Clinical and biological roles of Kelch-like family member 7 in breast cancer: a marker of poor prognosis

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ABSTRACT

BACKGROUND: The functions of many proteins are tightly regulated with a complex array of cellular functions including ubiquitination. In cancer cells, aberrant ubiquitination may promote the activity of oncogenic pathways with subsequent tumour progression. Kelch-like family member 7 (KLHL7) is involved in the regulation of ubiquitination and may play a role in breast cancer (BC). Present study aims to evaluate the biological and clinical usefulness of KLHL7 in BC utilising large well-characterised cohorts with long follow up term.

METHODS: The relationships between *KLHL7* gene copy number alteration (CNA) and mRNA expression and clinicopathological variables and clinical outcomes were evaluated in 1980 patients from the METABRIC BC cohort. Prognostic significance of KLHL7 mRNA was validated using the Breast Cancer Gene-Expression Miner v4.0 datasets (n = 5206). KLHL7 protein expression was assessed using immunohistochemistry in a large annotated series of early-stage BC (n=917) with long-term follow-up.

RESULTS: *KLHL7* CNA was significantly correlated with its mRNA expression. *KLHL7* mRNA expression was higher in luminal B and basal-like molecular subtypes and in higher grade tumours. Increased KLHL7 protein expression was significantly correlated with features of aggressive phenotype including lymphovascular invasion, high histological grade, hormonal receptor negativity, high PIK3CA and p53 expression. Outcome analysis showed that high KLHL7 expression is an independent predictor of shorter survival ($p = 0.0011$).

CONCLUSIONS: KLHL7 appears to play an important role in BC progression. High KLHL7 protein expression identified a subgroup of BC with aggressive behaviour and provided independent prognostic information.

INTRODUCTION

Advances in early detection, diagnosis and refinement of prognostic and therapy prediction have led to improvement of the outcome of invasive breast cancer however; approximately 20% of early-stage breast cancer patients still experience recurrence and metastasis [1, 2]. Identification of genes significantly associated with tumour progression providing potential therapeutic values remains as one of the main goals of breast cancer research. The functions of many proteins are controlled by ubiquitination [3, 4], and in cancer cells, abnormal ubiquitination may promote the activity of oncogenic pathways [5, 6], enhance tumour proliferation, migration, invasion, angiogenesis, epithelial–mesenchymal transition and metastasis [7, 8]. Kelch-like family member 7 (KLHL7), which is a member of the Kelch protein family associated with the development of retinitis pigmentosa [9, 10], forms a ubiquitin ligase complex by binding to the BTB and BACK domains of Cullin3 (CUL3) [10, 11]. This binding facilitates proteasome degradation of target proteins by enhancing E2 or E3 ligase activity and polyubiquitination [10-12]. KLHL7 is considered to be crucial for regulating the protein homeostasis and its aberrant expression has also been associated with cancer cell proliferation [13-16]. However; the role of KLHL7 in breast cancer has not been established.

In this study we investigated the biological and clinical significance of KLHL7 in breast cancer at the genetic transcriptomic and protein levels. *KLHL7* copy number alterations (CNA) and mRNA expression as well as KLHL7 protein expression assessed using immunohistochemistry was correlated with clinicopathological features and outcome using large well-characterised cohorts of early-stage breast cancer.

MATERIALS AND METHODS

KLHL7 gene copy number and mRNA expression

The Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset, containing 1980 invasive breast cancers [17, 18], was explored for genomic/transcriptomic profiling of *KLHL7*. In the METABRIC study, DNA and RNA extracted from primary tumour samples were hybridised using Affymetrix SNP 6.0 arrays (Affymetrix, Inc., Santa Clara, USA) and Illumina Human HT-12 v3 platforms (Illumina, Inc., San Diego, USA) . All patients were treated uniformly. Oestrogen receptor (ER)-positive breast cancer patients with lymph node-negative were not offered adjuvant chemotherapy. ER-negative or lymph node-positive patients were treated with adjuvant chemotherapy. No human epithelial growth factor 2 (HER2)-positive patients received therapy with trastuzumab. None of the patients included in the study received neoadjuvant treatment.

The prognostic value of *KLHL7* mRNA expression was further evaluated using the Breast Cancer Gene-Expression Miner v4.0 (bc-GenExMiner v4.0) database which includes 5861 breast cancer patients [19].

KLHL7 protein expression

KLHL7 protein expression was assessed in large (n=917) cohort of early-stage breast cancer from surgically treated patients presented to Nottingham City Hospital, UK between 1989 and 1998. Tissue microarray (TMA) sections were stained using specific anti-KLHL7 antibody (see below). No patients underwent neoadjuvant treatment before initial breast surgery. In this study, 917 cases were informative for the biomarker expression. Lymphovascular invasion (LVI) was evaluated by haematoxylin and eosin staining and immunostaining for CD34 or D2-40 as previously described [20]. Patient characteristics were shown in Supplementary Table 1. Data on ER, progesterone receptor (PR), HER2, Ki67, phosphatidylinositol 3-kinase (PIK3CA), p53 and phospho-Akt1 (pAkt) were available and

were assessed as previously described [21-26]. ER-positive/HER2-negative breast cancer patients with PR-positive and low Ki67 staining (labelling index of ≤ 10%) were classified as luminal A-like type; other ER-positive patients were classified as luminal B-like type. Patients who were ER-positive and/or PR-positive and HER2-positive were defines as luminal-HER2 [27].

