PRECLINICAL STUDY

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Expression of Lamin A/C in early-stage breast cancer and its prognostic value

I. M. Alhudiri[1,3] · C. C. Nolan[2] · I. O. Ellis[2] · A. Elzagheid[3] · E. A. Rakha[2] · Andrew R. Green[2] · C. J. Chapman[1]

Abstract

Purpose Lamins A/C, a major component of the nuclear lamina, play key roles in maintaining nuclear integrity, regulation of gene expression, cell proliferation and apoptosis. Reduced lamin A/C expression in cancer has been reported to be a sign of poor prognosis. However, its clinical significance in breast cancer remains to be defined. This study aimed to evaluate expression and prognostic significance of lamin A/C in early-stage breast cancer.

Methods Using immunohistochemical staining of tissue microarrays, expression of lamin A/C was evaluated in a large well-characterised series of early-stage operable breast cancer ($n = 938$) obtained from Nottingham Primary Breast Carcinoma Series. Association of lamin A/C expression with clinicopathological parameters and outcome was evaluated.

Results Positive expression rate of lamin A/C in breast cancer was 42.2% ($n = 398$). Reduced/loss of expression of lamin A/C was significantly associated with high histological grade ($p < 0.001$), larger tumour size ($p = 0.004$), poor Nottingham Prognostic Index score ($p < 0.001$), lymphovascular invasion ($p = 0.014$) and development of distant metastasis ($p = 0.027$). Survival analysis showed that reduced/loss of expression of lamin A/C was significantly associated with shorter breast cancer-specific survival ($p = 0.008$).

Conclusion This study suggests lamin A/C plays a role in breast cancer and loss of its expression is associated with variables of poor prognosis and shorter outcome.

Keywords Breast cancer · Lamin A/C · Immunohistochemistry · Tissue microarray · Prognosis

Introduction

Lamins, type V intermediate filament proteins, are the major components of the nuclear lamina [1] and are classified into A (lamin A and C) and B types. Lamin A/C are implicated in a multitude of functions within the cell [2] including binding to chromatin, maintaining nuclear integrity, apoptosis and regulating gene expression and cell proliferation. Lamin proteins are involved in regulating cell proliferation through

different mechanisms including oxidative stress and reactive oxygen species signalling pathways and modulation of phosphoinositide 3-kinase signalling pathway [3, 4]. A-type lamins also participate in many other cell functions through binding to a myriad of nuclear, signal transduction and gene regulatory proteins [5, 6].

Lamins are synthesised throughout the cell cycle and the lamina is depolymerised and re-polymerised during mitotic prophase and telophase, respectively [7, 8]. Cells deficient in LMNA gene exhibit enhanced proliferation, impaired cellular migration and nuclear orientation [9, 10]. Conversely, in vivo lamin A/C over-expression inhibits cell proliferation [11]. Mutations in the LMNA gene cause different degenerative disorders collectively called laminopathies which are associated with a wide range of heritable diseases including muscular dystrophies and premature ageing [12].

Nuclear changes are hallmarks of cancer and diagnosis of malignant transformation in pathological specimens depends on the presence of characteristic alterations in nuclear shape and heterochromatin and also used in evaluation of grade

✉ Andrew R. Green
andrew.green@nottingham.ac.uk

1 Division of Breast Surgery, The University of Nottingham, Nottingham, UK

2 Nottingham Breast Cancer Research Centre, Division of Cancer and Stem Cells, School of Medicine, Nottingham City Hospital, The University of Nottingham, Nottingham NG5 1PB, UK

3 Genetic Engineering Department, Biotechnology Research Centre, Tripoli, Libya

and hence prognosis in breast cancer [13, 14]. As lamins are thought to be a principal determinant of nuclear shape and architecture, it is hypothesised that they are responsible for the structural changes observed in cancer cells. Moreover, α-helical rod domain of A-type lamins bind to chromatin both directly and indirectly through other proteins, e.g. emerin and lamin-associated protein 2 (LAP2) [15–17].

