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PRECLINICAL STUDY

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 ($n=1980$) and The Cancer Genome Atlas (TCGA) dataset ($n=1039$) 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 to AR signalling pathway and is associated with BC prognosis.

Keywords Invasive breast cancer · Androgen receptor · Ankyrin 3 · Prognostic marker

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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 three 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] ($n = 1980$) and the Cancer Genome Atlas (TCGA) [26] dataset ($n = 1039$) provided by cBioPortal [27].

The normalisation method of mRNA expression in the METABRIC cohort was previously described [24]. TCGA mRNA data were $\log_2$ 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]. 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. 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. 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*, *RNF14*, *RNF6*, *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 (Fig. 1a, 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 *ANK*3 and *AR* mRNA was not a significant independent prognostic factor in BC (Supplementary Fig. 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, $p = 0.011$; Fig. 1c, 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 (Fig. 2a–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 (Fig. 2d–f).

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 ≤ 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 Fig. 2).

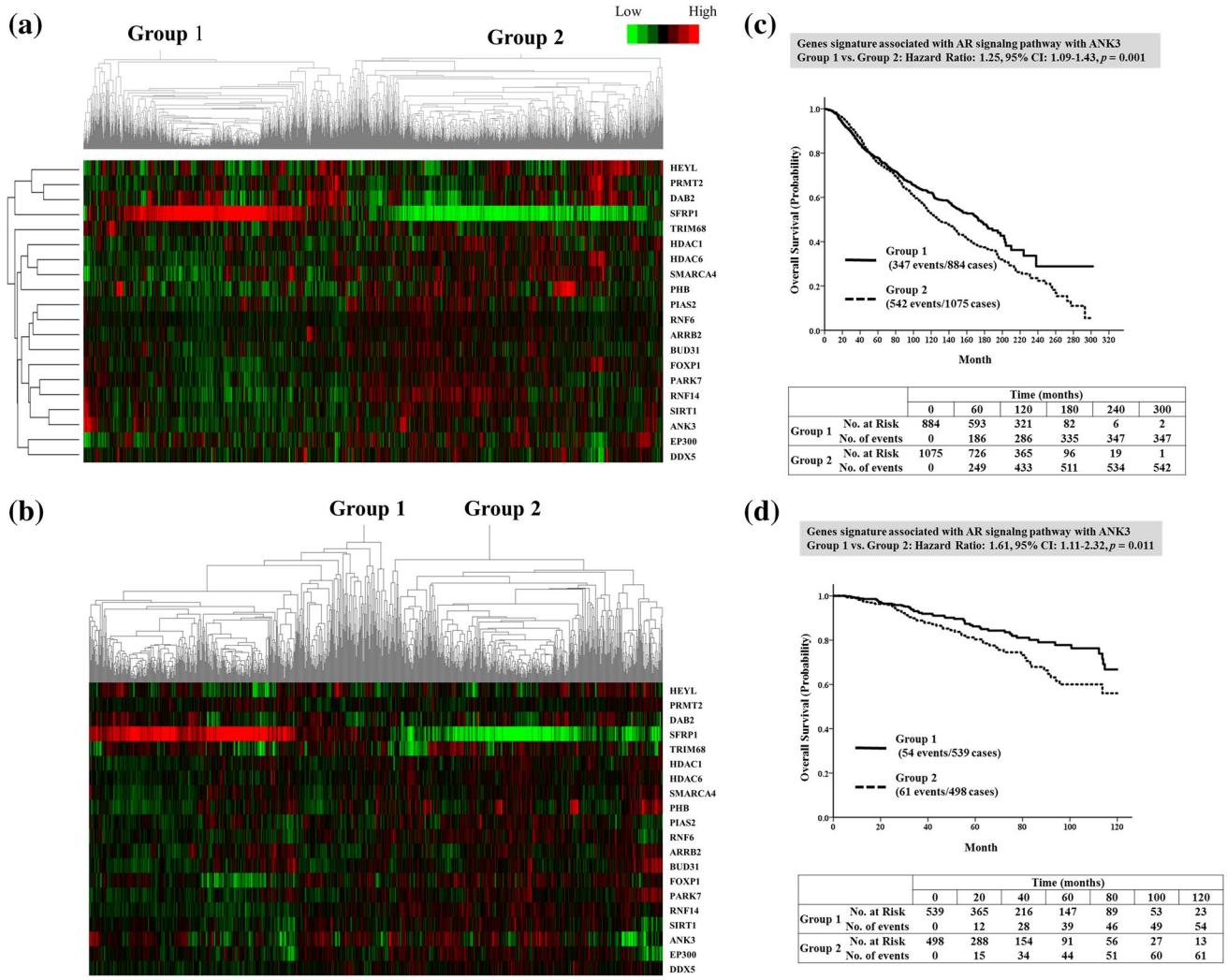

Fig. 1 Prognostic utility of an androgen receptor (AR)-related gene signature, including *ANK3* mRNA expression. Heat map of the AR-related gene signature for the **a** METABRIC and **b** TCGA cohorts generated by unsupervised cluster analysis, showing a clear division of cases between Group 1 and Group 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

