Utility of Ankyrin 3 as a Prognostic Marker in Androgen-Receptor-Positive Breast Cancer

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Abstract

Purpose. Androgen receptor (AR) and AR signaling pathways are thought to play a role in breast cancer (BC) and are potentially related to treatment responses and outcomes. Ankyrin 3 (ANK3) is associated with AR stability in cancer cells. In the present study, we investigated the clinicopathological utility of ANK3 expression with emphasis on AR and its associated signalling pathway at transcriptomic and proteomic phases.

Patients and Methods. The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) cohort (n = 1,980) and The Cancer Genome Atlas (TCGA) dataset (n = 1,039) were used to assess the expression and significance of ANK3 mRNA and other AR signalling pathway-associated gene signature. Using immunohistochemistry, ANK3 protein expression was evaluated in large (n = 982) cohort of early-stage BC with long-term follow-up and compared with clinicopathological characteristics and its prognostic value in the whole cohort and the subgroups stratified by AR protein expression.

Results. An AR-related gene signature was developed, comprising 20 genes, which included ANK3. This AR-related gene signature was significantly associated with AR mRNA expression, oestrogen receptor, human epidermal growth factor receptor 2 (HER2) status and the patients’ outcomes. In tumours with high AR protein expression (n = 614), high ANK3 protein expression was significantly associated with progesterone receptor positivity and it was independently associated with the good outcomes (p = 0.025).

Conclusions. This study indicates that ANK3 is related AR signalling pathway and is associated with BC prognosis.

Keywords: invasive breast cancer, androgen receptor, ankyrin 3, prognostic marker.
BACKGROUND

Treatments of breast cancer (BC) are generally determined on the basis of the molecular phenotype of the primary tumour [1, 2]. However, the biological heterogeneity of BC constitutes an important determinant of treatment sensitivity, success and outcomes. Hormone-dependent pathways, including androgen receptor (AR) signalling pathways, are thought to play an important role in BC cell proliferation [3, 4]. Previous studies have indicated that AR and AR signaling pathways are associated with treatment resistance and prognosis of BC [5, 6]. In previous research, we found that approximately 55% of BC had high AR expression, which was observed in 42% of human epidermal growth factor receptor 2 (HER2)-positive tumours and in 20% of triple negative BC (TNBC) [7]. Some studies indicate that high AR expression is a good prognostic factor in BC [7, 8]. However, in HER2-positive and TNBC subtypes, AR signalling pathways are considered to play an important role in tumour progression. He et al. suggested that AR promotes the growth of HER2-positive BC via crosstalk with the intracellular HER2 downstream pathway [9]. The luminal-AR BC subtype, a molecular subtype of TNBC, not only expresses AR but also has enriched hormone-dependent pathways, as demonstrated at the global transcriptomic level [10, 11]. It has also been shown in oestrogen receptor (ER)-positive and HER2-negative BC that aberrant AR-related oncogenic pathway activation is associated with resistance to endocrine therapy [12].

Ankyrin 3 (ANK3), a member of the ankyrin family of membrane-associated proteins, is believed to link integral membrane proteins to cytoskeletal components. Ankyrins are
associated with cytoplasmic structures and are also necessary in the regulation of cell migration and adhesion and for the maintenance of cellular membrane domains [13-15]. ANK3 has been suggested to play a role in regulating the stability and turnover of AR and is closely associated with AR genomic activities [16]. AR signaling pathway promotes cancer cell proliferation by increasing cyclin-dependent kinase activity [17, 18] and ANK3 regulates the expression of cell cycle components as cyclins A and B [16]. Hence, ANK3 may play an important role in AR signaling pathway in cancer. However, the association between ANK3 expression and AR signaling pathway in BC remains poorly defined. In this study, ANK3 was first evaluated as a component of the AR signaling pathway in BC, utilising well characterised large cohort transcriptomic databases. The clinicopathological and prognostic significance of ANK3 protein expression levels was assessed using immunohistochemistry (IHC) in a large series of BC patients’ specimens.

**MATERIALS AND METHODS**

*Cluster Analysis of AR-Signaling-Pathway-Associated Genes*

Gene Ontology (GO) Consortium is the large genomic annotation project and widely used as biological databases for annotating genes to the previous evidence regarding their biological role [19, 20]. GO terms are divided into 3 categories as biological process, molecular function and cellular component [21]. In GO terms of the biological process, gene symbols related to ‘Regulation Of Androgen Receptor Signaling Pathway (GO: 0060765)’ were accessed using the online database Gene Set Enrichment Analysis (http://software.broadinstitute.org/gsea/msigdb/cards/GO_REGULATION_OF_ANDROGEN_RECEPTOR_SIGNALING_PATHWAY) [22, 23].
The mRNA expression data of these genes, including ANK3, together with the clinicopathological characteristics and outcomes of patients with BC, were collected from the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset [24, 25] (n = 1,980) and the Cancer Genome Atlas (TCGA) [26] dataset (n = 1,039) provided by cBioPortal [27].