Mechanisms underlying the role of lamin in cancer have been speculated and this has led to the conclusion that A-type lamins contribute to tumorigenesis and progression. A-type lamins in fibroblasts have important interactions with nuclear proteins, emerin and LAP2α, influencing the activity of oncogene β-catenin and retinoblastoma protein (pRb) growth regulators [18].

A-type lamins are not only expressed in terminally differentiated mammalian cells; but are also found in embryonic stem cells but at low amounts [4, 19]. Lamins are differentially expressed in normal and cancer tissues. For instance, both lamin A and C are highly expressed in basal and squamous cell carcinoma but lamin C is expressed in all layers of the normal epidermis while lamin A expression was absent in the normal basal layers [20]. In contrast, decreased expression of lamin A/C is seen in small cell lung cancer (SCLC) but not in non-SCLC in which it was also aberrantly localised to the cytoplasm [21, 22]. Moreover, reduced expression of lamin A/C has also been reported in adenocarcinoma of stomach, colon and oesophageal carcinoma [23]. Reduced or negative lamin A/C expression is associated with poor prognosis in a number of cancers including gastric carcinoma, lymphomas, lung, colon and breast cancer [22, 24–28].

Some studies of lamin A/C expression in breast cancer have also reported decreased expression [23, 27–30]. However, most of these studies included small sample sizes and with scarce comment of the clinical outcome.

To assess the prognostic effect of lamin A/C in breast cancer and association with different clinical parameters, we investigated its expression in a large and well-characterised series of early-stage breast carcinoma with a long-term follow-up and correlated this with clinicopathological variables and outcome.

Materials and methods

Patients and tissue specimens

A total of 938 cases of operable invasive breast carcinoma obtained from Nottingham Tenovus Primary Breast Carcinoma Series were investigated. This is a well-characterised series of primary breast carcinoma with long-term follow-up that were routinely assessed for tumour type, tumour size, histological grade, vascular invasion and Nottingham

Prognostic Index (NPI). The median follow-up time was 146 months (range 1–244). Information on local and regional recurrence, presence of distance metastasis, lymph node status, survival and therapy was collected on a prospective basis. The patients were treated in a uniform way and the series has previously been used to study a wide range of biomarkers including oestrogen receptor (ER), progesterone receptor (PgR), HER2, EGFR, p53 and E-cadherin [31]. Breast cancer-specific survival (BCSS) was defined as the length of time (in months) from the date of the primary surgery to the time of breast cancer-related death, and disease-free interval (DFI) was defined as the length of time (in months) from the date of the primary surgery to the first locoregional recurrence or distant metastasis. NPI is derived as follows: $NPI = 0.2 \times$ pathological tumour size (cm) + histological grade (1–3) + lymph node stage. The NPI divides patients into three sub-groups; good (≤ 3.4), moderate (3.41–5.4) and poor (> 5.4) prognostic groups [32]. Patients were managed according to their hormonal status and NPI score; those with an NPI score ≤ 3.4 received no adjuvant therapy, those with a NPI score > 3.4 received tamoxifen if ER positive ($\pm$ zoladex if pre-menopausal) or classical cyclophosphamide, methotrexate and 5-fluorouracil if ER negative and fit enough to tolerate chemotherapy [33]. Patient details and clinicopathological characteristics are listed in Table 1.

Ethical approval for this study was granted by Nottingham Research Ethics Committee 2 under the title of "Development of a molecular genetic classification of breast cancer".