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 Fig. 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, $p = 0.020$; Fig. 3a–e, but not in those whose tumours had low AR expression (HR 0.89, 95% CI 0.62–1.28, $p = 0.53$; Supplementary Fig. 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

Table 1 Clinicopathological characteristics of breast cancer associated with AR signaling pathway-related gene signature

METABRIC cohort						TCGA cohort					
Factors		Genes signature associated with the AR pathway		*p* value		Factors		Genes signature associated with the AR pathway		*p*-value	
		Group 1	Group 2	Univariate	Multivariate			Group 1	Group 2	Univariate	Multivariate
Tumour size	≥2 cm	564 (42.2%)	774 (57.8%)	< 0.0001*	0.00065*	Tumour size	T2–4	388 (50.5%)	381 (49.5%)	0.079	0.098
	< 2 cm	319 (51.8%)	297 (48.2%)				T1	153 (56.7%)	117 (43.3%)		
Nodal status	Positive	413 (44.0%)	525 (56.0%)	0.28	0.66	Nodal status	Positive	261 (49.8%)	263 (50.2%)	0.11	0.70
	Negative	481 (46.5%)	554 (53.5%)				Negative	278 (54.8%)	229 (45.2%)		
Grade	Grade 3	444 (46.6%)	508 (53.4%)	0.22	0.0070*	Grade	Grade 3	233 (56.7%)	178 (43.3%)	0.020	0.0093*
	Grade 1, 2	412 (43.8%)	528 (56.2%)				Grade 1, 2	272 (49.1%)	282 (50.9%)		
ER	Positive	569 (37.8%)	937 (62.2%)	< 0.0001*	< 0.0001*	ER	Positive	340 (44.5%)	424 (55.5%)	< 0.0001*	< 0.0001*
	Negative	330 (69.6%)	144 (30.4%)				Negative	183 (79.9%)	46 (20.1%)		
PR	Positive	409 (39.3%)	631 (60.7%)	< 0.0001*	0.90	PR	Positive	303 (45.8%)	358 (54.2%)	< 0.0001*	0.80
	Negative	490 (52.1%)	450 (47.9%)				Negative	215 (65.7%)	112 (34.3%)		
HER2	Positive	104 (42.1%)	143 (57.9%)	0.27	< 0.0001*	HER2	Positive	57 (32.6%)	118 (67.4%)	< 0.0001*	< 0.0001*
	Negative	795 (45.9%)	938 (54.1%)				Negative	385 (56.6%)	295 (43.4%)		
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%)								

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						Factors		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	Hazard ratio	95% CI	*p*-value
AR related signature	Group 1	Reference			Reference			AR related signature	Group 1	Reference			Reference		
	Group 2	1.25	1.09–1.43	0.0013*	1.23	1.06–1.42	0.0066*		Group 2	1.61	1.11–2.32	0.011*	1.82	1.08–3.06	0.026*
Tumour size	< 2 cm	Reference			Reference			Tumour size	T2–4	Reference			Reference		
	≥ 2 cm	1.83	1.57–2.15	< 0.0001*	1.60	1.36–1.89	< 0.0001*		T1	1.67	1.07–2.62	0.026*	1.18	0.66–2.09	0.58
Nodal status	Negative	Reference			Reference			Nodal status	Negative	Reference			Reference		
	Positive	1.86	1.63–2.12	< 0.0001*	1.62	1.40–1.86	< 0.0001*		Positive	2.05	1.38–3.02	0.00033*	1.75	1.05–2.91	0.032*
Grade	Grade1-2	Reference			Reference			Grade	Grade1-2	Reference			Reference		
	Grade 3	1.42	1.24–1.63	< 0.0001*	1.10	0.94–1.28	0.25		Grade 3	1.36	0.92–2.00	0.13	0.93	0.54–1.60	0.78
ER	Positive	Reference			Reference			ER	Positive	Reference			Reference		
	Negative	1.37	1.18–1.59	< 0.0001*	1.08	0.88–1.32	0.46		Negative	1.69	1.13–2.52	0.011*	1.61	0.73–3.56	0.24
PR	Positive	Reference			Reference			PR	Positive	Reference			Reference		
	Negative	1.44	1.26–1.64	< 0.0001*	1.29	1.09–1.52	0.0025*		Negative	1.55	1.06–2.27	0.025*	1.18	0.57–2.47	0.66
HER2	Negative	Reference			Reference			HER2	Negative	Reference			Reference		
	Positive	1.57	1.31–1.89	< 0.0001*	1.27	1.04–1.56	0.022*		Positive	1.57	0.95–2.59	0.076	1.24	0.70–2.20	0.46

