This is the peer reviewed version of the following article:


which has been published in final form at [http://doi.org/10.1111/his.12948](http://doi.org/10.1111/his.12948)

This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.

This paper is made available in Western Sydney University [ResearchDirect](http://researchdirect.westernsydney.edu.au) in accordance with publisher policies.

Please cite the published version when available.

Access to the published version may require a subscription.
Increased expression of senescence markers p14ARF and p16INK4a in breast cancer is associated with increased risk of disease recurrence and poor survival outcome.

Running title: Senescence markers expression in breast cancer

Rahmawati Pare1, 2, Joo-Shik Shin1- 3, C. Soon Lee1-5*

1Discipline of Pathology, School of Medicine, Western Sydney University
2Ingham Institute for Applied Medical Research
3Department of Anatomical Pathology, Liverpool Hospital,
4Cancer Pathology, Bosch Institute, University of Sydney,
5South Western Sydney Clinical School, University of New South Wales, Sydney, Australia.

Address correspondence to: Professor C. Soon Lee, MBBS, MD, FRCPA, FRCPath (UK), MIAC, AFRACMA.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/his.12948
This article is protected by copyright. All rights reserved.
Abstract

Aims: Breast cancer is a hormonally driven disease. Cellular senescence is an age-related irreversible cell cycle arrest at the G1 phase upon induction. This study aimed to characterize the expression patterns of senescence markers p14<sup>ARF</sup>, p16<sup>INK4a</sup> and p21<sup>WAF1/Cip1</sup> during breast cancer progression in a large patient cohort.

Methods and results: We conducted a retrospective study of 1080 patients with invasive ductal carcinoma, no special type, over an 11 year period. We performed immunohistochemical staining on tissue microarrays that include normal, benign hyperplasia, ductal carcinoma in situ and invasive ductal carcinoma from each patient. Invasive ductal carcinomas demonstrated greater expression of p14<sup>ARF</sup> and p16<sup>INK4a</sup>, but lower expression of p21<sup>WAF1/Cip1</sup> when compared to non-malignant tissues. There was significant correlation between normal, benign, pre-invasive and malignant tissues with p14<sup>ARF</sup>, p16<sup>INK4a</sup> and p21<sup>WAF1/Cip1</sup> expression (p<0.05). Univariate comparison showed correlation between strong p16<sup>INK4a</sup> expression with poor survival (p=0.000) and increased risk of relapse (p=0.000), while high p14<sup>ARF</sup> expression correlated only with increased risk of relapse (p=0.038). Multivariate analysis showed p16<sup>INK4a</sup> as an important prognostic factor for overall survival (p=0.011) and disease free survival (p=0.004), with p14<sup>ARF</sup> also a significant prognostic factor for disease free survival (p=0.043).

This article is protected by copyright. All rights reserved.
Moreover, patients displaying both strong p16 and p14 expressions had an adjusted 3-fold increased risk of disease recurrence (p<0.05) and 2-fold increased risk of all-cause related death (p<0.05).

Conclusions: These finding suggest p16\textsuperscript{INK4a} and p14\textsuperscript{ARF} expression may play an important role in the progression of proliferative breast tissue to invasive cancer, and may be useful as prognostic factors.

Keywords: Breast cancer, Cellular senescence, p16\textsuperscript{INK4a} and p14\textsuperscript{ARF}

INTRODUCTION

Breast cancer is a heterogenous disease that can follow the evasion of various cell cycle checkpoints. Deregulation of the latter is a common pathway in the development of many human cancers, in which these normally fail-safe mechanisms are interrupted, resulting in genetic instability compounded by cell cycle progression\textsuperscript{(1)}.

Cellular senescence is an age-related irreversible cell cycle arrest at the G1 phase upon intrinsic or extrinsic induction. It is one of the critical responses for sustaining cellular integrity by irreversibly desisting to replicate after exposure to influences that promote the perpetuation of cell division and to environmental stress. Senescence subtypes can be categorised as replicative or oncogene-induced\textsuperscript{(2)}. Replicative senescence is triggered by the progressive telomere shortening to a critical length with cell divisions, while oncogene-induced senescence (OIS) is promoted by the activated RAS oncogene, DNA damage or other cellular stresses. OIS can be induced or maintained through two well established pathways: p16\textsuperscript{INK4a}–Rb and ARF-p53-p21\textsuperscript{(3)}.