Table 1 Clinicopathological criteria of study patients

Clinicopathological parameter	No. of patients (%)
Age ≤ 50 years	309 (32.9)
Age ≥ 50 years	629 (67)
Local recurrence	392 (41.8)
Distant metastasis	302 (32.2)
BCSS	
Lymph node negative cohort	123 (62.8)
Lymph node positive cohort	145 (28.6)
Tumour size < 1.5 cm	198 (21)
Tumour size > 1.5 cm	733 (78)
Definite vascular invasion	312 (33)
Received endocrine therapy	300 (32)
Received chemotherapy	178 (19)
Grade 1	140 (14.9)
Grade 2	269 (28.7)
Grade 3	522 (55.7)
NPI score	
Good prognosis	229 (24.4)
Moderate prognosis	503 (53.6)
Poor prognosis	197 (21)

Immunohistochemistry

Breast cancer tissue microarrays (TMAs) were prepared as previously described [33]. The monoclonal antibody to lamin A/C (Cell Signalling Mouse monoclonal antibody, #2032) was used for immunohistochemical detection. After deparaffinisation in xylene and rehydration through graded alcohol, sections were immersed in 0.1 M citrate buffer pH 6.0 and microwaved for 20 min in order to retrieve antigenicity. Once complete, slides were cooled and rinsed with Tris-buffered saline (TBS) pH 7.6. Endogenous peroxidase activity was inhibited using peroxidase block (Dako Real peroxidise blocking solution, S2023) for 5 min followed by TBS rinse. Sections were incubated with Ultra V block (Thermo Scientific TA-125-UB) to block non-specific staining by the primary antibody for 5 min at room temperature. The antibody was optimally diluted to 1:20 and incubated for 30 min at room temperature. After washing with TBS, all sections were incubated with a secondary antibody (dextran-coupled peroxidase molecules and goat anti-mouse/rabbit immunoglobulin; Dako REAL™ EnVision™/HRP, Rabbit/Mouse bottle A, K5007) for 30 min. Sections were washed in TBS and incubated with freshly prepared 3′3-diaminobenzidine (Dako Envision Kit, Bottle B and C, K5007) for 5 min and repeated once. After rinsing in TBS three times, sections were counterstained with haematoxylin (Dako Real Automation Haematoxylin, S3301) for 6 min. After washing in tap water, the sections were dehydrated in ethanol, cleared in xylene and mounted with DPX (BDH, Poole, UK). A formalin-fixed paraffin embedded tissue section from a primary ductal carcinoma was used as a positive control for lamin A/C staining.

Evaluation of immunohistochemical staining

Immunoreactivity of Lamin A/C in the TMA cores was evaluated semi-quantitatively by assessing both percentage of cells stained and intensity of staining. Nuclear staining of the TMA cores was measured using the modified histochemical score (H score) with a range from 0 to 300. H score was calculated as the sum of the percentage of cells with weak, moderate and strong staining. The staining intensity was scored as 0 (no staining), 1 (weak staining), 2 (moderate staining) and 3 (strong staining). Only staining of the invasive malignant cells within the tissue cores was considered. TMAs were manually assessed using high-resolution digital images (NanoZoomer; Hamamatsu Photonics, Welwyn Garden City, UK), at ×20 magnification, using a web-based interface (Distiller; Slidepath Ltd., Dublin, Ireland). All samples were scored by one observer (IA) and each core was scored twice and the average taken. The intraobserver agreement was good as suggested by Cohen's κ value of 0.80 and a 95% confidence interval between 0.93 and 0.68.

Statistical analysis

Statistical analysis was performed using SPSS v18 statistical software. χ^2 Analyses were used for correlations between Lamin A/C expression and clinicopathological parameters, steroid receptors and biomarkers. Possible correlation between Lamin A/C expression levels and BCSS and DFI was examined using Kaplan–Meier curves and the differences between the curves were analysed using the log-rank test. Cox regression models were used for multivariate analysis to test the effect of Lamin A/C expression and clinicopathological parameters on disease-free survival and BCSS as well as its statistical independence. A p value of < 0.05 was considered statistically significant.