ER oestrogen receptor, *PR* progesterone receptor, *CI* confidence interval, *HER2* human epidermal growth factor receptor 2, *AR* androgen receptor

*Significant difference, $p < 0.05$

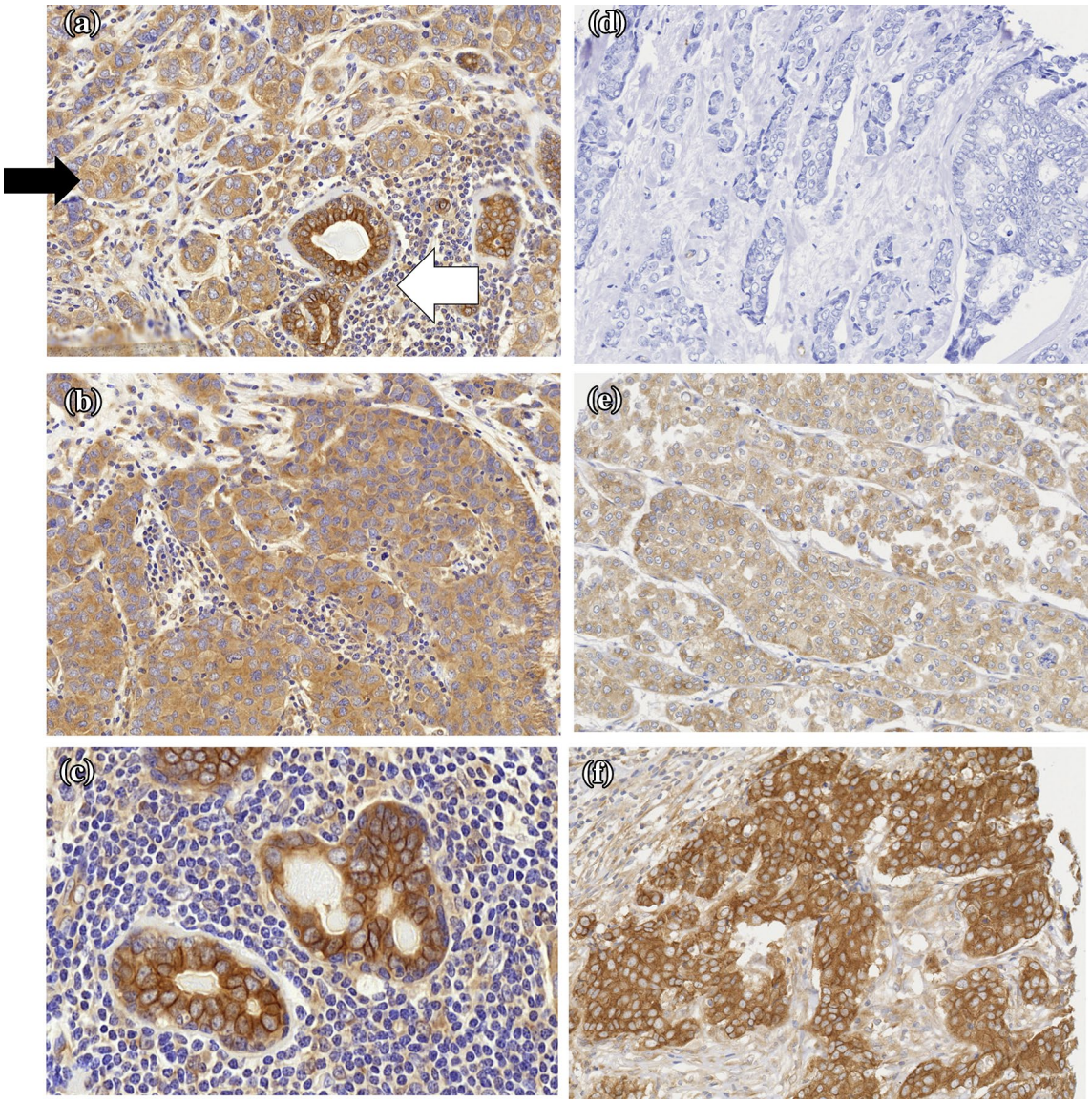

Fig. 2 Morphological characteristics of ANK3 immunohistochemistry in breast cancer tissue. **a** ANK3 immunoreactivity differs between invasive cancer cells and adjacent normal mammary glandular tissues (black arrow: invasive cancer cells; white arrow: normal mammary gland). Immunoreactivity in normal mammary gland cells is stronger than that in invasive cancer cells (magnification: ×100). **b** Invasive cancer cells showing uniform ANK3 immunoreactivity primarily in the cytoplasm (magnification: ×200). **c** ANK3 immunoreactivity is uniformly strong in normal epithelial cells and weaker in myoepithelial cells than in glandular cells (magnification: ×400). 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: ×200)