This article is protected by copyright. All rights reserved.
In this study, our interest is centered on the senescence markers involved in these Rb and p53 pathways, specifically p16$^{\text{INK4a}}$, p14$^{\text{ARF}}$ and p21. p16$^{\text{INK4a}}$ and p14$^{\text{ARF}}$ are encoded by the same INK4A/ARF gene locus. They share the same exons which only differ on the alternative reading frame. p16$^{\text{INK4a}}$ and p14$^{\text{ARF}}$ are interdependent and both are often altered in primary breast carcinoma (4). p21 protein is a product of WAF1/CIP1/SDI1 gene on chromosome 6p21. p21 can be activated via p53 dependent or independent pathways (5). The activation of p53 leads to transcriptional activation of p21 and blocks cyclin A, resulting in cell cycle arrest in G1 phase. Others have previously shown that p14$^{\text{ARF}}$, p16$^{\text{INK4a}}$ and p21 expression is interrupted in various tumours, including breast carcinoma (6-8). However, those studies only involve small cohorts or cell lines. Thus, there is a need for a large cohort study of primary breast cancers to ascertain the prognostic value of these biomarkers.

Senescence-associated markers are found to play an important role in the development of a wide range of human cancers. Recent studies have shown that expression of senescence-associated markers is increased in premalignant but lost in malignant lesions (9). Our study aims to compare the expression profiles of senescence markers p14$^{\text{ARF}}$, p16$^{\text{INK4a}}$ and p21 at different morphological stages of development of invasive breast carcinoma, and to determine their prognostic significance. We hypothesize senescence markers are expressed in ductal hyperplasia and carcinoma in situ (DCIS), but lost in malignant tumours. Such knowledge may be useful in the clinical management of breast cancer patients and may allow for better stratification for various treatment options.

This article is protected by copyright. All rights reserved.
MATERIALS AND METHODS

Patients

This retrospective study involves a cohort of female breast cancer patients diagnosed between 2000 to 2011 from the Department of Anatomical Pathology, South Western Sydney Area Pathology Service, Liverpool Hospital (New South Wales, Australia), which covers 6 regions in the South Western area of Sydney. The cohort consists of 1080 patients who were diagnosed with invasive ductal carcinoma (IDC) of the breast. Women who had pre-operative chemotherapy or recurrent disease at the time of diagnosis were excluded. This study was approved by the Human Research Ethics Committee of the South Western Sydney Local Health District Ethics and Research Governance Office (HREC/12/LPOOL/158). This study operated in accordance with the National Health and Medical Research Council’s National Statement on Ethical Conduct in Research involving Humans and the CPMPACH Note for Guidance on Good Clinical Practice. Patients’ demographic, clinicopathological and follow up information were obtained from the electronic medical records that include the pathology and medical oncology databases (Table 1). For all patients, the following data were known: age at diagnosis, tumour size, tumour grade, staging, presence of lymphovascular invasion (LVI) and lymph node involvement (LNI). The end of follow-up period was at 30 June 2014 with median follow-up overall survival of 4.96 years (0.15-14.86 years) and disease-free survival of 4.76 years (0.11-13.84 years).

Tissue Microarray Construction

Resected tumour tissues at surgery were fixed in 10% buffered formalin, prior to routine histopathology processing resulting in paraffin embedded tissue blocks for microscopic examination and storage. Using the light microscope, representative areas of interest were
marked on H&E stained sections of each of these archived donor blocks to assist in sampling the appropriate areas for incorporation into the tissue microarray recipient block. The latter included duplicate cores of normal, atypical ductal hyperplasia, DCIS and IDC tissues per patient using the 1.0mm punch. Concurrent atypical ductal hyperplasia and DCIS were present in 51.2% and 72.0% of the cases respectively, which were included in the tissue array along with the corresponding normal and malignant tumour tissues. Sections were cut from the arrays at 4-5µm thickness and mounted on Superfrost ultra plus® glass slides.

**Immunohistochemistry**

Tissue microarray sections were subjected to immunohistochemistry staining for protein expression assessment. Briefly, the sections were dried in the oven at 60°C for 1 hour. The array sections were deparaffinised in xylene three times and rehydrated through graded, decreasing concentrations of alcohol. The sections were then introduced into water prior to incubation in the pre-heated 98°C antigen retrieval buffer in hot water bath. The sections were immersed in citrate buffer pH6 (p14) Or Tris/EDTA pH9 (p21 and p16) for 45 minutes. Endogenous peroxidase was blocked with hydrogen peroxidase for 20 minutes before antibody incubation. Slides were incubated with primary antibody against p14^{ARF} (1:50 dilution for 1 hour at room temperature; Sigma-Aldrich, Saint Louis, USA), p21 (1:25 dilution for 45 minutes at room temperature; Dako, CA, USA) and p16^{INK4a} (1:4 dilution; CINTEC). Dako Mouse linker (Envision™ FLEX +Mouse Linker) was then added to the primary mouse antibody for 30 minutes to amplify the antigen signal. Goat secondary antibody was added for 15 minutes to detect any primary antibody-antigen complex, to which high sensitivity 3, 3’-diaminobenzidine tetrahydrochloride was then added, followed by hematoxylin counterstaining and mounted.
Scoring of immunohistochemically stained sections