Results

Immunohistochemical examination revealed positive staining of lamin A/C localised to the nuclear envelope of invasive breast cancer cells (Fig. 1). Stromal fibroblasts and vascular endothelial cells showed positive staining for nuclear lamin A/C which provided a positive control for expression. A total of 96% (900/938) of cases demonstrated varying degrees of nuclear staining to lamin A/C in the tumour cells, while 4% (38/938) showed complete absence of nuclear staining. Data were categorised into two groups according to frequency distributions and Kaplan–Meier curves of the effect on BCSS and cut-off was selected using X-tile software [34]. Low/absent nuclear expression was defined as H score ≤ 150 and high nuclear expression as H score > 150. High expression of lamin A/C was observed in 398/938 (42.3%) tumours while negative expression rate of lamin A/C was 540/938 (57.7%).
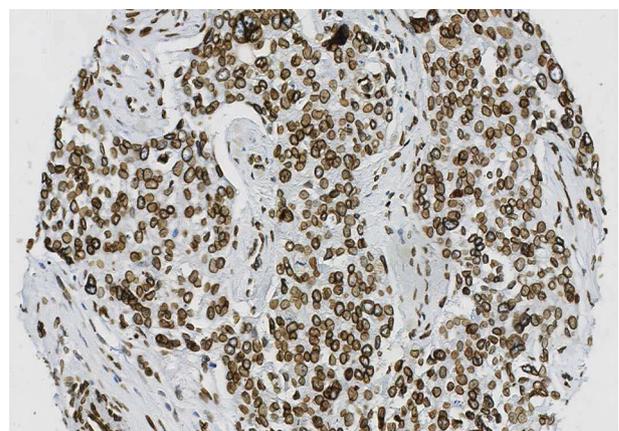

Fig. 1 Photograph showing positive lamin A/C nuclear staining in breast determined using immunohistochemistry. ×15 Magnification

Correlation of lamin A/C expression with clinicopathological parameters

Reduced/absent lamin A/C expression was associated with high mitotic frequency and nuclear pleomorphism ($p \leq 0.001$), high-grade tumours ($p < 0.001$), larger tumours ($p = 0.004$), vascular invasion ($p = 0.014$), poor NPI score ($p < 0.001$) and presence of distant metastases ($p = 0.027$; Tables 2, 3). Negative expression of lamin A/C was associated with invasive ductal/no special type and atypical medullary histological tumour types ($p = 0.002$). Lamin A/C expression was not associated with age at diagnosis, nodal stage or locoregional recurrence.

Association of lamin A/C with biomarkers expression

Relationships between lamin A/C and biomarkers are summarised in Table 4. Tumours with high expression for lamin A/C was significantly associated with ER ($p = 0.002$) and PgR positivity ($p = 0.001$). On the other hand, HER2 positive tumours had a negative association with lamin A/C expression ($p = 0.005$). Low lamin A/C immunoreactivity was further correlated with triple-negative status ($p = 0.026$). Low Lamin A/C expression also showed significant association with high p53 expression ($p = 0.001$). There was no correlation between lamin A/C with either E-cadherin or Ki67 expression.

Association of lamin A/C with patient outcome

Tumours with reduced/absent Lamin A/C expression showed significantly shorter BCSS compared with those showing high expression for the biomarker ($p = 0.008$; Fig. 2). On the other hand, low/absent expression of lamin A/C was associated with a trend for shorter DFI distant metastasis ($p = 0.057$) but there was no association with locoregional recurrence ($p = 0.360$; Figs. 3, 4). High Lamin A/C expression was significantly associated with BCSS in ER-positive ($p = 0.026$) but not ER-negative patients ($p = 0.442$). In multivariate analysis, expression of lamin A/C was not independent of tumour grade, size and stage for both BCSS and DFI (Table 5).

