Clinicopathological significance of lipocalin 2 nuclear expression in invasive breast cancer

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ABSTRACT

PURPOSE: The epithelial–mesenchymal transition (EMT) plays a key role in breast cancer progression and metastasis. Lipocalin 2 (LCN2) is involved in the regulation of EMT. The aim of this study was to investigate the clinicopathological significance of LCN2 expression in breast cancer.

METHODS: The expression of LCN2 protein was immunohistochemically assessed in two well-characterised annotated cohorts of breast cancer (discovery cohort, n = 612; validation cohort, n = 1,363). The relationship of LCN2 expression and subcellular location with the clinicopathological factors and outcomes of patients was analysed.

RESULTS: Absent or reduced nuclear LCN2 expression was associated with features of aggressive behaviour, including high histological grade, high Nottingham Prognostic Index, high Ki67 labelling index, hormone receptor negativity and human epidermal growth factor receptor 2 positivity. The high cytoplasmic expression of LCN2 was correlated with lymph node positivity. The nuclear downregulation of LCN2 was correlated with the overexpression of EMT associated proteins (N-cadherin and Twist-related protein 2) and basal biomarkers (cytokeratin 5/6 and epidermal growth factor receptor). Unlike the cytoplasmic expression of LCN2, the loss of nuclear expression was a significant predictor of poor outcome. The combinatorial expression tumours with high cytoplasmic and low nuclear expression were associated with the worst prognosis.

CONCLUSIONS: Tumour cell expression of LCN2 plays a role in breast cancer progression with loss of its nuclear expression is associated with aggressive features and poor outcome. Further functional analysis is warranted to confirm the relationship between the subcellular localisation LCN2 and behaviour of breast cancer.

INTRODUCTION

Breast cancer is a heterogeneous disease with variable morphology, metastatic behaviour and response to therapy [1]. Further investigations of new biomarkers are warranted to develop a personalised management of

this disease. Lipocalin 2 (LCN2) is a secreted glycoprotein that transports small lipophilic ligands and acts as an iron binding protein. It also acts as a member of the autocrine system via SLC22A17 (receptor of LCN2) in cancer cells [2-4]. LCN2 is expressed in the extracellular matrix of tumours, regulates the epithelial–mesenchymal transition (EMT), and is involved in the proliferation of cancer cells [5, 6]. Previous studies suggested that LCN2 expressed in stromal tissue may regulate tumour angiogenesis, oncogenesis and progression in various types of cancer by controlling EMT and proliferation [7, 8]. LCN2 has been associated with matrix metallopeptidase 9 (MMP9), which is an established factor related to EMT and tumour metastasis in the extracellular matrix [9]. When LCN2 forms a complex with MMP9, it increases MMP9-activity and prevents its auto-degradation. Since MMP9 degrades the extracellular matrix and basement membranes, LCN2/MMP9 complex contributes to tumour progression, invasion and metastasis [9].

Abnormal expression of LCN2 plays important role in various pathological conditions including inflammation and tissue injury. LCN2 is also considered as a potential biomarker and a modulator of human epithelial malignancy. The expression of LCN2 is dysregulated in a variety of cancers including hepatocellular [8], pancreatic [10], colorectal [11], and prostate [12] carcinomas. In the breast, it has been reported that stroma-secreted LCN2 promotes metastasis *in vitro* and *in vivo* and contributes to tumour progression [13]. Decreasing LCN2 expression has reduced the invasion and migration ability of HER2-positive breast cancer cells [14]. However, some authors have reported that overexpression of LCN2 does not affect cell proliferation or anchorage-independent growth *in vitro*, or primary tumor weight *in vivo* [15]. Data from The Cancer Genome Atlas (TCGA) using 1,075 cases indicates an association between loss of *LCN2* mRNA expression and poor prognosis [16]. Therefore, clinicopathological significance of LCN2 expression and its morphological characteristics a in breast cancer remain unclear. In the present study, we attempt to demonstrate the clinicopathological significance of LCN2 expression using immunohistochemistry (IHC). We assessed the relationship between LCN2 expression and its subcellular localisation and other clinicopathological factors, including breast cancer progression/metastasis-related biomarkers and breast cancer patient outcomes.

MATERIALS AND METHODS

Patients` characteristics

Two independent cohorts of patients with early invasive breast cancer were included in this study: a discovery set and a validation set comprising 612 and 1,363 patients, respectively. The clinicopathological significance of the nuclear, cytoplasmic and combined expression of LCN2 was evaluated using the former set. The clinicopathological and prognostic value of the nuclear expression of LCN2 was further validated using the

latter set. All patients underwent primary breast surgery without prior neoadjuvant therapy at the Nottingham University Hospitals (Nottingham, UK). Clinical and pathological data of patients (i.e. age at diagnosis, histological tumour type, grade, tumour size, lymph node status, Nottingham Prognostic Index and lymphovascular invasion) were collected. Survival data, including breast cancer-specific survival (BCSS; defined as the time [in months] from the date of the primary surgical treatment to the time of death from breast cancer), were retrieved. Data related to the expression of basic breast cancer markers, including oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor 2 (HER2), are available [17-19].

The expression of a large panel of breast cancer progression/metastasis-related biomarkers, including the Ki67 labelling index, E-cadherin, N-cadherin, P-cadherin, Twist-related protein 2 (TWIST2) and basal markers (cytokeratin 5/6 [CK5/6] and epidermal growth factor receptor [EGFR]), was also studied in a previous IHC analysis using the discovery cohort [20-25].