Expressions of senescence-associated biomarkers were assessed semi-quantitatively by two independent pathologists who were blinded to the patients’ details. The scorers underwent a period of training with a multiheader microscope to ensure consistent and reliable interpretation. Using a test series of at least 36 tissue core sections, intra- and inter-observer agreement were estimated using Kappa (κ) and Spearman rho (ρ). Training was ended when the desired level of agreement, consistent over time, was achieved (κ>0.6 and ρ>0.8). An average score was obtained from the duplicate cores of each tissue sample. Immunoreactivity in the nucleus and cytoplasm were individually graded. Intensity and distribution of the immunoreactivity were recorded. Scoring methods for p14ARF was performed as prescribed previously:(10) intensity score 0 (no staining), 1 (weak staining), 2 (moderate staining) or 3 (strong staining) were added to distribution score 0 (no staining), 1 (<1%), 2 (1-10%), 3 (11-33%), 4 (34-66%) or 5 (67-100%) resulting in a final score ranging from 0-8. Scores of ≥ 3 were considered increased expression. p16INK4a was scored based on the study by Milde-Langosch et al:(8) intensity score (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining) was multiplied with distribution score (0 = no staining, 1 = <10%, 2 = 10-50%, 3 = 51-80%, 4 = >80%). Final score was categorized into 0–4: weakly positive, 5–6: moderately positive, 8–12: strongly positive. Immunoreactivity for p21 was calculated based on the percentage of cells with nuclear staining, in which >5% was considered as positive(11).

Statistical analyses

Statistical analyses were performed using the IBM SPSS Statistics 21.0 (© Copyright IBM Corporation 1989, 2012) programme. The parameters were dichotomised where possible to assist
the analyses. P values of <0.05 were regarded as statistically significant. Associations between expression of each of the proteins and clinicopathological data were examined using Pearson chi-square test. Kaplan-Meier survival analysis was conducted to estimate the likelihood of survival at a specific point in time for the patient groups. Log-rank test was used to compare the subgroups. Univariate and multivariable analyses were performed using the Cox proportional hazard regression test. The assumption of time-independent effects was not violated. The clinical endpoints for overall survival (OS) defined as time from date of surgery until death by any cause and disease-free survival (DFS) defined as time from date of surgery until any recurrence (locoregional and/or distant, whichever came first), were calculated based on the documented dates of death, recurrence or last follow up. Patients who were still alive (for OS) or had no disease recurrence (for DFS) at last follow up were censored.

RESULTS

Patient demographics and tumour characteristics

All 1080 female patients were newly diagnosed cases with age ranging from 27 to 102 years (median 59). 72.0% of the cases had concurrent DCIS and 51.2% had accompanying ductal hyperplasia. 2.5% of the cases had inadequate amount of normal breast tissue for examination, due to age related atrophy. The mean tumour size was 21.5 ± 13.9 mm (mean ± SD), with median of 18.0mm and which ranged from 1 to 112 mm. The majority of tumours were ER and PR positive and HER2 negative, which accounted for 73.6%, 64.2% and 51.9% of the cases respectively. 37.7% of the cases had missing HER2 status, as these cases were from the earlier years in the cohort (2000 to 2005) when this marker not routinely assessed in breast carcinomas.
Expression of senescence markers

Breast cancers showed loss of nuclear expression of p21. Cases with negative p21 expression in the normal breast tissue were more likely to remain negative in the subsequent proliferative and malignant tissues. Overall however, p21 expression appeared to increase from normal to cancer tissues, albeit in each tissue type the percentage of positive cells were small. The distribution of p21 staining amongst tumour cells was heterogeneous, with expression often appearing in clusters. Only 5.2% (score range 5-60%) of invasive tumours had positive immunoreactivity in the nucleus (Table 2).

$p16^{INK4a}$ expression was predominantly seen in the nucleus than in the cytoplasm. Malignant tumours with strong immunoreactivity showed uniform staining throughout all tumour cells, whereas tumours with weak and moderate $p16^{INK4a}$ staining often showed a heterogeneous staining pattern (Figure 1). The number of positive cells and their staining intensity increased with progression from benign to neoplastic tissues. Invasive tumours showed higher $p16^{INK4a}$ expression compared to non-malignant tissues (p<0.05, Table 2).