The specificity of KLHL7 antibody (HPA029491, Merck, Germany) was confirmed using Western blotting and MCF-7 and Jurkat cell lines (The American Type Culture Collection; Rockville, MD, USA). This showed primary antibody specificity; with a single band observed at approximately 70 kDa in both cell lines (Supplementary Figure 2).

KLHL7 protein expression was characterized immunohistochemically (IHC) in 15 full-face breast cancer tissue sections prior to TMA application. IHC assays were performed using Novolink Max Polymer Detection System (RE7280-k, Leica, Newcastle, UK). The anti-KLHL7 primary antibody was diluted 1:100 in Bond Primary Antibody Diluent (Leica, Germany). A polyclonal rabbit anti-human beta-2-microglobulin antibody (diluted 1:2000; Dako, Glostrup, Denmark) was used as a positive staining control. Diaminobenzidine tetrahydrochloride (Novolink DAB substrate buffer plus) was used as the chromogen; sections were counterstained with Meyer's haematoxylin for 6 min.

Immunostained TMA sections were digitally scanned into high-resolution digital images (NanoZoomer, Hamamatsu Photonics, Tokyo, Japan) and viewed using Aperio Image Scope (Aperio Technologies, Milton Keynes, UK). KLHL7 expression was scored as none, weak, moderate or strong depending on the intensity of cytoplasmic staining (Figure 1). Cytoplasmic expression was assessed, and H-scores were calculated using the proportion of stained cells (0%–100%) and intensity scores (0, negative; 1, weak; 2, moderate; 3, strong) as previously described [28, 29].

Statistical analysis

Statistical analysis was performed with SPSS v22.0 (IBM Corp., Armonk, NY, USA). The association of *KLHL7* mRNA expression and CNA was assessed using the Kruskal–Wallis test. The significance of differences in CNA and mRNA expression in tumours stratified by PAM50 subtype, size, lymph node metastasis grade and histological grade was determined using the Chi-squared and Fisher's exact tests. For analysis, tissue samples were assigned to high- and low-expression groups using the median mRNA expression level as the cut-off. For KLHL7 expression, the cut-off was an H-score of 90 determined by X-Tile plotting (X-Tile Bioinformatics Software, Yale University, version 3.6.1), with the samples stratified to high and low groups based on patient outcome. For the association between KLHL7 and prognosis, Kaplan–Meier survival curves of 10-year breast cancer specific survival (BCSS) were plotted, and significance was determined by the log-rank test. As the patients in the Nottingham primary series were followed-up for at least 10 years, BCSS was defined as the interval from surgery to death from breast cancer. In univariate and multivariate analyses of clinicopathological factors and KLHL7 expression and prognosis, 95% confidence interval (CI) values were determined using the Cox proportional hazards regression model.

RESULTS

KLHL7 Copy number aberration and mRNA expression

A total of 150 of 1980 patients (7.6%) had a copy number gain whereas 23 (1.7%) had a loss of *KLHL7*. *KLHL7* mRNA expression was significantly higher in samples with CNA gain than in CNA-neutrals ($p < 0.0001$). The expression was significantly lower in tumours with CNA loss than in CNA-neutral tissues ($p = 0.042$). *KLHL7* CNAs were significantly correlated with molecular subtype ($p < 0.0001$); *KLHL7* gain was higher in HER2-enriched tumours, whereas *KLHL7* loss was more frequent in basal-like tumours than in other subtypes

(Table 1). High *KLHL7* mRNA expression was significantly related with high histological grade ($p = 0.010$) and molecular subtypes ($p < 0.0001$); high expression in HER2-enriched tumours and low in basal-like tumours (Table 1).

Although the association between *KLHL7* CNA and mRNA expression and outcome was not significant in the METABRIC series (Supplementary Figure 3), it was significant in the larger series of the Breast Cancer Gene-Expression Miner v4.0 with an association between high *KLHL7* mRNA expression and shorter survival (hazard ratio (HR) =1.3; $p<0.0001$; Figure 2-a).

KLHL7 protein expression

In full-face sections, the expression of KLHL7 protein was variable in the different components of tissues with increased expression in invasive tumours cells than DCIS and normal parenchymal cells (Figure 3). Fibroblasts and lymphocytes in the mammary stroma adjacent to the cancer cells showed negative to weak staining. In normal glandular epithelium, the reactivity of IHC was absent to weak and the reactivity of myoepithelial cells tended to be higher than those of glandular cells. The reactivity of myoepithelial cells surrounding DCIS was higher compared to the intraductal malignant epithelial cells. KLHL7 positivity was recognised in the cytoplasm of invasive cancer cells and the reactivity in both was substantially stronger compared to normal mammary gland cells if positive. Focal weak to moderate nuclear immunoreactivity was seen in the tumour cells simultaneously with cytoplasmic staining.