The normalisation method of mRNA expression in the METABRIC cohort was previously described [24]. TCGA mRNA data was log2-transformed prior to cluster analysis. For cluster analysis [28] and heat mapping construction, Cluster 3.0 and Java Treeview was used [29]. Data were filtered to remove all genes that did not have at least one observation with absolute values greater than 2.0 or whose maximum minus minimum values were less than 2.0.

**ANK3 Protein Expression**

A total of 982 BC patients who underwent surgery at Nottingham City Hospital in the UK between 1987 and 1998 (referred to as the Nottingham Primary Breast Cancer Series) were included in this study. All patients had undergone breast-conserving surgery or modified radical mastectomy without any neoadjuvant treatment. The availability and assessment of hormone receptors (AR, ER and progesterone receptor [PR]), HER2 and Ki67 were described in previous studies [7, 30-37]. The cohort was stratified on the basis of AR expression [7], with 614 patients (62.5%) with high and 368 patients (37.5%) with low AR expression (Supplementary Table 1).

ANK3 protein expression was assessed by IHC using an anti-ANK3 antibody (HPA055643; Merck, Darmstadt, Germany) diluted 1:300 as previously described [38–40]. In order to evaluate the pattern of ANK3 protein expression, 15 full-face BC tissue sections were
assessed prior to staining the whole cohort (n = 982) prepared as tissue microarrays (TMAs). Immunostained TMA sections were digitally scanned using a NanoZoomer (Hamamatsu Photonics, Tokyo, Japan). Cytoplasmic staining of ANK3 in cancer cells was assessed using the H-score method on the basis of intensity scoring (0 = negative, 1 = weak, 2 = moderate, 3 = strong) and proportion scoring (0–100) as previously reported [41, 42].

Statistical Analysis
Statistical analyses were conducted using SPSS v24.0 (IBM, Armonk, NY, USA). The relationship between ANK3 mRNA with ANK3 protein expression and AR mRNA expression was examined using Pearson’s correlation coefficient test. In order to assess the associations between AR mRNA expression and groups stratified by the AR-related gene signature, the Mann–Whitney U test was used. The chi-square test as univariate analysis and the logistic regression test as multivariate analysis were used to assess several clinicopathological factors, including tumour size, lymph node status, histological grade, ER, PR, HER2 and molecular subtypes, stratified by groups based on AR-related gene signature and levels of ANK3 protein expression. In order to assess the prognostic utility of ANK3 expression, Kaplan–Meier survival curves was used. In univariate and multivariate analyses, to assess the associations between clinicopathological factors, including ANK3 expression, and prognosis, 95% confidence intervals (CIs) were assessed using the Cox proportional hazards regression model. In these survival analyses, the median value (H-score = 120) was used as a cut-off point to divide the samples into high and low expression groups.

RESULTS
**ANK3 mRNA Expression and AR Signaling Pathway Gene Signature**

High ANK3 mRNA expression was significantly associated with high AR mRNA expression (METABRIC: $r = 0.019$, $p = 0.39$; TCGA: $r = 0.28$, $p < 0.0001$) in TCGA cohort. An AR-related gene signature was developed using genomic data filtering, and this comprised 20 genes, including ANK3 and 19 other relevant genes available in the databases: ARRB2, BUD31, DAB2, DDX5, EP300, FOXP1, HDAC1, HDAC6, HEYL, PARK7, PHB, Pias2, PRMT2, RNFL4, RNFL6, SFRP1, SIRT1, SMARCA4 and TRIM68 (Supplementary Table 2).

Using the dendrogram of cluster analysis, the METABRIC and TCGA cohorts were stratified into two groups on the basis of the AR-signaling-pathway-associated genes [Figs. 1(a) and 1(b)], where tumours in group 1 had significantly lower AR mRNA expression than that in Group 2 ($p < 0.0001$). Group 1 tumours included 899 (45%) from the METABRIC and 541 (52%) from TCGA cohort.

In the METABRIC and TCGA cohorts, multivariate analysis indicated that the AR-related gene signature in group 2 was significantly associated with lower grade ($p = 0.0070$, and $p = 0.0093$ respectively), ER positivity ($p < 0.0001$, and $p < 0.0001$ respectively), and HER2 positivity ($p < 0.0001$ and $p < 0.0001$; Table 1). In the METABRIC cohort, the AR-related gene signature was significantly associated with molecular subtype ($p < 0.0001$), with 83% of the basal-like tumours in group 1 and 90% of the luminal B tumours in group 2 (Table 1). Although the expression of ANK3 and AR mRNA was not a significant independent prognostic factor in BC (Supplementary Figure 1), there was an association between AR-related gene signature subgroups and patients’ outcomes, where patients with the AR-related gene signature group 2 showed significantly worse outcome than those with Group 1 tumours [METABRIC: hazard ratio (HR) 1.25, 95% CI: 1.09-1.43, $p = 0.0013$; TCGA: HR 1.61, 95%
CI: 1.11-2.32, \( p = 0.011 \); Figs. 1(c) and 1(d)]. On multivariate analysis, AR-related gene signature group 2 was an independent prognostic factor predicting poor outcomes in both cohorts (METABRIC: HR 1.23, 95% CI: 1.06-1.42, \( p = 0.0066 \); TCGA: HR 1.82, 95% CI: 1.08-3.06, \( p = 0.026 \); Table 2).