Interestingly, our study showed that $p14^{ARF}$ expression was primarily in the cytoplasm rather than in the nucleus (Figure 1). Homogenous staining was seen in the cytoplasm of each tissue types. The differences in $p14^{ARF}$ expression were statistically significant between each tissue types. Of interest, normal tissue showing $p14^{ARF}$ positivity was more likely to be associated with increased $p14^{ARF}$ expression in the matching malignant tissue (p<0.001, Table 2).

Association with clinicopathological parameters

Amongst the various clinicopathological variables, tumour differentiation was the only variable that correlated with all three biomarkers. Poor tumour differentiation correlated significantly
with overexpression of \( p14^{\text{ARF}} \) and \( p16^{\text{INK4a}} \) \((p<0.05 \text{ and } p<0.0001 \text{ respectively})\), while moderate differentiation correlated with loss of \( p21 \) expression \((p<0.0001)\) (Table 3). In addition, estrogen receptor positivity in invasive tumour correlated with \( p14^{\text{ARF}} \) expression \((p<0.05)\), while both estrogen receptor and progesterone receptor negativity correlated with strong \( p16^{\text{INK4a}} \) expression \((\text{both } p<0.0001)\). There were no significant associations between the expression of three markers and other clinicopathological parameters, including age, tumour size, staging, lymphovascular invasion, lymph node involvement and HER2 status (Table 3).

**Survival analyses**

OS and DFS were determined in 1033 and 1023 patients respectively, with a median follow-up 5 years (0.15-14.86 years). The estimated mean OS period for the entire cohort was 12.2 years (95% CI 11.8-12.6) with a total of 151 (14.6%) deaths and 880 (85.4%) still alive at the end of the follow up period. The 1 year survival rate was 98.5%, 2 year survival rate 96.5%, 3 year survival rate 93.1% and 5 year survival rate was 87.0%. Disease relapses occurred in 121 women, and of these, death followed in 62 patients with mean duration of 5.2 years.

Kaplan-Meier log rank test indicated significant association between strong \( p16^{\text{INK4a}} \) expression and poor survival \((\text{Log rank test, } p=0.000)\) and increased risk of recurrence \((\text{Log rank test, } p=0.000)\) (Figure 3). However, \( p14^{\text{ARF}} \) positivity correlated only with increased risk of recurrence \((\text{Log rank test, } P=0.038)\) (Figure 2). On the other hand, \( p21 \) expression showed no significant associations with survival outcomes.

Univariate analysis showed that patients with cancers displaying strong \( p16^{\text{INK4a}} \) expression were more likely to die of any cause \((p=0.000, \text{ HR } 2.498, 95\% \text{ CI } 1.739, 3.588)\) and to have disease recurrence \((p=0.000, \text{ HR } 2.943, 95\% \text{ CI } 1.970, 4.396)\) (Table 4) than those with low or absent \( p16 \) expression. Following adjustment for age, tumour size, tumour grade, TNM...
staging, lymphovascular invasion, hormone and HER2 receptor status, patients with strongly positive p16 cancers had a 2.5-fold increased risk of death (\(p=0.011\), HR 2.403, 95% CI 1.222, 4.724) and more than 3-fold increased risk of disease recurrence, compared to those with p16 negative cancers (\(p=0.004\), HR 3.107, 95% CI 1.434, 6.724).

On univariate analysis, p14\(^{ARF}\) expression did not show any influence on the prediction of overall patient survival, but patients with p14\(^{ARF}\) positive cancers were more likely to have disease recurrence (\(p=0.043\), HR 2.942, 95% CI 1.033 - 8.374). After adjustment for age, tumour size, tumour grade, TNM staging, lymphovascular invasion, hormone and HER2 receptor status, patients with strongly p14\(^{ARF}\) positive cancers had more than 3-fold increased risk of disease recurrence, compared to those with p14\(^{ARF}\) negative cancers (\(p=0.004\), HR 3.107, 95% CI 1.434, 6.724).

**Effect of combining p16 and p14 expression on outcomes.**

The effects of combining p16 and p14 expression on outcomes are shown in Fig 4, and were analysed using univariable models. Relative to patients with p16 and p14 negative cancers, patients with breast cancers displaying strong p16 and p14 expressions had an adjusted 3-fold increased risk of having disease recurrence (HR=3.283, 95% CI: 1.569,6.869) and 2-fold increased risk of all-cause related death (HR=1.935, 95% CI: 1.108,3.377) (Table 5).

