Cell cycle marker expression in benign and malignant intraductal papillary lesions of the breast

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ABSTRACT

Aims The diagnosis of intraductal papillary lesions of the breast on core biopsy remains challenging in pathology, with most patients requiring formal surgical excision for a definitive diagnosis. The aim of this study was to determine whether a representative panel of proliferative cell cycle immunohistochemical markers (cyclin A2, cyclin B1 and cyclin D1) could improve the specificity of pathological diagnosis of these lesions.

Methods A series of 68 surgically excised intraductal papillary lesion cases were retrospectively selected, and immunohistochemistry for cyclin A2, cyclin B1 and cyclin D1 was performed.

Results Cyclin B1 (OR 1.80, 95% CI 1.01 to 3.2, p=0.046) and cyclin D1 (OR 1.13, 95% CI 1.05 to 1.22, p=0.002) expression was independently associated with a diagnosis of malignancy in papillary lesions, although expression was frequently heterogeneous and only focal. Cyclin A2 expression (OR 0.76, 95% CI 0.41 to 1.4, p=0.38) was not associated with a malignant diagnosis in multivariable logistic regression models. All three cyclins displayed high sensitivity (80%–95%) for a diagnosis of malignancy, although cyclin B1 showed a superior specificity of 72.7% compared with the low specificity of cyclins A2 and D1.

Conclusions Our study has identified for the first time that the expression of key cell cycle markers differs between benign and malignant papillary breast lesions and identified changes to the mitotic marker, cyclin B1, as particularly significant. However, given the low level and heterogeneous nature of expression of these markers, there remains a significant risk of undersampling in core biopsies and thus they are unlikely to be useful in routine clinical practice.

INTRODUCTION

Intraductal papillary lesions of the breast are characterised by fibrovascular cores lined by epithelial cells within a duct space and encompass a heterogeneous group of benign and malignant lesions for which a wide variety of terms have been used. The most recent WHO classification[1] defines intraductal papilloma as a benign lesion characterised by fibrovascular cores covered by an epithelial and myoepithelial cell layer. Intraductal papillary carcinoma (previously termed papillary ductal carcinoma in situ (DCIS)) is a malignant non-invasive neoplastic epithelial proliferation with papillary architecture occurring in the lumen of the ductal-lobular system.[1] Intraductal papillomas may also contain areas of atypical ductal hyperplasia (ADH) (previously termed atypical papillomas, defined as involvement by an atypical epithelial proliferation of <3 mm).[1] Encapsulated papillary carcinomas are a group of papillary tumours surrounded by a fibrous capsule that generally lack a demonstrable myoepithelial cell layer within and surrounding the lesion and are thus regarded as an indolent form of invasive papillary carcinoma, although the behaviour and prognosis of these lesions is very similar to DCIS.[1] Papillary lesions represent a challenging diagnostic problem in breast pathology on core biopsy and hence provide difficulties in subsequent patient management. The recommended treatment for papillomas with ADH and malignant papillary lesions diagnosed on core biopsy is surgical excision. However, it can be difficult to make a definitive benign diagnosis of intraductal papilloma on core biopsy primarily given the risk of sampling errors in intraductal papillomas that may contain foci of atypia or carcinoma.[2 3] Excision of all papillary lesions is standard management given this risk. Although controversial, it has also been suggested that when the histological diagnosis is benign, papillary lesions may be safely managed with imaging follow-up rather than with surgical excision.[4]

Distinguishing benign from malignant papillary lesions based on H&E morphology can be difficult especially where there is limited or fragmented material as in core biopsies. Pathmanathan *et al*[5] investigated the utility of a combination of histopathological features and immunohistochemistry (IHC) in improving the diagnosis of benign from malignant papillary breast lesions in a series of 127 lesions, concluding that a combination of broad sclerotic fibrovascular cores and epithelial cytokeratin (CK) 5/6 staining was most commonly seen in benign papillomas. However, others have found that broad fibrovascular cores may also be seen focally in atypical and malignant papillary lesions.[6] Other markers such as oestrogen receptor in combination with CK 5/6 and/ or CK 14 expression have been used and can assist with the differential diagnosis between benign and malignant papillary lesions on core biopsy in some instances to improve diagnostic accuracy, but do not eliminate false-negative results.[3] To date, there are limited reliable ancillary markers to assist with this clinically important distinction and there is a significant need to identify better markers.