Immunohistochemistry

The specificity of the LCN2 antibody (LS-C405956; Lifespan Biosciences, Seattle, WA) (dilution: 1:500) was validated, and a specific band (70 kDa) was detected through western blotting of lysate obtained from the cell line HeLa (The American Type Culture Collection, Rockville, MD) (Supplementary Fig 1). Full-face tissue sections from patients with invasive breast cancer (n = 10) [24-26] were stained before tissue microarray (TMA) to assess the morphological characteristics of LCN2 expression and test the suitability of this method. TMA sections from the discovery and validation sets were IHC-stained to evaluate the expression of LCN2 protein. The H-score [27, 28] was used to assess the nuclear and cytoplasmic expression of LCN2.

Statistical analysis

SPSS statistical software version 24.0 (IBM Corp., Armonk, NY) was used for statistical analysis. The χ^2 and Fisher's exact tests were used to evaluate the relationships between LCN2 expression and clinicopathological factors. BCSS was used to assess the prognostic utility of LCN2 expression. The association with survival was evaluated using the Kaplan–Meier method and the log-rank test. Multivariate survival analyse was assessed using the Cox proportional hazards regression model. The median of the H-scores was used as a cutoff point to divide the samples into high- and low-LCN2 expression groups. A p-value <0.05 (two-tailed) denoted statistical significance.

RESULTS

Morphological characteristics of LCN2 protein expression

The morphological features of LCN2 expression using full-face tissue sections of invasive breast cancer are shown in Figure 1. LCN2 showed staining signals in both the cytoplasm and nucleus of malignant epithelial cells, with weak staining noted in the stroma. The expression of LCN2 in the nuclei of adjacent apparently normal terminal duct-lobular units was moderate to strong. In contrast, the LCN2 nuclear immunoreactivity of invasive cancer cells was lower than that of adjacent normal epithelial cells. The immunoreactivity of LCN2 in the cytoplasm of cancer cells was moderate compared with that reported in the nucleus.

In this study, 310 (51%) cases showed high cytoplasmic expression whereas 316 (52%) cases showed high nuclear expression. Regarding subcellular localisation, cases were classified into four combinatorial phenotypic groups as follows: (1) low nuclear and high cytoplasmic expression (21%); (2) high nuclear and low cytoplasmic expression (19%); (3) both with low expression (30%); and (4) both with high expression (29%) (Fig 2).

Clinicopathological characteristics of LCN2 protein expression

The low nuclear expression of LCN2 was significantly associated with high histological grade ($p < 0.0001$), high Nottingham Prognostic Index ($p = 0.00055$), ER negativity ($p < 0.0001$), PR negativity ($p = 0.0013$) and breast cancer subtypes ($p = 0.00034$) (Table 1). A total of 60% of HER2-positive and 65% of triple-negative cases showed a low nuclear expression of LCN2. Low nuclear expression was significantly associated with high Ki67 labelling index ($p < 0.0001$), high N-cadherin expression ($p = 0.00052$), high TWIST2 expression ($p = 0.039$), CK5/6 positivity ($p = 0.0088$) and EGFR positivity ($p = 0.019$). However, no correlation was identified between the nuclear expression of LCN2 and E-cadherin (Table 2). In addition, the low nuclear expression of LCN2 was associated with a shorter BCSS ($X^2 = 4.90$, $p = 0.027$) (Fig 3a).

The high cytoplasmic expression of LCN2 was significantly correlated with lymph node metastasis ($p = 0.039$; Table 1) and expression of E-cadherin ($p = 0.010$; Table 2). However, the cytoplasmic expression of LCN2 was not a significant predictor of outcome (Supplementary Fig 2).

Validation analysis for the clinicopathological significance of LCN2 nuclear expression

In the validation cohort, the low nuclear expression of LCN2 was significantly associated with high histological grade ($p < 0.0001$), high Nottingham Prognostic Index ($p < 0.0001$), ER negativity ($p < 0.0001$) and PR negativity ($p < 0.0001$). The downregulation of the nuclear expression of LCN2 was also significantly related to

large tumour size ($p = 0.00066$) and HER2 positivity ($p = 0.0042$; Supplementary Table 1). A total of 75% of triple-negative cases showed a low nuclear expression of LCN2 ($p < 0.0001$) (Supplementary Table 1).

The significance of the low nuclear expression of LCN2 as a poor prognostic marker was validated in 1,363 cases ($\chi^2 = 11.66$, $p = 0.00064$) (Fig 3b), although the low nuclear expression of LCN2 was not an independent prognostic marker in the multivariate analysis (Supplementary Table 2)

Combinatorial expression

Although no significant difference in the survival between these four groups, the tumours with low cytoplasmic and high nuclear expression showed the best outcome whereas the tumours with high cytoplasmic and low nuclear expression showed the worst outcome (Supplementary Fig 3).