**DISCUSSION**

Cellular senescence encompasses the two crucial tumor suppressor pathways: p53 and p16\(^{INK4a}\) signaling which converge at the Rb pathway\(^{12}\). By entering either one of the pathways, cancer cells may be directed to senescence and cancer progression is inhibited, as has been demonstrated by many in vivo and in vitro studies\(^{13, 14}\). Induction of either p53 or p16\(^{INK4a}\) in
response to abnormal mitogenic signaling promotes premature senescence, suggesting that it is a mechanism of tumour suppression\textsuperscript{(13)}. Hence, loss of p16\textsuperscript{INK4a} in the normal cell promotes hyperplasia which can lead to increased risk of tumour development\textsuperscript{(15)}. Induction of cancer formation has been associated with replicative senescence in normal human cells\textsuperscript{(16)} and with p21-induced senescence in tumour cells\textsuperscript{(6)}. The tumour suppressor gene p14\textsuperscript{ARF}, which acts as upstream regulator of p53, induces premature senescence through the activation of p21-dependent pathway.

Our study investigated the expression of senescence-associated proteins to assess for their potential as prognostic markers in a large series of breast cancer patients, many with matching normal, hyperplastic and premalignant neoplastic tissues. It has been hypothesized that expression of senescence-associated markers is increased in pre-invasive but is lost in malignant lesions. We found the expression level for each marker appears to increase from normal to atypical ductal hyperplasia to DCIS, then plateau or slightly drop from DCIS to IDC. Expression of senescence markers gradually increase as the tissues become more proliferative, as a natural response of tumour suppressor genes to counteract the growth, peaking at the pre-invasive stage. However, when the lesion become invasive (i.e. tumour cells are so called 'immortable' and can divide indefinitely) they are escaping senescence by acquiring mutations that either directly/indirectly negate tumour suppressor gene function, hence resulting in the senescence marker levels plateauing, as in this cohort. It has previously been shown that over-expression of p14\textsuperscript{ARF} was prominent in both pre-invasive and invasive breast cancers\textsuperscript{(10)}. To date, increased p16\textsuperscript{INK4a} expression in both pre-malignant and malignant tissues have been seen in several other human cancers, including esophageal\textsuperscript{(17)} and prostate carcinoma\textsuperscript{(18)}. Pathophysiologically, overexpression of senescence markers in a tumour cell may indicate that the tumour cell has
been activated to enter the senescence program, but it may also reflect disruption of a downstream mediator of the regulatory pathway (19).

In general, p14^{ARF} tumour suppressor activity can be activated either via or independently of p53. p14^{ARF} is a predominant nucleolar protein; however, we report a distinct cytoplasmic immunostaining. A similar finding was also reported in breast cancer by another group where cytoplasmic p14^{ARF} staining was an important prognostic factor (10). Moreover, cytoplasmic p14^{ARF} is evident in other types of human cancers such as non-small cell lung cancer, oral squamous cell carcinoma, pancreatic cancer and prostate cancer (20-22). Relocalisation of p14^{ARF} from the nucleus to the cytoplasm may possibly relate to functional inactivation similar to other tumour suppressor genes such as p21 and p53 (23, 24). However, at this stage we are not able to draw any conclusion on the functional meaning of cytoplasmic p14^{ARF} expression. Interestingly, p14^{ARF} overexpression in invasive ductal carcinoma is associated with established prognostic factors such as high grade tumour and positive estrogen receptor status. To our knowledge, such associations have not been reported in human breast cancer. Nevertheless, other cancer studies that have documented p14^{ARF} overexpression in association with aggressive tumour behavior suggested it as a sign of malfunction of the major cell-cycle regulatory pathways (25). There is no conclusive explanation for the significant association of p14^{ARF} overexpression with estrogen receptor positivity, although another study has documented downregulation of p14^{ARF} expression as a mediator of tumorigenesis in the presence of ER upregulation (26). In contrast to our finding of association between p14^{ARF} expression and increased risk of relapse, a study by Vestey et. al found that the presence of cytoplasmic p14^{ARF} confers significant survival advantage in breast cancer patients (10). This contradictory finding may be related to the dual function of p14^{ARF}, in which it acts as a tumour suppressor in some tumours but instead exhibit oncogenic activity in
Moreover, senescent cells have been shown to change their surrounding tissue environment which can in turn promote tumour formation in the adjacent cells (27).

\( p16^{\text{INK4a}} \) is a negative cell-cycle regulator that inhibits aberrant cells from proliferating at the late G1 phase (28). Although \( p16^{\text{INK4a}} \) share the same exons with \( p14^{\text{ARF}} \), downregulation of \( p16^{\text{INK4a}} \) in human breast cancer were more frequent than loss of \( p14^{\text{ARF}} \) expression (29). Nonetheless, we found a significantly higher level of \( p16^{\text{INK4a}} \) expression in invasive ductal carcinoma when compared to normal breast tissue. In addition, our study has shown that increased \( p16^{\text{INK4a}} \) expression in malignant breast tissue correlates with unfavourable prognostic factors of poor tumour differentiation and ER and PR negativity. A similar finding has been reported in a study by others with the same scoring system (8). Abnormally high level of \( p16^{\text{INK4a}} \) was proposed as a result of pRb inactivation or low pRb expression (30). On the other hand, a recent study reported replicative senescence may contribute to the stabilisation and increased expression of \( p16^{\text{INK4a}} \) in senescent cells (7). In the present study, we found that high \( p16^{\text{INK4a}} \) expression is a sign of poor overall and disease free survival with increased risk of relapse in breast cancer. This is supported by a study which reported association between over-expression of \( p16^{\text{INK4a}} \) mRNA with loss of survival advantage in primary breast cancer (30).