During oncogenesis, cell cycle control mechanisms become deregulated and cell division is uncontrolled.[7] Cyclins are cell cycle regulator proteins that activate cell cycle progression by binding to cyclin-dependent kinases (CDKs). CDK deregulation is

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considered one of the key events in tumour cells gaining unrestrained growth capacity. In breast cancer cells, the cell cycle machinery can be disrupted at different stages throughout the cell cycle, often through the differential expression and regulation of cyclin proteins. It is likely that cell cycle regulation may also be perturbed in papillary lesions of the breast.

Cyclin D1-CDK4/6 complex is a crucial regulator of G1/S transition through pRb phosphorylation and titration of p21 and p27 levels.[7] The primary function of cyclin D1 is to integrate stimuli from extracellular mitogenic factors, such as tyrosine kinases and hormones, to drive cell cycle progression during the G1 phase.[8] Cyclin D1 overexpression has also been detected in ductal hyperplasia and DCIS, indicating that it may have a role in the evolution of early breast cancer.[9]

Cyclin A2 expression in cells rises in early S phase, is essential for DNA replication and is also involved in G2/M transition.[10] Much like Ki67 and proliferating cell nuclear antigen, cyclin A2 is an excellent marker of cell proliferation.[11] Increased cyclin A2 expression has been observed in several malignancies, including breast cancer,[12 13] where it has been reported to be associated with poor prognosis[14 15] although not all studies have confirmed this.[16]

Cyclin B1/CDK1 complex controls G2-M phase transition and is needed for initiation of mitosis.[17] Cyclin B1 expression levels are often high in breast cancer with increased expression in advanced malignant breast lesions compared with benign and premalignant lesions.[18]

To date, there are no comparative studies of the expression of a panel of cell cycle markers in intraductal papillary lesions of the breast. The aim of this study was to survey changes in expression of cell cycle regulators representative of different phases of the cell cycle (cyclin D1: G1/S phase, cyclin A2: S phase, cyclin B1: G2/M phase). As each protein is a well-characterised immunohistochemical marker in studies of breast cancer, we evaluated whether each could potentially improve the diagnosis of intraductal papillary lesions based on core biopsies with the potential of reducing unnecessary surgery in benign lesions with attendant improvements in efficiency and resource allocation.

METHODS

Patient samples

Patients diagnosed with intraductal papillary lesions were retrospectively and consecutively selected from the electronic report databases of Royal Prince Alfred and Concord Repatriation General Hospitals in Sydney Australia. Formalin fixed paraffin embedded tissue samples for a total of 86 cases were originally included in the study who had definitive treatment in the form of surgical excision in 2001–2011. All cases were reviewed by a specialist breast pathologist (SO or CC) and the diagnostic category confirmed using standard criteria.[1] Only six papillomas with ADH were present in the series, and these were excluded from analysis due to their low numbers precluding meaningful statistical analysis. Encapsulated papillary carcinomas (n=5), confirmed as lacking a myoepithelial layer by p63 IHC, were excluded as was the single case of invasive papillary carcinoma given the aim of the study was to investigate intraductal papillary lesions.