DISCUSSION

EMT plays a key role in the metastasis of breast cancer [29]. Several previous studies have indicated the possible value of biomarkers associated with EMT as prognostic factors and have proposed their utility in predicting the response of invasive breast cancer to chemotherapy [30, 31]. In the present study, a low nuclear expression of LCN2 was significantly associated with a high expression of N-cadherin and TWIST2. N-cadherin, a member of the genetically related transmembrane glycoproteins, promotes tumour–stroma interactions and stimulates cell motility, invasion and metastasis [32, 33]. The overexpression of TWIST2 promotes metastasis of breast cancer by activating EMT and enhancing the self-renewal system of cancer stem-like cells [34]. Previous studies have suggested that LCN2 regulates the activity of the Ras pathway and regulates cell migration and EMT [35, 36]. Further functional studies are necessary to explore the association of aberrant LCN2 function with EMT in invasive breast cancer.

In the present study, the low nuclear expression of LCN2 was significantly associated with the positive expression of basal markers such as CK5/6 and EGFR. Several studies have suggested a strong correlation between the expression of basal cytokeratins and cell proliferation [37, 38]. EGFR is not a basal-like specific marker like CK5/6; however, it is expressed in the basal-like type and strongly assists in the immunohistochemical identification of this type [39, 40]. The Nottingham Prognostic Index+ is based on the assessment of the biological class combined with established clinicopathological prognostic variables, including CK5/6 and EGFR. This index provides improved stratification of patient outcomes for invasive breast cancer [41]. Recently, the use of molecular-targeted therapy against EGFR-positive breast cancer (e.g. neratinib [42],

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pertuzumab [43] and lapatinib [44]) has been proposed. The association of LCN2 expression with basal-like breast cancers may define a subset of patients more likely to benefit from treatment with these agents.

Several previous studies investigating the complex role of LCN2 in a variety of cancer types have suggested that LCN2 leads to apoptosis and suppresses proliferation/metastasis [8, 10, 45, 46]; however, a number of other studies have suggested that the expression of LCN2 promotes tumour growth, migration and invasion [47, 48]. The low nuclear expression of LCN2 has been significantly related to high tumour proliferation, hormonal receptor negativity, HER2 positivity and poor prognostic outcome. Notably, other studies have indicated that the high cytoplasmic expression of LCN2 is associated with the decreased disease-free survival in patients with invasive breast cancer [49, 50]. In the present study, the cytoplasmic expression of LCN2 was related to positivity for lymph node metastasis. The tumour microenvironment controls the LCN2-autocrine system of cancer cells via endoplasmic reticulum stress-dependent and -independent mechanisms [51]. Once released in the extracellular compartment, LCN2 drives iron sequestration and internalisation through established receptors, promoting cell survival and EMT [48, 51]. These mechanisms may be responsible for the clinicopathologically significant discrepancy observed between the nuclear and cytoplasmic expression of LCN2. Therefore, further investigations are warranted to examine the activity and functions of LCN2 based on its intracellular localisation.

CONCLUSIONS

Not only the expression but also subcellular localisation of LCN2 expression appears to play a role in breast cancer progression. Loss or reduced expression of nuclear LCN2 expression is related to the aggressive types and poor outcome in breast cancer. Further functional studies of LCN2 in breast cancer with consideration of its subcellular localisation in tumour cells are warranted.

Informed consent

Informed consent was obtained from all participants in the study.

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FIGURE LEGENDS

Fig. 1 Immunohistochemical analysis of the morphological characteristics of LCN2

(a) The normal terminal duct-lobular unit showed a high immunoreactivity of LCN2 in the nuclei (magnification: ×200). (b) The expression of LCN2 in the stromal cells was weak (magnification: ×100). (c) The nuclear immunoactivity of LCN2 in invasive breast cancer cells was weaker than that observed in normal epithelial cells. However, the cytoplasmic immunoreactivity of LCN2 in invasive breast cancer cells was moderate (magnification: ×200).

Fig. 2 Representative tissue microarray images of the expression of LCN2

(a) Low nuclear and low cytoplasmic expression of LCN2; (b) low nuclear and high cytoplasmic expression of LCN2; (c) high nuclear and low cytoplasmic expression of LCN2; and (d) high nuclear and high cytoplasmic expression of LCN2. Magnification: ×200 for all images.

Fig. 3 Breast cancer-specific survival stratified according to the nuclear expression of LCN2

(a) In the discovery cohort, breast cancer-specific survival was significantly worse in the LCN2-low expression group versus the LCN2-high expression group. (b) For the validation cohort, a significant difference was observed in the breast cancer-specific survival between LCN2-high and -low expression tumours.

Table 1 Associations between LCN2 nuclear/cytoplasmic expression and clinicopathological features

Factors	Nuclear expression			Cytoplasmic expression		
	High LCN2	Low LCN2	*p*-value	High LCN2	Low LCN2	*p*-value
	N (%)	N (%)		N (%)	N (%)	
Tumour size						
<2.0 cm	150 (52.3%)	137 (47.7%)	0.075	147 (51.2%)	140 (48.8%)	0.81
≥2.0 cm	146 (44.9%)	179 (55.1%)		163 (50.2%)	140 (48.8%)	
Nodal status						
Negative	181 (48.8%)	190 (51.2%)	0.80	175 (47.2%)	196 (52.8%)	0.039
Positive	115 (47.7%)	126 (52.3%)		135 (56.0%)	106 (44.0%)	
Nottingham Prognostic Index						
Good prognostic group	109 (60.2%)	72 (39.8%)	0.00055	89 (49.2%)	92 (50.8%)	0.21
Moderate prognostic group	149 (44.3%)	187 (55.7%)		165 (49.1%)	171 (50.9%)	
Poor prognostic group	38 (40.0%)	57 (60.0%)		56 (58.9%)	39 (41.1%)	
Histological grade						
Grades 1 and 2	189 (58.7%)	133 (41.3%)	<0.0001	156 (48.4%)	166 (51.6%)	0.26
Grade 3	107 (36.9%)	183 (63.1%)		154 (53.1%)	136 (46.9%)	
Lymphovascular invasion						
Negative	174 (50.9%)	168 (49.1%)	0.17	170 (49.7%)	172 (50.3%)	0.63
Positive	122 (45.2%)	148 (54.8%)		140 (51.9%)	130 (48.1%)	
Oestrogen receptor (ER)						
Negative	49 (33.6%)	97 (66.4%)	<0.0001	74 (50.7%)	72 (49.3%)	1.00