Currently, there is increasing use of two or more biomarkers in the assessment of patient prognosis and response to treatment. We have shown in this study that breast cancers that show a combination of \( p16 \) and \( p14 \) expression have increased risk of poor clinical outcome in the form of disease recurrence and poor overall survival, although the effect on the latter was not as strong.
Functionally, p21 plays an important role in the regulation of cell cycle, gene transcription, DNA repair and modulation of apoptosis \(^5\). Despite its function as a tumour suppressor, p21 is found to paradoxically induce cell cycle progression. In a recent finding, progestin, the synthetic progesterone, induces breast tumour progression via increased expression of p21 by formation of transcriptional complex with stat3, progesterone receptor and ErbB-2 \(^31\). Still, the significance of cyclin-dependent kinase inhibitor p21 expression in human breast cancer is uncertain with limited and conflicting data. While some studies report direct relationship between p21 expression and poor prognostic factors \(^32, 33\), others have shown correlation between p21 expression with good prognosis \(^34, 35\). In the present study, we found loss of p21 protein expression in 94.8% of the cases. A relationship is seen between absence of p21 with high tumour grade but not with other clinicopathological parameters. However, there are studies by other groups that have reported the insignificant value of p21 in relation to clinical parameters and in predicting the outcome of breast cancer, suggesting that p21 may not be an important prognostic factor in these tumours \(^36\). Similarly, we were unable to show any significant correlation between p21 expression and overall and disease free survival.

**CONCLUSION**

In conclusion, defining the expression profiles of senescence biomarkers in the morphological progression of proliferative breast lesions to invasive cancer in a large cohort may enhance the understanding of the fundamental aspects of breast tumourigenesis. We have shown that the expression of these markers plateau or slightly decreases from premalignant to malignant lesions, suggestive of tumour evasion of the senescence pathway. Therefore, induction of senescence may assist in therapies aimed at inhibiting tumour growth. Our results suggest p16 and p14 are
potentially useful prognostic biomarkers in breast cancer. Subsequent stratification of breast tumour patients may further optimize their treatment and counselling.

Conflict of interest
None declared

Financial Disclosure
None declared

ACKNOWLEDGEMENTS:
Rahmawati Pare is sponsored by Ministry of Education, Malaysian Government and the University of Malaysia Sabah. The authors are grateful to Irani Dissanayake, Vijini Abeyratne, Indika Liyanage, Nilusha Lakmalie and Menaka Weerasingha, who have assisted in the immunohistochemical scoring of the slides, and are supported by Cancer Institute New South Wales through the Centre for Oncology Education and Research Translation (CONCERT).

AUTHOR CONTRIBUTIONS:
Conceived and designed the research: RP, JSS, CSL. Performed the experiments: RP. Contributed essential reagents or tools: RP, JSS, CSL. Analysed the data: RP, JSS, CSL. Wrote the paper: RP, JSS, CSL.

Reference list


Table legends

**Table 1.** Clinicopathological parameters included in the study. LVI, lymphovascular invasion; LNI, lymph node invasion; ER, estrogen receptor; PR, progesteron receptor; ADH, atypical ductal hyperplasia; DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma; NA, not available

**Table 2.** Chi square P-value indicates the differences between: (a) Normal vs atypical ductal hyperplasia (ADH); (b) ADH vs DCIS; (c) DCIS vs IDC and (d) IDC vs Normal. Percentages represent ratio of positive or negative to the total number of cases.

**Table 3.** Association of senescence-associated markers with clinicopathological variables.

* Pearson $\chi^2$
* Statistically significant p<0.05

**Table 4.** Univariate and multivariate cox regression.
* Statistically significant p<0.05

**Table 5.** Association between P16 and P14 status on time to death and recurrence.
* Statistically significant p<0.05
† Cox proportional hazard regression
Figure legends

**Figure 1** Immunohistochemical staining of p14^{ARF}, p16^{INK4a} and p21 in the normal, hyperplastic, pre-malignant and malignant breast tissues, from left to right. A1-A4 shows positive nuclear staining of p21 in the respective tissues. B1-B4 shows positive nuclear and cytoplasmic staining of p16^{INK4a} in the respective tissues. C1-C4 shows positive cytoplasmic staining of p14^{ARF} in the respective tissues. Magnification x400.