Clinicopathological and prognostic significance of KLHL7 protein expression

Of the 917 patients, 407 (44.4%) had tumours with low KLHL7 expression whereas 510 (55.6%) had tumours with high expression (H-score $\geq$ 90). KLHL7 expression was positively

correlated with histological grade ($p = 0.0002$) and LVI status ($p = 0.030$) but inversely correlated with ER status ($p = 0.015$, Table 2). High KLHL7 expression was significantly related with molecular subtypes ($p = 0.026$), especially HER2-positive and triple-negative breast cancer (TNBC) classes (Table 2). High KLHL7 expression was significantly associated with high expression of PIK3CA ($p = 0.044$) and p53 ($p = 0.002$), but not pAkt expression (Table 2).

The 10-year BCSS of the subgroup with KLHL7 high expression was significantly shorter than that of the subgroup with KLHL7 low expression (HR = 9.1; $p = 0.0025$; Figure 2-b). Univariate analysis identified high KLHL7 expression (HR = 1.5; $p = 0.003$), ER negativity (HR = 1.9; $p < 0.0001$), PR negativity (HR = 2.3; $p < 0.0001$), HER2 positivity (HR = 2.3; $p < 0.0001$), large tumour size (HR = 2.3; $p < 0.0001$), positive nodal status (HR = 2.6; $p < 0.0001$), histological grade 3 (HR = 3.3; $p < 0.0001$), positive LVI (HR = 2.4; $p < 0.0001$) and high p53 expression (HR = 1.9; $p < 0.0001$) as poor prognostic factors.

In multivariate analysis including other prognostic variables, KLHL7 expression remained independently associated with poor prognosis (HR = 1.3; $p = 0.042$; Table 3).

DISCUSSION

KLHL7 forms a ubiquitin ligase complex and regulates ubiquitination [9, 10]; however, there is a critical lack of knowledge of the targets of KLHL7 ubiquitination in breast cancer. In the present study, we revealed the significant association of KLHL7 expression with p53 suggesting that aberrant KLHL7 ubiquitination may be responsible for decreased p53 function. Several E3 ubiquitin ligases are thought to regulate p53 expression in cancer [30, 31]. The function of p53 have been known to play important role in genomic stability, cell cycling and apoptosis [32,33] with p53 suppressing cell proliferation in breast cancer cells [34-36]. The function of p53 is also associated with activation of PI3K/Akt signalling

pathway [37]. In a previous study, high p53 protein expression significantly correlated with high PIK3CA protein expression [24]. Activation of PI3K pathway is regulated by growth factors through transmembrane receptor [38]. The breast cancer with PIK3CA mutations have frequently aberrant activation of PI3K pathway [38]. Approximately 20-25% of breast cancer have PIK3CA mutations [39]. Previous studies suggest that breast cancer become resistant to treatment by activating the PI3K/Akt signalling pathway [40, 41]. Recent clinical trials indicated that PI3K inhibitor [42] and mTOR inhibitor [43], which inhibit the activity of PI3K/Akt signalling pathway, were useful for the treatment of metastatic breast cancer patients. The current study indicates the positive relationship between KLHL7 and PIK3CA expression. Further functional studies are necessary to explore the association of aberrant ubiquitination caused by KLHL7 overexpression with PI3K/Akt signalling pathway activity in breast cancer.

LVI is involved in breast cancer metastasis and is a recognised prognostic factor [44-47]. In this study, KLHL7 expression was significantly associated with positive LVI status, negative ER status and TNBC. *KLHL7* is located at 7p15, which shows frequent copy number alteration in basal-like subtype [48]. A recent genetic analysis of TNBC cases identified mesenchymal and mesenchymal stem-like subtypes to be high expressers of epithelial mesenchymal transition (EMT) - and cancer stem cell-related genes [49, 50]. Ubiquitination is thought to influence cancer stem cell-like properties in breast cancer, and E3 ligases may be active in cancer stem cell growth [8]. A recent study reported that the C-terminus of heat shock cognate 71 kDa (HSC70) interacting protein (CHIP), a ubiquitin ligase present in the cytoplasm, inhibits cancer stem-like cell activity in breast tumours and suppresses proliferation and metastasis of cancer cells [51]. Reduced CHIP expression has also been associated with high histological grade, hormonal receptor negativity and poor prognosis in

breast cancer [52]. Aberrant ubiquitination may be responsible for enhanced cancer stem cell-like functions and EMT of breast cancer cells underlying LVI and metastasis.

KLHL7 positivity was recognised in the cytoplasm of invasive cancer cells. Although occasional cases showed nuclear staining the number was small for reliable statistical analysis and the expression was weak to moderate and seen simultaneously with cytoplasmic staining. High cytoplasmic staining of KLHL7 was associated with poor outcome in breast cancer patients. Importantly, the association with poor outcome was independent of other prognostic variables. Although no association between *KLHL7* CNA and outcome was identified in the METABRIC cohort, the number of cases with CNA was limited with less than 2% showing copy number loss. Using the large cohort of Breast Cancer Gene-Expression Miner, the association between KLHL7 mRNA expression and outcome was highly significant.

CONCLUSIONS

KLHL7 expression was related with molecular subtypes of breast cancer at genomic, transcriptomic and proteomic levels, and was strongly correlated with poorly differentiated tumours with LVI and with expression p53 and PIK3CA. KLHL7 may be released into the cytoplasm and ubiquitinates proteins involved in oncogenic pathways with the other factors involved in PI3K pathway. This may explain at least in part why the cytoplasmic expression of KLHL7 is an indicator of aggressive features and poor outcome in breast cancer.