Discussion

Lamins have been implicated in a variety of diseases and cancers and reported as having prognostic significance. In breast cancer, a study of lamin A/C expression on 56 invasive ductal carcinoma in TMAs showed that lamin A/C immunostaining was completely absent in 21/56 (38%) of cases [29]. Our study showed that the majority of the breast

Table 2 Correlation of Lamin A/C expression with clinicopathological parameters

Parameters	Lamin A/C		
	Negative $n = 540$ (%)	Positive $n = 398$ (%)	p values
Grades			
1	60 (11.2)	81 (20)	**< 0.001**
2	134 (25)	137 (34.9)	
3	346 (64.3)	176 (44.8)	
Total	540	394	
LN stages			
1	311 (57.9)	245 (62.2)	0.354
2	173 (32.2)	118 (30)	
3	53 (10)	31 (7.9)	
Total	538	393	
Tumour size			
< 1.5 cm	98 (18.2)	100 (25.4)	**0.004**
≥ 1.5 cm	440 (81.8)	293 (74.6)	
Total	538	393	
Distant metastases			
No	352 (65.3)	278 (70.7)	0.027
Definite	187 (34.7)	115 (29.3)	
Total	539	393	
Nottingham Prognostic Index			
Good	107 (20)	122 (31)	**< 0.001**
Moderate	294 (54.7)	209 (53.3)	
Poor	136 (25.3)	61 (15.6)	
Total	537	392	
Tubules			
1	21 (4)	26 (6.9)	**0.001**
2	150 (28.5)	142 (37.6)	
3	355 (67.5)	210 (55.6)	
Total	526	378	
Pleomorphism			
1	3 (0.6)	8 (2.1)	**< 0.001**
2	154 (29.3)	157 (41.8)	
3	369 (70.2)	211 (56)	
Total	526	376	
Mitosis			
1	130 (24.7)	144 (38)	**< 0.001**
2	84 (16)	82 (21.7)	
3	312 (59.3)	152 (40.2)	
Total	526	378	
Vascular invasion			
Negative	337 (63)	279 (70.8)	**0.014**
Probable	197 (36.9)	115 (29.2)	
Total	534	394	

Bold values indicate statistcally significantly

cancer tissues demonstrated the expression of lamin A/C; however, strong nuclear staining of invasive breast carcinoma cells was also evident in some cancer tissues.

Table 3 Association of lamin A/C expression in breast cancer with histological type

Tumour types	Lamin A/C	
	Negative (%)	Positive (%)
Invasive ductal/no special type	367 (67.6)	215 (54.0)
Tubular mixed	72 (13.3)	79 (19.8)
Atypical medullary	21 (3.9)	5 (1.3)
Classical lobular	21 (3.9)	21 (5.3)
Lobular mixed	10 (1.9)	17 (4.3)
Mixed NST and lobular	15 (2.8)	16 (4)
Tubular	11 (2)	13 (3.3)
Mixed NST and a special type	6 (1.1)	7 (1.8)
Mucinous	4 (0.7)	2 (0.5)
Typical medullary	1 (0.2)	2 (0.5)
Solid lobular	1 (0.2)	1 (0.3)
Tubulolobular	0	2 (0.5)
Invasive papillary	2 (0.4)	1 (0.3)
Miscellaneous types	10 (1.9)	16 (4.0)
Total	540	398

$p = 0.002$

Table 4 The relationship between Lamin A/C expression, hormonal receptors and other tumour markers expression

Patients	Lamin A/C		
	Negative (%)	Positive (%)	p values
ER			
Negative	175 (33.3)	88 (22.8)	**0.002**
Positive	353 (67.0)	299 (77.0)	
Total	528	387	
PgR			
Negative	246 (47.2)	134 (35.4)	**0.001**
Positive	275 (53.0)	244 (64.5)	
Total	521	378	
HER2			
Negative	442 (83)	346 (89.5)	**0.005**
Positive	92 (17.0)	41 (10.5)	
Total	534	387	
Triple negative			
No	412 (76)	329 (82.5)	**0.026**
Yes	116 (21.5)	58 (15)	
Total	528	387	
ER–PgR status			
Both absent	166 (32.3)	78 (21)	**0.002**
ER absent	6 (1.16)	6 (1.6)	
Both positive	267 (52)	237 (64)	
ER only	74 (14.4)	50 (13.5)	
Total	513	370	
p53			
Negative	319 (67)	268 (77.2)	**0.001**
Positive	157 (33)	79 (22.8)	
Total	476	347	
Ki67			
Negative	139 (34.3)	121 (38.8)	0.218
Positive	266 (65.7)	191 (61.2)	
Total	405	312	
E-cadherin			
Negative	75 (15.7)	50 (14.4)	0.609
Positive	404 (84.3)	298 (85.6)	
Total	479	348	