**Figure 2. Left:** Overall survival probability based on p14 expression (Kaplan-Meier curve, P=0.456). **Right:** Recurrence free survival probability based on p14 expression (Kaplan-Meier curve, p=0.038).

**Figure 3. Left:** Overall survival probability based on p16 expression (Kaplan-Meier curve, Weak vs Moderate p=0.022, Strong vs Weak p=0.000). **Right:** Recurrence free survival probability based on p16 expression (Kaplan-Meier curve, Moderate vs Strong p=0.013, Strong vs Weak p=0.000)

**Figure 4. Left:** Overall survival probability based on joint effect of p16 and p14 expression. P16+/P14+ vs P16-/P14- (p=0.022) and P16+/P14+ vs P16-/P14+ (p=0.000) showed significant differences on overall survival. The worst outcome was seen in patients P16+/P14+. **Right:** Recurrence free survival probability based on joint effect of p16 and p14 expression. P16+/P14+ vs P16+/P14- (p=0.034), P16+/P14+ vs P16-/P14+ (p=0.000) and P16+/P14+ vs P16-/P14- (p=0.001) showed significant differences in DFS. The worst outcome was seen in patients with P16+/P14+ tumours.
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
</tr>
<tr>
<td>≤60 years</td>
<td>572 (53.0)</td>
</tr>
<tr>
<td>&gt;60 years</td>
<td>508 (47.0)</td>
</tr>
<tr>
<td><strong>Tumour size</strong></td>
<td></td>
</tr>
<tr>
<td>≤20mm</td>
<td>641 (59.4)</td>
</tr>
<tr>
<td>&gt;20mm</td>
<td>439 (40.6)</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>441 (40.8)</td>
</tr>
<tr>
<td>Poor</td>
<td>378 (35.0)</td>
</tr>
<tr>
<td>I</td>
<td>451 (41.8)</td>
</tr>
<tr>
<td>II</td>
<td>503 (46.6)</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>115 (10.6)</td>
</tr>
<tr>
<td>IV</td>
<td>11 (1.0)</td>
</tr>
<tr>
<td><strong>LVI</strong></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>711 (65.8)</td>
</tr>
<tr>
<td>Positive</td>
<td>369 (34.2)</td>
</tr>
<tr>
<td><strong>LNI</strong></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>679 (62.9)</td>
</tr>
<tr>
<td>Positive</td>
<td>401 (37.1)</td>
</tr>
<tr>
<td><strong>ER</strong></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>795 (73.6)</td>
</tr>
<tr>
<td>Missing</td>
<td>58 (5.4)</td>
</tr>
<tr>
<td>Negative</td>
<td>330 (30.6)</td>
</tr>
<tr>
<td><strong>PR</strong></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>693 (64.2)</td>
</tr>
<tr>
<td>Missing</td>
<td>57 (5.3)</td>
</tr>
<tr>
<td>Negative</td>
<td>561 (51.9)</td>
</tr>
<tr>
<td><strong>HER2</strong></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>112 (10.4)</td>
</tr>
<tr>
<td>Missing</td>
<td>407 (37.7)</td>
</tr>
<tr>
<td><strong>Normal tissue</strong></td>
<td></td>
</tr>
<tr>
<td>Available</td>
<td>1053 (97.5)</td>
</tr>
<tr>
<td>NA</td>
<td>27 (2.5)</td>
</tr>
<tr>
<td><strong>ADH</strong></td>
<td></td>
</tr>
<tr>
<td>Available</td>
<td>553 (51.2)</td>
</tr>
<tr>
<td>NA</td>
<td>527 (48.8)</td>
</tr>
<tr>
<td><strong>DCIS</strong></td>
<td></td>
</tr>
<tr>
<td>Available</td>
<td>778 (72.0)</td>
</tr>
<tr>
<td>NA</td>
<td>302 (28.0)</td>
</tr>
<tr>
<td></td>
<td>p21 Expression</td>
</tr>
<tr>
<td>---------------</td>
<td>----------------</td>
</tr>
<tr>
<td></td>
<td>Negative (&lt;5%)</td>
</tr>
<tr>
<td>Normal</td>
<td>988 (99.6%)</td>
</tr>
<tr>
<td>ADH</td>
<td>492 (98.6%)</td>
</tr>
<tr>
<td>DCIS</td>
<td>709 (94.9%)</td>
</tr>
<tr>
<td>IDC</td>
<td>1004 (94.8%)</td>
</tr>
<tr>
<td></td>