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Conflict of interest

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There were no competing interests for all other authors.

Figure legends

Figure 1. Immunohistochemical staining of KLHL7 to assay protein expression in breast cancer tissue showing (a) no staining, (b) weak staining, (c) moderate staining and (d) strong staining in the cytoplasm of cancer cells.

Figure 2. Cumulative survival of breast cancer patients stratified by KLHL7 expression in breast tumours. (a) Significant differences were noted in survival of patients with high and low *KLHL7* mRNA expressions using Breast Cancer Gene Expression Miner v4.0 [19] ($p < 0.0001$). (b) Ten-year breast cancer specific survival was significantly worse in the KLHL7 protein expression-positive group than in the expression-negative group ($p = 0.0011$).

Figure 3. Morphological characteristics of KLHL7 immunohistochemistry in breast cancer tissue. (a) The immunohistochemical expression of KLHL7 was different between invasive carcinoma, intraductal cancer cells, and normal mammary gland adjacent to the tumour (Black arrow: invasive carcinoma, Grey arrow: intraductal cancer cells and white arrow: normal mammary gland). (b) Normal mammary gland cells showed absent or weak KLHL7

staining. The reactivity of normal myoepithelial cells around epithelium (Black arrow) tended to be higher than those of normal epithelium. (c) Invasive cancer cells showed strong KLHL7 staining. The reactivity was mainly recognized in the cytoplasm. (d) The degree of KLHL7 immunohistochemical expression in invasive cancer cells was stronger than those of intraductal cancer cells.

Table 1. Association of *KLHL7* copy number and *KLHL7* mRNA expression with clinicopathological characteristics

Characteristic		Expression of *KLHL7* (Copy number)				*p*	Characteristics		Expression of *KLHL7* (mRNA)			*p*
		Loss	Neutral	Gain	Total				≥Median	<Median	Total	
Tumour size	≥ 2 cm	16 (1.2%)	1208 (90.4%)	113 (8.5%)	1337	0.13	Tumour size	≥ 2 cm	670 (50.0%)	668 (50.0%)	1338	0.72
	< 2 cm	7 (1.1%)	573 (93.0%)	36 (5.8%)	616			< 2 cm	303 (49.3%)	313 (50.7%)	616	
Nodal status	Positive	8 (0.9%)	846 (90.3%)	83 (8.9%)	937	0.069	Nodal status	Positive	462 (49.3%)	476 (50.7%)	938	0.51
	Negative	15 (1.4%)	953 (92.1%)	67 (6.5%)	1035			Negative	525 (50.7%)	510 (49.3%)	1035	
Histological grade	Grade 3	14 (1.5%)	865 (90.9%)	73 (7.7%)	952	0.45	Histological grade	Grade 3	445 (46.8%)	507 (53.3%)	952	0.010*
	Grade 1, 2	8 (0.9%)	860 (91.6%)	71 (7.6%)	939			Grade 1, 2	495 (52.7%)	445 (47.3%)	940	
Molecular subtype	Luminal A	5 (0.7%)	665 (92.6%)	48 (6.7%)	718	<0.0001*	Molecular subtype	Luminal A	396 (55.2%)	322 (44.8%)	718	<0.0001*
	Luminal B	8 (1.6%)	446 (91.4%)	34 (7.0%)	488			Luminal B	223 (45.7%)	265 (54.3%)	488	
	HER2-enriched	0 (0.0%)	205 (85.4%)	35 (14.6%)	240			HER2-enriched	133 (55.4%)	107 (44.6%)	240	
	Basal-like	10 (3.0%)	296 (90.0%)	23 (7.0%)	329			Basal-like	130 (39.5%)	199 (60.5%)	329	
	Normal-like	0 (0.0%)	189 (95.5%)	9 (4.5%)	198			Normal-like	104 (52.3%)	95 (47.7%)	199	

Some variables do not add up to 1980 for all patients due to missing data.

* Significant difference p<0.05.

Table 2. Correlation between KLHL7 expression and clinicopathological characteristics

Characteristic		Expression of KLHL7			*p*
		Low	High	Total	
ER	Positive	306 (46.9%)	346 (53.1%)	652	0.015*
	Negative	101 (38.1%)	164 (61.8%)	265	
PgR	Positive	244 (47.2%)	273 (52.8%)	517	0.051
	Negative	163(40.8%)	237 (59.3%)	400	
HER2	Positive	48(37.2%)	81 (62.8%)	129	0.077
	Negative	359 (45.6%)	429 (54.4%)	788	
Tumour size	≥ 2cm	219 (42.4%)	297 (57.6%)	516	0.18
	< 2cm	188 (46.9%)	213 (53.1%)	401	
Nodal status	Positive	160 (42.3%)	218 (57.7%)	378	0.29
	Negative	247 (45.8%)	292 (54.2%)	539	
Histological grade	Grade 3	193 (38.7%)	306 (61.3%)	499	0.00015*
	Grades 1, 2	214 (51.2%)	204 (48.8%)	418	
Lymphovascular invasion	Positive	157 (40.3%)	233 (59.7%)	390	0.030*
	Negative	250 (47.4%)	277 (52.6%)	527	
Intrinsic Subtype	Luminal A-like	117 (52.2%)	107 (47.8%)	224	0.026*
	Luminal B-like	166 (45.1%)	202 (54.9%)	368	
	HER2 (non Luminal)	24(35.3%)	44 (64.7%)	68	
	Luminal-HER2	24 (39.3%)	37 (60.7%)	61	
	Triple negative	76(38.8%)	120 (61.2%)	196	
PIK3CA	Low	81 (52.3%)	74 (47.7%)	155	0.044*
	Moderate	87 (47.8%)	95 (52.2%)	182	
	High	156 (41.1%)	224 (58.9%)	380	
pAKT	Low	65 (43.3%)	85 (56.7%)	150	0.82
	High	206 (42.3%)	281 (57.7%)	487	