Immunohistochemical Expression of ANK3 Protein

The assessment of ANK3 in full-face tissue sections indicated that the pattern of ANK3 expression in cancer cells was homogeneous, but it differed from that in normal mammary glands [Figs. 2(a)–2(c)]. ANK3 expression was observed in the normal glandular and luminal epithelial cells, where it was stronger than the surrounding myoepithelial cells. ANK3 immunopositivity was observed in the cytoplasm of invasive cancer cells and was typically weaker than in the adjacent normal epithelial cells [Figs. 2(c)–2(e)].

In 198 cases in the METABRIC dataset, which overlapped with the Nottingham Primary Series, \( ANK3 \) mRNA and ANK3 protein expression were significantly correlated (\( r = 0.15, p = 0.039 \)). In the Nottingham series, 579 (59%) tumours had low ANK3 expression (H-score \( \leq \) 120) and 403 (41%) had high ANK3 expression (H-score > 120). High AR expression was present in 614 (63%) tumours and low AR expression was present in 368 (37%). Among those with high AR expression, 250 (41%) also had high ANK3 expression. A similar proportion (153, 42%) had high ANK3 expression in the low AR expression group (\( n = 368 \)). AR expression was not associated with ANK3 expression on proteomic analysis (\( p = 0.79 \)). When all 982 cases were combined (i.e. not stratified according to AR expression), ANK3 was not a significant prognostic factor (Supplementary Figure 2).
In tumours with high AR expression, high ANK3 expression was significantly associated with PR positivity ($p = 0.014$; Supplementary Table 3). In terms of BC-specific survival, high AR protein expression was a significant good prognostic factor (HR 0.66, 95% CI: 0.52-0.84, $p = 0.00066$; Supplementary Figure 3). Low ANK3 protein expression was a poor prognostic factor in patients with high AR expression [HR 1.49, 95% CI: 1.07-2.09, $p = 0.020$; Figs. 3(a)–3(e)], but not in those whose tumours had low AR expression (HR 0.89, 95% CI: 0.62-1.28, $p = 0.53$; Supplementary Figure 4). In high-AR-expressing BC patients, univariate analysis using the Cox proportional hazards regression analysis identified low ANK3 expression, large tumour size (HR 2.61, $p < 0.0001$), positive nodal status (HR 2.84, $p < 0.0001$) and high histological grade (HR 3.27, $p < 0.0001$) as poor prognostic factors. On multivariate analysis, low ANK3 protein expression was an independent prognostic factor predicting poor outcomes in BC with high AR expression (HR 1.47, $p = 0.025$; Table 3).

**DISCUSSION**

AR expression is a crucial factor in the progression of BC, as it controls the expression of various genes and proteins through a genomic pathway [5, 6]. In this pathway, AR mediates intracellular steroid hormone-related signaling pathways to regulate the transcription of target genes in conjunction with other transcription factors, such as signal transducers and activators of transcription [43, 44]. As a mechanism involved in the development of BC, AR expression might be involved in the crosstalk with epidermal growth factor receptor pathways, such as human epidermal growth factor receptor 1 (EGFR) and HER2 signaling [45]. In this study, there were a significant correlation between ANK3 and AR mRNA and ANK3 was one of the gene component of the AR-related gene signature. When BC was classified into 2 groups
based on the expression of AR-related gene signature, the group 2 gene signature, which was associated with high $AR$ mRNA expression and present in 90% of luminal B tumours, was a significant prognostic factor indicating poor outcomes in BC. This finding suggests that aberrant AR-related oncogenic pathway activation is associated with a number of factors that portend a poor BC outcome.