<td>p21 Expression</td>
</tr>
<tr>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td></td>
<td>Low</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
</tr>
<tr>
<td>≤60 years</td>
<td>536</td>
</tr>
<tr>
<td>&gt;60 years</td>
<td>468</td>
</tr>
<tr>
<td><strong>Tumour size</strong></td>
<td></td>
</tr>
<tr>
<td>≤20mm</td>
<td>595</td>
</tr>
<tr>
<td>&gt;20mm</td>
<td>409</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>256</td>
</tr>
<tr>
<td>Moderate</td>
<td>398</td>
</tr>
<tr>
<td>Poor</td>
<td>350</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>425</td>
</tr>
<tr>
<td>II</td>
<td>463</td>
</tr>
<tr>
<td>III</td>
<td>107</td>
</tr>
<tr>
<td>IV</td>
<td>9</td>
</tr>
<tr>
<td><strong>LVI</strong></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>662</td>
</tr>
<tr>
<td>Present</td>
<td>342</td>
</tr>
<tr>
<td><strong>LNI</strong></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>638</td>
</tr>
<tr>
<td>Present</td>
<td>366</td>
</tr>
<tr>
<td><strong>ER</strong></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>205</td>
</tr>
<tr>
<td>Positive</td>
<td>745</td>
</tr>
<tr>
<td><strong>PR</strong></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>300</td>
</tr>
<tr>
<td>Positive</td>
<td>651</td>
</tr>
<tr>
<td><strong>HER2</strong></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>527</td>
</tr>
<tr>
<td>Positive</td>
<td>100</td>
</tr>
<tr>
<td>Variable</td>
<td>Univariate analysis</td>
</tr>
<tr>
<td>-------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td></td>
<td>HR</td>
</tr>
<tr>
<td><strong>Overall survival (OS)</strong></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1.367</td>
</tr>
<tr>
<td>Tumour size</td>
<td>1.892</td>
</tr>
<tr>
<td>Grade</td>
<td>2.368</td>
</tr>
<tr>
<td>Staging</td>
<td>16.302</td>
</tr>
<tr>
<td>LVI</td>
<td>3.143</td>
</tr>
<tr>
<td>LNI</td>
<td>2.429</td>
</tr>
<tr>
<td>ER</td>
<td>0.377</td>
</tr>
<tr>
<td>PR</td>
<td>0.399</td>
</tr>
<tr>
<td>HER2</td>
<td>1.382</td>
</tr>
<tr>
<td>p16</td>
<td>2.498</td>
</tr>
<tr>
<td>p14</td>
<td>1.179</td>
</tr>
<tr>
<td>p21</td>
<td>0.650</td>
</tr>
<tr>
<td><strong>Disease-free survival (DFS)</strong></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.815</td>
</tr>
<tr>
<td>Tumour size</td>
<td>1.975</td>
</tr>
<tr>
<td>Grade</td>
<td>4.093</td>
</tr>
<tr>
<td>Staging</td>
<td>12.790</td>
</tr>
<tr>
<td>LVI</td>
<td>4.003</td>
</tr>
<tr>
<td>LNI</td>
<td>2.798</td>
</tr>
<tr>
<td>ER</td>
<td>0.293</td>
</tr>
<tr>
<td>PR</td>
<td>0.432</td>
</tr>
<tr>
<td>HER2</td>
<td>1.649</td>
</tr>
<tr>
<td>p16</td>
<td>2.943</td>
</tr>
<tr>
<td>p14</td>
<td>1.767</td>
</tr>
<tr>
<td>p21</td>
<td>0.503</td>
</tr>
<tr>
<td></td>
<td>Time to death</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------</td>
</tr>
<tr>
<td></td>
<td>HR</td>
</tr>
<tr>
<td>a) Joint effect</td>
<td></td>
</tr>
<tr>
<td>P16+/P14+</td>
<td>1.935</td>
</tr>
<tr>
<td>P16+/P14-</td>
<td>1.140</td>
</tr>
<tr>
<td>P16-/P14+</td>
<td>0.771</td>
</tr>
<tr>
<td>P16-/P14-</td>
<td>reference</td>
</tr>
<tr>
<td>b) Stratified effect</td>
<td></td>
</tr>
<tr>
<td>P16+/P14+</td>
<td>1.681</td>
</tr>
<tr>
<td>P16+/P14-</td>
<td>reference</td>
</tr>
<tr>
<td>P16-/P14+</td>
<td>0.787</td>
</tr>
<tr>
<td>P16-/P14-</td>
<td>reference</td>
</tr>
</tbody>
</table>
This article is protected by copyright. All rights reserved.