IHC for cyclin A2, B1 and D1

Three micron tissue sections were cut and deparaffinised for IHC. IHC was performed using a Dako autostainer and Envision detection system (Dako) for all antibodies. IHC for cyclin D1 was performed using rabbit polyclonal anti-cyclin D1 (Clone SP4, Thermo Scientific) at 1:100 dilution for 60 min. Antigen retrieval was performed with Dako buffer pH 6 (S1699, Dako) at 100°C for 20 min in a waterbath. Squamous epithelial basal cells within tonsil tissue were used as a positive tissue control with lymphoid cells within the tonsil tissue serving as a negative tissue control. IHC for cyclin B1 was performed using mouse monoclonal anti-cyclin B1 (Clone 7A9, Novocastra/Leica) at 1:40 dilution for 90 min. Antigen retrieval was performed with Dako buffer pH 9 (S2367, Dako) at 125°C for 2 min in a Pascal pressure cooker (Dako). Tonsil tissue was used as a positive tissue control and normal breast as a negative tissue control. IHC for cyclin A2 was performed using mouse monoclonal anti-cyclin A2 (Clone 6E6, Novocastra/Leica) at 1:100 dilution for 60 min. Antigen retrieval was performed with Dako buffer pH 6 (S1699, Dako) at 125°C for 2 min in a Pascal pressure cooker. Tonsil tissue was also used as a positive tissue control and normal breast as a negative tissue control. Matched host immunoglobulin G controls were also included as a negative control for each antibody.

IHC expression of each cyclin (A2, B1 and D1) was calculated as a percentage and independently scored by two breast specialist clinicians (SFL and CC) including a specialist breast pathologist (CC). Discrepancies in scoring were reviewed and a final score given in consensus conference. Heterogeneity was also assessed by a specialist breast pathologist (CC), who evaluated whether there was uniform distribution of positive nuclei throughout the lesion.

Statistical analysis

The percentage expression of cell cycle markers was compared between groups using Student t test. Marker expression was dichotomised to positive or negative to calculate sensitivity and specificity of each biomarker for the outcome of papillary DCIS. The clinical utility of each immunohistochemical marker as continuous scores was determined using logistic regression. Each marker was tested in a univariable model, then all three were included in a multivariable 'full' model, then a backward selection procedure was used to remove non-significant predictors, giving a 'final' model. A receiver operating characteristic (ROC) curve analysis was used to determine the power of the final model's predicted values to discriminate between benign and malignant cases, quantified by the area under the ROC curve analysis. Statistical analyses were performed using SPSS software V.22 (IBM SPSS Statistics) by a qualified statistician (EHB).

RESULTS

Of the 75 cases selected for inclusion in this study, the results of seven were unavailable due to inadequate lesion remaining in the sections on which cell cycle IHC was performed. Of the final 68 patients included in this study, there were 46 intraductal papillomas and 22 intraductal papillary carcinomas. Eleven papillomas had superimposed usual ductal hyperplasia.

The age and size of each of the two categories of intraductal papillary lesions are shown in table 1.

Nuclear staining for each cell cycle marker was evaluated in intraductal papillomas and intraductal papillary carcinoma cases (figure 1). The percentage of positive cases was found to be higher in intraductal papillary carcinoma (cyclin A2 median score 2% (IQR 1%–5%), B1 median score 1.5% (IQR 1%–3%) and D1 median score 20% (IQR 1%–40%)) compared with intraductal papillomas for all three cell cycle markers analysed (cyclin A2 median 1% (IQR 0%–2%), B1 median 0% (IQR 0%–1%) and D1 median 1% (range 0%–5%)) (figure 1). In addition, the percentage of cells positive for cyclin B1

Table 1 Characteristics of intraductal papillary lesions showing median values and IQR for age and size

Variable	Median (IQR) overall	Median (IQR) intraductal papilloma	Median (IQR) intraductal papillary carcinoma	p Value intraductal papilloma versus intraductal papillary carcinoma
Age	55 (45–66)	54 (45–63)	50 (43–67)	0.77
Size (mm)	11 (6–24)	7 (5–16)	24 (13.5–46.5)	<0.001

(p<0.001) and D1 (p<0.001) was found to be significantly higher in intraductal papillary carcinoma compared with intraductal papillomas.