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,

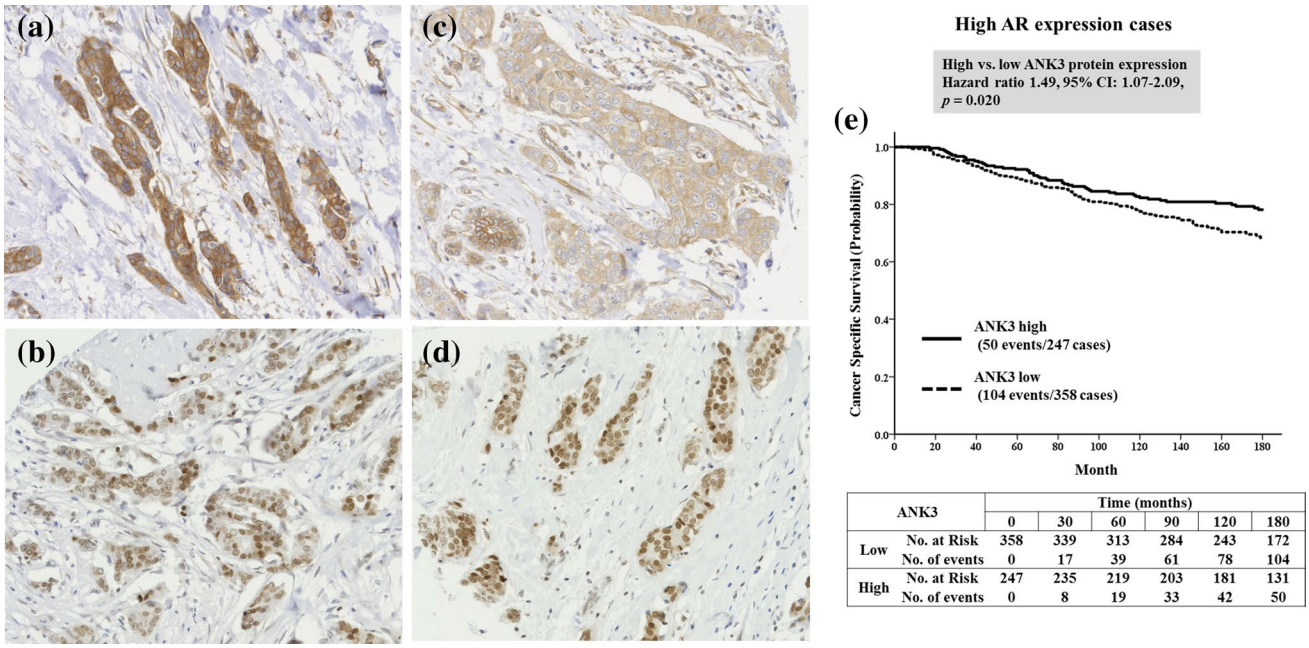

Fig. 3 ANK3 protein expression in breast cancer and cumulative survival rates stratified by ANK3 expression. **a**–**d** ANK3 and AR expression in breast cancer. Case 1: high ANK3 (**a**) and high AR (**b**) expression. Case 2: low ANK3 (**c**) and high AR (**d**) expression (magnification: ×200 for all images). **e** With high AR expression, BC-specific survival was significantly worse in those with low than high ANK3 expression

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*
Tumour size	< 2 cm	Reference			Reference		
	≥ 2 cm	2.61	1.86–3.69	< 0.0001*	1.75	1.22–2.51	0.0024*
Nodal status	Negative	Reference			Reference		
	Positive	2.84	2.05–3.92	< 0.0001*	2.22	1.58–3.11	< 0.0001*
Histological grade	Grades 1 and 2	Reference			Reference		
	Grade 3	3.27	2.36–4.53	< 0.0001*	2.23	1.57–3.16	< 0.0001*
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

ANK3 ankyrin 3, *AR* androgen receptor, *CI* confidence interval, *HER2* human epidermal growth factor receptor 2, *HR* hormone receptor
*Significant difference, $p < 0.05$

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 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

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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