In a previous study using microarray gene expression analysis, the downregulation of $ANK3$ was included in an 11-gene signature associated with poor prognosis in patients with various cancers including BC [46]. In a meta-analysis of gene expression signatures in BC, the downregulation of $ANK3$ appeared to enhance cancer cell differentiation, proliferation and metastasis [47]. Previous research using microarray data of prostate cancer suggested that low $ANK3$ expression is related to positivity for ERG, member of the erythroblast transformation-specific family [48]. ERG is correlated with AR activity [49], transcriptional stability [50] and stem cell maintenance [51] in multiple cancers. Prostate cancer cells with $ANK3$ knockdown exhibit significant increases in cell invasion through an AR-dependent mechanism as a regulator of AR protein stability [16]. In the present study, the association between $ANK3$ protein expression and outcomes was highly significant in BC with high AR expression. In addition, high $ANK3$ protein expression was associated with PR positivity. These findings suggest that $ANK3$ may play an important role in the maintenance of hormonal activity, and AR stabilisation by $ANK3$ may therefore be related to the improved outcomes in BC patients with high AR expression. A proportion of ER-negative BC are generally considered to retain active AR signaling [6, 52]. Several prospective clinical trials of AR-targeted therapies have been conducted on TNBC with high AR expression. These trials indicated that treatment with an AR inhibitor is feasible, with a clinical benefit rate of
approximately 20% in TNBC [53-55]. The upregulation of ANK3 may increase AR stability and improve the response to an AR inhibitor in TNBC. Further functional and translational research is necessary in order to explore the association of ANK3 with AR stability with the efficacy of treating BC with an AR inhibitor.

In conclusion, the AR signaling pathway and ANK3 mRNA expression are associated with AR mRNA expression and BC prognosis. High ANK3 protein expression is an independent prognostic factor in BC with high AR expression. Overall, these findings indicate that ANK3 may play an important role in breast tumour progression and, in conjunction with AR, may be related to BC outcomes.

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Compliance with Ethical Standards

Funding

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Conflict of Interest

Ibraheem Alshankyty is a consultant/advisory board in Molecular Diagnostics Lab, College of Applied Med. Sci., KAU.

All authors of this work declare that they have no conflict of interest.
Ethical approval

This study was approved by the Nottingham Research Ethics Committee 2 (Reference title: Development of a molecular genetic classification of breast cancer). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from the participants included in the study.

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**Figures Captions**

**Fig. 1.** Prognostic utility of an androgen receptor (AR)-related gene signature, including ANK3 mRNA expression.

Heat map of the AR-related gene signature for the (a) METABRIC and (b) TCGA cohorts generated by unsupervised cluster analysis, showing a clear division of cases between Group 1 and Group 2 on the basis of the AR-related gene expression. The overall survival of patients
with breast cancer with the AR-related Group 2 gene signature was significantly worse than that of those with the Group 1 gene signature in the (c) METABRIC and (d) TCGA cohorts.

**Fig. 2.** Morphological characteristics of ANK3 immunohistochemistry in breast cancer tissue. (a) ANK3 immunoreactivity differs between invasive cancer cells and adjacent normal mammary glandular tissues (black arrow: invasive cancer cells; white arrow: normal mammary gland). Immunoreactivity in normal mammary gland cells is stronger than that in invasive cancer cells (magnification: x100). (b) Invasive cancer cells showing uniform ANK3 immunoreactivity primarily in the cytoplasm (magnification: x200). (c) ANK3 immunoreactivity is uniformly strong in normal epithelial cells and weaker in myoepithelial cells than in glandular cells (magnification: x400). Tissue microarray images of breast cancer tissue samples immunohistochemically stained for ANK3, showing (d) no staining, (e) weak staining and (f) strong staining in the cytoplasm of cancer cells (magnification: x200).

**Fig. 3.** ANK3 protein expression in breast cancer and cumulative survival rates stratified by ANK3 expression. (a–d) ANK3 and AR expression in breast cancer. Case 1: high ANK3 (a) and high AR (b) expression. Case 2: low ANK3 (c) and high AR (d) expression (magnification: x200 for all images). (e) With high AR expression, BC-specific survival was significantly worse in those with low than high ANK3 expression.
Table 1. Clinicopathological characteristics of breast cancer associated with AR signaling pathway-related gene signature.