Positive	247 (53.0%)	219 (47.0%)	<0.0001	236 (50.6%)	230 (49.4%)	1.00
Progesterone receptor (PR)						
Negative	99 (40.2%)	147 (59.8%)	0.0013	116 (47.2%)	130 (52.8%)	0.16
Positive	197 (53.8%)	169 (46.2%)		194 (53.0%)	172 (47.0%)	
Human epidermal growth factor receptor 2 (HER2)						
Negative	260 (49.9%)	261 (50.1%)	0.070	267 (51.2%)	254 (48.8%)	0.50
Positive	36 (39.6%)	55 (60.4%)		43 (47.3%)	48 (52.7%)	
Breast cancer subtypes						
ER- and/or PR-positive/HER2-negative	223 (53.9%)	191 (46.1%)	0.00034	216 (52.2%)	198 (47.8%)	0.55
HER2-positive	36 (39.6%)	55 (60.4%)		43 (47.3%)	48 (52.7%)	
Triple negative	37 (34.6%)	70 (65.4%)		51 (47.7%)	56 (52.3%)	

Table 2 Association of LCN2 nuclear/cytoplasmic expression with the expression of breast cancer progression/metastasis-related biomarkers

Factors	Nuclear expression			Cytoplasmic expression		
	High LCN2	Low LCN2	p-value	High LCN2	Low LCN2	p-value
	N (%)	N (%)		N (%)	N (%)	
Ki67 labelling index						
Low (<10%)	110 (59.5%)	75 (40.5%)	<0.0001	93 (50.3%)	92 (49.7%)	0.93
High (≥10%)	114 (40.1%)	170 (59.9%)		145 (51.1%)	139 (48.9%)	
E-cadherin						
Low	150 (45.3%)	181 (54.7%)	0.099	151 (45.6%)	180 (54.4%)	0.010
High	135 (52.3%)	126 (47.7%)		149 (56.4%)	115 (43.6%)	
N-cadherin						
Low	83 (60.6%)	54 (39.4%)	0.00052	75 (54.7%)	62 (45.3%)	0.48
High	140 (42.7%)	188 (57.3%)		167 (50.9%)	161 (49.1%)	
P-cadherin						
Low	117 (43.7%)	151 (56.3%)	0.66	137 (51.1%)	131 (48.9%)	0.79
High	109 (45.8%)	129 (54.2%)		125 (52.5%)	113 (47.5%)	
TGF-β						
Low	92 (44.4%)	115 (55.6%)	1.00	100 (48.3%)	107 (51.7%)	0.21
High	134 (44.8%)	165 (55.2%)		162 (54.2%)	137 (45.8%)	
TWIST2						
Low	106 (58.6%)	75 (41.4%)	0.039	97 (53.6%)	84 (46.4%)	0.92
High	118 (48.2%)	127 (51.8%)		129 (52.7%)	116 (47.3%)	

CK5/6						
Negative	257 (51.1%)	246 (48.9%)	**0.0088**	256 (50.9%)	247 (49.1%)	**1.00**
Positive	37 (36.6%)	64 (63.4%)		51 (50.5%)	50 (49.5%)	
EGFR						
Negative	245 (51.0%)	235 (49.0%)	**0.019**	248 (51.7%)	232 (48.3%)	**0.61**
Positive	47 (38.8%)	74 (61.2%)		59 (48.8%)	62 (51.2%)	

Supplementary Table 2 Survival analysis based on clinicopathological characteristics of breast cancer, including LCN2 nuclear expression

Factors		Multivariate analysis		
		Hazard Ratio	**95% CI**	*p* value
LCN2 nuclear expression	Low	Reference		
	High	0.88	0.68-1.15	0.36
Tumour size	< 2cm	Reference		
	≥ 2cm	1.38	1.05-1.79	0.018
Nodal status	Negative	Reference		
	Positive	2.11	1.79-2.48	<0.0001
Histological grade	Low	Reference		
	High	1.91	1.51-2.41	<0.0001

Supplementary Table 1 Associations between LCN2 nuclear expression and clinicopathological features in the validation set