All three markers were associated with malignancy in univariable models, but when included together cyclin A2 was no longer significant, possibly due to its high correlation with cyclin B1. In the final model, the odds of a diagnosis of malignancy increased by 80% (OR 1.80, 95% CI 1.01 to 3.2, p=0.046) for each unit of increase in cyclin B1 expression, and the odds of a diagnosis of malignancy in an intraductal papillary lesion rather than intraductal papilloma (table 2) increased by 13% (OR 1.13, 95% CI 1.01 to 1.22, p=0.002) for each unit of increase in cyclin D1 expression. Cyclin A2 was not a significant predictor of malignancy when included in a multivariable model with the other two markers.

The sensitivity and specificity of cell cycle markers were assessed (table 3). All three markers displayed high sensitivity (80%–95%); however, only cyclin B1 showed reasonable specificity of 73%. Cyclins A2 and D1 showed specificity of only 43% and 33%, respectively. An ROC curve was plotted for cyclin B1

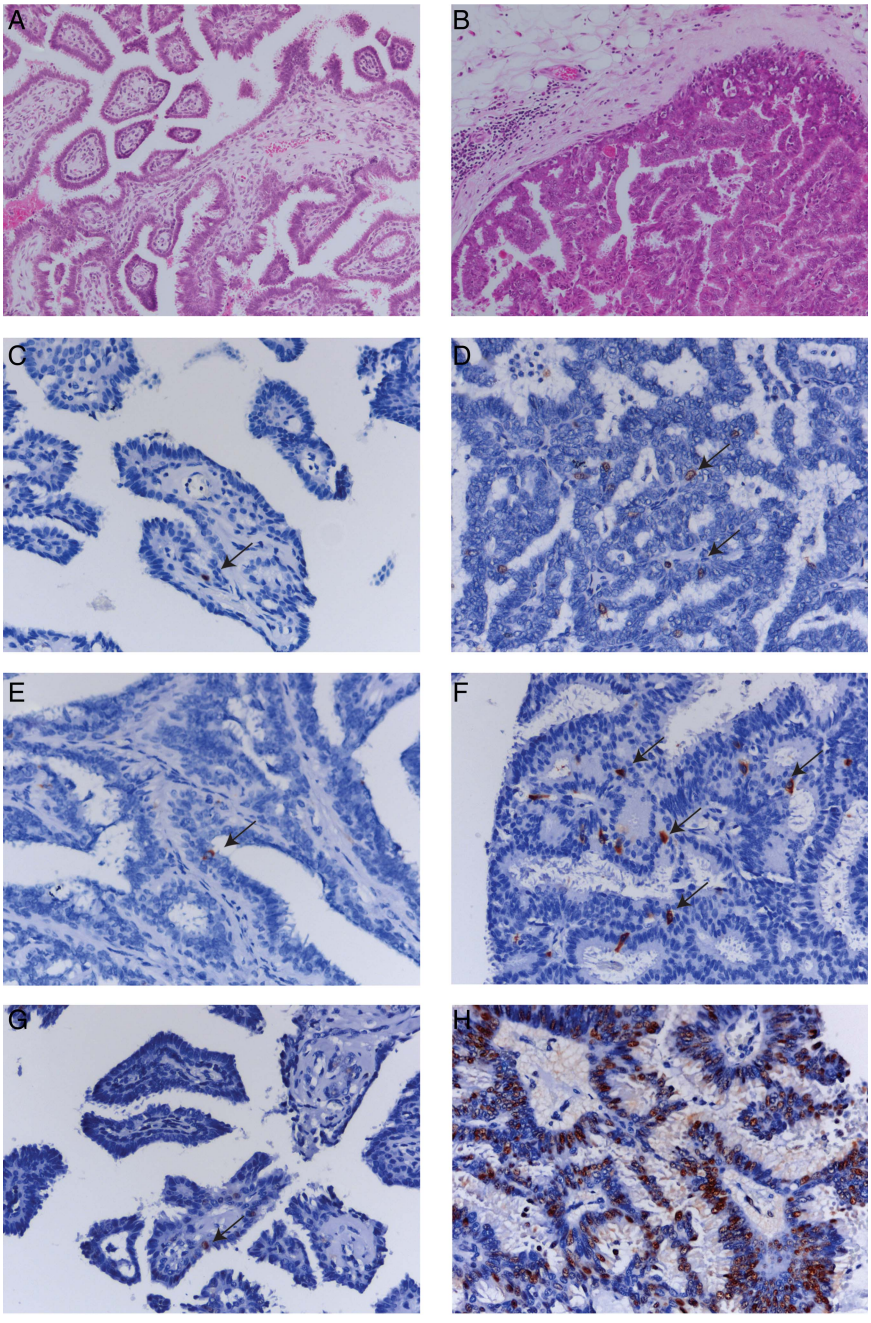

Figure 1 Papillary lesion cell cycle marker expression, representative H&E sections of intraductal papilloma (A) and intraductal papillary carcinoma (B), as well as matching immunohistochemistry staining for cell cycle markers: (C) cyclin A2 in an intraductal papilloma, (D) cyclin A2 in intraductal papillary carcinoma, (E) cyclin B1 in an intraductal papilloma, (F) cyclin B1 in the intraductal papillary carcinoma, (G) cyclin D1 in an intraductal and (H) cyclin D1 in intraductal papillary carcinoma. All images 100× magnification, C-H 200× magnification.

Table 2 Logistic regression of intraductal papillary carcinoma on cell cycle marker (percentage immunohistochemistry (IHC) expression)

Marker (% IHC expression)	Odds ratio (95% CI); p Value		
	Univariable models	Multivariable full model	Multivariable model (backward selection)
Cyclin A2	1.41 (1.07 to 1.86); 0.016	0.76 (0.41 to 1.4); 0.38	