| Factors                | METABRIC cohort | TCGA cohort | p-value    | Factors                | METABRIC cohort | TCGA cohort | p-value    |
|------------------------|-----------------|-------------|------------|------------------------|-----------------|-------------|------------|
|                        | Group 1 | Group 2   |            |                        | Group 1 | Group 2   |            |
|                        | Univariate | Multivariate |            |                        | Univariate | Multivariate |            |
| **Tumour size**        |          |            |            |                        |          |            |            |
| > 2cm                  | 564 (42.2%) | 774 (57.8%) | < 0.0001* | T2-4                   | 388 (50.5%) | 381 (49.5%) | 0.079      |
| < 2cm                  | 319 (51.8%) | 297 (48.2%) | 0.00065*  | T1                     | 153 (56.7%) | 117 (43.3%) | 0.098      |
| **Nodal status**       |          |            |            |                        |          |            |            |
| Positive               | 413 (44.0%) | 525 (56.0%) | 0.28       | Positive               | 261 (49.8%) | 263 (50.2%) | 0.11       |
| Negative               | 481 (46.5%) | 554 (53.5%) | 0.66       | Negative               | 278 (54.8%) | 229 (45.2%) | 0.70       |
| **Grade**              |          |            |            |                        |          |            |            |
| Grade 3                | 444 (46.6%) | 508 (53.4%) | 0.22       | Grade 3                | 233 (56.7%) | 178 (43.3%) | 0.020      |
| Grade 1, 2             | 412 (43.8%) | 528 (56.2%) | 0.0070*    | Grade 1, 2             | 272 (49.1%) | 282 (50.9%) | 0.0093*    |
| **ER**                 |          |            |            |                        |          |            |            |
| Positive               | 569 (37.8%) | 937 (62.2%) | < 0.0001*  | ER                     | 340 (44.5%) | 424 (55.5%) | < 0.0001*  |
| Negative               | 330 (69.6%) | 144 (30.4%) | < 0.0001*  | Negative               | 183 (79.9%) | 46 (20.1%)  | < 0.0001*  |
| **PR**                 |          |            |            |                        |          |            |            |
| Positive               | 409 (39.3%) | 631 (60.7%) | < 0.0001*  | PR                     | 303 (45.8%) | 358 (54.2%) | < 0.0001*  |
| Negative               | 490 (52.1%) | 450 (47.9%) | 0.90       | Negative               | 215 (65.7%) | 112 (34.3%) | 0.80       |
| **HER2**               |          |            |            |                        |          |            |            |
| Positive               | 104 (42.1%) | 143 (57.9%) | 0.27       | HER2                   | 57 (32.6%)  | 118 (67.4%) | < 0.0001*  |
| Negative               | 795 (45.9%) | 938 (54.1%) | < 0.0001*  | Negative               | 385 (56.6%) | 295 (43.4%) | < 0.0001*  |
| **Subtypes**           |          |            |            |                        |          |            |            |
| Luminal A              | 328 (45.7%) | 390 (54.3%) | < 0.0001*  | -                      | -           | -          |            |
| Luminal B              | 47 (9.6%)  | 441 (90.4%) |            | -                      | -           | -          |            |
| HER2-enriched          | 60 (25.0%) | 180 (75.0%) |            | -                      | -           | -          |            |
| Basal-like             | 274 (83.3%) | 55 (16.7%)  |            | -                      | -           | -          |            |
| Normal-like            | 186 (93.5%) | 13 (6.5%)   |            | -                      | -           | -          |            |

Abbreviations: ER: oestrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; AR: androgen receptor.

* Significant difference, \( p < 0.05 \).
Table 2. Survival analysis based on clinicopathological characteristics of breast cancer, including AR signaling pathway-related gene signature.

| Factors | METABRIC cohort | TCGA cohort |
|---------|----------------|-------------|
|         | Univariate analysis | Multivariate analysis | Univariate analysis | Multivariate analysis |
|         | Hazard Ratio | 95% CI | p-value | Hazard Ratio | 95% CI | p-value | Hazard Ratio | 95% CI | p-value |
| AR related signature | Group 1 Reference | Reference | 1.25 | 1.09-1.4 3 | 0.0013* | 1.23 | 1.06-1.4 2 | 0.0066* | Group 1 Reference | Reference | 1.61 | 1.11-2.3 2 | 0.011* | 1.82 | 1.08-3.0 6 | 0.026* |
| Tumour size | < 2cm Reference | Reference | 1.83 | 1.57-2.1 9 | < 0.0001* | 1.60 | 1.36-1.8 9 | < 0.0001* | Tumour size T2-4 Reference | Reference | 1.67 | 1.07-2.6 2 | 0.026* | 1.18 | 0.66-2.0 9 | 0.58 |
|          | ≥ 2cm Reference | Reference | 1.86 | 1.63-2.1 2 | < 0.0001* | 1.62 | 1.40-1.8 6 | < 0.0001* |
| Nodal status | Negative Reference | Reference | 1.42 | 1.24-1.6 3 | < 0.0001* | 1.10 | 0.94-1.2 8 | 0.25 |
|          | Positive Reference | Reference | 1.37 | 1.18-1.5 9 | < 0.0001* | 1.08 | 0.88-1.3 2 | 0.46 |
| Grade | Grade1-2 Reference | Reference | 1.10 | 0.94-1.2 8 | 0.25 |
|          | Grade 3 Reference | Reference | 1.44 | 1.26-1.6 4 | < 0.0001* | 1.29 | 1.09-1.5 2 | 0.0025* |
| ER | Positive Reference | Reference | 1.57 | 1.31-1.8 9 | < 0.0001* | 1.27 | 1.04-1.5 6 | 0.022* |
|          | Negative Reference | Reference | 1.57 | 1.31-1.8 9 | < 0.0001* | 1.27 | 1.04-1.5 6 | 0.022* |
| PR | Positive Reference | Reference | 1.57 | 1.31-1.8 9 | < 0.0001* | 1.27 | 1.04-1.5 6 | 0.022* |
|          | Negative Reference | Reference | 1.55 | 1.06-2.2 7 | 0.025* | 1.18 | 0.57-2.4 7 | 0.66 |
| HER2 | Negative Reference | Reference | 1.55 | 1.06-2.2 7 | 0.025* | 1.18 | 0.57-2.4 7 | 0.66 |
|          | Positive Reference | Reference | 1.57 | 0.95-2.5 9 | 0.076 | 1.24 | 0.70-2.2 0 | 0.46 |