Factors	High LCN2 (nuclear)	Low LCN2 (nuclear)	*p*-value
	N (%)	N (%)	
Tumour size			
<2.0 cm	404 (49.9%)	406 (50.1%)	**0.00066**
≥2.0 cm	217 (40.3%)	321 (59.7%)	
Nodal status			
Negative	397 (48.2%)	427 (51.8%)	**0.052**
Positive	222 (42.6%)	299 (57.4%)	
Nottingham Prognostic Index			
Good prognostic group	266 (61.4%)	167 (38.6%)	**<0.0001**
Moderate prognostic group	283 (41.5%)	399 (58.5%)	
Poor prognostic group	70 (46.1%)	159 (69.4%)	
Histological grade			
Grades 1 and 2	427 (59.2%)	294 (40.8%)	**<0.0001**
Grade 3	194 (30.9%)	433 (69.1%)	
Lymphovascular invasion			
Negative	455 (47.7%)	498 (52.3%)	**0.063**
Positive	166 (42.0%)	229 (58.0%)	
Oestrogen receptor			
Negative	69 (24.4%)	214 (75.6%)	**<0.0001**
Positive	553 (51.8%)	514 (48.2%)	

Supplementary Table 2 Survival analysis based on clinicopathological characteristics of breast cancer, including LCN2 nuclear expression

Factors		Multivariate analysis		
		Hazard Ratio	95% CI	*p* value
LCN2 nuclear expression	Low	Reference		
	High	0.88	0.68-1.15	0.36
Tumour size	< 2cm	Reference		
	≥ 2cm	1.38	1.05-1.79	0.018
Nodal status	Negative	Reference		
	Positive	2.11	1.79-2.48	<0.0001
Histological grade	Low	Reference		
	High	1.91	1.51-2.41	<0.0001

	Progesterone receptor		
Negative	201 (35.7%)	362 (64.3%)	**<0.0001**
Positive	417 (53.6%)	361 (46.4%)	
	Human epidermal growth factor receptor 2		
Negative	555 (47.6%)	610 (52.4%)	**0.0042**
Positive	67 (36.2%)	118 (63.8%)	
	Breast cancer subtypes		
Triple negative	53 (25.5%)	155 (74.5%)	**<0.0001**
Non-triple negative	569 (49.8%)	573 (50.2%)	

Fig 3

a)

b)

c)

Fig 1

a)

b)

Fig 3

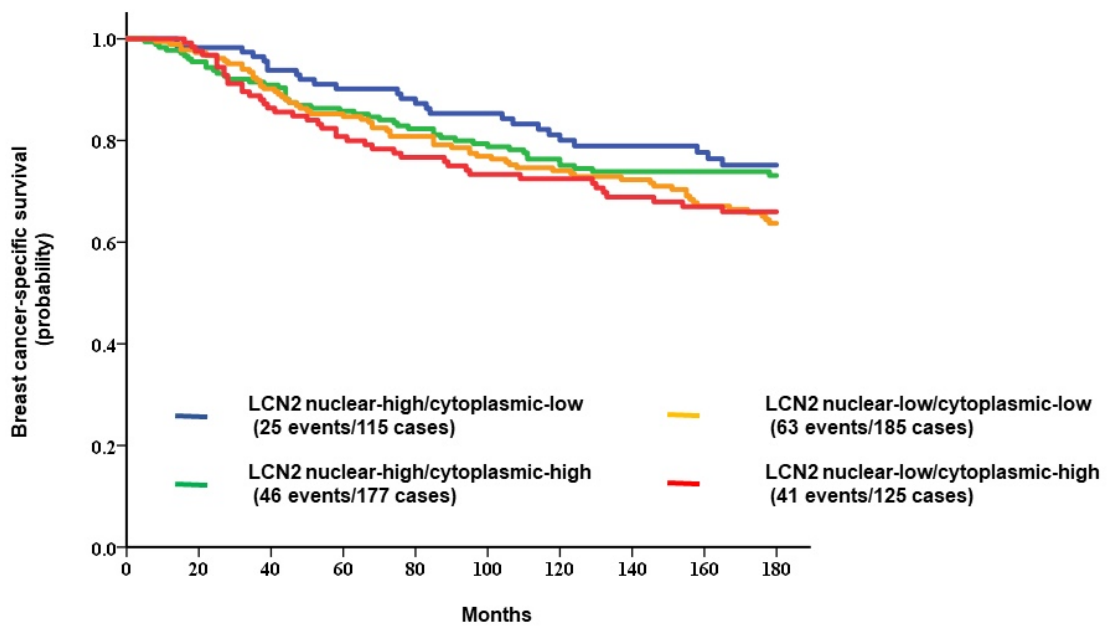a)

c)

b)

d)