p53	Low	294 (47.5%)	325 (52.5%)	619	0.0015*
	High	102 (36.2%)	180 (63.8%)	282	

The variables of PIK3CA, pAKT and p53 do not add up to 917 for all patients due to missing data.

*** Significant difference p<0.05.**

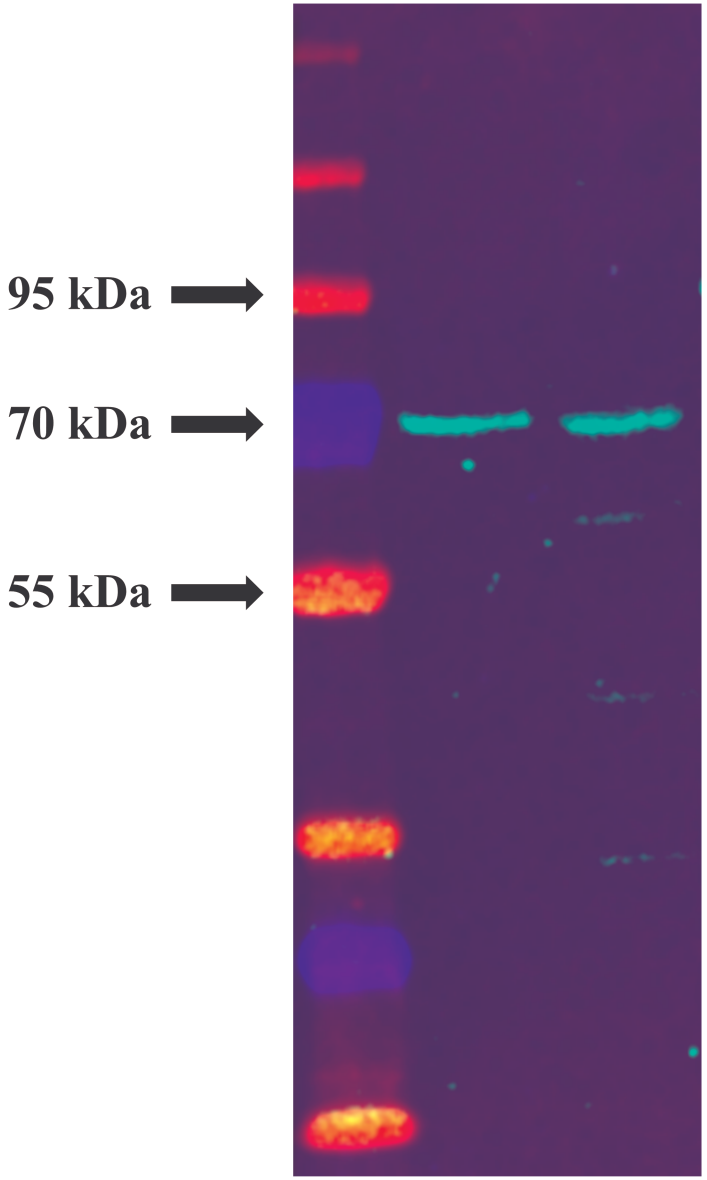
Table 3. Survival analysis based on clinicopathological characteristics, including KLHL7 expression

Characteristics		Univariate analysis			Multivariate analysis		
		Hazard Ratio	95% CI	*p*	Hazard Ratio	95% CI	*p*
KLHL7	Low	Reference			Reference		
	High	1.53	1.18–1.99	0.0011*	1.32	1.02–1.71	0.037*
ER	Positive	Reference			Reference		
	Negative	1.97	1.53–2.54	<0.0001*	0.83	0.59–1.18	0.30
PR	Positive	Reference			Reference		
	Negative	2.33	1.81–3.01	<0.0001*	1.80	1.27–2.53	0.00085*
HER2	Negative	Reference			Reference		
	Positive	2.27	1.69–3.04	<0.0001*	1.44	1.06–1.96	0.019*
Tumour size	< 2cm	Reference			Reference		
	≥ 2cm	2.34	1.77–3.08	<0.0001*	1.62	1.22–2.16	0.00085*
Nodal status	Negative	Reference			Reference		
	Positive	2.59	2.01–3.34	<0.0001*	2.31	1.79–3.00	<0.0001*
Histological grade	Grade 1-2	Reference			Reference		
	Grade 3	3.37	2.51–4.52	<0.0001*	2.28	1.64–3.15	<0.0001*

* Significant difference p<0.05.