Cyclin B1	2.27 (1.32 to 3.92); 0.0031	2.63 (0.92 to 7.52); 0.07	1.80 (1.01 to 3.2); 0.046
Cyclin D1	1.09 (1.04 to 1.15); <0.001	1.13 (1.05 to 1.23); 0.002	1.13 (1.05 to 1.22); 0.002

and cyclin D1 (figure 2), with the area under the curve calculated to be 0.87, indicating that the combination of cyclin B1 and D1 possesses strong discrimination for a malignant diagnosis.

All three cell cycle markers showed heterogeneity of expression (defined as non-uniform distribution of positive cells) within papillary lesions; 63% of all intraductal papillary lesions showed a heterogeneous distribution of cyclin A2, 77% of lesions showed a heterogeneous expression pattern of cyclin B1 and 87% of lesions showed a heterogeneous expression pattern of cyclin D1. No pattern of concordance of either heterogeneity or homogeneity was observed between markers for the same case.

DISCUSSION

This is the first study surveying the expression of a representative panel of cell cycle markers, cyclins A2, B1 and D1, in a cohort of intraductal papillary lesions of the breast. Due to the difficulties in differentiating benign from malignant lesions in core biopsies, better ways to improve histological diagnosis are required. This study investigated the utility of cell cycle immunohistochemical markers to improve the diagnosis of intraductal papillary lesions of the breast.

Despite significantly higher expression of cyclins A2, B1 and D1 in intraductal papillary carcinoma compared with intraductal papillomas, the overall utility of each cell cycle marker individually for predicting malignancy in papillary lesions was found to be insufficiently selective. This is because expression of each marker was heterogeneous and, other than cyclin B1, lacked specificity for malignancy. The combination of cyclin B1 and D1 showed the greatest overall specificity and sensitivity, but again the heterogeneous nature of expression means this combination is unlikely to be a useful test clinically.