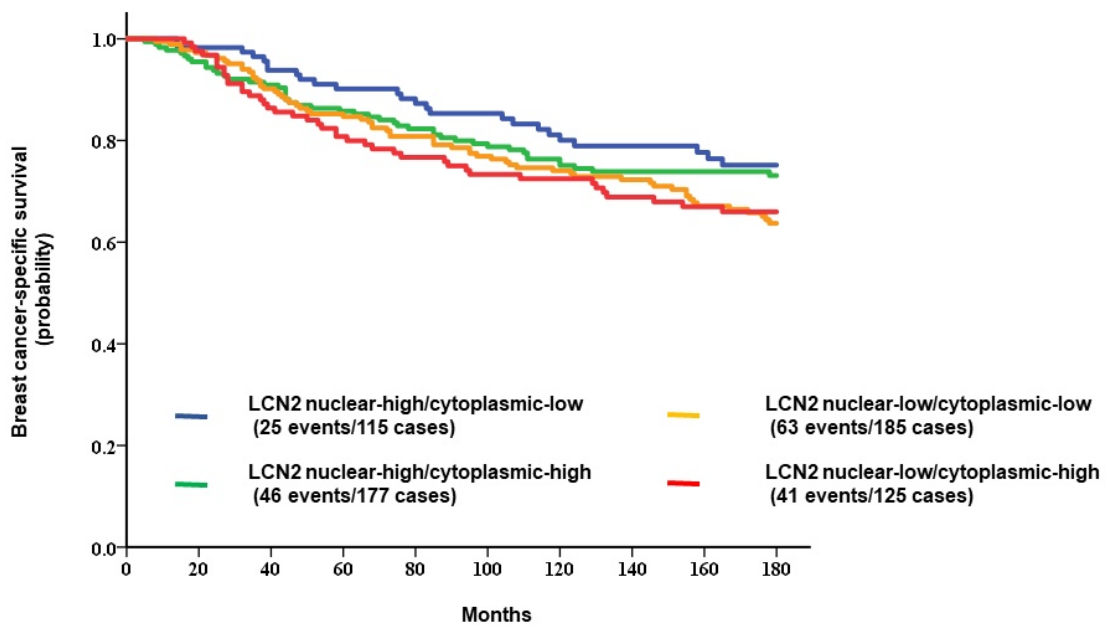Fig 2

HeLa

70 kDa

55 kDa

40 kDa

LCN2
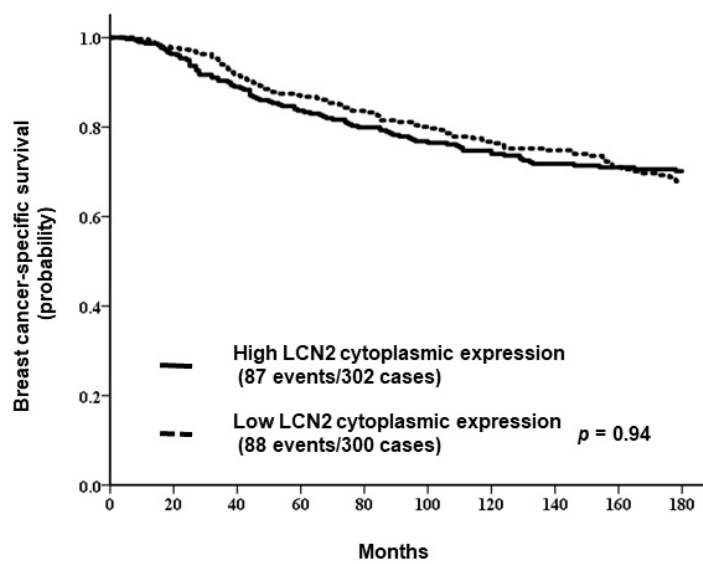
β-Actin
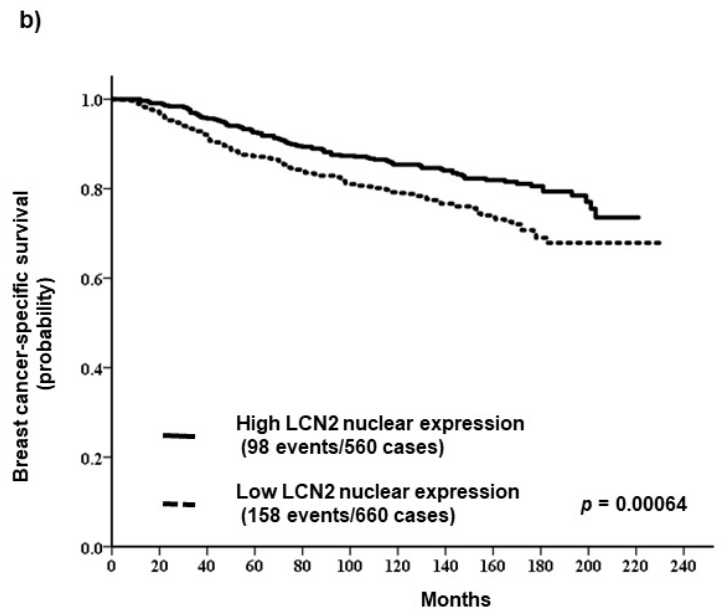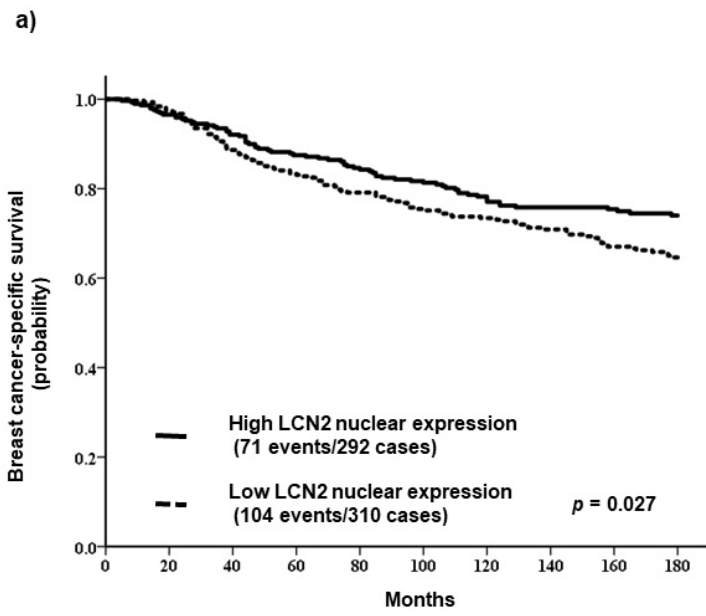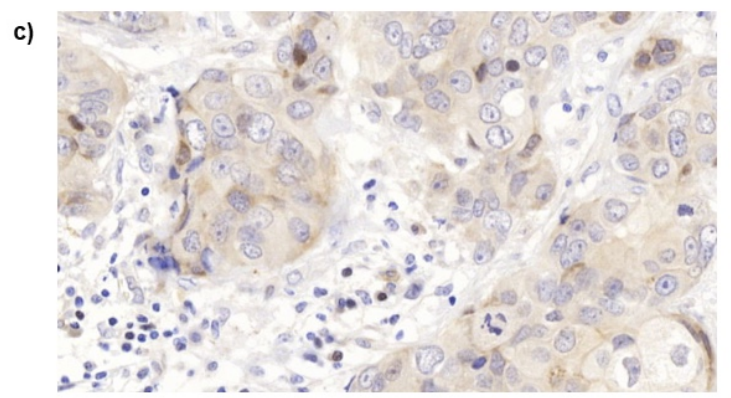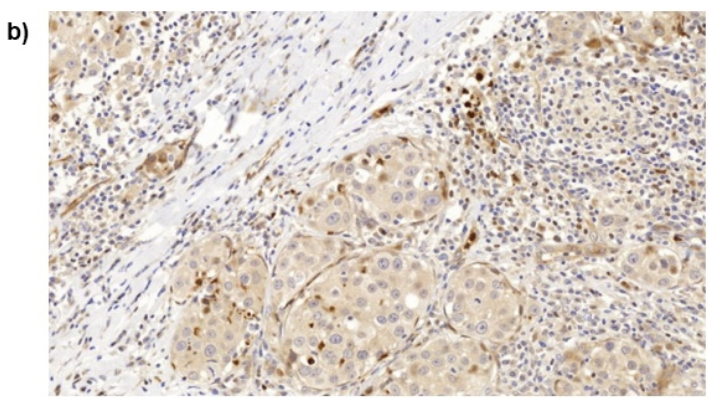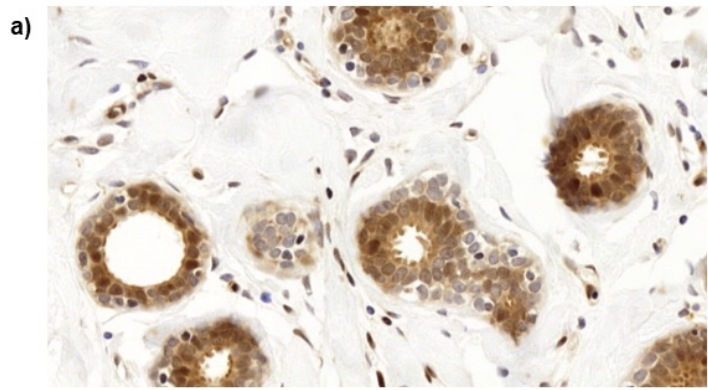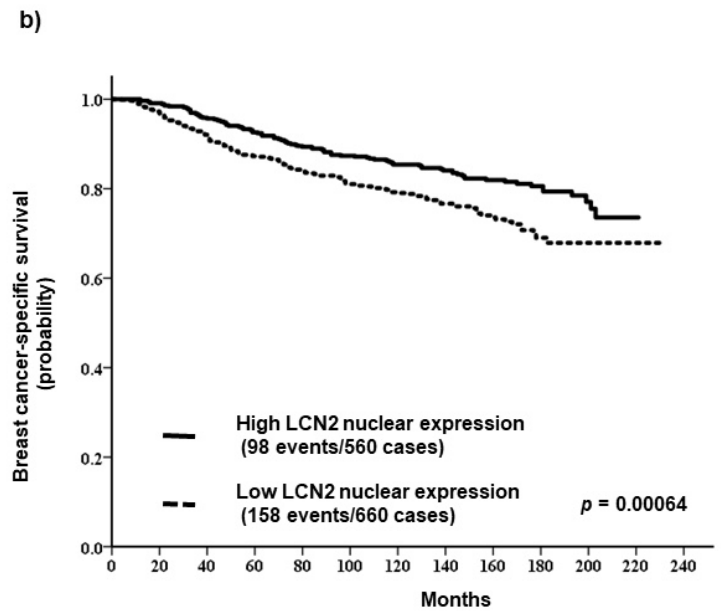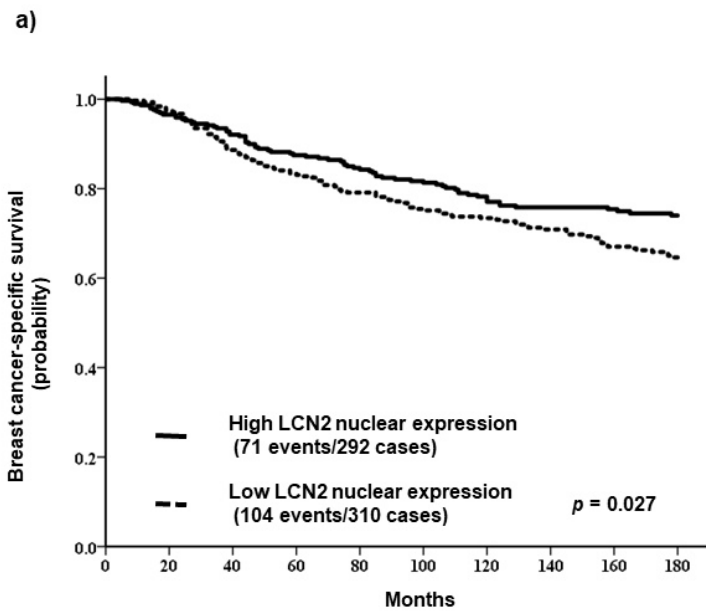