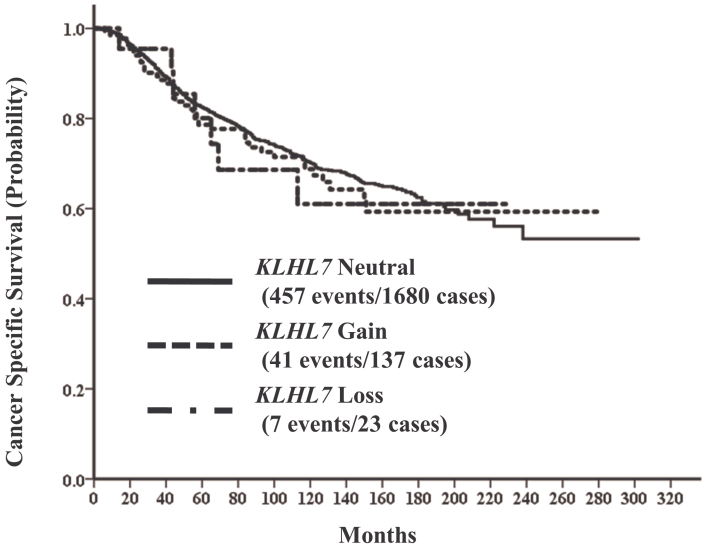
Supplementary File 1. Characteristics of the Nottingham primary cohort patients

Age range (years)	**Patients (n)**
24–40	104
41–59	535
60 and over	278
Menopausal status	
Pre	393
Post	524
Tumour size	
< 2.0cm	401
≥ 2.0 cm	516
Nodal status	
Negative	539
Positive	378
Lymphovascular invasion	
Negative	527
Positive	390
Type of breast surgery	
Breast-conserving surgery	372
Mastectomy	545
Axillary surgery	
Sampling alone	500
Axillary lymph node dissection	351
No surgery	5
Unknown	61
Chemotherapy	
Yes	199
No	671
Unknown	47
Endocrine therapy	
Yes	337
No	533
Unknown	47



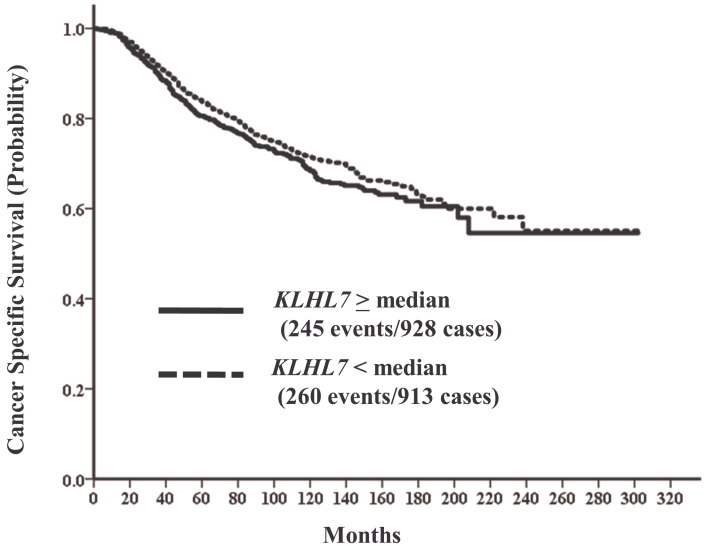
Supplementary File 2

a)



For copy numbers of *KLHL7*
Gain vs. Neutral: Hazard Ratio: 0.3, p= 0.56
Loss vs. Neutral: Hazard Ratio: 0.1, p= 0.75

b)