Bold values indicate statistcally significantly

In addition, we observed a correlation between high lamin A/C expression and breast cancer clinicopathological parameters associated with a good prognosis. Therefore, breast tumours negative for lamin A/C expression were more likely to have an aggressive phenotype. Many poorly differentiated tumours from various sites have been described to show reduced lamin A/C expression [23, 25, 27], which supports our findings, whereas low lamin A/C was associated with the poor prognostic NPI group, HER2-positive and triple-negative tumours, vascular invasion, larger tumours and poor differentiation implying that measurement of lamin A/C could provide an additional prognostic information.

However, multivariate analysis showed that lamin A/C was not prognostically independent from tumour size, tumour grade and nodal stage. Two previous studies on lamin A/C in breast cancer demonstrated that patients with tumour cells down-regulating Lamin A/C had a poorer prognosis than those expressing the gene. However compared with our study, these studies had a much smaller sample size ($n = 115$ and 73), shorter follow-up time and few clinicopathological criteria [27, 28].

Studies of lamin A/C in primary gastric carcinoma has also shown similar results to our findings, suggesting that tumour cells with low lamin A/C expression had a poor prognosis and this was also an independent prognostic factor [25]. It has also been demonstrated that loss of lamin A/C expression correlated with decreased overall survival in nodal diffuse large B-cell lymphoma [35].

Different mechanisms has been proposed for loss of lamin A/C expression in cancer. LMNA gene silencing by

CpG island promoter hypermethylation has led to inactivation of the gene and loss of lamin A/C mRNA and protein expression in hematologic malignancies [35]. In normal cells, CpG islands are not subject to methylation at any stage of cell cycle and they permit the expression of that particular gene if the appropriate transcription factors are present and the chromatin is available to them. However, CpG islands of tumour suppressor genes, in tumour cells, become hypermethylated [36, 37]. Gene silencing caused by methylation is a common and early event in breast tumorigenesis [38–40]. Furthermore, methylation of the
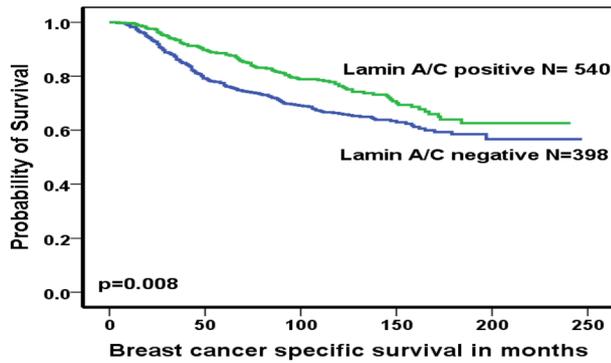
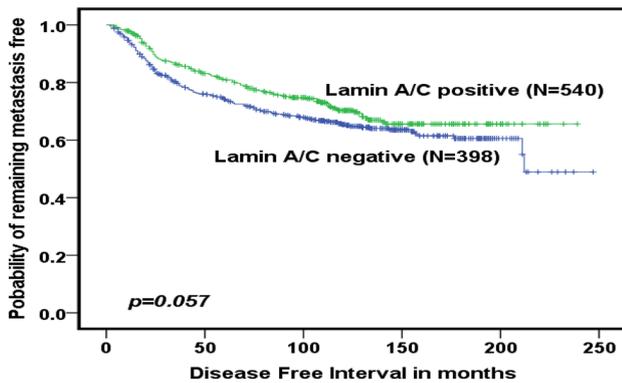**Fig. 2** Lamin A/C expression in relation to BCSS in breast tumours