Other immunohistochemical markers that have been used in previous studies include CK 5/6 and p63 (summarised in ref. 1). CK 5/6 can be helpful in differentiating benign papillomas from papillomas with ADH as lack of expression of high-molecular-weight CK is found in papillomas with ADH and intraductal papillary carcinoma.[1]

Table 3 Cell cycle marker expression in intraductal papillomas versus intraductal papillary carcinoma

		Papilloma	Intraductal papillary carcinoma	Sensitivity (%)	Specificity (%)
Cyclin A2	–	19 (43%)	1 (5%)	95	43.2
	+	25 (57%)	19 (95%)		
Cyclin B1	–	32 (73%)	4 (20%)	80	72.7
	+	12 (27%)	16 (80%)		
Cyclin D1	–	14 (33%)	3 (14%)	86.4	32.6
	+	29 (67%)	19 (86%)		

While our study has not identified a superior marker of intraductal papillary carcinoma, we have identified a general trend for an increase in proliferative markers in intraductal papillary carcinoma. However, it is notable that cyclin A2, which is normally an excellent marker of proliferation, was the least specific and sensitive of our cell cycle markers. This mirrors the data of Pathmanathan *et al*,[5] who found that there was heterogeneous staining of the proliferative marker Ki67 and that it was not useful in differentiating benign from malignant papillary lesions. Cyclin D1 did associate with a malignant diagnosis, which is consistent with a previous small study of 14 patients which found cyclin D1 expression was significantly higher in malignant papillary lesions compared with papillomas.[19]

Biologically our most interesting finding was that cyclin B1, the master regulator of transit through mitosis, was the most specific and sensitive marker of malignant intraductal papillary lesions. This suggests that mitotic control is particularly important in papillary lesions in contrast to other breast cancer subtypes where dysregulation of G1/S phase (often via cyclin D1) appears to be the most severe cell cycle lesion and correlated with poor prognosis.[20] Future studies of malignant intraductal papillary lesions should include other markers of mitotic dysfunction such as BubR1, Aurora kinase, Mad1/2 and Brca1/Brca2 to confirm that mitosis is disrupted and potentially identify improved markers, especially as these proteins are frequently dysregulated in cancer.[21]

In summary, our study has identified for the first time that the expression of key cell cycle markers differs between intraductal papilloma and intraductal papillary carcinoma and identified changes to the mitotic marker, cyclin B1, as particularly significant. However, given the low level and heterogeneous nature of expression of these markers, there remains a significant risk of undersampling in core biopsies and thus they are unlikely to be useful in routine clinical practice.

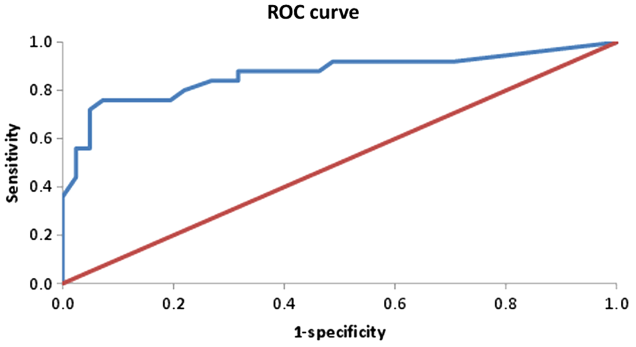

Figure 2 Receiver operating characteristic (ROC) curve from the multivariable logistic regression model containing cyclin B1 and cyclin D. Area under ROC curve (95% CI) 0.87 (0.77 to 0.97).

Take home messages

- Due to the difficulties in differentiating benign from malignant lesions in core biopsies, better ways to improve histological diagnosis are required.
- This study evaluates for the first time the expression and utility of the cell cycle immunohistochemical markers cyclin A2, B1 and D1 to improve the diagnosis of papillary lesions of the breast.
- The mitotic marker, cyclin B1, was the most specific and sensitive marker of intraductal papillary carcinoma providing potential biological insights into the pathogenesis of these lesions. However, given the low level and heterogeneous nature of cyclin B1 expression in papillary lesions, it is unlikely to be useful in routine clinical practice.

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Contributors SFL helped to design and carry out experimental studies and drafted the paper. CC helped to design the study, analysed the data and also drafted the paper. CIS helped to carry out experimental studies, analysed data and revised the paper. EHB analysed the data, performed statistical analysis and revised the paper. CC, HC, RW, LG and JMB helped with planning, contributing samples for analysis and revision of the paper. CEC analysed data and revised the paper. SO helped to design the study, analyse the data, write and revise the paper and is the guarantor.

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