For mRNA expression of *KLHL7*
> median vs. < median: Hazard Ratio: 1.9, p= 0.17

Supplementary File 3

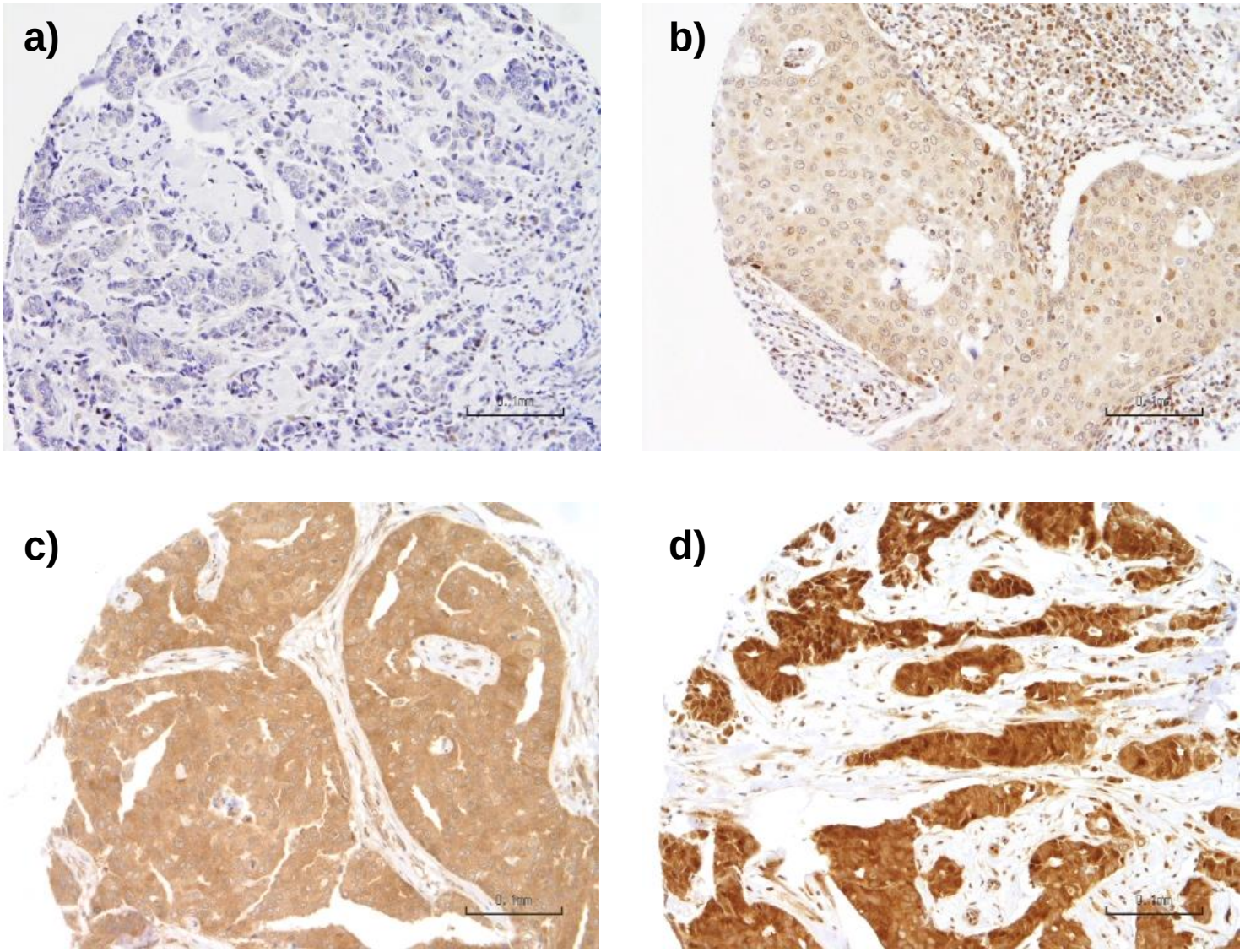
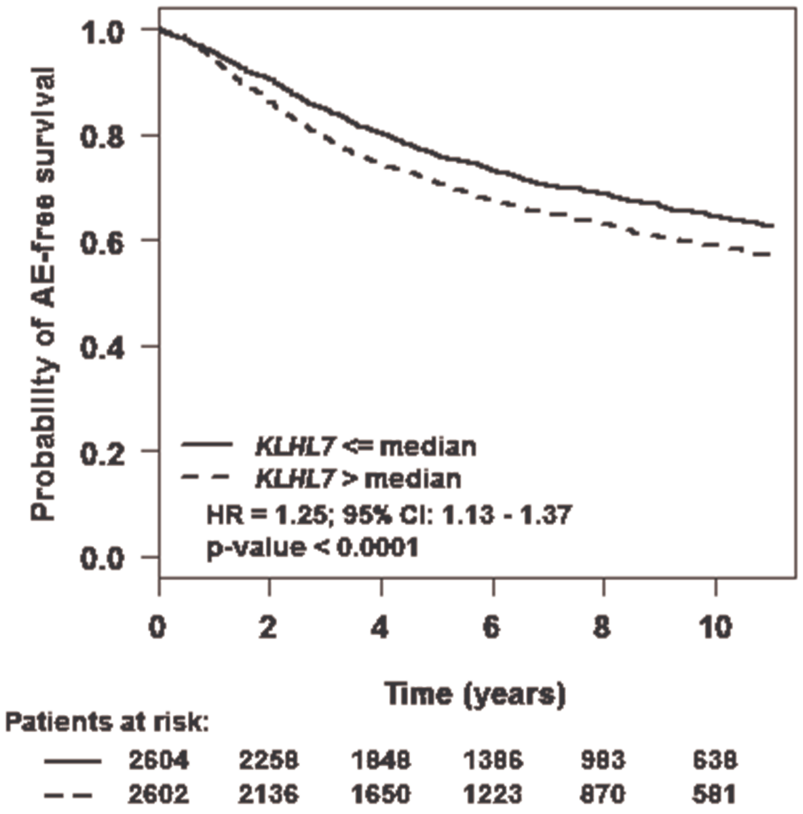
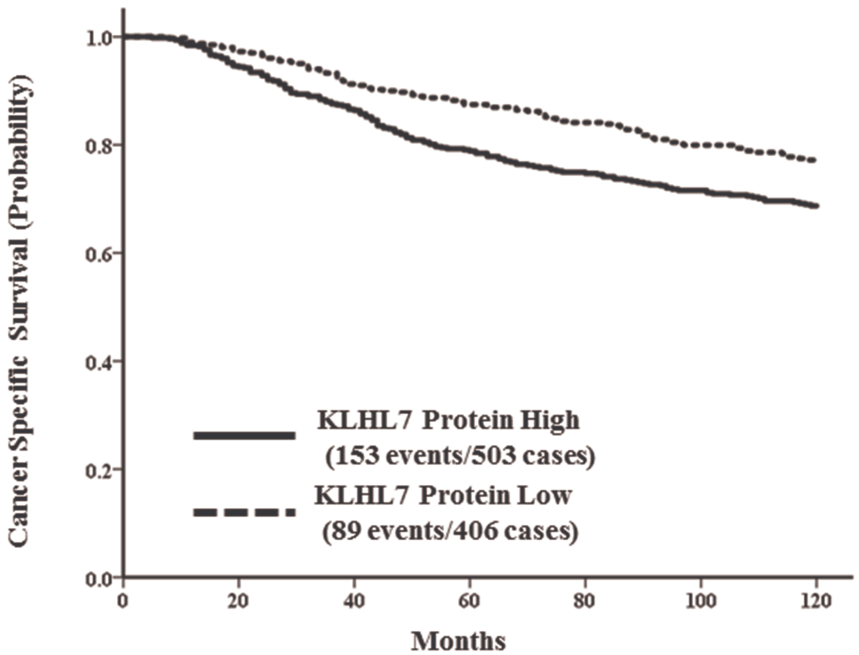