Abbreviations: ER: oestrogen receptor; PR: progesterone receptor; CI: confidence interval; HER2: human epidermal growth factor receptor 2; AR: androgen receptor.
* Significant difference, p < 0.05.
Table 3. Survival analysis based on clinicopathological characteristics of breast cancer, including ANK3 expression in tumours with high AR expression group.

| Factors            | Univariate analysis | Multivariate analysis |
|--------------------|---------------------|-----------------------|
|                    | Hazard Ratio | 95% CI | p-value | Hazard Ratio | 95% CI | p-value |
| ANK3 expression    | High        | Reference | Reference | Low        | 1.49    | 1.07–2.09 | 0.020* | 1.47    | 1.05–2.07 | 0.025* |
|                    | Low         | 2.61    | 1.86–3.69 | <0.0001* | 1.75    | 1.22–2.51 | 0.0024* |
| Tumour size        | <2 cm       | 2.84    | 2.05–3.92 | <0.0001* | 2.22    | 1.58–3.11 | <0.0001* |
|                    | ≥2 cm       | 3.27    | 2.36–4.53 | <0.0001* | 2.23    | 1.57–3.16 | <0.0001* |
| Nodal status       | Negative    | Reference | Reference | Positive  | 3.49    | 2.40–5.08 | <0.0001* | 2.31    | 1.55–3.44 | <0.0001* |
| Histological grade | Grades 1 and 2| 1.6     | 0.83–3.05 | 0.16 | 1.27    | 0.65–2.47 | 0.49 |
|                    | Grade 3     | 1.6     | 0.83–3.05 | 0.16 | 1.27    | 0.65–2.47 | 0.49 |
| Subtypes           | HR-positive/HER2-negative | Reference | Reference | HER2-positive | 3.49    | 2.40–5.08 | <0.0001* | 2.31    | 1.55–3.44 | <0.0001* |
|                    | Triple negative | 1.6     | 0.83–3.05 | 0.16 | 1.27    | 0.65–2.47 | 0.49 |

Abbreviations: ANK3: ankyrin 3; AR: androgen receptor; CI: confidence interval; HER2: human epidermal growth factor receptor 2; HR: hormone receptor.
*Significant difference, \( p < 0.05 \).
Figure 1

a) Gene expression heatmap for Groups 1 and 2, showing differential expression levels.

b) Heatmap with gene expression data for Groups 1 and 2.

c) Kaplan-Meier survival analysis for Group 1 vs. Group 2. Hazard Ratio: 1.25, 95% CI: 1.09-1.40, p = 0.001.

| Time (months) | Group 1 No. at Risk | No. of events |
|---------------|---------------------|---------------|
| 0             | 884                 | 0             |
| 60            | 593                 | 186           |
| 120           | 321                 | 286           |
| 180           | 82                  | 335           |
| 240           | 6                   | 347           |
| 300           | 2                   |               |

| Time (months) | Group 2 No. at Risk | No. of events |
|---------------|---------------------|---------------|
| 0             | 1975                | 0             |
| 60            | 726                 | 148           |
| 120           | 365                 | 266           |
| 180           | 96                  | 335           |
| 240           | 19                  | 347           |
| 300           | 1                   |               |

d) Kaplan-Meier survival analysis for Group 1 vs. Group 2. Hazard Ratio: 1.61, 95% CI: 1.11-2.32, p = 0.011.

| Time (months) | Group 1 No. at Risk | No. of events |
|---------------|---------------------|---------------|
| 0             | 539                 | 0             |
| 20            | 365                 | 12            |
| 40            | 216                 | 28            |
| 60            | 147                 | 39            |
| 80            | 89                  | 46            |
| 100           | 53                  | 49            |
| 120           | 23                  |               |

| Time (months) | Group 2 No. at Risk | No. of events |
|---------------|---------------------|---------------|
| 0             | 498                 | 0             |
| 20            | 288                 | 12            |
| 40            | 154                 | 28            |
| 60            | 91                  | 39            |
| 80            | 56                  | 46            |
| 100           | 27                  | 49            |
| 120           | 13                  |               |
Figure 3
### Supplementary Table 1. Patients’ characteristics of high and low androgen receptor expression groups in the Nottingham primary cohort