Fig. 3 Lamin A/C expression in relation to DFI (metastasis) in breast tumours
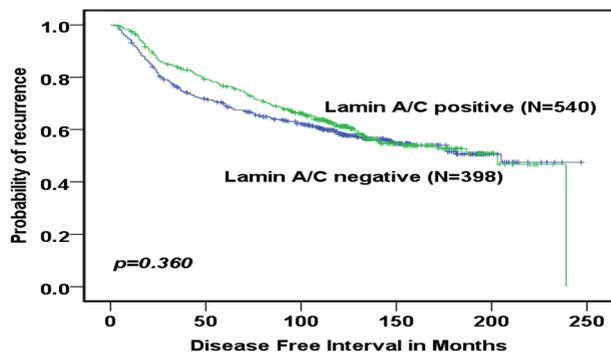

Fig. 4 Lamin A/C expression in relation to DFI (Locoregional Recurrence) in breast tumours

tumour suppressor genes in breast cancer is linked with poor prognostic indicators and hormonal receptor status [41–45]. Lamin A/C might thus serve as a tumour suppressor based on these findings [46]. There are reports on hypermethylation of the regulatory regions of many breast tumour-related genes such as p53, Cyclin D1, ER, PgR, E-cadherin, H-cadherin, Caspase-8, BRCA1, HOXA7, RASSF1 and HOXB13 further supporting the suggestion of lamin A/C hypermethylation [38, 47–49].

To determine whether lamin A/C expression was correlated with cellular proliferation, we analysed co-expression of lamin A/C and Ki67 where the majority of highly proliferative tumours showed low expression for lamin A/C; however, the association was not statistically significant. In a study of basal cell carcinoma, reduced expression of lamin A was shown in most hyperproliferative tumours expressing Ki67. It seems that decreased A-type lamin expression is inversely proportional to proliferation in cancer cells.

Lamins are required for growth of the nuclear envelope and for increase of nuclear volume during the cell cycle and progression into S phase is dependent on the possession of a certain nuclear volume [50]. In addition, the down-regulation of lamins leads to abnormal condensation of chromatin. This may explain the high association of lamin A/C negative expression with high-grade mitosis.

This study suggests lamin A/C expression as a potential prognostic biomarker for early operable invasive breast cancer. Our findings indicate that lamin A/C expressing tumours are associated with better BCSS and reduced lamin A/C expression is associated with more aggressive tumours. Further analysis of lamin A and C expression would be useful to investigate if there is differential expression and effect on clinical outcome comparing with normal breast samples. DNA methylation and gene-silencing studies of LMNA gene is interesting to study the role of lamin A/C in breast tumourigenesis.

Table 5 Cox proportional hazards analysis for predictors of breast cancer-specific survival (BCSS) and disease-free survival (model including lamin A/C expression)

Variables	Breast cancer-specific survival			Disease-free survival (recurrence)		
	Hazard ratios	p values	95% CI	Hazard ratios	p values	95% CI
Tumour size	2.153	< 0.001	1.408–3.290	1.475	0.008	1.105–1.967
Tumour stage	1.750	< 0.001	1.480–2.068	1.651	< 0.001	1.424–1.914
Tumour grade	1.965	< 0.001	1.584–2.437	1.271	0.002	1.092–1.479
Lamin A/C	0.876	0.304	0.680–1.128	0.988	0.905	0.803–1.214

Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of interests.

Ethical approval 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. This study was approved by the Nottingham Research Ethics Committee 2 under the title "Development of a molecular genetic classification of breast cancer".

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

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