Figure 1

a)



b)



For protein expression of KLHL7
≥ H-socre 90 vs. < H-score 90:
Hazard Ratio: 9.1, p= 0.0025

Figure 2

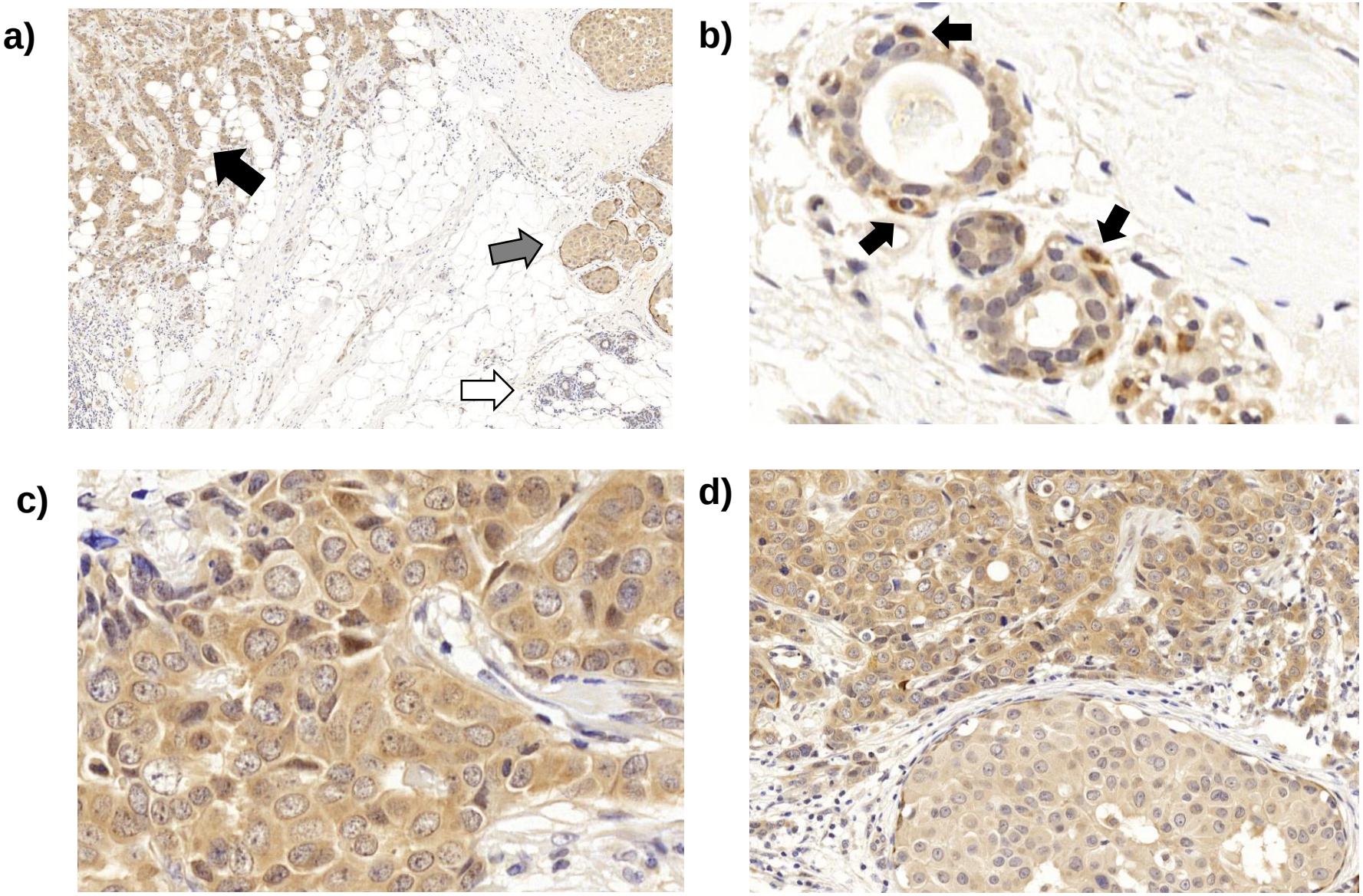


Figure 3