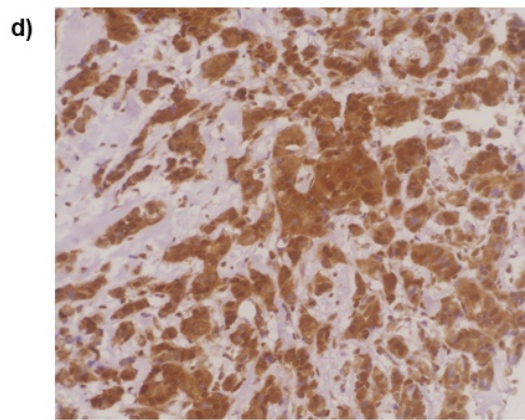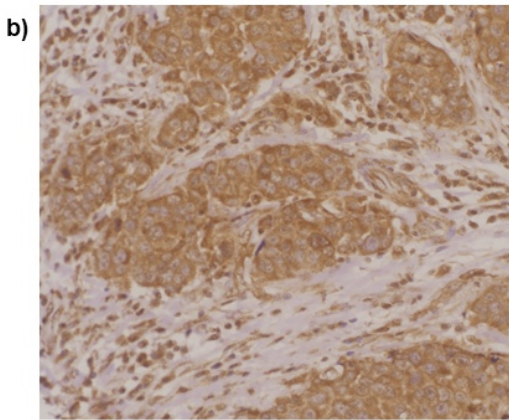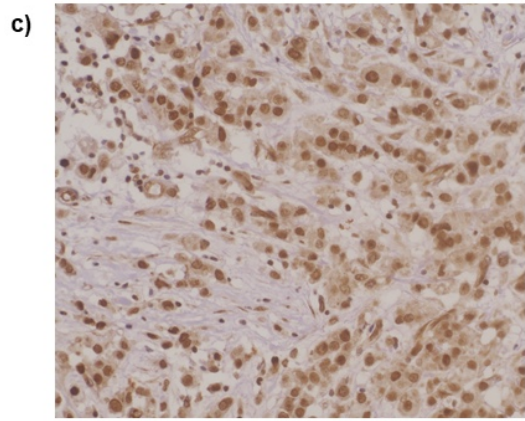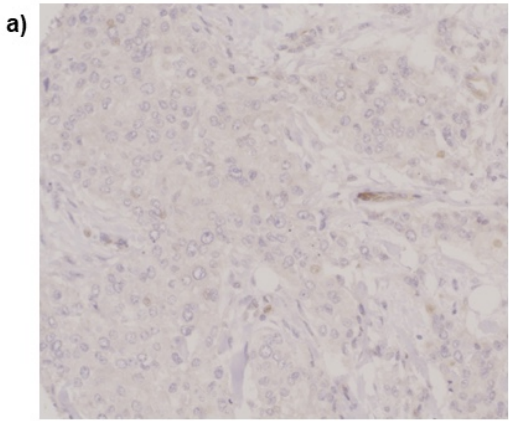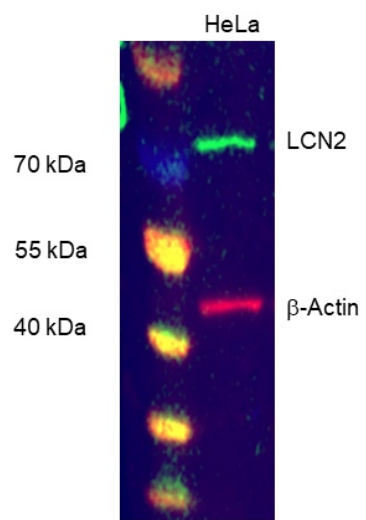
Supplementary Fig 1

LCN2 nuclear-high/cytoplasmic-low vs. LCN2 nuclear-high/cytoplasmic-high: $p = 0.48$
LCN2 nuclear-high/cytoplasmic-low vs. LCN2 nuclear-low/cytoplasmic-low: $p = 0.056$
LCN2 nuclear-high/cytoplasmic-low vs. LCN2 nuclear-low/cytoplasmic-high: $p = 0.079$

Supplementary Fig 3

LCN2 nuclear-high/cytoplasmic-low vs. LCN2 nuclear-high/cytoplasmic-high: p = 0.48
LCN2 nuclear-high/cytoplasmic-low vs. LCN2 nuclear-low/cytoplasmic-low: p = 0.056
LCN2 nuclear-high/cytoplasmic-low vs. LCN2 nuclear-low/cytoplasmic-high: p = 0.079

Supplementary Fig 3

Supplementary Fig 2