| Factors                        | Expression of AR | $p$-value |
|--------------------------------|------------------|-----------|
|                                | High group       | Low group |           |
| Age range in year              | 24-70 (median: 54) | 25-70 (median: 51.5) | <0.0001* |
| ANK3                           |                  |           |
| High                           | 250 (62.0%)      | 153 (38.0%) | 0.79     |
| Low                            | 364 (62.9%)      | 215 (37.1%) |           |
| Menopausal status              |                  |           |
| Pre-                           | 236 (58.0%)      | 171 (42.0%) | 0.013*   |
| Post                           | 378 (65.7%)      | 197 (34.3%) |           |
| Tumour size                    |                  |           |
| ≥2cm                           | 313 (58.8%)      | 219 (41.2%) | 0.0094*  |
| <2cm                           | 301 (66.9%)      | 149 (33.1%) |           |
| Nodal status                   |                  |           |
| Positive                       | 239 (62.6%)      | 143 (37.4%) | 0.98     |
| Negative                       | 375 (62.5%)      | 225 (37.5%) |           |
| Histological grade             |                  |           |
| Grade 3                        | 232 (46.1%)      | 271 (53.9%) | <0.0001* |
| Grade 1 and 2                  | 382 (79.7%)      | 97 (20.3%)  |           |
| Subtypes                       |                  |           |
| HR-positive/HER2-negative      | 510 (76.7%)      | 155 (23.3%) | <0.0001* |
| HER2-positive                  | 72 (56.7%)       | 55 (43.3%)  |           |
| Triple negative                | 32 (16.8%)       | 158 (83.2%) |           |
| Type of breast surgery         |                  |           |
| Breast-conserving surgery      | 282 (65.6%)      | 148 (34.4%) | 0.081    |
| Mastectomy                     | 332 (60.1%)      | 220 (39.9%) |           |
| Axillary surgery               |                  |           |
| Sampling alone                 | 371 (62.2%)      | 225 (37.8%) | 0.92     |
| Axillary lymph node dissection | 239 (62.6%)      | 143 (37.4%) |           |
|                | No surgery | Unknown |
|----------------|------------|---------|
| No surgery     | 4 (100.0%) | 0 (0.0%) |

|                | Yes        | No       | Unknown |
|----------------|------------|----------|---------|
| Chemotherapy   | 83 (39.9%) | 512 (69.8%) | 19 (47.5%) |
|                | 125 (60.1%) | 222 (30.2%) | 21 (52.5%) |

<0.0001*<br>

|                | Yes        | No       | Unknown |
|----------------|------------|----------|---------|
| Endocrine therapy | 258 (69.2%) | 337 (59.2%) | 19 (47.5%) |
|                | 115 (30.8%) | 232 (40.8%) | 21 (52.5%) |

0.0020*<br>

Abbreviations: ANK3: ankyrin 3; HER2: human epidermal growth factor receptor 2; HR: hormone receptor.<br>* Significant difference p < 0.05.
| Gene symbol | Gene name                                                                 |
|-------------|---------------------------------------------------------------------------|
| ANK3        | Ankyrin 3                                                                 |
| ARRB2       | Arrestin Beta 2                                                           |
| BUD31       | BUD31 Homolog                                                             |
| DAB2        | DAB2, Clathrin Adaptor Protein                                             |
| DDX5        | DEAD-Box Helicase 5                                                       |
| EP300       | E1A Binding Protein P300                                                   |
| FOXP1       | Forkhead Box P1                                                           |
| HDAC1       | Histone Deacetylase 1                                                     |
| HDAC6       | Histone Deacetylase 6                                                     |
| HEYL        | Hes Related Family BHLH Transcription Factor With YRPW Motif-Like          |
| PARK7       | Parkinsonism Associated Deglycase                                           |
| PHB         | Prohibitin                                                                |
| PIAS2       | Protein Inhibitor Of Activated STAT 2                                     |
| PRMT2       | Protein Arginine Methyltransferase 2                                      |
| RNF14       | Ring Finger Protein 14                                                    |
| RNF6        | Ring Finger Protein 6                                                     |
| SFRP1       | Secreted Frizzled Related Protein 1                                       |
| SIRT1       | Sirtuin 1                                                                 |
| SMARCA4     | SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A, Member 4 |
| TRIM68  | Tripartite Motif Containing 68 |
### Supplementary Table 3. Correlation between ANK3 expression and clinicopathological characteristics of breast cancer stratified by AR expression.

