 2D) and went forward to analyse the *ER-α* expression. We found downregulation of *ER-α* at both mRNA (*ESR1*) and protein levels (Fig2 E & F), which indicate that *NBR2* has a regulative control over *ER-α*.

Based on the successful outcomes in TCGA and *in-vitro* analysis, we orthotopically implanted the MCF7 cells with or without *NBR2* knockdown into the fourth mammary fat-pad of female NSG mice. Details of the animals used in the experiment can be found in Fig 3F. Post-harvest, we found the *NBR2* knockdown tumours to be significantly bigger in size. Further, histopathological analysis showed that in comparison to wildtype tumours, *NBR2* knockdown tumours showed reduced differentiation ability into ducts and had signs of anisokaryosis, neovascularization, and invasion to the surrounding fat-pad (Fig 3 C & D). At last, we analysed the *ER-α* status of the explanted tumours and found the *ER-α* levels to be significantly downregulated in *NBR2* knockdown cells. From these results, we could finally conclude that *NBR2* deficiency leads to the transformation of breast tumours into an *ER-α* negative phenotype.

Given the success rate of anti-endocrine therapies involving ER modulators (tamoxifen), aromatase inhibitors (anastrozole, letrozole), and selective ER degraders

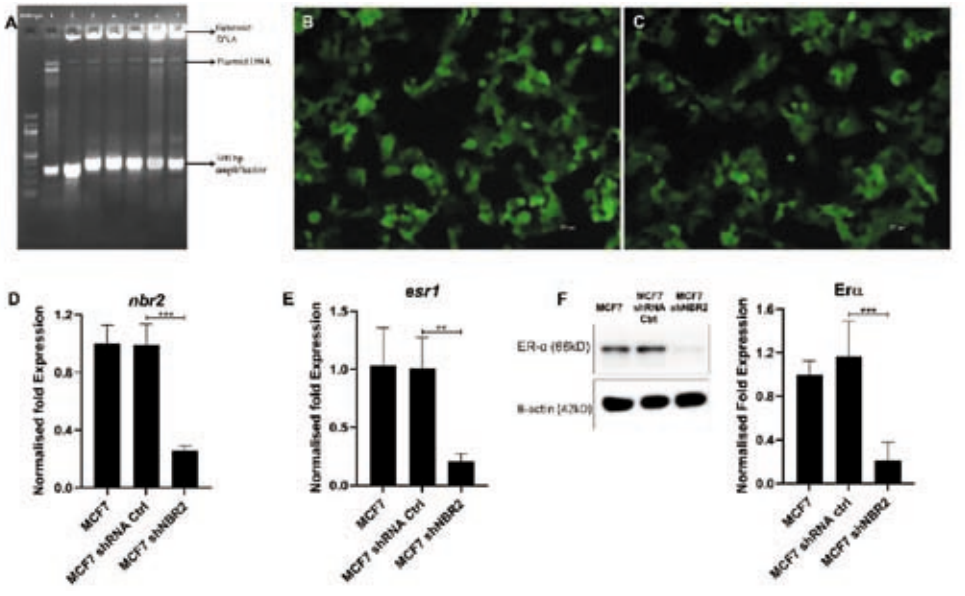
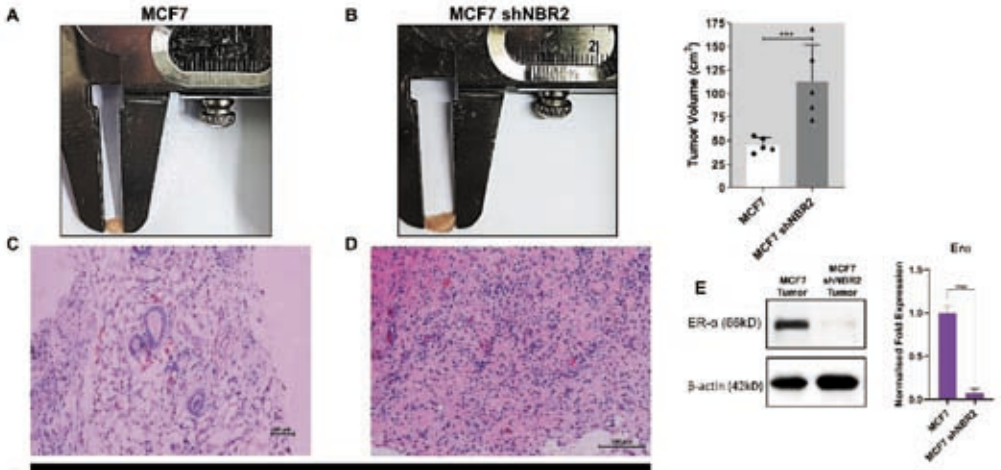


Figure. 2. A shows colony PCR image confirming the cloning of shRNA against NBR2. **B** & **C** represents the transfection of shRNA control and shRNA targeting *NBR2* respectively (GFP tagged). D confirms the downregulation of *NBR2* at RNA level. **E** & **F** show the *ER-α* expression upon *NBR2* downregulation in mRNA and protein level, respectively.



F

Groups	MCF7	MCF7 shNBR2
No of mice per expt	2	2
No of expt	3	3
No of successful implants	6	6
Total	6	6

Figure. 3. A & **B** represent the difference in tumour volume generated from wildtype and NBR2 knockdown MCF7 cells, respectively. **C** & **D** represent H & E images of wildtype and NBR2 knockdown tumours, respectively. **E** shows ER-α expression in NBR2 knockdown tumours analysed via immunoblotting. **F** shows the no of mice used for *in vivo* experiments.

(fulvestrant), the ER status is a vital factor in the prognosis of breast and ovarian cancers. Since, more than a quarter of breast and ovarian cancers turn ER negative, search for better treatment modalities for this subgroup is indispensable. With regards to this, our current findings suggest that ER negative tumours should be screened for NBR2 status, as tumours with NBR2 deficiency could have a better response if targeted via NBR2 signalling by drugs like metformin and phenformin (Liu and Gan 2016).

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Conflict of Interest

The authors declare no conflict of interest

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