| Factors          | High AR group |         | Low AR group |         |       |       |
|------------------|---------------|---------|--------------|---------|-------|-------|
|                  | Expression of ANK3 | p-value | Expression of ANK3 | p-value |
|                  | High  | Low  |    | High  | Low  |    |
| Tumour size      |       |       |       |       |       |       |
| ≥2 cm            | 120 (38.3%) | 193 (61.7%) | 0.22 | 95 (43.4%) | 124 (56.6%) | 0.40 |
| <2 cm            | 130 (43.2%) | 171 (56.8%) |     | 58 (38.9%) | 91 (61.1%) |       |
| Nodal status     |       |       |       |       |       |       |
| Positive         | 95 (39.7%) | 144 (60.3%) | 0.70 | 62 (43.4%) | 81 (56.6%) | 0.58 |
| Negative         | 155 (41.3%) | 220 (58.7%) |     | 91 (40.4%) | 134 (59.6%) |       |
| Histological grade |       |       |       |       |       |       |
| Grade 3          | 96 (41.4%) | 136 (58.6%) | 0.80 | 119 (43.9%) | 152 (56.1%) | 0.13 |
| Grades 1 and 2   | 154 (40.3%) | 228 (59.7%) |     | 34 (35.1%) | 63 (64.9%) |       |
| ER               |       |       |       |       |       |       |
| Positive         | 225 (40.8%) | 326 (59.2%) | 0.86 | 64 (36.4%) | 112 (63.6%) | 0.052 |
| Negative         | 25 (39.7%) | 38 (60.3%) |     | 89 (46.4%) | 103 (53.6%) |       |
| PR               |       |       |       |       |       |       |
| Positive         | 195 (43.7%) | 251 (56.3%) | 0.014* | 51 (38.9%) | 80 (61.1%) | 0.44 |
| Negative         | 55 (32.7%) | 113 (67.3%) |     | 102 (43.0%) | 135 (57.0%) |       |
| HER2             |       |       |       |       |       |       |
| Positive         | 29 (40.3%) | 43 (59.7%) | 0.94 | 25 (45.5%) | 30 (54.5%) | 0.53 |
| Negative         | 221 (40.8%) | 321 (59.2%) |     | 128 (40.9%) | 185 (59.1%) |       |
| Subtypes         |       |       |       |       |       |       |
| HR-positive/HER2-negative | 208 (40.8%) | 302 (59.2%) | 1.00 | 56 (36.1%) | 99 (63.9%) | 0.20 |
| HER2-positive    | 29 (40.3%) | 43 (59.7%) |     | 25 (45.5%) | 30 (54.5%) |       |
| Triple negative  | 13 (40.6%) | 19 (59.4%) |     | 72 (45.6%) | 86 (54.4%) |       |
| Ki67             |       |       |       |       |       |       |
| High (≥10%)      | 120 (44.4%) | 150 (55.6%) | 0.12 | 100 (44.1%) | 127 (55.9%) | 0.59 |
| Low (<10%)       | 85 (37.6%) | 141 (62.4%) |     | 27 (40.3%) | 40 (59.7%) |       |

Abbreviations: ANK3: ankyrin 3; AR: androgen receptor; ER: oestrogen receptor; PR: progesterone receptor; HR: hormone receptor.

Some variables have missing data.

*Significant difference, $p < 0.05$. 
Survival analysis stratified by \( AR \) mRNA expression

**METABRIC cohort**

- High \( AR \) group (495 events/983 cases)
- Low \( AR \) group (394 events/976 cases)

**TCGA cohort**

- High \( AR \) group (59 events/519 cases)
- Low \( AR \) group (56 events/516 cases)

\( AR \) mRNA expression
High group vs. Low group: Hazard Ratio: 1.12, \( p = 0.097 \)

Survival analysis stratified by \( ANK3 \) mRNA expression

**METABRIC cohort**

- High \( ANK3 \) group (446 events/975 cases)
- Low \( ANK3 \) group (443 events/984 cases)

**TCGA cohort**

- High \( ANK3 \) group (61 events/519 cases)
- Low \( ANK3 \) group (54 events/518 cases)

\( ANK3 \) mRNA expression
High group vs. Low group: Hazard Ratio: 0.97, \( p = 0.62 \)

Supplementary Figure 1
For protein expression of ANK3
High vs. Low: Hazard Ratio: 0.85, 95% CI: 0.67-1.08, 
\( p = 0.19 \)
Supplementary
Figure 3

For protein expression of AR
High vs. Low: Hazard Ratio: 0.66, 95% CI: 0.52-0.84
p = 0.00066
Low AR expression cases

High vs. low ANK3 protein expression
Hazard ratio 0.89, 95% CI: 0.62-1.28, p = 0.53

Cancer Specific Survival (Probability)

Month

ANK3 high
(53 events/151 cases)

ANK3 low
(68 events/210 cases)

| ANK3 | Time (months) |
|------|--------------|
|      | 0  | 30 | 60 | 90 | 120 | 180 |
| Low  | No. at Risk | 210 | 181 | 157 | 140 | 132 | 94 |
|      | No. of events | 0  | 23  | 44  | 55  | 58  | 68 |
| High | No. at Risk  | 151 | 125 | 107 | 99  | 86  | 63 |
|      | No. of events | 0  | 23  | 39  | 45  | 48  | 53 |